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Begonia hooveriana Wiriad. photo by Andini Agustiani

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Chapter in the book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds.). Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

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Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from the internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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Morphological and flowering phenology characterization of wild *Begonia* (*B. hooveriana* and *B. hijauvenia*) collected at Bogor Botanic Gardens, Indonesia

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Abstract. *Agustiani A, Garvita RV, Siregar HM, Windarsih G. 2025. Morphological and flowering phenology characterization of wild* Begonia *(*B. hooveriana *and* B. hijauvenia*) collected at Bogor Botanic Gardens, Indonesia. Nusantara Bioscience 17: 1-10. Begonia hooveriana* and *B. hijauvenia* are known for their varied morphology. This study aimed to describe the morphological characteristics of three *Begonia* accessions and determine their flowering phenology and pollen viability *B. hooveriana* with green and red color and *B. hijauvenia*. The *Begonia* were compared to characterize the plants, stomata on the underside of the leaves were examined, flowering phenology was observed daily during the flowering phase, and pollen viability was observed at the anthesis and post-anthesis phases. Data were recorded and analyzed descriptively. Based on morphological characterization data, the only morphology that distinguishes the two accessions of *B. hooveriana* is the color. The most prominent difference between *B. hijauvenia* and the previous two comparators is the type of plant. Accession *B. hooveriana* has anisocytic stomata, while *B. hijauvenia* has parasitic stomata. Based on the results of flowering phenology, the two accessions of *B. hooveriana* have a flowering period of 10-21 days, and *B. hijauvenia* has a flowering period of 17 days. The highest percentage of pollen viability in both flowering phases was found in *B. hooveriana* red (anthesis: 96.60±4.08%, post-anthesis:96.30±4.46%). The varied morphological characteristics of these three plants can be used as a basis for plant breeding programs.

Keywords: *Begonia hijauvenia, B. hooveriana*, Bogor Botanic Gardens, flowering phenology, morphology, pollen viability

INTRODUCTION

Begonia is one of the genera of Angiosperms with the highest number of species, comprising more than 3.000 plant species (Wahyu and Lucyanti 2019). Most members of the genus *Begonia* have the potential to become ornamental plants. In addition, some *Begonia* species can also be used as medicines, and the parts used are the leaves and stems. The efficacy of this plant is as a medicine for ulcers, to cure dizziness and fever, and to remove phlegm (Hartutiningsih 2017, et al. 2018; Wahyu and Lucyanti 2019). *Begonia* has a varied habitat type; this plant can thrive in forest areas, humid areas, mossy soils, and rather shady areas at altitudes up to 2.400 m asl. (Munawaroh and Hartutiningsih 2018). Its wide distribution causes *Begonia* to have variations and a wide range of morphological characteristics and results in complex species boundaries. Indonesia has a variety of habitats suitable for the growth of wild *Begonia* and almost all parts of Indonesia can be found wild *Begonia*. Sulawesi and Sumatra are rich in *Begonia* diversity (Thomas et al. 2013).

Begonia hooveriana Wiriad. is discovered in Sulawesi, Indonesia in 1998. The name was given in honor of Mr. Walter Scott Hoover of the Tropical Conservatory of New England, USA, who sponsored an expedition to search for a new species of *Begonia* in Indonesia (Wiriadinata 2013). Meanwhile, *Begonia hijauvenia* Girm., Ardi & M.Hughes. is found in West Sumatra, Indonesia, especially in lowland forest areas. The name hijauvenia comes from the Indonesian word meaning green, referring to the green veins found on its leaves (Girmansyah et al. 2022).

Research about those two species of *Begonia* is poorly reported. Wiriadinata (2013) reported that the characteristics of *B. hooveriana* are its flat-shaped leaves and shiny dark green color, while Alvitasari (2022) reported that *B. hooveriana* has morphological variations in the form of size, shape, and color in its morphology. Then Girmansyah et al. (2022) also reported that *B. hijauvenia* is unique in male flowers, which have pink color in the adaxial part and white in the abaxial part.

Bogor Botanic Gardens, Indonesia, managed 134 species of *Begonia*, consisting of 97 species of wild *Begonia* and 37 species of exotic *Begonia* in 2019. *Begonia* cultivation and breeding at Bogor Botanic Gardens has produced 17 new varieties (Riswati 2019). The data is certainly subject to change as research on the genus increases. Efforts to explore the potential utilization of *Begonia* as ornamental plants have been carried out at Bogor Botanic Gardens, including characterization activities (Hartutiningsih et al. 2018). Taxonomic approaches are useful in uncovering the diversity of plant species based on morphological characteristics. Therefore, other approaches are needed to complement existing morphological data, such as anatomical characterization of stomata and flowering phenology that can also be important characters in *Begonia* characterization (Chuine and Régnière 2017). To support breeding programs in flowering plants and create new cultivars, information on flowering phases is crucial, particularly regarding stigma receptivity duration and pollen viability.

Data on morphology, flowering phenology, and pollen viability are very important for plant breeding programs. However, studies on the phenology of those species of *Begonia* are still limited. Therefore, it is necessary to conduct a more in-depth study of the morphological characterization, flowering phenology, and pollen viability of *B. hooveriana* and *B. hijauvenia* so that it is hoped that this research can provide new data on morphological diversity and flowering phenology and pollen viability so that it can help in the plant breeding process.

MATERIALS AND METHODS

Plant materials

The study was conducted from September to December 2023 at the nursery greenhouse and laboratory of Bogor Botanic Gardens, National Research and Innovation Agency (BRIN), Bogor, Indonesia. The samples consisted of 3 wild *Begonia* accessions maintained at the Bogor Botanic Gardens (Table 1), and each accession consisted of 3 individuals.

Procedures

Morphological characterization

Morphological characters were characterized to compare the three wild *Begonia* accessions used. Secondary data was obtained through literature studies referencing Tjitrosoepomo (2020) and the General Guide for Describing Ornamental Plants (PVTPP 2021). Meanwhile, quantitative data were obtained by measuring morphological characteristics in the three *Begonia* accessions. The characteristics observed were leaf size, stem size, stalk size, male flower size, and rhizome size. The method used involved direct observation of all samples used.

Stomatal preparation was conducted by creating a mold using dental resin on the lower surface of the leaf (Weyers and Johansen 1985). Once the mold dried, it was coated with transparent nail polish. After drying, the nail polish was carefully peeled off so that the stomata adhered to the layer of nail polish. It comprised an objective magnification of 40x and an ocular magnification of 10x to observe the types and sizes of stomata using a light microscope. The observed stomatal characteristics include stomatal type as well as the length and width of the guard cells. Stomata were observed on the abaxial leaf surface in all three *Begonia* accessions observed. The stomata were collected on the abaxial surface of the leaf because the surface has more stomata compared to the adaxial surface. This is suspected due to the adaxial surface being directly exposed to sunlight, which can lead to damage to the stomata (Papuangan et al. 2014).

Flowering phenology

Observations of flowering phenology were made by following the method of Arteca (2013). The flowering phenological observation was conducted daily from the onset of flower bud emergence until flower blooming and wilting. The length and width of petals were measured each day. Each stage was photographed and documented. Data on flower development phases observed included the number of days of flower development, the duration from flower bud to blooming, and the time from blooming to wilting.

Pollen viability

Pollen viability testing of the three *Begonia* accessions was conducted using staining with 1% acetocarmine solution following the method of Warid and Palupi (2009). Pollen viability was assessed at two different flower maturation times, including: (i) On the first day during flower blooming and (ii) On the 4th day after blooming. The pollen was placed on a microscope slide using a brush, then stained with acetocarmine, and incubated at room temperature for 15 minutes. Subsequently, the preparation was observed under a light microscope with a total magnification of 100x, comprising an objective magnification of 10x and an ocular magnification of 10x. Pollen grains were deemed viable based on the color formed as an indicator. Partial staining of pollen grains after acetocarmine staining indicated non-viable pollen grains, whereas complete staining was categorized as viable pollen grains (Shivakumar et al. 2014). Pollen grains were considered normal if they absorbed at least 70% of the stain, resulting in a dark red color (Dewi et al. 2015).

Table 1. *Begonia* samples observed

Species	Location origin	Collection origin	Code number
Begonia hooveriana Wiriad. with green color	Indonesia, South Sulawesi, Tana Toraja, Makale, 590-1109 m asl,	Bogor Botanic Gardens	B2013070053
Begonia hooveriana Wiriad. with red color	Indonesia, South Sulawesi, Tana Toraja, Makale, 590-1109 m asl,	Bogor Botanic Gardens	MHS20
Begonia hijauvenia Girm., Ardi & M.Hughes	Indonesia, West Sumatra	Bogor Botanic Gardens	MHS15

Data analysis

Data analysis was carried out using a comparative descriptive method with qualitative and quantitative approaches. Morphological analysis was carried out directly by comparing data from observations and measurements; then, a score was made. Data on pore size and stomatal protective cells were taken from images taken using a microscope, then measured using ImageJ software, and analyzed using standard deviation using SPSS 23.00. The determination of mean and standard deviation was performed using Microsoft Office Excel 2010.

Data on flowering phenology were taken from the results of measurements and documentation carried out directly and analyzed for size and the length of the flowering phase from budding to wilting and falling flowers (Arteca 2013). Data on pollen viability were taken from microscope images, then measured the length and width of pollen visible in the field of view with imageJ software. Determination of pollen viability is based on the formula:

$$\text{Pollen viability} = \frac{\text{TOTAL POLLEN STAINED}}{\text{TOTAL POLLEN IN THE FLIED OF VIEW}} \text{x } 100\%$$

RESULTS AND DISCUSSION

Morphological characterization

The environment can affect the morphological and anatomical structures of plants. One of the methods to identify morphological characteristics in plants is by directly observing particular organs, such as roots, stems, leaves, flowers, and fruit (Susetyarini et al. 2020). The wild *Begonia* collection at Bogor Botanic Gardens, namely *B. hooveriana* with green color, *B. hooverianaa* with red color, and *B. hijauvenia*, were suspected to have varying characteristics. This hypothesis is determined based on the diverse morphological variations observed among those three *Begonia* accessions. The most notable morphological variations are the shape, size, and color of the plants and their parts, such as leaves and flowers. A comparison of these variations among the three *Begonia* accessions can be seen in Table 2.

Begonia hooveriana *with green color*

The plant is shrubby, and the upper surface of the leaves is shiny. The petiole is positioned at the edge of the leaf blades. It has a type of inflorescence, protandrous, where male flowers mature earlier than female flowers, and male flowers grow clustered in one peduncle (Figure 1).

Begonia hooveriana *with red color*

The morphological characteristics of *B. hooveriana* with red color are nearly identical to the *B. hooveriana* with green color. The plant is shrubby, the upper leaf surface is shiny, with two color variations, i.e., green and red as spots; has a type of inflorescence, protandrous where male flowers appear earlier than female flowers; male flowers have two color variations, with the petals being light pink when in the bud, then turning white when fully bloomed, leaving pink spots at the tips of the petals; growing clustered in one peduncle (Figure 2).

Begonia hijauvenia

Begonia hijauvenia is an endemic species in Sumatra, with has interesting leaf patterns, brownish with green veins, stiff leaf surfaces, long-haired petioles, grow directly from rhizomes, have an inflorescence type, male flowers have four petals, two larger petals reddish on the outside of the petals and white on the inside of the petals, the outer surface of the petals is hairy with moderate intensity. At the time of observation, female flowers could not be identified because they had not yet appeared. After all, this plant has protandus flowers, where male flowers appear first before female flowers (Figure 3).

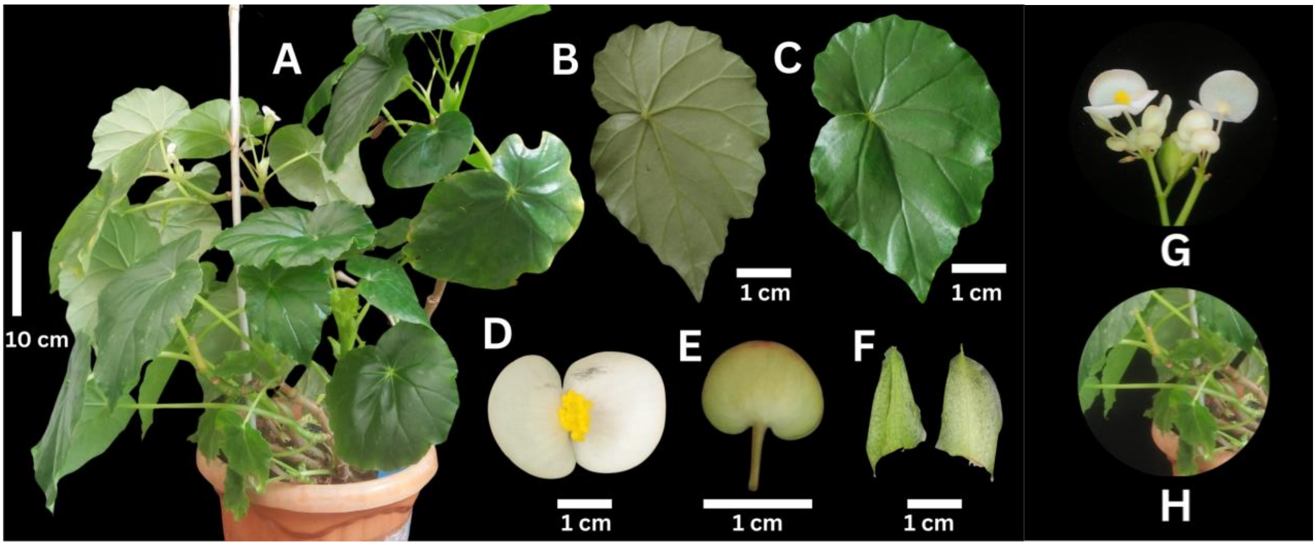

Figure 1. Morphology of *Begonia hooveriana* with green color: A. Habitus; B. Abaxial leaf surface; C. Adaxial leaf surface; D. Male flower in bloom; E. Male flower bud; F. Stipule; G. Inflorescence; H. Petiole. Bar = 10 cm and 1 cm

Table 2. Comparison of morphological characters of 3 accessions of *Begonia* collected at Bogor Botanic Gardens, Bogor, Indonesia

Character	*B. hooveriana*		*B. hijauvenia*
	Green	Red	
Plant type	having a stem	having a stem	rhizome
Plant height (cm)	13.1-80.3	55.0-78.8	14.9-19.7
Leaf type	single	single	single
Leaf shape	ovate	ovate	orbicular
Leaf wrinkles	none	none	finely creased
Leaf base	cordate	cordate	emarginate
Leaf tip	tapered (acuminate)	tapered (acuminate)	tapered (acuminate)
Leaf incision	shallow	shallow	none
Leaf margin	undulate	undulate	entire
Color of leaf blade surface	green	green, red	green, red
Primary color of upper surface of leaf blade	Dark Yellow Green A Green Group 139	Moderate Olive Green A Yellow Green Group 147	Strong Yellow Green A Green Group 143
Secondary color of upper surface of leaf blade	none	Strong Yellow A Green Group 143	Greyish Red A Greyed-Red Group 178
Base color of lower surface of leaf blade	Moderate Olive Brown A Gray Brown Group 199	Greyish Red A Greyed Red Group 178	Strong Yellow Green D Green Group 143
Secondary color of lower surface of leaf blade	none	Strong Yellow Green C Green Group 143	Greyish Brown A Greyed-Orange Group 166
Hair distribution on leaf blade	none	none	entire leaf surface
Intensity of hairs on leaf blade surface	none	none	medium
Petiole length (cm)	7.0-20.5	6.0-16.3	3.5-9.5
Petiole color	Strong Yellow Green A Yellow Green Group 145	Light Yellow Green A Yellow Green Group 145	Moderate Reddish Brown B Greyed-Orange Group 166
Petiole shape	cylinder	cylinder	cylinder
Intensity of hairs on petiole surface	none	none	dense
Flower type	compound	compound	compound
Color of petals	white	white, pink	white, red
Base color of the outer petals of male flowers	White C White Group NN15	White C White Group NN155	Moderate Red B Greyed Red Group 180
Male petal inner base color	White C White Group NN15	White C White Group NN155	Moderate Purplish Pink D Red Purple Group 170
External secondary color male flower petals	none	Vivid Purplish Pink A Red Purple Group N66	none
Secondary color of the inside of male petals	none	Vivid Purplish Pink A Red Purple Group N66	White B White Group NN155
Intensity of hairs on the outer surface of male flowers	none	none	medium
Stem length (cm)	21.6-76.7	20.4-106.4	none
Rhizome length	none	none	4.0-37.5 cm
Color of stem	Strong Yellow Green A Yellow Green Group 145	Strong Yellow Green A Yellow Green Group 145	none
Rhizome color	none	none	Strong Yellow Green C Yellow Green Group 145

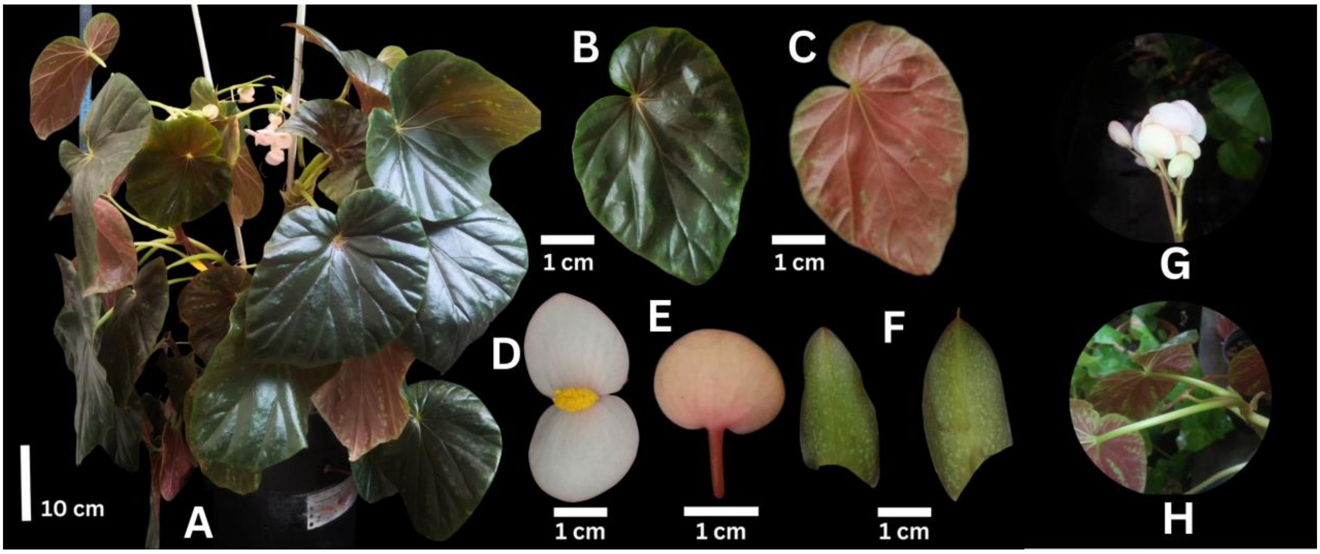

Figure 2. Morphology of *Begonia hooveriana* with red color: A. Habitus; B. Adaxial leaf surface; C. Abaxial leaf surface, D. Male flower in bloom; E. Male flower bud; F. Stipule; G. Inflorescence; H. Petiole. Bar = 10 cm and 1 cm

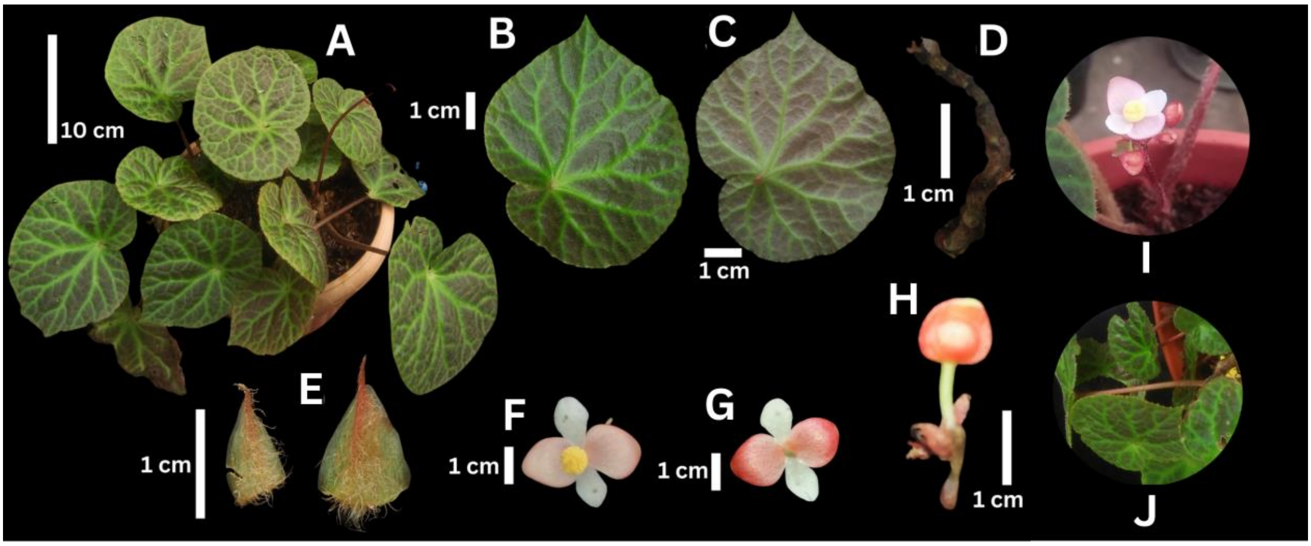

Figure 3. Morphology of *Begonia hijauvenia* with green color: A. Habitus; B. Abaxial leaf surface; C. Adaxial leaf surface; D. Rhizome; E. Stipule; F. Upper surface of male flower when blooming; G. Lower surface of male flower when blooming; H. Male flower bud; I. Inflorescence; J. Petiole. Bar = 10 cm and 1 cm

The morphological characters in plants can change because it is controlled by genetic traits under the influence of environmental factors, such as climate, temperature, soil type, soil conditions, altitude, and air humidity. Red color variations on the stem can occur due to plant responses to abiotic factors, such as low temperature (Hadiyanti et al. 2018). In *B. hijauvenia*, there is a rhizome that groups up to 30 cm long (Girmansyah et al. 2022). Serrated leaf margin variation was found in *B. hooveriana*; this serrated leaf margin variation can show the link between species and their habitat (Nicotra et al. 2011). However, the function of serrations on the edge of *Begonia* leaves is not yet known with certainty because more serrated leaf edges are also found in lowland locations and vice versa; at high altitudes, individuals found have leaf edges with wider serrations (Ayu et al. 2019). While *B. hijauvenia* has flat leaf edges and there are hairs with moderate intensity.

There is no difference in the structure of male flowers in *B. hooveriana* with green color and *B. hooveriana* with red color. Male flowers of both accessions have two petals. However, the male flower petals on *B. hooveriana* with green color are larger than male flower petals on *B. hooveriana* with red color. The male flowers of *B. hijauvenia* have 4 petals with 2 petals that have a larger size, and there are hairs with medium intensity on the outside of the petals. The color of the petals on the three *Begonia* accessions varied from white to white with a pink tinge and red.

Stomatal characteristics

Stomatal type

The results showed that *B. hooveriana*, both with green and red colors, have anisocytic stomatal type. Anisocytic stomata are characterized by three neighboring cells of different sizes (Figure 4). This result supported Efendi (2019), which indicates that *B. hooveriana* has anisocytic stomatal type. Meanwhile, *B. hijauvenia* has a parasitic stomatal type, characterized by each guard cell joining with one or more neighboring cells and the long axis of neighboring cells parallel to the guard cell without a gap (Efendi 2019).

Stomatal size

The stomata of the three *Begonia* accessions observed have different sizes, with the longest guard cells found in the green-colored *B. hooveriana* (5.09±0.27 µm), which is not significantly different from the red-colored *B. hooveriana*, but significantly different from *B. hijauvenia*; followed by *B. hijauvenia* (4.38±1.04 µm), which is significantly different from the other two accessions, and the red-colored *B. hooveriana* (3.73±0.17 µm), respectively (Table 3). As reported by Efendi (2019), the stomatal size characteristics in *Begonia* can not be used to differentiate between species. According to Hong et al. (2018), stomatal size can directly affect transpiration and photosynthesis rates. High stomatal density and size can increase conductance and photosynthetic gas exchange before the leaves undergo final senescence (Sabina and Sameena 2022). Plants with small stomatal density and size will have the ability to maintain high gas exchange rates under high-temperature stress by opening the stomatal pores (Caine et al. 2019).

Flowering phenology

Phenology is one of the plant physiological processes related to biological event timing and environment (Lestari et al. 2023). Observation of flowering phenology was conducted to determine the flowering period in one period, from bud initiation to flower withering and subsequent shedding. The development of flowers in a plant can also be used to determine whether the plant exhibits self-pollination or cross-pollination characteristics (Rahayu et al. 2015). Information about flowering and fruiting phenology is essential to increase productivity and understanding plant physiology and environmental response (Lestari 2019).

When the male flower buds first appear, the petals of *B. hooveriana* have a greenish-white color, while the petals of *B. hijauvenia* are red. On the 2nd day, the color of the petals on red *B. hooveriana* undergoes a color change where a pink color appears at the tip of the petals. When the flowers begin to bloom fully, green *B. hooveriana* petals have a white color until the flowers fall, red *B. hooveriana* petals have a red, white, and pink color at the tip of the petals until the flowers fall, while *B. hijauvenia* petals have two color variations on the 4 petals; the outside and inside of the two large petals have a red color, while the small petals have a white color (Figure 5).

Table 3. Stomatal characteristics observation of the three accessions of *Begonia* collected at Bogor Botanic Gardens, Indonesia

Species	Stomatal type	Length of guard cell (µm)	Width of guard cell (µm)
B. hooveriana with green color	Anisocytic	5.09±0.27^{a}	1.32±0.08^{b}
B. hooveriana with red color	Anisocytic	3.73±0.17^{a}	1.31±0.03^{b}
B. hijauvenia	Parasitic	4.38±1.04^{ab}	1.14±0.08^{a}

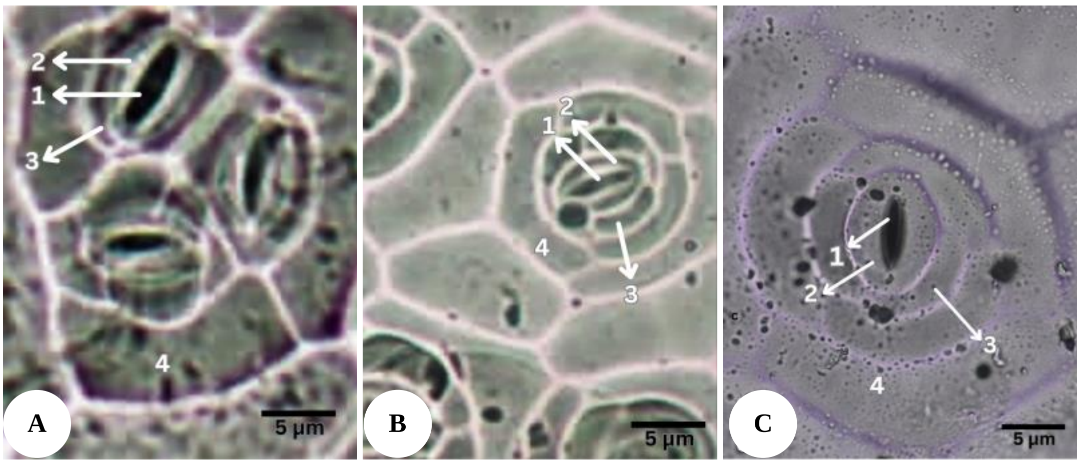

Figure 4. Stomatal anatomy with anisocytic stomatal type in: A. *Begonia hooveriana* with green color; B. *Begonia hooveriana* with red color, and parasitic stomatal type in: C. *Begonia hijauvenia*: 1. Stomatal pore; 2. Guard cell; 3. Neighbor cell; 4. Epidermal cell. Bar = 5 µm

Figure 5. Growth stages of: A. *Begonia hooveriana* with green color; B. *Begonia hooveriana* with red color; and C. *Begonia hijauvenia*: 1. First appearance of flower bud; 2. Flower bud on the 2nd day; 3. Flower bud on the 5th day; 4. Flower bud on the 7th day; 5. Fully open flower on the 1st day; 6. Fully open flower on the 2nd day; 7. Fully open flower on the 4th day; 8. Fully open flower on the 5th day; a. Flower bud; b. Sepals; c. Flower bud changing color; d. Stamen. Bar = 1 cm

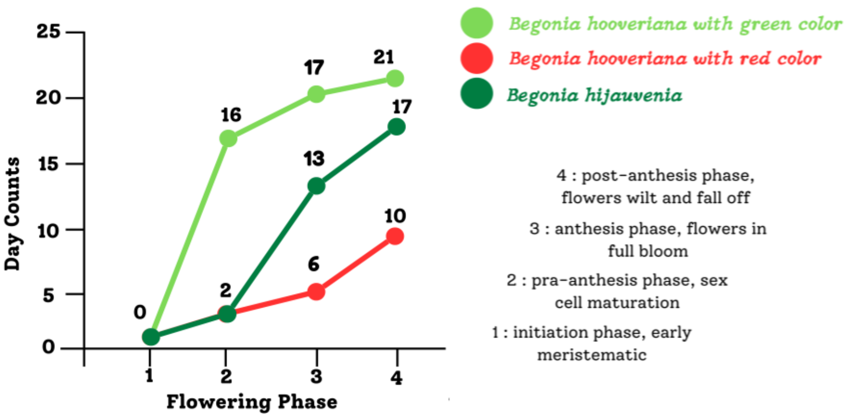

Figure 6. The flowering phase of *Begonia hooveriana* with green color, *Begonia hooveriana* with red color, and *Begonia hijauvenia*

Begonia hooveriana *with green color*

The initiation phase is marked by the appearance of a peduncle with bracts, which are small leaf-like structures that are green in color. This is followed by the emergence of oval-shaped, green organs in the center of the bract, which represent the flower buds. Sepals are the first organs to appear after the bracts. Initially, these are small spheres measuring 9.06-17.15 mm in length and 7.40-14.80 mm in width. The pre-anthesis phase lasts from day 2nd to day 16th, during which the corolla remains in bud form, measuring 10.08-22.50 mm in length and 8.96-14.96 mm in width, with a whiter coloration. The anthesis phase occurs from day 17th to day 20th, during which the flower is fully open, the corolla is white, and the pollen is matured. The final phase, post-anthesis, occurs on day 21st when the flower begins to wither and eventually falls off (Figure 6).

Begonia hooveriana *with red color*

The initiation phase is marked by the emergence of a peduncle with bracts, which are small leaf-like structures that are green in color. This is followed by the appearance of oval-shaped, green organs in the center of the bract, representing the flower bud. Sepals are the first organs to appear after the bracts, initially forming small spheres measuring 10.22-11.80 mm in length and 8.07-9.53 mm in width. The pre-anthesis phase lasts from day 2nd to day 5th, during which the corolla remains in bud form, measuring 12.26-15.44 mm in length and 9.59-12.25 mm in width. The corolla exhibits a whiter and pinkish coloration on the edges. The anthesis phase occurs from day 6th to day 9th, during which the flower is fully open, the corolla is white, and the pollen is matured. The post-anthesis phase occurs on day 10th when the flower begins to wither and eventually falls off (Figure 6).

Begonia hijauvenia

The initiation phase is characterized by the emergence of a red-colored peduncle with fine hairs on its surface. This is followed by the appearance of oval-shaped, red organs at the tip of the stalk, which represent the flower bud. Sepals are the first organs to appear, initially forming small spheres measuring 2.64-4.10 mm in length and 3.26-5.44 mm in width. The pre-anthesis phase lasts from day 2nd to day 12th, during which the corolla remains in bud form, measuring 4.90-6.12 mm in length and 5.56-6.10 mm in width. The corolla is red with some whitish areas. The anthesis phase occurs from day 13th to day 16th when the flower is fully open, the corolla is white, and the pollen is matured. The post-anthesis phase occurs on day 17th when the flower begins to wither and eventually falls off (Figure 6). Based on the results of observation, each flower cluster can consist of 4-10 flower buds, with the flowers blooming asynchronously, alternating between each other from top to bottom. In cross-pollinating plants, this increases the chance of genetic diversity in their offspring, thus beneficial for maintaining their species to prevent extinction (Reed and Frankham 2003). The process of flowering is fundamentally influenced by the interaction of external factors, such as temperature, light, humidity, rainfall, and nutrient availability, with internal factors, such as genetic factors and hormones (Yang et al. 2016; Singh et al. 2018; Navas-Lopez et al. 2019). When plants grow in an environment with optimal conditions, these conditions will induce the plant's flowers to bloom and undergo fertilization.

Pollen viability

Stamens and pistils ripen at different times; the stamens ripen first, and then after a few days, the pistils will ripen. This implies bidirectional movement of the two organs as the pistil elongates and the stigma enlarges (Middleton 2016). The temporal difference in the maturity of male and female organs is termed dichogamy (Cardoso et al. 2018). The quality and quantity of pollen produced by flowers are crucial components for the sustainability of a plant species. This relates to the function of pollen as male gametes are responsible for fertilizing the egg cells, enabling pollination and fertilization to produce fruit and seeds (Hasrianda et al. 2020). Moreover, preservation of the viability and longevity of pollen is very important for plant breeding to overcome the obstacles of hybridization of species with different flowering times (Baninasab et al. 2017). Observations of viable pollen are conducted by observing color changes that serve as indicators of pollen viability, assuming the nutritional content within the pollen is sufficient. Pollen is considered viable if its nutritional content meets certain criteria. Viable pollen typically exhibits a deep red color when stained with 1% acetocarmine, while non-viable pollen remains transparent. The red color observed in viable pollen test results is due to acetocarmine reacting with the exine structure and nucleus. The pollen viability test results indicate varying percentages of pollen viability among the three observed *Begonia* accessions.

The observation of colored pollen showed that the pollen decreased in the post-anthesis phase compared to the colored pollen in the anthesis phase. The highest viability value resulted from red *B. hooveriana* pollen, and the lowest viability value resulted from *B. hijauvenia* pollen (Table 4). This result shows that pollen can experience damage and quality decline along with the length of storage (Samudra and Herawati 2020).

The viability of pollen tested based on coloring also gives diverse results in each *Begonia* with different storage times. In the anthesis phase, the highest percentage of pollen viability is owned by *B. hooveriana* with red color as well as in the post-anthesis phase *B. hooveriana* with red color has the highest percentage of pollen viability (Table 4). This level of fertility is determined by the pollen staining reaction that produces a dark red color after being treated with 1% acetocarmin solution. In comparison, the unstained pollen remains transparent and does not have any reaction (Figures 7-8).

The appearance of red color in pollen is caused by the reaction between acetocarmine and starch content in pollen (Ernawiati et al. 2018). Based on the average percentage of pollen stained with acetocarmine, pollen viability decreases in the post-anthesis phase. This is suspected due to the influence of flower maturation time; the longer the pollen is stored, the more the growth ability decreases. This is related to the water and nutrient content in the pollen; the longer it is stored, the more the water and nutrients deteriorate (Ulfa et al. 2016).

Table 4. Pollen viability in the three *Begonia* accessions from the Bogor Botanic Gardens, Bogor, Indonesia collection

Species	Pollen viability (%)*	
	Anthesis	Post-anthesis
Begonia hooveriana with green color	$92.15{\pm}6.34^{ab}$	$91.31{\pm}6.53^{b}$
Begonia hooveriana with red color	$96.60{\pm}4.08^{b}$	$96.30{\pm}4.46^{b}$
Begonia hijauvenia	$84.00{\pm}10.20^{a}$	$83.30{\pm}7.84^{a}$

Note: *The anthesis phase is observed when the flowers blossom; the post-anthesis phase is observed on the 4th day after the flowers blossom

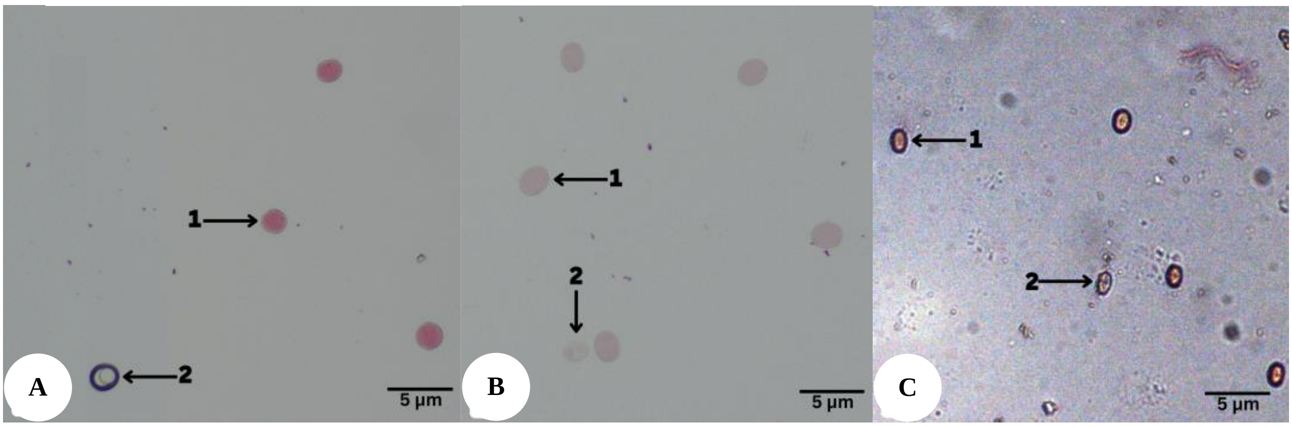

Figure 7. Pollen viability at the anthesis stage: A. *Begonia hooveriana* with green color; B. *Begonia hooveriana* with red color; and C. *Begonia hijauvenia*; 1. Viable pollen; 2. Non-viable pollen. Bar = 5 µm

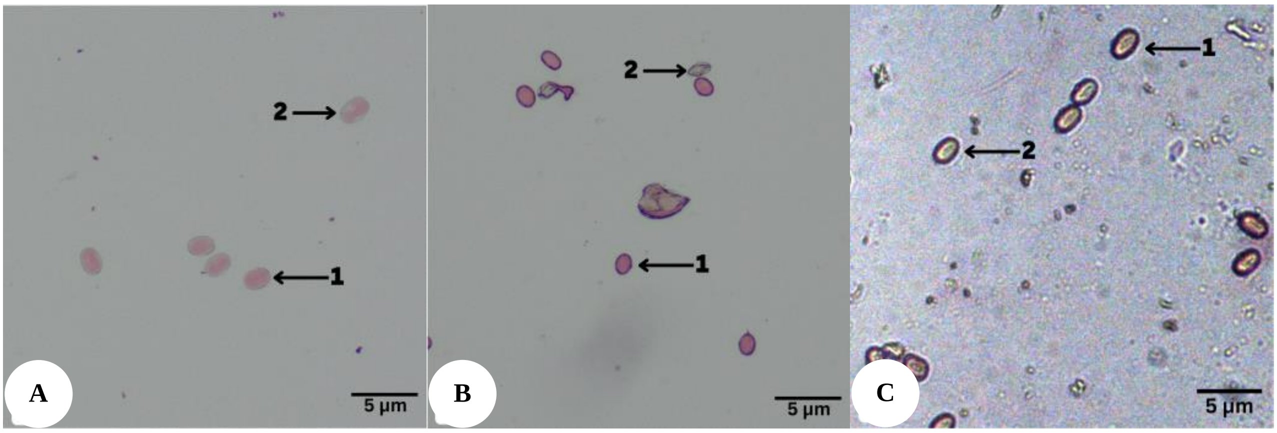

Figure 8. Pollen viability at the post-anthesis stage: A. *Begonia hooveriana* with green color; B. *Begonia hooveriana* with red color; C. *Begonia hijauvenia*; 1. Viable pollen; 2. Non-viable pollen. Bar = 5 µm

The decrease in pollen viability is suspected to occur because the pollen dries out, caused by prolonged storage, leading to a further decrease in viability and loss of germination capacity. Additionally, this is indicated by the wilting of the stamen and the drying of the pollen. In an open environmental condition during flower blooming, pollen is in optimal viable condition when the flower has just started to bloom. These findings are significant as they provide a deeper understanding of the factors influencing pollen viability, which is crucial for plant reproduction and conservation in the field of botany.

In conclusion, three accessions of wild *Begonia* collected at the Bogor Botanic Gardens have morphological variations in stems, leaves, and flowers. Stomata characters reveal differences in size and kind, with *B. hooveriana* possessing the biggest protective cell size and green hue. Anisocytic and parasitic stomata, which are present in all three *Begonia* species, may play a supporting role in the section-level grouping of Begonias. The flowering period of *B. hooveriana* with green color and *B. hooveriana* with red color has a longer flowering period from flower buds to fall compared to *B. hijauvenia*, which is about 10-21 days. At the same time, *B. hijauvenia* flowers have a blooming period of about 17 days. Acetocarmin dye gave high viability values to the three plants. Especially in *B. hooveriana* with red color, in both phases used, namely the anthesis and post-anthesis phases, this plant has the highest viability value among the other two comparisons. *B. hooveriana* has the potential to be used as an ornamental plant because it can flower throughout the year, and the length of bloom is relatively long. This research is an initial study of the diversity of *Begonia*. The data presented can be used as basic information in plant breeding efforts and Begonia conservation. Furthermore, other approaches, such as molecular, are needed to add complete diversity information about *Begonia*.

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Potential of a mixed extract of ginger, lemongrass, turmeric and black cumin as an immunostimulatory candidate

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Abstract. *Zilhaya U, Sari W, Syifa SB, Chusna SA, Rosnizar. 2025. Potential of a mixed extract of ginger, lemongrass, turmeric and black cumin as an immunostimulatory candidate. Nusantara Bioscience 17: 11-20.* Many Indonesian herbal plants show potential as immunostimulants, capable of enhancing the immune system's activity. Some synthetic immunomodulators are known to cause side effects, and create demand for safer and be more effective alternatives. Ginger (*Zingiber officinale*), lemongrass (*Cymbopogon citratus*), turmeric (*Curcuma longa*), and black cumin (*Nigella sativa*) are four plants recognized for their potential as immunostimulants. These plants contain metabolites such as alkaloids, terpenoids, flavonoids, phenolics, and tannins, which can enhance immune activity. This study aims to evaluate the effectiveness of mixed extracts from these plants as immunostimulants, particularly in boosting immune responses weakened by pathogens. The research employed a completely randomized design, utilizing phytochemical tests, in vitro assays, and in vivo evaluations against *Staphylococcus aureus*. The results demonstrated increased activity and phagocytic capacity of macrophages following treatment with extract solutions at various doses. In vitro tests with 100 ppm and 1000 ppm concentrations, as well as in vivo tests at 650 mg/kg body weight, significantly improved macrophage phagocytic activity and capacity. The mixed extract effectively enhanced the immune system.

Keywords: Black cumin, ginger, immunostimulant, lemongrass, macrophages, turmeric

Abbreviation: EGLTB: Mixed extracts of rhizome ginger, lemongrass, turmeric, and seed black cumin

INTRODUCTION

The human body has a natural defense mechanism against diseases caused by viruses and bacteria, known as the immune system. This system enables the body to combat pathogens by rejecting or destroying various foreign substances (antigens) that enter the body (Irianto 2012). The cellular innate immune system, responsible for neutralizing antigens, includes T cells, B cells, Natural Killer (NK) cells, and macrophages. The activity and development of these cells can be enhanced by immunomodulators (Faradilla and Iwo 2014; Sukmayadi et al. 2014).

Immunomodulators are compounds that can enhance the immune system's function, helping protect the body from disease (Rosida and Handojo 2019). These compounds boost the body's defense mechanisms, both specific and non-specific, and regulate cellular and humoral responses. Immunomodulators can act as immunoregulators (to balance the immune system), immunostimulants (to activate the immune system), or immunosuppressants (to suppress an overactive immune response). A variety of synthetic, recombinant, and natural materials can serve as immunomodulators to maintain immunity. When the body's resistance weakens, pathogens such as bacteria and viruses can invade more easily, which is why immunostimulants are essential. Immunostimulants enhance macrophage activity and capacity, allowing them to better phagocytize invading pathogens. Macrophages play a crucial role in the immune system by engulfing and digesting foreign particles (Aldi et al. 2015). The immune system can be modulated by immunosuppressants or enhanced by immunostimulants. Natural compounds that can boost immunity include curcumin, limonoids, vitamins C and E, flavonoids, and catechins (Alquraisi et al. 2021).

Several chemical immunomodulators, such as prednisone, hydrocortisone, and dexamethasone, are commercially available for treating inflammatory diseases. However, these drugs often cause side effects. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) frequently lead to gastric and intestinal mucosal damage, while corticosteroids can cause bone marrow depletion and skin fragility. Therefore, safer and more effective alternatives are needed. Natural products remain a valuable source of new, safe anti-inflammatory agents (Yuandani et al. 2021).

Various herbal ingredients are traditionally used to boost immunity. Ginger is widely used in herbal medicine due to its active compounds-gingerol, beta-carotene, capsaicin, curcumin, and salicylate-which have anti-inflammatory and antioxidant properties. Turmeric is valued for its antioxidant, antibacterial, antiviral properties, and its ability to boost immunity (Kusumo et al. 2020). Black cumin seeds are also commonly used to strengthen the immune system. According to Gunawati et al. (2020), black cumin seeds are known for their potential to treat asthma, bronchitis, and diabetes, and have antihistamine, antioxidant, antitumor, and antibacterial. Lemongrass offers antimicrobial, antifungal, antioxidant, antihyperlipidemic, and anticholesterol benefits, as well as detoxifying and insomnia-relief properties. According to research by Ernis et al. (2021), lemongrass can enhance lymphocyte proliferation in mice.

The impact of using herbal plants to enhance the immune system can be tested both in vivo and in vitro. In vivo testing is conducted directly on living test animals, allowing researchers to observe the biological or natural responses of the animals. In contrast, in vitro tests are performed on cells or tissues outside of living organisms, where the conditions can be controlled. In vivo testing can serve as a validation for in vitro or other laboratory models. In vitro studies allow for early-stage risk assessment, offering a more direct evaluation of an extract or drug, as they focus on its absorption. However, in vivo testing may introduce complications due to the indirect nature of the approach (Polli 2008; Rohr et al. 2016). In this study, *Staphylococcus aureus* was used as it is a common pathogenic bacterium found on human skin and mucosal surfaces. It is also capable of infecting healthy individuals, with an infection rate of 30-50%, making it likely to invade body tissues (Mutmainnah et al. 2020).

Herbal plants can be used in combination for improved efficacy. Kusumo et al. (2020) found that the combination of turmeric and ginger boosts the immune system and helps prevent microbial transmission, including bacteria and viruses. Akrom et al. (2015) demonstrated that ethanol extract from black cumin seeds (*Nigella sativa* L.) enhances immunity by increasing phagocytic activity and the secretion of Reactive Oxygen Intermediates (ROI) by macrophages in Swiss strain mice infected with *Listeria monocytogenes*. Furthermore, Ernis et al. (2021) concluded that lemongrass essential oil (*Cymbopogon citratus* (DC.) Stapf.) promotes lymphocyte cell proliferation in mice. The testing of herbal plant combinations aims to assess their effectiveness and impact on the immune system. Based on the evidence of the benefits of ginger, turmeric, lemongrass, and black cumin seeds as immunostimulators, a study was conducted to examine the combined effect of ethanol extracts from ginger (*Zingiber officinale* Rosc.), lemongrass (*C. citratus*), turmeric (*Curcuma longa* L.), and black cumin seeds (*N. sativa*) as potential immunostimulatory agents.

MATERIALS AND METHODS

Tools and materials

This research was conducted at the Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Syiah Kuala (USK), Banda Aceh, Indonesia. The equipment used included analytical scales, blenders, glass maceration jars, vials, test kits, animal cages, drinking bottles for test animals, sterile surgical instruments (scalpels, scissors, tweezers), trays, oral probes, autoclaves, cuvettes, spectrophotometers, light microscopes, cameras, micropipettes (20-200 µL), magnetic stirrers, hemocytometers, needle tubes, slide boxes, incubators, Laminar Air Flow (LAF) cabinets, Bunsen burners, Rotary Evaporators (RE), Ohaus scales, dye jars, glass slides, 2.5 mL cuvettes, vortex mixers, tally counters, and other glassware.

The materials used consisted of male Balb/C strain white mice (*Mus musculus* Linnaeus, 1758), aged 6-8 weeks and weighing 25-30 grams, mouse feed, bark, lemongrass rhizome (*C. citratus*, ginger rhizome (*Z. officinale*), turmeric rhizome (*C. longa*), black cumin seeds cumin (*N. sativa*), *Staphylococcus aures* ATCC 25923 and NaCl (0,9 %). Other materials included microtubes, pipette tips, 96% ethanol, 70% alcohol, methanol, 0.2 M Na_2EDTA solution, Phosphate Buffered Saline (PBS) solution (pH 7.8), 0.4% trypan blue solution, 10% Giemsa solution, dimethyl sulfoxide (DMSO), Nutrient Agar (NA) media, Nutrient Broth (NB) media, Stimuno (Imboost Force), immersionion oil, plastic wrap, aluminum foil, and filter paper.

Procedure

Herbal extraction and sample preparation

Ginger rhizomes, turmeric and lemongrass fronds were obtained from farms located in Aceh Besar and Banda Aceh, black cumin was obtained from Bandung, Indonesia. The identification process was carried out by the research team using the identification guidebook in the herbarium of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala and have been confirmed by expert.

After identification, each herbal material was peeled and cleaned. The samples were then cut into small pieces and air-dried for seven days (Rosnizar et al. 2022). Dried seeds were used for the black cumin sample. The ginger, lemongrass, turmeric rhizomes, and black cumin seeds were ground into a fine powder. The sample from each plant were then weighed 500 grams in a ratio of 1:1:1:1. Each powdered sample was macerated with sufficient 96% ethanol in sealed glass maceration jars. The entire sample was submerged and raised to a height of ±2 cm above the surface of the samples. The jars were left to stand for 3×24 hours, with occasional stirring. The mixture was then filtered using filter paper to separate the filtrate from the residue. The resulting filtrate was evaporated at a temperature of 55°C to yield a thick extract, which was then tested for metabolite content using phytochemical tests for alkaloids, steroids, terpenoids, saponins, and flavonoids (Chusna et al. 2024).

Phytochemical test

Herbal extracts were tested for their secondary metabolite content using phytochemical tests. Phytochemical tests include:

Alkaloid phytochemical analysis. Alkaloid content analysis was done using 2 mL extract solution with 1 mL of 2 N HCl and 6 mL of distilled water. Then heated for 2 minutes cooled and filtered. Alkaloid compounds were tested using Mayer and Wagner reagents. A total of 4 mL of filtrate was put into a test tube, then 1 mL of Mayer reagent was added. The alkaloid test is declared positive if a white or cream-colored precipitate is formed. The addition of the Wagner reagent states that the alkaloid test is positive if an orange to reddish-brown precipitate is formed (Illing et al. 2017).

Steroids and terpenoids phytochemical analysis. Phytochemical analysis of steroids and terpenoids was carried out using the Liebermann-Burchard test. A sample of ± 2 mL of extract was put into a test tube. The steroid test shows a positive result if it produces a green or blue color. Terpenoid test shows positive results if it produces red or purple color (Illing et al. 2017).

Flavonoid phytochemical analysis. 2 mL of extract was added to hot water at 90°C and then filtered. The filtrate was added with Mg powder and 1 mL of concentrated HCl then shaken. A positive flavonoid test was indicated if a red, yellow, or orange color was formed (Illing et al. 2017).

Saponin phytochemical analysis. 2 mL of extract was added to hot water at 90°C and a little concentrated HCl was added. The saponin test was positive if permanent foam was formed for ±15 minutes (Illing et al. 2017).

Tannins and phenolic compound phytochemical analysis. 2 mL of extract was added a few drops of H_2SO_4 reagent and gelatin. The tannin test is positive if it shows a white precipitate. Phenolic compound testing is carried out by adding $FeCl_3$ reagent to 2 mL of extract. Positive results of phenolic compounds are indicated by the appearance of a blackish-blue color in the solution (Koparde et al. 2019).

Test animal care

The test animals, male Balb/C strain mice aged 8-12 weeks with a body weight of 25-35 grams were housed in the Vivarium of the Department of Biology, FMIPA, USK. A total of 60 mice were placed in cages lined with husks and covered with wire. Mice were fed 'All Feed-4' type pellets and water were given ad libitum or as needed, and the husks were replaced twice a week. Prior to the experiment, the mice were acclimatized for one week. This research received ethical approval from the Research Ethics Committee of the Faculty of Veterinary Medicine, Universitas Syiah Kuala (No: 282/KEPH/XII/2023).

Preparing the sample test

The test solution was prepared by weighing the extract according to the specified dose and multiplied by the body weight of the mice. Weighed extracts are diluted with distilled water with the help of a magnetic stirrer. extract solution is made stock solution for 14 days of oral administration to mice. According to Dewi et al. (2021) the extract solution is given as much as 0.5 mL/20 gBW.

Culturing of Staphylococcus aureus *bacteria*

S. aureus bacteria used in the study were re-cultured on NA media and incubated for 24 hours at 37°C. After incubation, the bacterial colonies were transferred to NB media and incubated again for 24 hours at 37°C. Following incubation, 1 mL of the NB media containing bacteria was mixed with 9 mL of fresh NB media. The bacterial culture was then centrifuged at 2500 rpm for 25 minutes. The resulting pellet was resuspended in 1 mL of PBS. The bacterial cell density was determined using a spectrophotometer at 580 nm, targeting a transmittance of 25%, corresponding to a cell density of 10^9 cells/mL (Perdana 2022).

In vitro testing

To obtain intraperitoneal fluid, the mice were euthanized via cervical dislocation. The abdomen of mice was cleaned with 70% alcohol. If intraperitoneal fluid was insufficient, 3 mL of sterile PBS was injected into the peritoneal membrane, gently stirred for 1-3 minutes, and collected using a micropipette. The macrophage cell density was determined using a hemocytometer to achieve a concentration of 2.5 x 10^6 cells/mL. Viability was assessed using 0.4% trypan blue solution, with viable cells remaining.

The phagocytosis test was conducted by mixing 200 µL of bacterial suspension, 200 µL of macrophage cells, and 200 µL of test solution at concentrations of 0.1 ppm (P2), 1 ppm (P3), 10 ppm (P4), 100 ppm (P5), and 1000 ppm (P6). As a negative control (P0), 200 µL of bacterial suspension, 200 µL of macrophage cells, and 200 µL of PBS (without the test extract) were mixed. As a positive control (P1), 200 µL of bacterial suspension, 200 µL of macrophage cells, and 200 µL of Stimuno solution were mixed (Kirana et al. 2023). Samples were incubated at 37°C for 30 minutes (Handayani et al. 2018), followed by the addition of 50 µL of 0.2 M Na2EDTA solution and homogenized. Three replicate preparations were made for each treatment, then fixed with methanol for 5 minutes. Staining was done using 10% Giemsa solution (Hariyanti et al. 2015) for 5-20 minutes. The samples were then observed under a 1000x magnification and the activity and phagocytosis capacity of macrophages were calculated.

In vivo testing

Administration of the test extract was carried out once a day for 14 days. The test solution was administered according to the specified doses, which were adapted from the research by Masniah et al. (2021). The doses used in this study were 150 mg/kgBW (P2), 300 mg/kgBW (P3), 450 mg/kgBW (P4), and 600 mg/kgBW (P5). The negative control group (P0) received distilled water, while the positive control group (P1) received a commercial immunostimulant solution.

The test solutions were given orally using a sonde as much as 0.5 mL/20 gBW. On the 15^{th} day, mice were intraperitoneally injected with syringe as much as 0.5 mL of a bacterial suspension (Dewi et al. 2021). After the injection, the mice were left for 1 hour. To collect intraperitoneal fluid, the mice were euthanized by cervical dislocation, followed by dissection of their abdomens. The peritoneal fluid was extracted using a syringe.

Three slides were prepared for each treatment group, intraperitoneal fluid was dripped on a glass slide and smears were made with a tilt angle of 30°C and fixed with methanol for 5 minutes. The glass slides were stained with 10% Giemsa solution for 5-20 minutes. The smear preparations were rinsed using running distilled water and air dried. After drying, the slides were examined under a microscope at 1000x magnification.

Calculation of macrophage phagocytic activity and capacity values

Macrophage cell activity and phagocytosis capacity were then calculated. Phagocytosis activity value is the percentage of macrophage cells that are active in the phagocytosis process per 100 macrophage cells. Phagocytosis capacity was determined based on the total number of test bacteria in 50 active macrophage cells. The calculation values of macrophage phagocytosis activity and capacity were obtained through the following formula (Dewi et al. 2021):

$$\text{Phagocytosis Activity} = \frac{\text{number of activated macrophage cells}}{\text{100 macrophage cells}} \times 100\%$$

Phagocytosis Capacity = Total number of test bacteria (in 50 activated macrophage cells)

Data analysis

The results of the macrophage cell counts and phagocytic activity were analyzed using Analysis of Variance (ANOVA), assuming normal distribution, with a 95% confidence level and a 5% error rate (α=0.05) (Dewi et al. 2021). To further assess differences between treatment groups, a Duncan post-hoc test was conducted (Manurung and Mose 2019).

RESULTS AND DISCUSSION

Phytochemical test

Based on the phytochemical testing of the ethanol extract mixture of ginger, lemongrass, turmeric, and black seed (EGLTB), alkaloids, terpenoids, flavonoids, phenolic compounds, and tannins were identified, as shown in Table 1. The results of the phytochemical tests show that the metabolite compounds identified in the mixed rhizome and seed extract (MRE) are alkaloids, terpenoids, flavonoids, phenolics, and tannins. Alkaloids were derived from ginger (Dhanik et al. 2017), lemongrass (Asif and Khoadadi 2013), and black cumin (Ouattar et al. 2022). Terpenoids were found in turmeric (Sun et al. 2017) and lemongrass (Asif and Khoadadi 2013). Flavonoids were present in turmeric, ginger (Dhanik et al. 2017), lemongrass (Asif and Khoadadi 2013), and black cumin (Ouattar et al. 2022). Phenolic compounds were identified in turmeric (Dhanik et al. 2017), ginger (Sun et al. 2017), and black cumin (Ouattar et al. 2022), while tannins were found in turmeric (Dhanik et al. 2017) and lemongrass (Asif and Khoadadi 2013).

Research by Sun et al. (2017) indicated that turmeric contains terpenoids, flavonoids, phenolic compounds, organic acids, anthocyanins, tannins, and other organic compounds. According to Yuandani et al. (2021), the major compounds in turmeric rhizomes are polyphenols and terpenoids, with curcumin, a key polyphenol, demonstrating immunomodulatory, antioxidant, anti-inflammatory, and antitumor activities. Shabana et al. (2020) also reported that turmeric improves immune function by reducing proinflammatory cytokine levels in diabetic rats.

Besides its immune-boosting properties, the in vivo administration of CEERPB extract can produce pharmacological effects. Peng et al. (2019) found that alkaloids can reduce inflammation and mitigate colonic damage. Tanfil et al. (2023) demonstrated that alkaloids prevent significant increases in total cholesterol, triglycerides, Low Density Lipoprotein (LDL) cholesterol, Very Low Density Lipoprotein (VLDL) cholesterol, and the atherogenic index, while increasing High Density Lipoprotein (HDL) cholesterol levels. Belete (2019) also explained that alkaloids exhibit antibacterial properties by inhibiting bacterial growth and destruction.

Terpenoids also act as immunostimulants, with pharmacological effects demonstrated in vivo. Retnowati et al. (2011) noted that terpenoids interact with porins (transmembrane proteins) in the bacterial cell wall, forming strong polymer bonds that damage the porins. This reduces bacterial cell wall permeability, depriving the bacteria of essential nutrients, which ultimately inhibits bacterial growth or causes cell death. Research by Sari (2016) showed that terpenoids help synthesize organic substances and aid in cell recovery.

Phenolic compounds enhance the immune system and provide pharmacological benefits. According to Diniyah and Lee (2020), phenolics bind free radicals, decompose oxidation products, and chelate harmful metal ions. Puspitasari et al. (2016) found that phenolic compounds also serve as antibacterials by destroying cell walls and precipitating microorganism proteins, leading to cell dysfunction.

Table 1. Phytochemical screening results of mixed ethanol extracts of ginger, lemongrass, turmeric rhizome, and black cumin seeds

Metabolite content	Reagent	Results	Information
Alkaloids	Mayer	+	A white precipitate formed
	Wagner	+	A brown precipitate formed
	Dragendorff	+	A red precipitate formed
Steroids	Liebermann-Burchard test	-	No green color formed
Terpenoids	Liebermann-Burchard test	+	A red color formed
Saponin	Shuffling	-	No foam formed
Flavonoids	HCl and Mg Metal	+	A purple color formed
Phenolic	$FeCl_3$	+	A blue color formed
Tannin	Gelatin + H_2SO_4	+	A white precipitate formed

Flavonoids improve immune response and provide various pharmacological effects. These effects include protecting the intestinal mucosa, optimizing nutrient absorption (Mistiani et al. 2020), and acting as anti-inflammatories by inhibiting COX-2 and nitric oxide synthase enzymes (Rosa et al. 2001). Wang et al. (2011) found that flavonoids reduce asthma symptoms. They also act as anticancer agents by inducing apoptosis, regulating carcinogen metabolism, and inhibiting cancer development (Khoirunnisa and Sumiwi 2019).

Tannins, too, boost the immune system and offer pharmacological effects. According to Farha et al. (2020), tannins have been found to inhibit bacterial growth using various mechanisms of action including iron chelation, inhibition of cell wall synthesis, disruption of cell membranes, and inhibition of fatty acid biosynthetic pathways. Fauziah et al. (2021) demonstrated tannins' antioxidant capabilities, as they neutralize free radicals.

Both flavonoids and alkaloids play critical roles in improving immune function. Sholikhah and Rahayungsih (2015) explained that these compounds act as immunomodulators by increasing IL-2 (interleukin 2) activity and lymphocyte proliferation. Activated Th1 cells influence the Specific Macrophage Arming Factor (SMAF), including IFNγ (interferon gamma), which activates macrophages. When an antigen enters the body, T lymphocytes and macrophages collaborate to eliminate the bacteria. Macrophages phagocytize the bacteria, while T lymphocytes differentiate into CD4+ and CD8+ cells. CD4+ cells further differentiate into Th1 cells, producing IFNγ and TNFα cytokines, which activate Natural Killer cells. CD8+ cells also produce IFNγ cytokines, which activate macrophages, leading to the production of nitric oxide, a compound that kills bacteria.

In vitro test

The research began with a macrophage cell viability test to determine the number of live and dead cells, as well as the number of cells eligible for further testing. The Trypan Blue (TB) method is a very common assay to evaluate cell viability where dead cells absorb TB into the cytoplasm due to loss of membrane selectivity, while live cells remain unstained (Avelar-Freitas et al. 2014). Macrophage cell density was also measured using a hemocytometer to ensure that the number of macrophage cells was sufficient for the phagocytosis test. The percentage of viability and macrophage cell density are presented in Tables 2 and 3, respectively.

Based on the observations from five viability tests, the average cell viability was 96.61%. Only cell cultures with a viability of at least 95% were used for further experiments. This viability value should not be less than 95% (Chairul et al. 2009). The macrophage cell density measured over five repetitions averaged 4.632×10^6, exceeding the minimum required macrophage population density (2.5×10^6).

Macrophage phagocytic activity and capacity were used as indicators of immune system enhancement. Macrophages, which are phagocytic cells, play a critical role in the non-specific immune response and function as Antigen Presenting Cells (APCs). The immunostimulatory potential of EGLTB was assessed by observing the ability of macrophages to phagocytize *S. aureus*. According to Jensch-Junior et al. (2006), macrophage phagocytosis can be measured by calculating the percentage of phagocytic activity and capacity. The average activity and phagocytic capacity after EGLTB administration are shown in Table 4.

The mean values of macrophage cell phagocytic activity from the negative control to the highest concentration were $37.4^a\pm2.191$, $79.0^e\pm15.81$, $41.4^b\pm3.082$, $53.4^c\pm42.19$, $74.0^d\pm43.93$, $89.4^{f\pm}2.408$, and $98.6^g\pm1.342$, respectively. The mean values of phagocytic capacity of macrophage cells from the negative control to the highest concentration were $365.60^a\pm6.656$, $843.60^e\pm13.557$, $491.40^b\pm5.595$, $646.00^c\pm15.110$, $791.40^d\pm8.803$, $897.40^f\pm10.526$, and $988.00^g\pm7.176$, respectively. The highest percentage of phagocytic activity and capacity was found in the P6 treatment, while the lowest was found in the P0 treatment. Statistical analysis indicated that the data were normally distributed (P>0.05) and homogeneous (P>0.05). An ANOVA test was conducted to evaluate the effect of each treatment on increasing macrophage phagocytic activity and capacity.

Table 2. Macrophage cell viability test results

Test	Living cells	Dead cells	Viability (%)
1	94	3	96.9%
2	93	4	95.87%
3	89	3	96.7%
4	89	2	97.8%
5	91	4	95.78%
Average			96.61%

Table 3. Macrophage cell density in laboratory mice (using a hemocytometer)

Test	Density (cells/mL)
1	4.86×10^6
2	4.8×10^6
3	4.42×10^6
4	4.45×10^6
5	4.63×10^6
Average	4.632×10^6

Table 4. Mean phagocytic activity and capacity of macrophages after administration of EGLTB

Treatment	Cell average	
	Activity (%)	Capacity (cells/50 active macrophages)
P0	$37.4^a\pm2.191$	$365.60^a\pm6.656$
P2	$41.4^b\pm3.082$	$491.40^b\pm5,595$
P3	$53.4^c\pm42.19$	$646.00^c\pm15,110$
P4	$74.0^d\pm43.93$	$791.40^d\pm8,803$
P1	$79.0^e\pm15.81$	$843.60^e\pm13.557$
P5	$89.4^f\pm2.408$	$897.40^f\pm10.526$
P6	$98.6^g\pm1.342$	$988.00^g\pm7,176$

Note: P0: Negative control (no extract); P1: Positive control (comparative immunostimulant); P2: EGLTB 0.1 ppm; P3: EGLTB 1 ppm; P4: EGLTB 10 ppm; P5: EGLTB 100 ppm; P6: EGLTB 1000 ppm

The increased macrophage cell activity and capacity following EGLTB administration may result from the metabolite compounds present in EGLTB, which induce macrophage cells to become more active and phagocytize more *S. aureus* bacteria. EGLTB contains metabolite compounds such as alkaloids, terpenoids, flavonoids, phenolics, and tannins (see Table 1). The flavonoid content in EGLTB exhibits antioxidant activity, which can improve immune system function. This is because antioxidants can neutralize free radicals produced by the innate immune system, such as neutrophils and macrophages. According to Canton et al. (2021), these two phagocytic cells possess cell membranes bound to the NADPH-oxidase system, enabling macrophages to produce Reactive Oxygen Species (ROS), including hydrogen peroxide, hydroxyl radicals, and superoxide radicals. Additionally, macrophages produce prostaglandins, leukotrienes, interleukin-1, and interferons.

In addition to flavonoids, the phenolic and terpenoid contents in EGLTB can also boost the immune system. Tohma et al. (2017) reported that phenolic compounds in ginger possess antioxidant properties that support immune system improvement. El Gazzar et al. (2006) also noted that terpenoid compounds, such as thymoquinone, can enhance the immune system in patients with bronchial asthma caused by allergies.

The bioactive compounds in EGLTB, which are secondary metabolites, can attach to bacteria and act as chemoattractants for macrophage cells, making it easier for macrophages to phagocytize bacteria. These bioactive compounds can also bind to the macrophage cell membrane and influence signaling pathways (Elmowalid et al. 2007) to form pseudopodia, increasing the cells' activity and phagocytic capacity against bacteria.

Observation of macrophage cell phagocytic activity and capacity against *S. aureus* was performed using a trinocular microscope. Macrophage cells were observed after Giemsa staining, which allowed differentiation of the macrophage cell nucleus, cytoplasm, and *S. aureus* bacterial cells. The macrophage cell nucleus absorbed the stain more intensely than the cytoplasm, while *S. aureus* bacteria appeared as concentrated stains, making them easy to observe. According to Mokobi (2016), Giemsa is a specialized stain used for blood smear examinations to detect parasitic infections, particularly malaria, and can also be employed to observe macrophage cell phagocytic activity. In addition, Giemsa staining is used to distinguish various blood cells such as erythrocytes, platelets, and leukocytes, as well as cellular components such as cell nuclei and cytoplasm. The results of macrophage and *S. aureus* staining are shown in Figure 1.

In vivo test

The calculation of activity values and phagocytic capacity of mice macrophages was conducted to assess the ability of mixed rhizome and seed extracts of ginger, turmeric, lemongrass, and black cumin to enhance immunity. The results of statistical analysis showed that the data for phagocytic activity and capacity were normally distributed ($P>0.05$) and homogeneously varied ($P>0.05$), and were then analyzed using Analysis of Variance (ANOVA). Based on the ANOVA results, it was found that there was a significant effect ($P<0.05$) with a P-value of 0.00 on the activity and phagocytic capacity of macrophages treated with EGLTB. These results indicate that EGLTB acts as an immunostimulant. To further determine the effect of each dose on macrophage activity and phagocytic capacity, a Duncan test was performed. The activity values and phagocytic capacity of macrophages in mice treated for 14 days are presented in Table 5.

Based on the further test results presented in Table 5, the macrophage activity in the negative control (P0) was 31%, in the positive control (P1) it was 45.6%, for the 150 mg/kgBW treatment (P2) it was 37.6%, for the 300 mg/kgBW treatment (P3) it was 49%, for the 450 mg/kgBW treatment (P4) it was 62.6%, and for the 600 mg/kgBW treatment (P5) it was 69%. The treatments P2, P3, P4, and P5 showed a significant increase compared to the negative control, indicating a boost in macrophage activity and capacity as a result of natural immunity. Phagocytic activity reflects the immune response to foreign bodies, carried out by macrophages. This aligns with Coico and Geoffrey (2015), who stated that immunity functions to protect the body from pathogens, and that innate immunity can be considered a natural immune response present from birth. Additionally, Rosales and Eileen (2017) emphasized the key role macrophage cells play in natural immunity through phagocytosis, enabling the body to defend itself against antigens. The P0 treatment reflects the body's natural response to exposure to *S. aureus* bacteria, a common pathogen.

Table 5 also shows that macrophage phagocytic capacity values differed significantly between the negative control (P0), the positive control (P1), and the various treatment groups. Administering EGLTB at 300 mg/kgBW (P3) resulted in activity levels similar to P1 and P2, but the phagocytic capacity differed from the other treatments. EGLTB at 450 mg/kgBW (P4) and 600 mg/kgBW (P5) showed comparable activity levels but significantly different capacity values compared to other doses. The activity value represents the percentage of active macrophages among 100 macrophages, while the capacity value reflects the number of bacteria successfully phagocytosed by 50 active macrophages (Sari et al. 2016).

The differences in these results highlight the immune-boosting effects of EGLTB. Turmeric contains curcumin, which reduces inflammation and oxidative stress in the body, supporting immune function (Hewlings and Kalman 2017), and modulates cytokine production, which regulates immune responses (Aggarwal et al. 2013). Ginger inhibits free radicals and exhibits antitumor, anti-inflammatory, anticarcinogenic, antibacterial, antimutagenic, and antioxidant activities, thereby inhibiting pathogens (Munadi 2018). Lemongrass acts as an immunomodulator, increasing the activity and proliferation of lymphocytes in laboratory mice (Ernis et al. 2021). Black cumin also acts as an immunostimulant by increasing lymphocyte and monocyte numbers, though it does not reduce the numbers of eosinophils and neutrophil stem cells. Black cumin has immunomodulatory activity through both immunostimulant and immunosuppressant mechanisms (Gunawati et al. 2020).

The administration of EGLTB increases the activity and phagocytic capacity of macrophages. The results of macrophage and *Staphylococcus aureus* staining (in vivo test) are shown in Figure 2. Based on Figure 2.C, at a dose of 150 mg/kgBW, there is an increase in the number of bacterial cells phagocytosed by macrophages compared to the negative control (P0). Figure 2.A (negative control, P0) shows fewer phagocytosed bacteria than the other doses. Figure 2.B (positive control) shows almost the same amount of phagocytosis as Figures 2.D (P3), 2.E (P4), and 2.F (P5). The higher the dose, the greater the phagocytic ability of macrophages treated with EGLTB. This suggests that the EGLTB mixture can enhance macrophage function and the phagocytosis of pathogens. According to Hirayama et al. (2018), macrophages are activated by pathogen invasion and present peptide antigens from ingested bacteria to the Major Histocompatibility Complex (MHC), which activates T helper cells.

An increase in macrophage phagocytic activity and capacity indicates an improvement in the immune system. This mixture of ginger, turmeric, lemongrass, and black cumin functions as an immunostimulant. According to Purkon et al. (2021), immunostimulants stimulate the immune response by enhancing the activity of both non-specific and specific immune components against infection. In this study, macrophages act as part of the non-specific immune response, acting quickly as the body's first line of defense. This aligns with Hirayama et al. (2018), who stated that macrophages play an essential role in the innate (non-specific) immune system by recognizing and destroying pathogens. When this system fails to eliminate pathogens, adaptive immunity is activated. Upon recognizing a particle, phagocytes activate several signaling pathways to initiate phagocytosis.

Phagocytosis is the process by which cells engulf and eliminate particles larger than 0.5 µm in diameter, including microorganisms, foreign substances, and apoptotic cells. This process occurs in almost all cell types in multicellular organisms (Uribe-Querol and Rosales 2020). Phagocytic cells eliminate microorganisms and present antigens to lymphocytes, aiming to activate the adaptive immune response (Gordon 2016). The process of phagocytosis consists of several stages: (i) Detection of the particle by phagocytic cells; (ii) Activation of the internalization process; (iii) formation of phagosomes, which are specialized vacuoles; and (iv) maturation of phagosomes. During this process, foreign particles are incorporated into phagosomes, which then mature and fuse with lysosomes to form phagolysosomes. These contain enzymes that destroy and decompose the particles. Phagocytosis can target various cells, including apoptotic cells and microbes (Uribe-Querol and Rosales 2020). The process of antigen presentation is described in Figure 3.

Table 5. Mean macrophage phagocytic activity and capacity in vivo

Treatment (mg/kgBW)	**Mean**	
	Activity (%)	**Capacity (cells /50 macrophages active)**
P0	$31.0^{a}\pm7.6$	$137.4^{a}\pm27.94$
P1	$45.6^{b}\pm8.3$	$281.8^{d}\pm34.01$
P2 (150)	$37.6^{ab}\pm7.6$	$180^{b}\pm30.27$
P3 (300)	$49.0^{b}\pm15.17$	$219^{c}\pm24.87$
P4 (450)	$62.6^{c}\pm5.4$	$396.6^{e}\pm19.85$
P5 (600)	$69.0^{c}\pm2.5$	$537.6^{f}\pm20.26$

Notes: P0: Negative control treatment without extracts; P1: Positive control treatment (Imboost Force 0.975 mg/kgBW); P2: EGLTB 150 mg/kgBW; P3: EGLTB 300 mg/kgBW; P4: EGLTB 450 mg/kgBW; P5: EGLTB 600 mg/kgBW

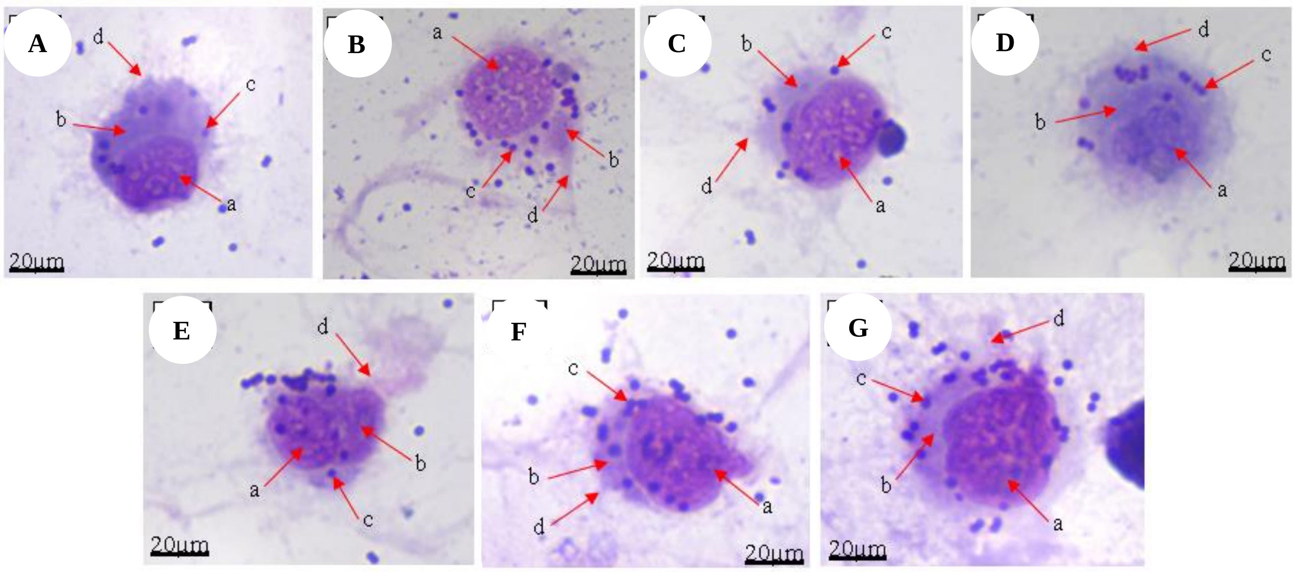

Figure 1. Mice's intraperitoneal macrophage cells after adding EGLTB and *Staphylococcus aureus* bacterial infection. A. Negative control; B. Positive control; C. EGLTB concentration 0.1 ppm; D. EGLTB concentration 1 ppm; E. EGLTB concentration 10 ppm; F. EGLTB concentration 100 ppm; G. EGLTB concentration 1000 ppm. a: Cell nucleus; b: Cytoplasm; c: Phagosome; d: Pseudopodia. Magnification 10x100

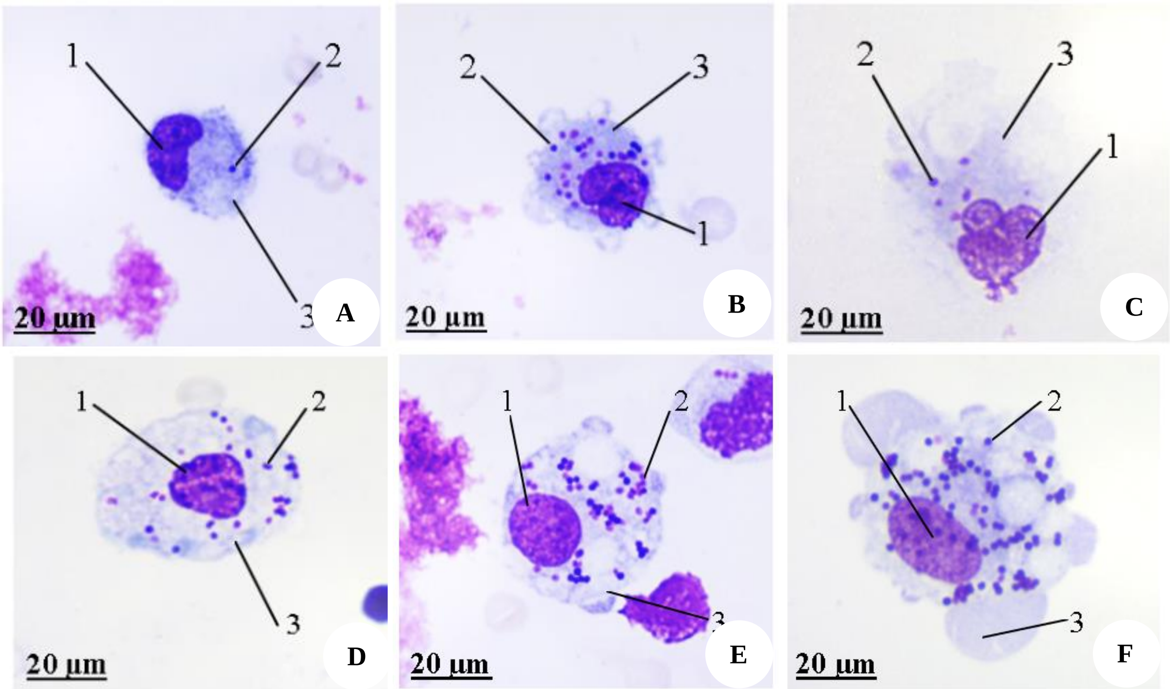

Figure 2. The administration of EGLTB increases the activity and phagocytic capacity of macrophages. A. P0; B. P1; C. P2; D. P3; E. P4; F. P5; 1. Macrophage; 2. Fagosom; 3. Pseudopod

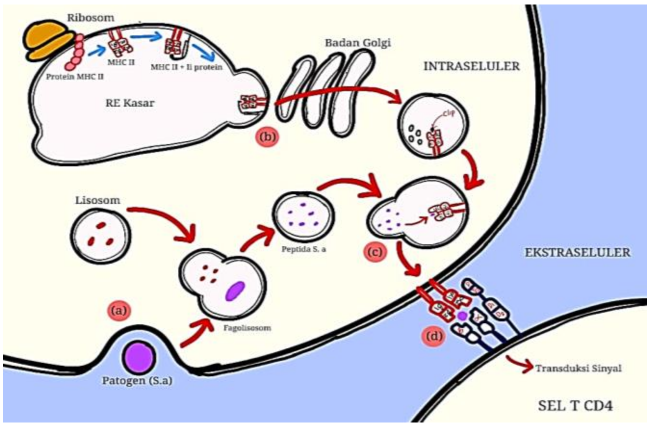

Figure 3. Antigen presentation process. A. Phagocytosis, B. MHC II formation, C. Bacterial peptide fusion with MHC II, D. Signaling of MHC II with bacterial antigen to CD4 T cells

When macrophages phagocytize *S. aureus* (Figure 3.A), lysosomes fuse with phagosomes to form phagolysosomes, producing small bacterial peptides. Meanwhile, the rough Endoplasmic Reticulum (ER) forms major histocompatibility complex II (MHC II) (Figure 3.B). MHC II passes through the Golgi body, producing a clip on MHC II. The MHC II then binds to bacterial peptides (Figure 3.C). The CD4 T cell receptor verifies that the cell has indeed produced MHC II. Upon confirmation, signal transduction occurs, activating CD4 T cells (Figure 3.D). This process is consistent with the findings of Mufidah et al. (2013), who demonstrated that *S. aureus* is phagocytosed and recognized by MHC II, which then presents the peptide antigens to T lymphocytes. The effector CD4+ T cells secrete interferon-gamma (IFN-γ), a cytokine that activates macrophages.

The signaling pathway is initiated when macrophages sense danger signals through Pattern Recognition Receptors (PRRs), which allow immune cells to rapidly respond to various pathogens with common structures, known as Pathogen-Associated Molecular Patterns (PAMPs). For example, components of bacterial cell walls, such as lipopolysaccharide (LPS), and viral double-stranded RNA are produced during infection (Marshall et al. 2018). *S. aureus* has a cell wall composed of lipopolysaccharide (LPS), which is the primary component of Gram-negative bacterial cells and causes septic shock. *S. aureus* has been shown to trigger the secretion of cytokines and chemoattractants (TNF-α, IL-1β, IL-10, IL-12, IL-8, leukotriene B4, complement factor 5a, MCP-1, MIP-1α, and granulocyte colony-stimulating factor) from monocytes and macrophages. Mohammad et al. (2022) explained that *S. aureus* expresses several molecules, including bacterial lipoproteins (Lpps), which play a role in both the immune response and disease pathogenesis. Lpps, the primary ligand for TLR2, are critical for bacterial survival by maintaining bacterial metabolic activity and contributing to host cell invasion during various infections. Nguyen et al. (2017) further explained that the lipid moiety of Lpps in *S. aureus* functions as part of Microbe-Associated Molecular Patterns (MAMPs), alerting the innate immune system via PRR detection, particularly through TLR2 in host cells. This lipid moiety serves as an important danger signal to the host.

In conclusion, the phytochemicals obtained from this study were a mixture of ethanol extracts of ginger rhizome, turmeric, lemongrass, and black cumin seeds, including alkaloids, terpenoids, flavonoids, phenolics, and tannins. The administration of a mixture of ethanol extracts from ginger rhizomes, turmeric, lemongrass fronds, and black cumin seeds positively affects the immune system. This is evidenced by an increase in the activity and phagocytic capacity of intraperitoneal macrophages in mice after induction with *S. aureus*. The enhancement of the immune system corresponds with the dose administered. Optimal doses for increasing macrophage activity and phagocytic capacity are concentrations of 100 and 1000 ppm in vitro tests, and 450 mg/kgBW and 600 mg/kgBW in in vivo tests. The signaling process of macrophages occurs from the activation of CD4 T cells by MHC II. To further explore the immunomodulatory potential of the ethanol extract mixture (EGLTB), additional research is recommended, focusing on test animal organs to evaluate cellular effects.

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Comparing poultry manure and cow dung on *Arachis hypogaea* growth in savanna environment

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Abstract. *Mahmud AA, Abdulrahman MD. 2025. Comparing poultry manure and cow dung on* Arachis hypogaea *growth in Savanna environment. Nusantara Bioscience 17: 21-29.* The study investigated the effects of poultry manure and cow dung on the growth of *Arachis hypogaea* L. (groundnut) in a controlled experimental setup at the botanical garden of Department of Plant Science and Biotechnology, Federal University Dutsin-Ma, Katsina State, Nigeria. The experiment followed a Completely Randomized Block Design (CRBD) with two organic fertilizer as treatments; poultry manure cow dung, and a control sample with no fertilizer. Two groundnut seeds varieties; pure red and light tan mixed each separately with cow dung, and poultry manure, and were subjected to viability test before planting. Growth parameters, including the number of leaves and plant height, were measured monthly for five months. The data obtained were analyzed using SPSS statistical software version 21, and significant differences were identified with Duncan's multiple range tests at a 5% probability level. Results indicated a significant impact of organic fertilizers on the growth parameters. The results revealed that cow dung promotes early growth while poultry manure contributes more to reproductive success and yield. The control group showed significantly lower leaf development, with an average of (43.4) leaves. The findings highlight the role of organic fertilizers in enhancing plant leaf production, as they provide essential nutrients that are often deficient in untreated soils. In terms of plant height, the light tan variety treated with cow dung achieved the greatest average height (16.53 cm), surpassing both the control (15.4 cm) and the pure red variety treated with poultry manure (13.45 cm). Overall, the study emphasizes the importance of using organic fertilizers like poultry manure and cow dung to improve groundnut growth, reduce dependence on synthetic fertilizers, and enhance soil health. These organic amendments are beneficial for sustainable agricultural practices, contributing to improved crop yield and environmental sustainability.

Keywords: *Arachis hypogaea*, cow dung, groundnut growth, organic fertilizer, poultry manure

INTRODUCTION

Groundnut (*Arachis hypogaea* L.), also known as peanut, is a vital legume crop grown widely across tropical and subtropical regions due to its nutritional and economic value. It serves as a key source of protein, oil, and income for many farmers, particularly in Sub-Saharan Africa, where it is cultivated on small and large scales (Ramatsetse et al. 2023). Groundnut plays a significant role in improving soil fertility through nitrogen fixation, making it an essential crop in various agricultural systems. However, its productivity is often hindered by inadequate soil nutrients, particularly in regions where synthetic fertilizers are either unaffordable or unavailable to smallholder farmers. As a result, there has been increasing interest in the use of organic fertilizers as a sustainable alternative to synthetic inputs (Ramatsetse et al. 2023). Organic fertilizers, such as poultry manure and cow dung, have been widely recognized for their ability to enhance soil fertility, improve plant growth, and contribute to sustainable agricultural practices (Ke et al. 2024). These organic amendments contain essential nutrients, including nitrogen, phosphorus, and potassium, which are vital for plant development. Additionally, they improve soil structure, water retention, and microbial activity, all of which contribute to healthier plant growth (Leopold 2022). The use of organic fertilizers can also reduce reliance on chemical fertilizers, which are known to degrade soil quality and pose environmental risks when overused. In this context, understanding the effects of different organic fertilizers on crop performance is crucial for promoting sustainable farming practices, especially for crops like groundnut that are widely cultivated.

Numerous studies have highlighted the benefits of organic fertilizers in enhancing crop productivity, these studies include that of (Bergstrand 2022; Govindasamy et al. 2023; Sathiparan et al. 2023). Cow dung, for instance, has been found to significantly increase plant growth due to its high nutrient content and rapid nutrient release (Shalaby et al. 2022). It improves soil organic matter, boosts microbial activity, and provides a steady supply of nutrients, making it particularly effective for enhancing leaf production and overall plant vigor. Similarly, cow poultry, though slower in nutrient release compared to poultry manure, improves soil texture and water-holding capacity, and contributes to long-term soil fertility. These benefits make both poultry manure and cow dung valuable inputs for groundnut cultivation, where improved growth metrics such as leaf number and plant height are critical indicators of productivity (Ho et al. 2022). Despite the documented benefits of organic fertilizers, their effects can vary depending on several factors, including the type of fertilizer, crop variety, and local environmental conditions (Gao et al. 2023). Different groundnut varieties may respond differently to specific fertilizers due to variations in their nutrient requirements and growth habits (Abebe et al. 2022). Therefore, it is important to investigate the comparative effects of organic fertilizers on different groundnut varieties to determine the most effective fertilization strategy for maximizing growth and yield.

This study aimed to evaluate the effects of two organic fertilizers; poultry manure and cow dung mixed each separately with two varieties of groundnut seeds (pure red and light tan), focusing on evaluating key growth parameters such as the number of leaves and plant height. The experiment was carried out in Completely Randomized Block Design (CRBD) at the botanical garden of the Department of Plant Science and Biotechnology, Faculty of Life Science, Federal University Dutsin-Ma, Katsina State, Nigeria. The two groundnut seeds varieties were subjected to viability tests before planting, and growth parameters were measured monthly for five months. Data obtained were analyzed using SPSS statistical software version 21, and significant differences between the treatments were identified using Duncan's multiple range tests at a 5% probability level. By comparing the effects of poultry manure and cow dung on different groundnut varieties, this study provides valuable insights into the role of organic fertilizers in promoting groundnut growth. Understanding these effects is essential for smallholder farmers seeking sustainable alternatives to synthetic fertilizers, as organic inputs offer both economic and environmental benefits. The findings of this research are expected to contribute to the broader knowledge of organic fertilization strategies for groundnut cultivation and offer practical recommendations for farmers to improve crop yield through sustainable agricultural practices.

MATERIALS AND METHODS

Study area

The study was conducted within five-month period at the botanical garden of the Department of Plant Science and Biotechnology, Federal University Dutsin-Ma, Katsina State. Dutsin-Ma is situated at a longitude of 7°11'14.86" East of the Greenwich Meridian and a latitude of 7°28'51.39" North. Katsina State lies within the derived Savanna Zone. In Dutsin-Ma, the growth of groundnut (*A. hypogaea*) is influenced by several environmental factors such as; temperature, rainfall and soil texture. Groundnuts thrive in warm temperatures, with an ideal range between 25-30°C. Dutsin-Ma's climate typically supports groundnut growth, as it experiences warm temperatures for much of the year. High temperatures, however, can stress the plant, especially if they exceed 35°C consistently. In terms of rainfall, groundnuts require moderate rainfall, with an optimal range of 500-700 mm during the growing season. Dutsin-Ma has a semi-arid climate with a rainy season between May and September, which provides a suitable amount of water for groundnut cultivation.

However, irregular rainfall patterns and prolonged dry spells can impact yields, making supplementary irrigation beneficial. Whereas in terms of soil texture, groundnuts grow best in well-drained sandy or sandy-loam soils. Dutsin-Ma has predominantly sandy to loamy soil, which is suitable for groundnut cultivation as it facilitates good root development and easy penetration for pods to develop underground. Proper soil management is essential to ensure adequate nutrient availability, particularly for nitrogen and phosphorus (Mahmud et al. 2023). These conditions collectively support groundnut growth in Dutsin-Ma (Mahmud et al. 2023), but careful management practices are necessary to optimize yields and address any limitations in climate or soil quality.

Procedures

Sample collection

Two dry groundnut seeds varieties were purchased from Dutsin-Ma market and transported to the laboratory of the Department of Biological Sciences for analysis. The organic fertilizers (cow dung and poultry manure) were sourced from the animal farm of the Faculty of Agriculture, Federal University Dutsin-Ma, Katsina State, Nigeria.

Experimental design

The experiment was carried out in Completely Randomized Block Design (CRBD) (Figure 1). A polythene bag filled with two organic fertilizer treatments separately (manure and cow dung) and a control sample were randomly placed on the fertilized plots. The control sample received no manure treatment (Figure 2). The two groundnut seeds varieties: pure red and light tan were mixed separately with both poultry manure and cow dung and the control variety was randomly planted in polythene bags (Azad et al. 2022). The experiment was conducted in triplicate.

Viability test

For the viability test, the seeds that floated were discarded, while the viable ones were used for planting (Pradhan et al. 2022).

Seeds planting

The viable groundnut seeds were sown separately in each polythene bag containing soil mixed with either poultry manure, cow dung, or only soil (without fertilizer which served as control) (Chowdary et al. 2022).

Measurement of growth parameters

Growth parameters, including the number of leaves per plant and plant height, were recorded throughout the planting trial (Paradiso and Proietti 2022).

Numbers of leaves

The number of leaves for each replicate was counted weekly, and the average leaf count was recorded from each organic fertilizer treatment and groundnut variety (Sapre et al. 2022).

Data analysis

The collected data were analyzed using One-Way Analysis of Variance (ANOVA), and significant differences were identified through Duncan's Multiple Range Test (DMRT) at a 5% significance level. The data analysis was performed using the SPSS statistical software, version 21.

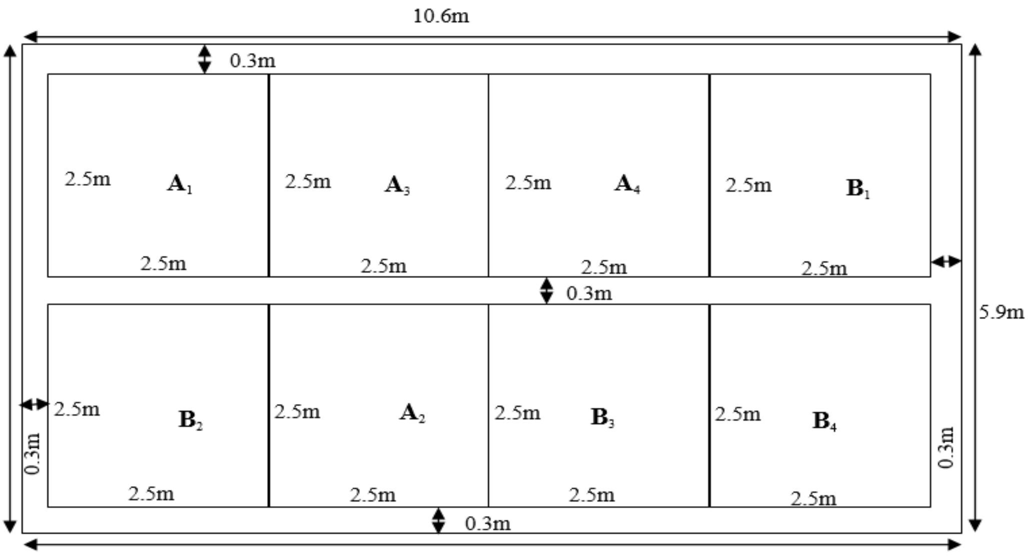

Figure 1. Complete randomized block design illustrating the plot configuration in Dutsin-Ma, Katsina State, Nigeria

Figure 2. Groundnut seeds treated with cow dung and poultry manure

RESULTS AND DISCUSSION

Yield analysis was conducted to assess flowering and pod development. Table 1 summarizes the flowering time and pod development across various treatments. Poultry manure-treated plants exhibited the earliest flowering and highest pod count, with a significant difference ($p < 0.05$) compared to cow dung-treated and control plants.

The yield analysis revealed significant differences among the treatments. Poultry manure-treated plants exhibited the highest yield (2,800 kg/ha), significantly outperforming cow dung-treated plants (2,200 kg/ha) and the control group (1,400 kg/ha). The gradual nutrient release from poultry manure likely contributed to its superior performance during the reproductive phase, resulting in more pods and higher pod weight. In contrast, cow dung provided a rapid nutrient boost, which favored early vegetative growth but was less effective in sustaining yield. These results are consistent with previous studies, such as those by Ho et al. (2022) and Shalaby et al. (2022), which demonstrated the benefits of organic fertilizers in enhancing crop productivity. The findings emphasize the importance of choosing fertilizers that match the crop's nutrient requirements at different growth stages. The yield analysis indicated that poultry manure provided a significant advantage in pod number and weight compared to cow dung and control. The gradual nutrient release of poultry manure likely contributed to these long-term benefits. In contrast, cow dung facilitated rapid vegetative growth, which may explain its early advantages in plant height and leaf production.

On the other hand, following the procedure of Ho et al. (2022), a post-harvest soil analysis revealed significant improvements in soil quality in plots treated with organic fertilizers compared to the control. Poultry manure-treated soils showed the highest levels of organic matter (3.0%), nitrogen (0.18%), phosphorus (20 ppm), and potassium (150 ppm). These improvements reflect the gradual nutrient release and superior nutrient density of poultry manure. Cow dung-treated soils also exhibited notable enhancements, particularly in organic matter (2.5%) and potassium (120 ppm), though to a lesser extent than poultry manure. Control plots, lacking any fertilizer, had the lowest nutrient levels, underscoring the necessity of organic amendments for soil fertility in the savanna environment (Table 2).

In contrast, the control plots exhibited poor soil quality, with low organic matter and nutrient levels. This reinforces the need for organic fertilizers to maintain soil fertility and achieve sustainable agricultural production in nutrient-depleted soils common in the savanna region. The study underscores the need to select fertilizers based on crop growth stages. Cow dung is more effective during the vegetative phase, providing immediate nutrient availability. However, poultry manure excels in sustaining growth and yield, as supported by previous studies (Ho et al. 2022; Shalaby et al. 2022).

Table 1. Yield analysis values

Treatment	Time to first flowering (days)	Number of pods	Yield (kg/ha)
Cow dung	42	20	2,800
Poultry manure	38	25	2,200
Control	50	12	1,400

Table 2. Soil analysis values

Parameter	Control	Cow dung	Poultry manure
Organic matter (%)	1.2	2.5	3.0
Nitrogen content (%)	0.05	0.12	0.18
Phosphorus (ppm)	8	20	20
Potassium (ppm)	70	150	150
Soil pH	6.2	6.8	6.8

Table 3. Number of leaves per groundnut samples

Sample	1st Month	2nd Month	3rd Month	4th Month	5th Month	Total mean
Pac	18.75±1^{d}	30.25±1^{c}	35± 1^{c}	42.00±1^{b}	53.5±1^{a}	35.9±1^{ba}
Pap	18.0± 1^{d}	24.75±1^{c}	29.75±1^{c}	33.75±1^{b}	55.75±1^{a}	32.0±4^{b}
Lac	18.5±1^{d}	26.25± 1^{c}	32.25±1^{c}	34.25±1^{b}	48.00±1^{a}	31.0±85^{c}
Lap	15.0±1^{d}	23.75 ±1^{c}	28.0± 1^{c}	33.00±1^{b}	49.00±1^{a}	29.0±75^{d}
Control	12.0±1^{d}	21.00±1^{b}	27.6± 1^{b}	31.4± 1^{a}	43.40± 1^{a}	27.±085^{e}

Note: Data presented as a mean variable ± standard error of the mean; PAC: pure red + cow dung, Pap: pure Red + Poultry droppings, Lac: Light tan + Cow dung; Lap: Light tan + Poultry droppings

From Table 3, a detailed progression of leaf development in *A. hypogaea* (groundnut) samples treated with poultry manure, cow dung, and a control (sample with no fertilizer) over a five-month period. The data provides insights into how different organic fertilizers impact plant growth, revealing patterns of early development and sustained growth benefits. Two groundnut seeds varieties were used for planting; pure red and light tan. These two varieties were mixed each with cow dung and poultry droppings individually as follows; pure red groundnut treated with cow dung (Pac), pure red groundnut treated with poultry droppings (Pap), light tan groundnut treated with cow dung (Lac), light tan groundnut treated with poultry droppings (Lap), and finally, an untreated control variety.

In the first 1st month, the pure red variety treated with cow dung (Pac) shows the highest number of leaves (18.75), followed closely by light tan treated with cow dung (Lac) with 18.5 leaves. These two groups outperformed both the poultry manure-treated groups and the control group, which had the lowest leaf count (12 leaves). This early dominance of cow dung-treated plants suggests that cow dung may release essential nutrients more rapidly, providing an initial growth boost. Conversely, the poultry manure-treated groups (Pap and Lap) lag behind with 18 and 15 leaves, respectively, indicating a slower release of nutrients from this fertilizer in the early growth stage. The control group, unsurprisingly, shows the slowest growth, with only 12 leaves, emphasizing the impact of organic amendments in enhancing early leaf development.

In the 2nd month, all samples show notable growth, but the pattern from 1st month persists. Pac continues to lead with 30.25 leaves, while Lac follows with 26.25 leaves. The poultry droppings-treated samples, Pap and Lap, maintain a slower growth trajectory, with 24.75 and 23.75 leaves, respectively. The control group shows moderate progress, with 21 leaves.

This phase of growth suggests that cow dung-treated plants maintain their early advantage, likely due to its faster nutrient release and better soil amendment properties. Poultry manure, however, remains behind in the initial phases of growth, highlighting its slower, more gradual nutrient availability. The control group, while progressing, continues to be significantly less vigorous, reinforcing the value of organic fertilizers in promoting leaf production. This is in line with the study of Oritsejafor et al. (2022).

By the 3rd month, there is a more distinct divergence in growth patterns. Cow dung-treated plants, Pac (35 leaves) and Lac (32.25 leaves), continue to show superior growth. The poultry manure-treated plants, Pap (29.75 leaves) and Lap (28 leaves), display gradual but consistent improvement, closing the gap with the cow dung-treated samples. The control group, although growing, lags behind with 27.6 leaves. This stage demonstrates the sustained effect of cow dung in maintaining higher growth rates, whereas poultry manure starts to exert its influence, especially in Pap, which shows gradual improvement. The steady growth of the control sample reflects the slower progress in nutrient-deficient soil, underscoring the critical importance of fertilizers in ensuring optimal growth conditions (Bashir et al. 2022).

4th month marks an interesting shift where the gap between cow dung-treated and poultry manure-treated plants begins to narrow. Pap, previously behind Pac, now shows a significant leap with 33.75 leaves compared to Pac's 42 leaves. Similarly, Lap catches up with Lac (33 versus 34.25 leaves). This indicates that the poultry manure, while slower to release nutrients initially, begins to provide sustained growth benefits in the later stages of development. Cow dung continues to support steady growth, but its early advantage diminishes as poultry manure-treated plants gain momentum. The control group shows continued growth (31.4 leaves), but remains behind the fertilized plants, reinforcing the consistent trend of underperformance in untreated soil. This findings disagrees with Janani and Jebakumar (2023) where they reported an opposite case.

Through the 5th month, the poultry manure-treated plants (Pap and Lap) demonstrate strong growth, with Pap reaching the highest number of leaves (55.75), surpassing even the Pac sample (53.5). This is as indicated by Okpanachi et al. (2022) that poultry manure, despite a slower start, ultimately supports more robust leaf production than cow dung. Lap also outperforms its cow dung-treated counterpart (49 leaves versus 48 leaves), confirming the long-term benefits of poultry manure in enhancing leaf production. The control group, which received no organic treatment, ends with 43.4 leaves, significantly lower than the fertilized plants. This outcome underscores the critical importance of organic fertilizers in maximizing leaf growth and overall plant vigor.

The total mean leaf count reflects the cumulative growth trends over the five-month period. Pac, treated with cow dung, leads with a mean of 35.9 leaves, followed by Pap (32.4 leaves), Lac (31.85 leaves), and Lap (29.75 leaves). The control group has the lowest total mean of 27.08 leaves, highlighting its consistent underperformance throughout the study. This data suggests that cow dung promotes faster early-stage growth, making it an effective organic amendment for plants that require a quick start. However, poultry manure, while slower in the initial stages, provides more sustained benefits, leading to superior leaf production in the long term. This makes poultry manure a better option for crops that require gradual nutrient availability over time.

The observed trends show clear differences in the effects of cow dung and poultry manure on the growth of groundnut leaves. Cow dung appears to have a faster nutrient release rate, leading to more immediate growth benefits in the first few months. This rapid response could be beneficial for crops that need a strong initial boost. However, as the development progresses, poultry manure-treated plants begin to outperform cow dung-treated plants, particularly in terms of leaf count, suggesting that poultry manure might offer more sustained and consistent nutrient availability. The control group's consistently lower performance throughout the study reinforces the necessity of organic fertilizers for maximizing crop growth. Untreated soils, as shown by the control group's slower growth, are less capable of supporting robust plant development, particularly in terms of leaf production, which is a key indicator of overall plant health.

We deduced from the above data that, both cow dung and poultry manure positively influence leaf development in groundnut plants, but their effects vary over time. Cow dung is more effective in promoting early growth, while poultry manure leads to greater long-term benefits, particularly in terms of leaf production. This data highlights the need for farmers to consider not only the type of fertilizer but also the growth stage of the plant when selecting organic amendments for optimal results. It is paramount to note that, while cow dung-treated plants led in leaf count, poultry manure have supported other metrics such as pod yield and overall plant health

Table 4 captures the growth patterns of various plant samples across five weeks, represented by the height measurements (in centimeters) of each sample group; Pac, Pap, Lac, Lap, and a Control group. As we examined this data month by month, we recorded a clear progression in growth as well as significant differences between the samples in terms of their overall performance.

In the 1st month, all samples demonstrate relatively low growth, which is expected as plants are in the early stages of establishment. The heights are close to each other across the samples: Pac (4.32 cm) shows the highest initial height, indicating early vigor, while Pap (4.08 cm) and Control (3.48 cm) follow closely behind.

Lac (3.38 cm) and Lap (3.25 cm) display the lowest heights, suggesting these samples may be slower to establish their root systems or have faced some form of early stress. This initial stage is critical because it lays the foundation for how well the plants can absorb nutrients and grow in the subsequent months (Bigatton et al. 2024). By the 2nd month, there is a noticeable increase in height for all samples, signaling the onset of accelerated growth. Pap (7.8 cm) exhibits the most significant growth spurt, jumping from 4.08 cm to 7.8 cm, reflecting favorable conditions or better nutrient uptake during this stage. Lac (6.1 cm) and Lap (6.1 cm) also show significant improvements, nearly doubling their heights, while Pac (4.93 cm) shows more moderate growth. Interestingly, Control (4.02 cm) grows the least in the 2nd month, hinting at possible constraints, such as nutrient limitation or less favorable conditions compared to the treated samples. This stage is crucial because it highlights the different growth potentials, with Pap emerging as the frontrunner, showing the highest growth increment. In the 3rd month, growth remains steady across all samples, although the rates vary between them. Pac (5.2 cm), Pap (9.5 cm), Lac (8.23 cm), and Lap (9.13 cm) continue to grow, but Pap remains ahead in terms of overall height. This consistent performance suggests it has more favorable growing conditions or genetic advantages. This consistence performance of pap was also reported by Abdelghany et al. (2022) in their study. Control (8.96 cm), on the other hand, experiences a sudden growth burst, surpassing Pac and nearly matching Lap. This could indicate delayed but robust growth once the plant has overcome any initial constraints. The comparative performance of Pac lags slightly behind, which might suggest that, although it had an early lead in the 1st month, its growth is now plateauing compared to other samples. This month showcases a leveling-off for some samples while others, particularly Pap and Control, are catching up or surpassing the initially strong performers.

Table 4. Plant height per groundnut samples

Sample (cm)	1st	2nd	3rd	4th	5th	Mean
Pac	4.32±0.4[d]	4.93±0.4[c]	5.2±0.4[b]	8.5±0.4[b]	13.65±0.4[a]	7.0±32[a]
Pap	4.08±0.4[d]	7.8±0.4[c]	9.5±0.4[b]	9.75±0.4[b]	13.45±0.4[a]	8.0±92[b]
Lac	3.38±0.4[d]	6.1±0.4[c]	8.23±0.4[b]	10.17±0.4[b]	16.53±0.4[a]	8.0±89[c]
Lap	3.25±0.4[d]	6.1±0.4[c]	9.13±0.4[b]	10.88±0.4[b]	14.6±0.4[a]	8.0±79[d]
Control	3.48± 0.4[d]	4.02± 0.4[c]	8.96±0.4[b]	13.04±0.4[b]	15.24±0.4[a]	8.0±95[e]

The 4th month introduces even more substantial growth for most samples. Lac (10.17 cm), Lap (10.88 cm), and Control (13.04 cm) show significant growth, with Control now taking the lead at 13.04 cm. Pap (9.75 cm) continues to perform well but doesn't match the dramatic growth observed in Control or Lap. Pac (8.5 cm) remains somewhat behind, suggesting that it may be reaching its growth capacity earlier than the others, or environmental factors may be hindering its development (Zhao et al. 2023). This month is pivotal because it illustrates how growth rates can vary, with some samples experiencing delayed but powerful spurts (as seen in control sample) while others, like Pac, may begin to slow down. By the 5th month, the plants appear to be reaching their peak growth or maturity, with clear differentiation between the samples in terms of final height: Lac (16.53 cm) shows the most substantial final height, reflecting sustained growth throughout the experiment. This indicates that Lac has benefited from optimal conditions, resulting in its peak performance. Control (15.24 cm) and Lap (14.6 cm) also exhibit strong final growth, closely following Lac, which shows that despite some variability in earlier months, these samples caught up and finished strong. Pap (13.45 cm), although it started off strong in earlier months, finishes at a lower height than Lac, Lap, and Control, which suggests that its growth rate slowed towards the end. Pac (13.65 cm), although initially a strong performer, finishes in the lower range, consistent with its slower growth from the 3rd month onward.

When we examine the total mean values, the differences in long-term growth performance become clearer: Pac (7.32 cm) has the lowest total mean despite its early lead in the 1st month, indicating that it did not maintain consistent growth over time. This could be due to nutrient depletion, water stress, or other environmental factors that hampered its long-term development. Pap (8.92 cm) and Lac (8.89 cm) are close in mean height, but Pap takes a slight lead. This suggests that Pap benefited from stronger early growth, but Lac caught up due to its stronger performance in the later months. Lap (8.79 cm) and Control (8.95 cm), while both showing delayed growth in the first two months, achieved higher means in the later months, with Control surpassing even Pac and Lap in the final analysis. When connecting the dots across the months, the growth trends indicate that initial height does not always guarantee the best long-term performance (Kumar et al. 2022). For example, Pac started off with the highest 1st month height but had the lowest total mean. On the other hand, Control, which had the lowest in the 1st month height, ended up with one of the highest final heights. Lac, Lap, and Pap showed more consistent growth over the period, with Lac particularly standing out due to its significant late-stage growth that pushed its final height and mean above most of the other samples. The Control sample's growth trajectory suggests that it may have faced some initial limitations but, once those were overcome, grew robustly in the later months, culminating in a strong final height. Later growth, such as delayed nutrient uptake from inherent soil reserves has reduced competition compared to fertilized plots. While the control group showed some recovery, it did not match the overall performance of fertilized plants in yield or total leaf count.

The data suggests that while some samples (such as Pac) may have a strong initial start, their long-term performance can be outpaced by samples that exhibit more consistent and sustainable growth (such as Lac and Control). This highlights the importance of environmental factors, treatment consistency, and possibly genetic resilience in determining final plant height. The interplay of early vigor and sustained growth is key in determining overall plant performance, with later-stage growth often playing a decisive role in final outcomes. This is as implied by Zang et al. (2023) and Kamendra et al. (2023) on their studies.

Data presented as a mean variable ± standard error of the mean

Tables 5 and 6 present statistical analyses of changes in plant height and leaf count across five months period using pairwise comparisons, with significant mean differences observed in both variables as time progresses. In Table 5, plant height consistently increases, with significant changes noted across monthly intervals, especially between 1st and 5th months, where the mean height difference reaches (10.776) units. Similarly, Table 6 shows an upward trend in the number of leaves, with 5th month demonstrating a substantial increase from 1st month, reaching a mean difference of (29.194) leaves. Both tables indicate statistically significant differences ($p < 0.5$) for most month-to-month comparisons, especially between non-consecutive months, suggesting substantial growth over time. Additionally, confidence intervals support these differences, highlighting the consistent growth pattern and reliability of the findings across intervals in both plant height and leaf count.

Table 5. Dependent variable versus plant height

	(J) Time interval	Mean difference (I-J)	Std. error	Sig.^{d}	95% Confidence interval for difference^{d}	
					Lower bound	Upper bound
Month 1	Week 2	-1.745^{*,b,c}	.872	.050	-3.488	-.002
		-3.235^{*,b,c}	.872	.000	-4.978	-1.492
	(I) Time interval	-4.640^{*,b,c}	.872	.000	-6.383	-2.897
	Month 5	-10.776^{*,b,c}	.822	.000	-12.421	-9.131
Month 2	Month 1	1.745^{*,b,c}	.872	.050	.002	3.488
	Month 3	-1.490^{b,c}	.872	.093	-3.233	.253
	Month 4	-2.895^{*,b,c}	.872	.002	-4.638	-1.152
	Month 5	-9.031^{*,b,c}	.822	.000	-10.676	-7.386
Month 3	Month 1	3.235^{*,b,c}	.872	.000	1.492	4.978
	Month 2	1.490^{b,c}	.872	.093	-.253	3.233
	Month 4	-1.405^{b,c}	.872	.112	-3.148	.338
	Month 5	-7.541^{*,b,c}	.822	.000	-9.186	-5.896
Month 4	Month 1	4.640^{*,b,c}	.872	.000	2.897	6.383
	Month 2	2.895^{*,b,c}	.872	.002	1.152	4.638
	Month 3	1.405^{b,c}	.872	.112	-.338	3.148
	Month 5	-6.136^{*,b,c}	.822	.000	-7.781	-4.491
Month 5	Month 1	10.776^{*,b,c}	.822	.000	9.131	12.421
	Month 2	9.031^{*,b,c}	.822	.000	7.386	10.676
	Month 3	7.541^{*,b,c}	.822	.000	5.896	9.186
	Month 4	6.136^{*,b,c}	.822	.000	4.491	7.781

Note: *: The mean difference is significant at the .05 level; b: An estimate of the modified population marginal mean (I); c: An estimate of the modified population marginal mean (J)

Table 6. Dependent variable versus number of leaves per plant

(I) Time interval	(J) Time interval	Mean difference (I-J)	Std. error	Sig.^{d}	95% Confidence interval for difference^{d}	
					Lower bound	Upper bound
Month 1	Month 2	-4.280^{a,b}	2.150	.051	-8.581	.021
	Month 3	-7.775^{a,b,*}	2.150	.001	-12.076	-3.474
	Month 4	-12.205^{a,b,*}	2.150	.000	-16.506	-7.904
	Month 5	-29.194^{a,b,*}	2.029	.000	-33.252	-25.135
Month 2	Month 1	4.280^{a,b}	2.150	.051	-.021	8.581
	Month 3	-3.495^{a,b}	2.150	.109	-7.796	.806
	Month 4	-7.925^{a,b,*}	2.150	.000	-12.226	-3.624
	Month 5	-24.914^{a,b,*}	2.029	.000	-28.972	-20.855
Month 3	Month 1	7.775^{a,b,*}	2.150	.001	3.474	12.076
	Month 2	3.495^{a,b}	2.150	.109	-.806	7.796
	Month 4	-4.430^{a,b,*}	2.150	.044	-8.731	-.129
	Month 5	-21.419^{a,b,*}	2.029	.000	-25.477	-17.360
Month 4	Month 1	12.205^{a,b,*}	2.150	.000	7.904	16.506
	Month 2	7.925^{a,b,*}	2.150	.000	3.624	12.226
	Month 3	4.430^{a,b,*}	2.150	.044	.129	8.731
	Month 5	-16.989^{a,b,*}	2.029	.000	-21.047	-12.930
Month 5	Month 1	29.194^{a,b,*}	2.029	.000	25.135	33.252
	Month 2	24.914^{a,b,*}	2.029	.000	20.855	28.972
	Month 3	21.419^{a,b,*}	2.029	.000	17.360	25.477
	Month 4	16.989^{a,b,*}	2.029	.000	12.930	21.047

Note: *: The mean difference is significant at the .05 level. b: An estimate of the modified population marginal mean (I). b: An estimate of the modified population marginal mean (J). d: Adjustment for multiple comparisons: Least significant difference (equivalent to no adjustments)

To conclude, the analysis of the growth patterns of the different groundnut samples over five months period reveals important insights into the effects of treatments on plant height. Based on what we observed, it was evident that while initial growth rates varied among the samples, their long-term performance was influenced by both early vigor and sustained growth. Lac emerged as the most resilient sample, showcasing substantial growth, particularly in the later weeks, which contributed to its highest final height and mean. This indicates its potential as a favorable option for cultivation where robust growth is desired. Control demonstrated the ability to overcome early limitations, achieving impressive growth in the later months. This suggests that under certain conditions, even plants that initially lag can exhibit strong recovery and growth potential. Pap and Lap displayed strong performances but experienced some variability in growth rates. While they performed well initially, their inability to maintain momentum in the later weeks highlights the need for monitoring growth conditions closely. Conversely, Pac, despite having a strong initial growth phase, concluded with the lowest total mean height. This indicates that early advantages do not guarantee long-term success and that consistent growth and resilience to stressors are crucial.

Furthermore, the study demonstrates that poultry manure and cow dung have distinct but complementary roles in enhancing groundnut growth and yield. While cow dung accelerates early growth, poultry manure provides sustained benefits, particularly for yield and soil health. Future studies should explore integrating these fertilizers over multiple growing cycles and assess their economic feasibility for smallholder farmers. Therefore, based on the results, it is recommended to prioritize Lac and Control varieties for future cultivation of groundnut due to their robust growth patterns and ability to adapt to changing conditions. These samples demonstrated not only initial growth but also sustained development over time. Farmers should implement regular monitoring of growth conditions to identify any stressors that may impede growth, especially for varieties like Pac and Pap, which showed variability in their growth rates. Adjustments in nutrient supply, water management, and pest control can significantly enhance performance. Given the importance of consistent growth, soil health and nutrient availability should be prioritized. Conducting soil tests and amending the soil based on specific nutrient deficiencies can lead to improved plant health and productivity. Following the completion of the growth period, further analysis of the plants physiological responses and root development would be beneficial. Understanding these factors can provide insights into how different samples respond to environmental stressors and can guide future planting strategies. Future studies should consider extending the growth period beyond five months to assess the long-term viability and yield potential of the different samples. Additional trials can provide more comprehensive data on growth patterns and the overall resilience of these varieties under varying environmental conditions. Encourage sustainable agricultural practices that promote plant health and reduce the need for chemical inputs. Integrating cover cropping, organic amendments, and integrated pest management can enhance the overall growth performance of these samples in the long term. It is our belief that, by following these recommendations, growers can optimize their cultivation strategies, leading to improved plant health and better yields in future growing seasons.

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Morphological differentiation of *Pennahia aneus* (Bloch, 1793) populations from Northern Peninsular Malaysia using geometric morphometrics

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Abstract. *Kachi JB, Binashikhbubkr K, Naim DM. 2025. Morphological differentiation of* Pennahia aneus *(Bloch, 1793) populations from Northern Peninsular Malaysia using geometric morphometrics. Nusantara Bioscience 17: 30-38.* The investigation of geometric morphometrics offers evidence for the adaptive traits shown by fish in response to their aquatic environment, which may be attributed to evolutionary processes. The examination of body morphometry has significant value in assessing the structure of fish populations, which is a fundamental necessity for the implementation of conservation measures and the management of fisheries in a sustainable manner. The study used geometric morphometrics to differentiate three populations of *Pennahia aneus* from Northern Peninsular Malaysia. A sum of 110 samples were obtained from Pulau Pinang, Kedah, and Perak states in Malaysia. Photographs of fish samples were taken, and 13 homologous landmarks were digitized using the tpsUtil and tpsDig2 software. All data were analyzed using multivariate statistical tests such as canonical variate analysis, cluster analysis, principal component analysis, and discriminant function analysis. Results showed significant morphological variations among the population of *P. aneus* with two morphological groups: Batu Maung group and Pantai Remis and Kuala Kedah group. The overall reclassification grouping for the populations of *P. aneus* in this study was 67.3% successful. These findings are crucial for conservation and management efforts and contribute to formulating efficient conservation policies to maintain the species populations in Northern Peninsular Malaysia, highlighting the potential impact of this research on future conservation policies. Further investigations in terms of expanding the study scope and application of molecular tools will give a holistic understanding of *P. aneus* population structure in Malaysia. This study represents the first to compare morphological differences in *P. aneus* populations from Malaysian waters using geometric morphometric analysis.

Keywords: Geometric morphometrics, multivariate analyses, *Pennahia aneus*

INTRODUCTION

Fish, shaped by physical and biological selection in their aquatic environments, presents a diverse range of morphologies, forming the basis of observable biodiversity (Dwivedi et al. 2020; Martinez et al. 2021; De Brito et al. 2022; Thambithurai and Kuparinen 2024; Rincon-Sandoval et al. 2024). Morphological features, including meristic and morphometric characters, have a significant historical background in biology for population identification (Sidiq et al. 2021; Jawad et al. 2022; Binashikhbubkr et al. 2023; Ainsworth et al. 2024; Xiao and Yang 2024). Variations in species populations can indicate habitat and behavioral disparities, especially for aquatic organisms like fish. These variations underscore the need for fish to adapt to their environment to optimize their biological systems, making them crucial for survival (Jalili et al. 2015; Wilson et al. 2019; Mawer et al. 2023; Akther et al. 2024; Bernos et al. 2024).

Researchers employ various methods to identify stocks, including life-history stages, morphological characteristics, otolith chemistry, and molecular markers like protein allozymes, microsatellite DNA, and mitochondrial DNA (Imtiaz et al. 2016). Among these, morphological measurements are the most popular and economical technique for identifying stocks (Sibinamol et al. 2020; Binashikhbubkr et al. 2023). A recent addition to this tool is Geometric Morphometrics (GM) powerful and popular technique that quantitatively analyzes biological shapes using cartesian coordinates of anatomical landmarks, maintaining the geometric information of the specimen throughout the study (Shukri et al. 2024). Data from GM not only reveal the relationships between landmarks and species but also provide spatial correlations, further enhancing its relevance and popular technique today (Hoff et al. 2020; Iqbal et al. 2024; Shukri et al. 2024).

Pennahia aneus (Donkey croaker) is a commercially important demersal croaker species of the Sciaenidae found in Malaysia. It is widely distributed in the Indo-West Pacific, from the Persian Gulf east to western Indonesia, and occurs in shallow coastal waters to depths of 60 m (Froese and Pauly 2024). *Pennahia aneus* is one of the five species of *Pennahia* genus, collectively called Pennah croakers (Lim et al. 2021). This species is a valuable Asian seafood component due to its delicate flavor and high economic value, and it is marketed in fresh, dried, and salty forms. It predominates at fish landing sites, particularly from muddy coastal fishing areas, where it is often caught as a by-catch using a variety of fishing gear (Wagiyo et al.

2020). The International Union for the Conservation of Nature (IUCN) considers *P. aneus* a species of Least Concern (LC), but one of the major threats it faces is overfishing, especially as by-catch (Chao et al. 2020), which needs to be considered.

Prior reports on *P. aneus* in Malaysia are limited, but the most recent report only focused on their distribution and abundance in the Strait of Pulau Pinang (Hamid et al. 2023), where they were reported to be the third dominant species. In Indonesia, studies on *P. aneus* have been on the aspects of their biological characteristics, abundance index, and fishing in Tangerang waters (Wagiyo et al. 2020), and the discrimination between the males and females using the Truss morphometric network and meristic characters in Central Java's fish auction center (Utarini et al. 2021). The contributions of *P. aneus* to the marine fisheries sector in Malaysia make them susceptible to anthropogenic factors like overfishing, pollution, and habitat degradation (Lim et al. 2021). Understanding the population structure of this species is important to enable sustainable use, and morphological characterization provides a rapid assessment of this, which has not yet been fully explored and documented. As a preliminary to studying the sciaenid fish in Malaysia, this study aims to address the gap of little or no information on the body shape and size variations of these important species using geometric morphometric analysis. Also, this study seeks to validate the verity of geometric morphometric analysis in intraspecies population discrimination. Hence, this study, for the first time, used geometric morphometrics techniques to compare the morphological variations among *P. aneus* populations from Northern Peninsular Malaysia, with the belief that the results will help give foremost insights into their population structure for effective conservation plans and sustainable use.

MATERIALS AND METHODS

Area of study and sample collection

All fish samples were collected from designated fish landing sites in Northern Peninsular Malaysia. The sampling sites were the fishing Jetty locations at Batu Maung (Penang), Kuala Kedah (Kedah), and Pantai Remis (Perak) (Figure 1). In all, 110 fish samples were collected from the sampling sites (Table 1). Cool boxes with ice were used to transport samples from the landing sites to the Molecular Ecology Laboratory, School of Biological Sciences, Universiti Sains Malaysia. Upon arrival at the laboratory, samples were cleaned, drained, and placed on a black background surface to take clear photographs using a Nikon D90 Digital Camera. The proper origin and insertion of fins were captured and erected using pins. Every sample was labeled, photographed, and digitized. Geometric morphometric analyses used only purified images of the samples, and morphometric traits were measured using a digital caliper (Figure 2).

Table 1. Sampling locations and sample size description

Sampling location	Coordinates	No of samples (N)
Kuala Kedah	6º 6’ 25.98’’ N; 100º 17’ 36.56’’ E	43
Batu Maung, Penang	5º 16’ 60.00’’ N; 100º 16’ 60.00’’ E	42
Pantai Remis, Perak	4º 27’ 28.74’’ N; 100º 37’ 45.91’’ E	25
Total		110

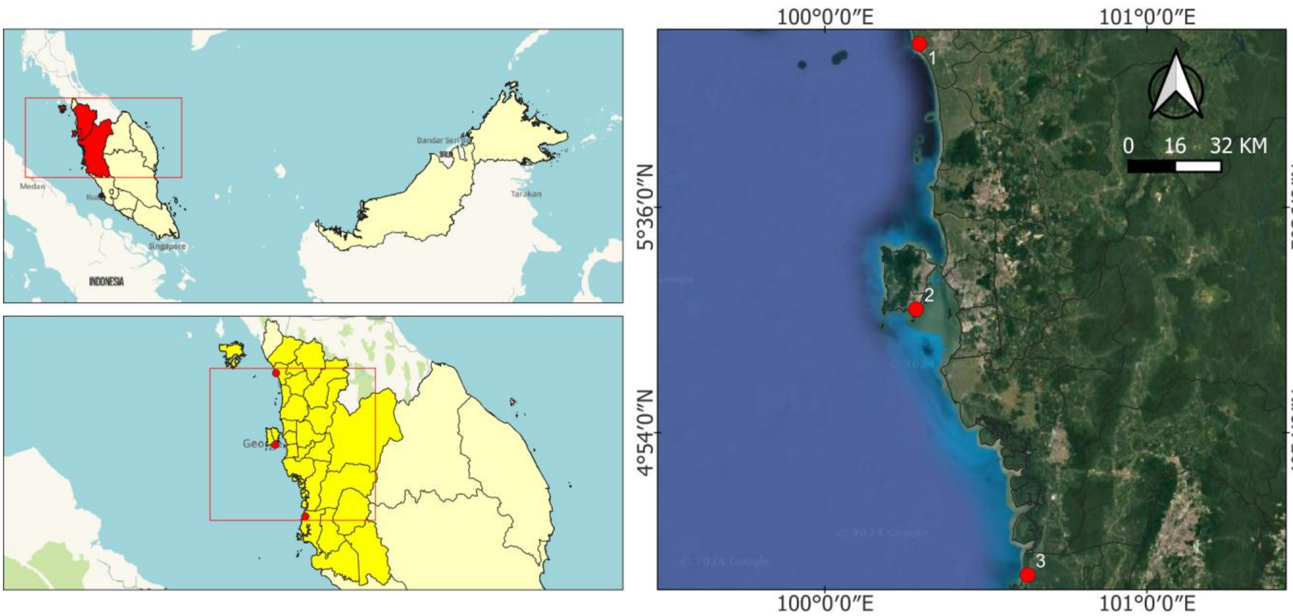

Figure 1. Map of the sampling sites of *Pennahia aneus* in Northern Peninsular Malaysia. Note: 1. Kuala Kedah (Kedah); 2. Batu Maung (Penang); 3. Pantai Remis (Perak)

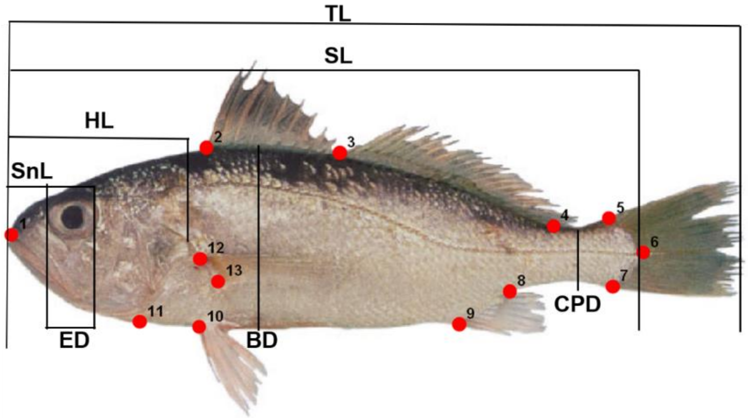

Figure 2. The 13 defined homologous landmark points used for body shape data extraction in populations of *Pennahia aneus*: 1. Anterior-most point of the snout; 2. Anterior end of dorsal fin base; 3. Notch between dorsal fin spines and rays; 4. Posterior end of dorsal fin base; 5. Posterodorsal end of caudal peduncle at its connection to caudal fin; 6. Posterior end of the body lateral line; 7. Posteroventral end of caudal peduncle at its connection to the caudal fin; 8. Posterior end of the anal fin base; 9. Anterior end of the anal fin base; 10. Anterior end of the pelvic fin; 11. Ventral edge of the head; 12. Anterior origin of pectoral fin; 13. Posterior end of pectoral fin. Source: Utarini et al. (2021)

Analyses of geometric morphometrics

Therefore, to describe the actual shape and dimension of every sample, twelve homologous landmarks were utilized (Figure 2). The input file for the data acquisition program was created using the tpsUtil software version 1.79. Images were retrieved to digitize landmarks that would later be used to determine the respective coordinates (x, y) of the landmarks using version 2.31 of the tpsDig2 software (Rohlf 2015). The data generated were analyzed using version 1.07 of the MorphoJ software, as described by Klingenberg (2011). MorphoJ minimizes dimension changes from digitalizing images by adjusting landmarks and creating a consensus configuration (Binashikhbubkr et al. 2022). Shape variations were analyzed using a wireframe designed by linking landmarks, which were measured and registered. The maximum amount of body shape variations was determined by conducting Principal Component Analysis (PCA) to estimate the differentiation in species (Klingenberg 2011). The Procrustes distances among the populations of *P. aneus* were utilized to conduct a Cluster Analysis (CA) based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and presented as Bray-Custis similarity, using the Paleontological Statistical (PAST) software version 4.03 (Hammer et al. 2001).

Seven morphometric traits were measured, including Head Length (HL), Snout Length (SnL), Eye Diameter (ED), Total Length (TL), Standard Length (SL), Body Depth (BD), and Caudal Peduncle Depth (CPD) (Figure 2). The mean Total Length (TL) in millimeters (mm) of the individual populations were Kuala Kedah (199.19 ± 15.53), Batu Maung (206.53 ± 16.58), and Pantai Remis (194.46 ± 8.40), respectively. The equation $Madj = M(Ls/L0)^b$ by Elliot et al. (1995) was used to normalize the morphometric variables accounting for variations in sizes across populations, where Madj equals the adjusted measurement in size, M equals the raw measurement, Ls equals the mean standard length of all samples, L0 equals the standard length for each sample. The value b was determined for each character from the observed data as the slope of log M on log L0 regression across populations for all fish species (Binashikhbubkr et al. 2024). Discriminant Function Analysis (DFA) was performed on the transformed morphometric characters to find a combination of traits that best maximizes population differentiations of the species. Discriminant function analysis was utilized to find the correct percentage classification for the populations using Wilks' lambda to distinguish between groups. Version 29 of the SPSS was used to determine the Discriminant Function Analysis (DFA).

RESULTS AND DISCUSSION

Data sampling

One hundred and ten samples of *P. aneus* were collected from three sampling sites (Batu Maung, Kuala Kedah, and Pantai Remis) within Northern Peninsular Malaysia (Table 1; Figure 1).

Principal Component Analysis (PCA)

Principal Components Analysis (PCA) showed that 22 principal components were used to explain the differences in body shape and size of the 110 samples. Although the first Principal Component (PC1) had a low eigenvalue of 0.078% in comparison with the (>0.3) considered significant by Imtiaz and Naim (2018), it contributed a percentage variance of 45.84% of the total body shape and size variations among the study populations. However, the first four Principal Components (PCs) combine to account for 75.86% total variance of the two-dimension body shape and size differences with 12.57% (PC2), 9.8% (PC3), and 7. 87% (PC4) respectively (Figures 3 and 4).

The patterns of overlap in the PC1 and PC2 scatter plots (Figure 4) revealed limited body size and shape differences among the populations of *P. aneus* which was not significant to distinguish the populations based on the 0.078% eigenvalue obtained. In PC1, the body shape differences were evident in the head, body depth, pectoral, dorsal, anal, and caudal regions, while in PC2, the differences were reflected in the head, body depth and length, dorsal, pectoral, and caudal regions (Figure 5).

Canonical Variate Analysis (CVA)

Compared to PCA, the Canonical Variate Analysis (CVA) provides a better resolution for intra-genus discrimination of the study populations, as revealed in this study. The canonical variate analysis revealed that two canonical components were used to describe the body shape and size differences among the study populations of *P. aneus* species. The first canonical variate component (CV1) accounted for 58.37% of the variation with a significant eigenvalue of 0.81, while the second canonical variate (CV2) addressed the remaining 41.63% with an eigenvalue of 0.58 (Figure 6). The body shape and size variations among populations of *P. aneus* were demonstrated in the CVA analysis (Figure 6) with some degree of overlap.

The variations observed from the grid transformation graphs (partial warp) (Figure 7) showed maximum variation in the head region, entire body shape, dorsal, pectoral, anal, and caudal regions (CV1). The overall variations in CV2 were revealed in the head and entire body length, pectoral, anal and caudal parts. The Procrustes distances among the populations as generated by CVA were subjected to Cluster Analysis (CA) based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Figure 8), which further revealed two distinct morphological groups: Batu Maung group and Pantai Remis and Kuala Kedah group of *P. aneus* from Northern Peninsular Malaysia.

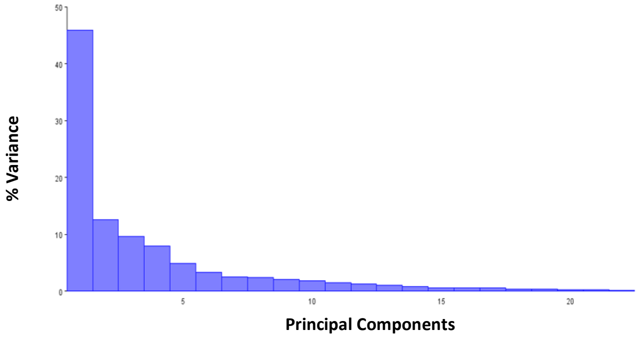

Figure 3. Shows a plot of all 22 principal components against the variance (%) that were involved in analyzing body shape variations among *P. aneus* populations from Northern Peninsular Malaysia

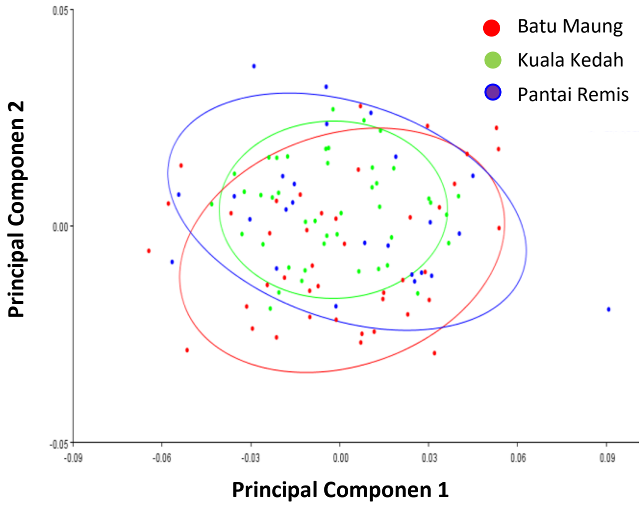

Figure 4. Principal components analysis of body shape and size variations among *P. aneus* populations with equal frequency ellipses shows PC1: 45.84%, PC2: 12.57%, PC3: 9.58%, and PC4: 7.87 % accounting for 75.86 % of the total variation

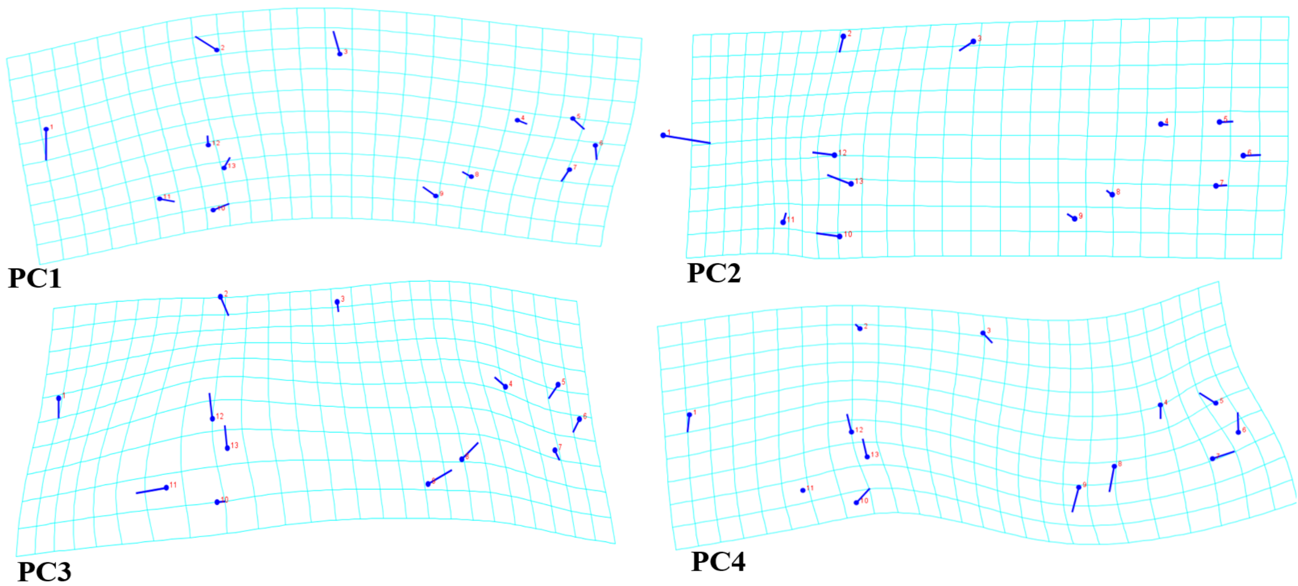

Figure 5. Visualization of body shape differences of *Pennahia aneus* populations via grid transformation graphs for PC1, PC2, PC3, and PC4. PC1 reveals variations in the head, body depth, dorsal, anal and caudal regions. PC2 reveals variations in the head, body depth, and length, dorsal, pectoral, and caudal regions. PC3 reveals variations in the head, body depth, dorsal, anal and caudal regions. PC4 reveals variations in the head, dorsal, pectoral, anal and caudal regions

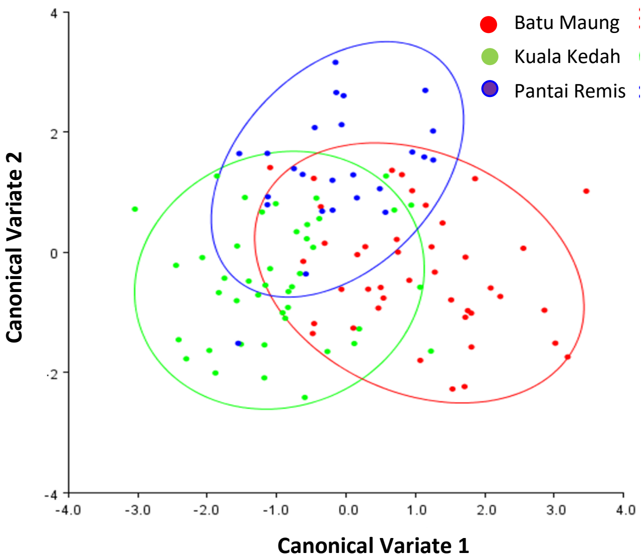

Figure 6. Canonical Variate Analysis (CVA) of body shape and size variations among *Pennahia aneus* populations with equal frequency ellipses showing (CV1: 58.37 %, eigenvalue: 0.81) and (CV2: 41.63 %, eigenvalue: 0.58) accounting for 100.00 % of the total variations among 110 individuals

Discriminant Function Analysis (DFA)

The DFA analyzed seven morphometric variables obtained from *P. aneus* populations. Only two canonical discriminant functions (1 and 2) were used for the analysis, with percentage variances of 73.9% and 26.1 %, respectively, accounting for 100% variations in morphometrics (Figure 9). In all, function 1 was the most contributor to identifying populations of *P. aneus* in the discriminant function analysis of the morphometric traits with an eigenvalue of 0.52. The best predicting morphometric variables for discriminating among *P. aneus* populations were the Standard Length (SL), Body Depth (BD), Total Length (TL), Snout Length (SnL), and Eye Diameter (ED) with correlations of -0.518, -0.471, -0.458, 0.338, and -0.137 respectively in function 1. The Caudal Peduncle Depth (CPD) and Head Length (HL) were the least predicting morphometric variables with 0.547 and -0.206 correlations, as shown in function 2 (Table 2).

The plot of the canonical discriminant function 1 against function 2 (Figure 9) revealed that the populations of *P. aneus* from Kuala Kedah, Batu Maung, and Pantai Remis formed a clustered group with overlapping morphometric characteristics. This attests to their homogeneity in morphology and further confirms the results of the PCA and CVA.

The DFA concluded by predicting the correct classification group of *P. aneus* populations (Table 3). Overall, 67.3% of the original group membership was correctly classified based on the morphometrics, while the remaining 32.7% could have been due to factors other than morphology. The Kuala Kedah population had the best classification success of 79.1%, followed by 69.0% of the Batu Maung population, while the Pantai Remis population had the lowest classification success of 44.0%.

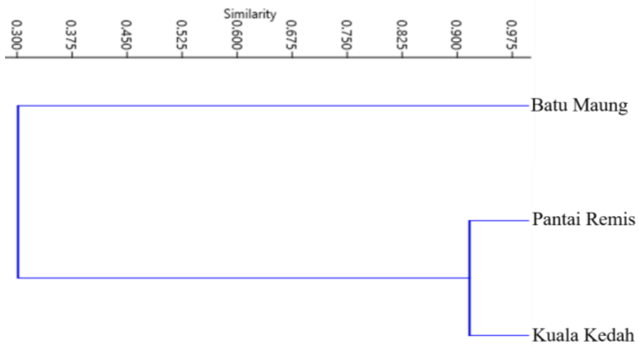

Figure 8. Bray-Curtis similarity cluster analysis of body shape and size variation among populations of *Pennahia aneus* based on UPGMA from the generated Procrustes distances

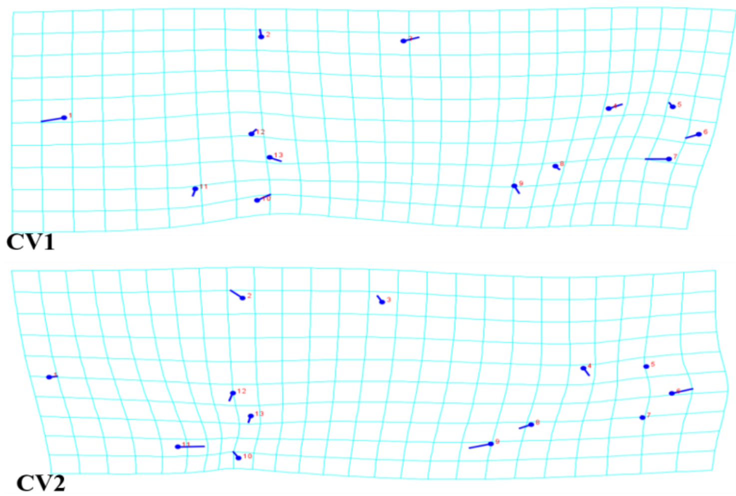

Figure 7. Visualization of body shape differences among *Pennahia aneus* populations via transformation grid graphs for CV1 and CV2. CV1 reveals variations around the head, body depth and length, and pectoral, anal, and caudal regions. CV2 reveals variations in the head, body depth and length, and pectoral, anal, and caudal regions

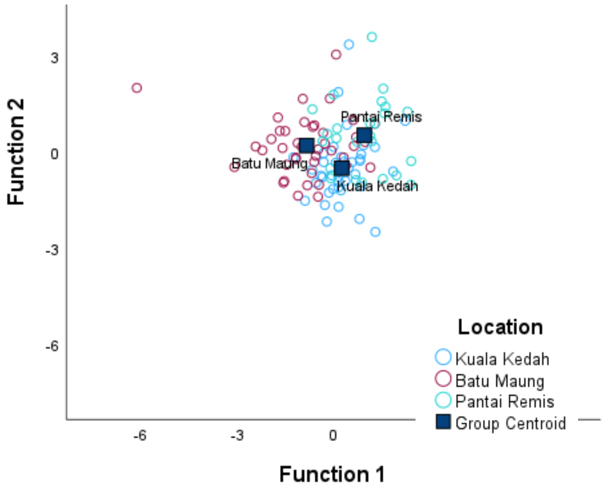

Figure 9. Discriminant function analysis shows (function 1: 73.9 %, eigenvalue: 0.52) and (function 2: 26.1 %, eigenvalue: 0.18) accounting for 100% total discrimination among *Pennahia aneus* populations from Northern Peninsular Malaysia based on morphometric measurements

Table 2. Distributed correlation structure matrix among morphometric variables with discriminant functions among *Pennahia aneus* populations

Morphometric traits	Function	
	1	2
SL	-0.518*	-0.153
BD	-0.471*	-0.087
TL	-0.458*	0.000
SnL	0.338*	-0.013
ED	-0.137*	-0.020
CPD	0.257	0.547*
HL	-0.192	-0.206*

Note: *: Largest absolute correlation between each variable and any discriminant function. SL: Standard Length; BD: Body Depth; TL: Total Length; SnL: Snout Length; ED: Eye Depth; CPD: Caudal Peduncle Depth; HL: Head Length

Table 3. Reclassification of group membership of *Pennahia aneus* populations from Northern Peninsular Malaysia with 67.3% correctly classified

		Predicted group membership			
	Location	Kuala Kedah	Batu Maung	Pantai Remis	Total
Original count	Kuala Kedah	34	4	5	43
	Batu Maung	9	29	4	42
	Pantai Remis	9	5	11	25
%	Kuala Kedah	**79.1**	9.3	11.6	100.0
	Batu Maung	21.4	**69.0**	9.5	100.0
	Pantai Remis	36.0	20.0	**44.0**	100.0
The total percentage of correct reclassification is					67.3%

Discussion

Geometric morphometrics has shown that body size and shape are vital for assessing the relationship between populations of similar taxa (same species) or different taxa, especially by revealing morphological variations (Imtiaz and Naim 2018; Shukri et al. 2024). This study, using Procrustes superimposition, has successfully correlated landmark coordinate variables, revealing subtle shape variation patterns (Shukri et al. 2024). Two morphologically distinct groups were identified in this study.

The PCA results revealed some degree of homogeneity in morphology among the populations of *P. aneus* (Figure 4). The first four Principal Components (PCs) accounted for more than 50 % variation in body shape and size of the 110 individuals considered. This similarity in morphology was also reported by Asaduzzaman et al. (2024) in their study of body divergence in populations of *Polynemus paradiseus* Linnaeus, 1758 from southern Bangladesh's coastal habitats, providing a reassuring alignment with previous studies. Other past reports of Imtiaz and Naim (2018) on *Nemipterus* species within Peninsular Malaysia and surrounding Seas, Moreira et al. (2020) on *Trachurus picturatus* (Bowdich, 1825) (blue jack mackerel) populations in the North Atlantic and Binashikhbubkr et al. (2022) on *Euthynnus affinis* (Cantor, 1849) populations in Peninsular Malaysia agree with the morphological homogeneity observed in this study. Specifically, the 75.86% of the total variance accounted for by the first four PCs observed in this study corroborates the 75.3% body shape variations reported in reef fishes (Claverie and Wainwright 2014), 71.3% body shape variation reported in *Euthynnus alletteratus* and *Thunnus thynnus* (Karakulak et al. 2016), and the 81.3% body shape variation reported in *Barbus* species (Geiger et al. 2016) from combining the first four PCs. The findings of Imtiaz and Naim (2018) and Binashikhbubkr et al. (2022) also reported relatively high percentages in the first four PCs of body shape variation of 80% in *Nemipterus* species and 65.69 % in *E. affinis*, respectively, from Peninsular Malaysia. The morphological variations observed around the body length, depth, and head region of *P. aneus* populations in this study are in congruence with the findings of (Bilici et al. 2015), who asserted that variations observed in the cyprinid fish *Cyprinion macrostomus* Heckel, 1843 revolved around the fish's body size and head region. The body shape variations discovered and reported in *Nemipterus* species (Imtiaz and

Naim 2018) and *E. affinis* (Binashikhbubkr et al. 2022) were all attributed to changes around the head region and the whole body which agrees with our findings.

The morphological variations among populations of *P. aneus* were well-detailed in the Canonical Variate Analysis (CVA) (Figure 6) with some intersections. In this study, the grid transformation graphs of CV1 and CV2 (Figure 7) showed that variations were pronounced around the head region and general body shape. This was consistent with the morphometric research undertaken by Kasinath et al. (2024), who revealed morphological differentiation around the head region among *Rastrelliger kanagurta* (Cuvier, 1816) (Indian mackerel) populations from the eastern Indian Ocean. Our findings also supported the geometric morphometric discrimination analysis conducted by Imtiaz and Naim (2018) on Nemipterid fish species (*Nemipterus* spp) found in Malaysia and the neighboring Seas. Their study demonstrated that body depth, body length, and head orientation were critical factors in the identification of *Nemipterus* species. The body shape variations observed in this study's CVA align with previous reports comparing populations of *Alburnus filippii* Kessler, 1877 in rivers Aras and Ahar-Chai in Iran (Jalili et al. 2015), *Capoeta trutta* (Heckel, 1843) in the Tigris River basin (Iran) (Keivany and Arab 2017), *Squalius namak,* an endemic fish in Iran's Namak inland basin (Saleh et al. 2017), and *Planiliza abu* (Heckel, 1843) (Abu Mullet) in Iran's Bushehr basin (Shabaninejad et al. 2021), using geometric morphometric techniques.

Our DFA findings corroborated the reports by Verma et al. (2014), Binashikhbubkr et al. (2022), and Rahayu et al. (2023), revealing that the first two functions (1 and 2) sufficed to separate among the populations of *Clupisoma garua* (Hamilton, 1822), *E. affinis,* and snapper species (Lutjanidae) in their respective studies. The Standard Length (SL), Body Depth (BD), and Total Length (TL) were shown to be the morphometric traits that significantly contributed to discrimination among *P. aneus* populations (Table 2), which further confirmed the general body form variation in the PCA and CVA results. However, our findings did not align with the reports of Karakulak et al. (2016) and Aminan et al. (2020), who showed that the head length was among the best two predicting morphometric traits to distinguish among populations of *Thunnus thynnus* (Linnaeus, 1758), *Euthynnus alletteratus* (Rafinesque, 1810), and *Rasbora* species, respectively. The DFA was concluded by the Jackknifed cross-validation reclassification of the original group membership of *P. aneus* populations, revealing moderate percentage (67.3%) of the original group members were classified correctly (Table 3). The analysis utilized the significant resemblance among the examined components to predict the group membership data (Aminan et al. 2020). Our findings did not conform with the high accuracy rates reported in previous studies. For instance, Binashikhbubkr et al. (2022) achieved a correct classification rate of 88.6% for seven populations of *E. affinis* in Peninsular Malaysia. Similarly, Moreira et al. (2020) achieved a correct classification rate of 83% for six populations of *Trachurus picturatus* (Bowdich, 1825) in the North-East Atlantic. Additionally, Hoff et al. (2020) reported a reclassification rate of 79% for five populations of the Sciaenid, *Isopisthus parvipinnis* (Cuvier, 1830), in the South-West Atlantic Ocean. Recently, Shahana et al. (2024) reported a reclassification rate of 88.01% for the crescent perch (*Terapon jarbua* (Forsskål, 1775)) population along the Indian coast, which was higher than that obtained in our result. This could be due to overlapping or similar habitat requirements for the different populations, which encourage the inhabiting of wider habitat ranges, bringing about population mixing.

Generally, fish are more prone and sensitive to environmental changes that alter their morphological features than other vertebrates (Verma et al. 2014). Although it is difficult to comprehend how these variations in morphology come about, previous researchers have opined that interactions between genetic and environmental factors could have aided the emergence of such morphological traits (Verma et al. 2014; Bilici et al. 2015; Aminan et al. 2020; Binashikhbubkr et al. 2022; Binashikhbubkr et al. 2024). According to Adibah et al. (2015), the Malay Peninsular specifically poses a significant physical challenge for various species, including fish, whose movement relies on ocean currents. Habitat stratification along the Strait of Malacca is believed to have elicited specific fish morphological responses in the strategic feeding patterns and modified body shapes for maneuvering their habitats (Jagerroos 2016; Simbolon et al. 2019). The morphological changes observed around the head, body length, depth, and caudal regions, as depicted (Figures 5 and 7; Table 2), could have been influenced by a combination of factors like food availability, temperature, radiation, salinity, water depth, and current velocity (Mustikasari et al. 2020). These variations bordering on body size and shape could contribute to their balance and movement, ultimately adapting them to navigating the water currents (Cantabaco et al. 2015). Also, the observed differences in the head morphological pattern could be due to prey types and the variety of ecological niches available in the respective habitats (Tripathy 2020; Binashikhbubkr et al. 2022). The Batu Maung population forming a distinct morphological group from those of Pantai Remis and Kuala Kedah (Figure 8) is not much of a surprise because Batu Maung is on the Island of Penang state, whose shorelines have been reported to be subjected to different anthropogenic activities, which eventually result in loss of habitat, pollution, and siltation (Hamid et al. 2023). We reasoned that these factors could have aided the observed morphological variations.

In conclusion, our study is a pioneering use of geometric morphometric techniques to study morphological variations among populations of *P. aneus* from Northern Peninsular Malaysia. The application of multivariate analyses (PCA, CVA, CA, and DFA) has shown significant morphological variations among *P. aneus* populations. Most importantly, GM has unveiled that the three populations of *P. aneus* species from Northern Peninsular Malaysia form two distinct groups morphologically. This study has demonstrated the potential of GM as a powerful tool for uncovering morphological differences within populations of the same species. Looking ahead, we are excited about the future of this research. To further our understanding of the stock structure of *P. aneus*, we plan to extend our research to other coastal areas within Peninsular Malaysia and incorporate molecular characterization of the species. We believe that these future steps will significantly advance our knowledge in this area.

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Diversity of macrofungi (Ascomycota and Basidiomycota) in the Banyak Mountain Forest Area, Sragen District, Indonesia

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Manuscript received: 22 November 2024. Revision accepted: 8 February 2025.

Abstract. *Nurzahra VY, Muzazzinah, Indrowati M. 2025. Diversity of macrofungi (Ascomycota and Basidiomycota) in the Banyak Mountain Forest Area, Sragen District, Indonesia. Nusantara Bioscience 16: 39-48.* Macroscopic fungi are high-value forest components that play an essential functional role in forest ecosystems. However, studies focusing on the diversity of macroscopic fungi in Indonesian forests are still limited and underexplored. The forest located in Banyak Mountain is one example of a forest area in Indonesia that has significant macroscopic fungal diversity. This study aims to examine the diversity of macroscopic fungi species in the Banyak Mountain Forest Area, Sragen District. The research employed an exploratory method by establishing 2×2 m plots using purposive sampling techniques and conducting descriptive analyses of the collected data. The results of the study in the Banyak Mountain Forest Area identified 33 species of macroscopic fungi from two divisions, namely Basidiomycota and Ascomycota, which are classified into four classes, six orders, twelve families, and twenty-two genera. Most macroscopic fungi found were substrate-dependent on decayed wood, with fewer species located on the soil. The environmental factors influencing their growth include air temperatures ranging from 28 to 32°C, air humidity between 67 and 80%, and soil pH levels of approximately 6.0 to 7.5, which are highly conducive to fungal proliferation. These findings provide a valuable checklist for future research on fungal distribution in tropical regions and contribute significantly to biodiversity conservation efforts.

Keywords: Ascomycota, Banyak Mountain Sragen, Basidiomycota, diversity, macrofungi

INTRODUCTION

Indonesia is a country with vast tropical rainforests that boast remarkable species diversity, including macroscopic fungi. The diversity of fungi is one of the most crucial indicators of forest health and biodiversity (Rakić et al. 2023). The presence of various macrofungi species indicates a rich and complex ecosystem where each organism plays a unique role in maintaining the overall balance and functioning of the forest. Macroscopic fungi are fungi with large fruiting bodies visible to the naked eye without requiring a microscope, encompassing members of the divisions Basidiomycota and Ascomycota (Roda et al. 2010). These fungi display a wide range of colors, including white, yellow, orange, light or dark brown, pink, yellowish white, and black. The fruiting bodies of macrofungi take on various forms, such as trumpet-shaped, fan-shaped, umbrella-shaped, kidney-shaped, and semicircular (Norfajrina et al. 2021).

Ecologically, macroscopic fungi can be classified into three groups: saprophytic, parasitic, and symbiotic species (mycorrhiza) (Kutszegi et al. 2015). Most terrestrial macroscopic fungi are either saprophytic or mycorrhizal symbionts, although some are plant pathogens or parasitic fungi. Fungi thrive in moist environments with limited sunlight due to their negative phototropism, which demonstrates their tendency to grow in darkness (Rahma et al. 2019). The habitats of Ascomycota and Basidiomycota fungi in forests typically include decayed or dead tree trunks, soil, and leaf litter, which provide various organic materials as nutrients for the fungi (Lailiyah et al. 2019). Fungal diversity depends on the variety of organisms present in an environment, such as plants and other animals (biotic factors), as well as pH levels, humidity, temperature, salinity, and climatic conditions (abiotic factors) (López-Bucio et al. 2015). Fungal diversity results from complex interactions between biotic and abiotic factors. For instance, optimal humidity and temperature can support plant growth, which in turn enhances fungal diversity (Tedersoo et al. 2014). Conversely, extreme climate change can disrupt these interactions and reduce fungal diversity (Hawksworth and Lücking 2017).

Fungi are among the elements that enrich biodiversity. In terms of utilization, fungi have been widely studied in the fields of industry, agriculture, medicine, food, textiles, and bioremediation (Hawksworth et al. 2017). Macroscopic fungi are consumed as food, supplements, and medicines, while also participating in nutrient cycling, carbon sequestration, pollution degradation, and ecosystem restoration (Tie et al. 2023). Additionally, macroscopic fungi are used as bioindicators of environmental quality (Alem et al. 2021). Some macroscopic fungi can accumulate heavy metals and other hazardous compounds, making them useful for monitoring pollution levels in the environment (Ediriweera et al. 2022). Mycorrhizal fungi, for example, are used to assess soil health and environmental pollution levels (Smith 2008). Additionally, decomposer fungi such as *Pleurotus ostreatus* (Jacq.)

P.Kumm. can be utilized to evaluate organic matter quality and decomposition processes within ecosystems (Chang and Lee 2004). These fungi produce composite enzymes capable of degrading environmental pollutants (Uddin et al. 2020). Furthermore, they generate organic compounds that bind soil particles, enhancing soil formation, structure, and nutrient absorption (Oregon State University Extension Service 2021).

Despite their diverse functions and benefits, macroscopic fungi are threatened by various factors, including human activities. Rapid development and urbanization are considered the most harmful human actions, as they destroy macrofungi and their natural habitats. Scientists and conservationists have emphasized the importance of recognizing fungi as a key component of ecosystems and have called for targeted management actions to protect fungal species. These efforts include preserving old-growth forests, maintaining habitats to meet the specific needs of threatened fungal species, and allowing the decomposition of dead wood and leaf litter to enhance biodiversity (da Silva et al. 2019). One positive outcome of these conservation efforts is the increased awareness and understanding of fungi's critical roles in supporting the environment and biodiversity.

Fungi represent one of the most diverse groups of organisms on earth (Tedersoo et al. 2014). Tropical regions are known for their high biodiversity of flora and fauna. However, the total number of described fungal species remains very low and uncertain (Hawksworth et al. 2017). Fungal diversity in tropical regions, including Indonesia's protected forests, has not been fully explored and documented. To date, data and literature on macroscopic fungal diversity in Indonesia remain scarce. According to Cannon et al. (2018), more than 93% of fungal species worldwide are still unknown. In Indonesia, it is estimated that approximately 200,000 fungal species have been discovered (Roosheroe et al. 2006). Mueller et al. (2007) estimated that the number of macrofungal species in tropical Asia ranges between 10,000 and 25,000 species worldwide.

The forest area in Banyak Mountain is one example of a forest in Indonesia that harbors a significant diversity of macroscopic fungi. To date, no research has been conducted to identify the species of fungi in this forest area. Considering the essential role of macroscopic fungi in forest ecosystems, it is crucial to document the fungal diversity in this region as part of conservation efforts, ensuring the preservation of endangered species and their habitats. By exploring the diversity of macroscopic fungal species in Banyak Mountain, this study not only contributes to scientific knowledge but also provides valuable insights for conservation strategies and sustainable management. Additionally, information on the diversity of macroscopic fungi can serve as an educational resource for teaching biology concepts related to fungi. Considering the potential utilization of these fungi, it is necessary to conduct an inventory of fungal species. This study aims to identify the diversity of macroscopic fungal species present found in the forest area of Banyak Mountain.

MATERIALS AND METHODS

Study period and area

Sampling of macroscopic fungi was conducted from May to August 2024 in the Banyak Mountain Forest Area, located along the hillside road. Administratively, Banyak Mountain is situated in Gesi Sub-district, Sragen District, Central Java, Indonesia. The Banyak Mountain area spans a total of approximately 54.50 hectares, encompassing various land uses such as forests, fields, plantations, and rice paddies. This study focused on the dark green forested area, which is more relevant to fungal ecosystems, covering approximately 8.90 hectares. The forest in Banyak Mountain features gently sloping topography, latosol soil type, sandy clay soil texture with slight rocky elements, humus-rich soil structure, and moderate soil fertility. A map showing the research location in presented (Figure 1).

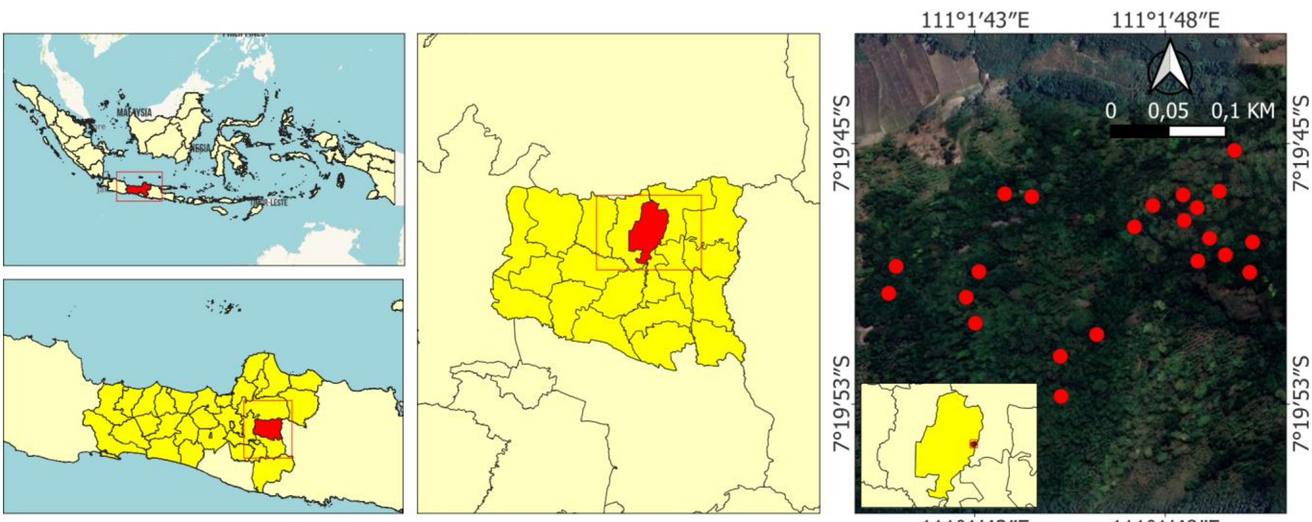


Figure 1. Map of the study area in Banyak Mountain Forest, Gesi Sub-district, Sragen District, Central Java Province, Indonesia

Procedures

The research employed an exploratory survey method involving direct observation of macroscopic fungi in the Banyak Mountain Forest Area. Sampling was conducted using a purposive sampling technique, with plots measuring 2×2 m established in areas where macroscopic fungi were found within the study site. The macromorphological characteristics such as fruiting body shape, fruiting body color, fruiting body size, cap surface texture, type of hymenophore, and type of substrate (Putra 2021)**,** habitat information, including humidity, temperature, soil pH, and fungal substrates such as wood, leaf litter, and soil, were observed on-site and recorded. Samples were photographed in their natural habitats using a digital camera for identification purposes. The collection site, date, specimen count, and habitat of each species were documented in a field diary.

Data analysis

The data collected from the field were analyzed using a descriptive-explorative method and presented in tables and figures. The stages were as follows: data were gathered through exploration of the research area, and macroscopic fungi found were visually observed based on characteristics. The fungi were documented to facilitate the identification process. Whenever possible, objects were identified directly in the field; if not, they were collected for further observation. For collection purposes, fungi samples were carefully taken, especially those with soft fruiting bodies, to ensure intact specimens. The samples were then wrapped in newspaper or placed in jars/storage boxes, labeled, and stored in large plastic bags with harder and heavier fungi placed at the bottom. Next, to preserve macroscopic fungi during identification, a drying process was conducted. For optimal results, macroscopic fungi were dried using an oven and then observed for their macroscopic characteristics. The macroscopic characteristics observed included the dimensions and shapes of the fruiting bodies. Identification of macroscopic fungi was performed after their macroscopic characteristics had been thoroughly documented. This process utilized relevant literature such as McKnight (1998), Arora (1986), and Desjardin (1950), research journals focusing on macroscopic fungi, and several online mushroom databases, including www.mushroomexper.com and https://www.indexfungorum.org/Names/Names.asp.

RESULTS AND DISCUSSION

Macroscopic fungi species in the Banyak Mountain Forest Area

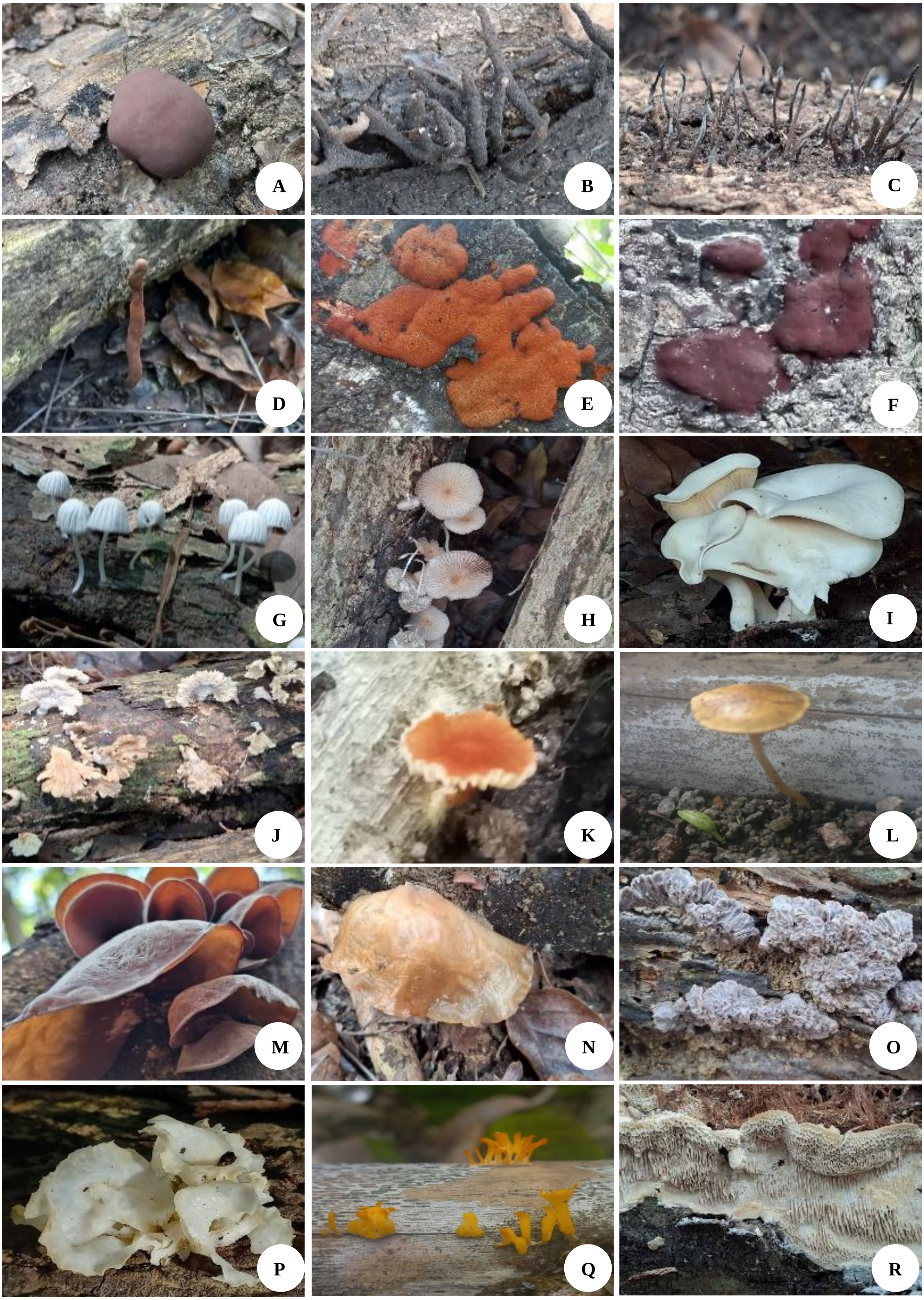
A total of 33 species of macroscopic fungi were identified in the Banyak Mountain Forest Area, Tangen Sub-district, Sragen District. These fungi were classified into the phyla Ascomycota and Basidiomycota. In total, 22 genera of macroscopic fungi were identified, including *Daldinia* Ces. & De Not., 1863, *Xylaria* Hill ex Schrank, 1789, *Hypoxylon* Bull., 1791, *Dacryopinax* G.W.Martin, *Tremella* Pers., *Coprinellus* P.Karst., *Parasola* Redhead, Vilgalys & Hopple, *Schizophyllum* Fr., 1815, *Marasmius* Fr., 1836, *Clitopilus* (Fr. ex Rabenh.) P.Kumm., *Tubaria* (W.G.Sm.) Gillet, *Auricularia* Bull., 1780, *Antrodia* P.Karst., *Fomitopsis* P.Karst., 1881, *Panus* C.J.Schoenherr, 1823, *Lentinus* Fr., *Polyporus* P.Micheli, 1729, *Trametes* Fr., 1836, *Hexagonia* Fr., *Tyromyces* P.Karst, *Earliella* Murrill, and *Ganoderma* P.Karst., 1881 (Figure 2). Most of the macroscopic fungi discovered were saprophytic species, often found growing in colonies (Table 1).

The results of this study revealed 27 species belonging to the division Basidiomycota and only 6 species classified under the division Ascomycota. Basidiomycota fungi were more frequently encountered than Ascomycota fungi, suggesting that the environmental conditions in the study area were more conducive to the proliferation of Basidiomycota species (Widyastuti and Yeni 2022). The population distribution of Basidiomycota fungi is strongly associated with the abundance of trees and leaf litter, which serve as substrates, along with high levels of moisture and water that promote the growth of macroscopic fungi (Rudawska et al. 2022).

This study identified 5 species of fungi with soft, jelly-like fruiting bodies. Surprisingly, these five fungi originated from three different orders. *Auricularia auricula* (L.) Underw. (Figure 2.M), *A. delicata* (Mont. ex Fr.) Henn. (Figure 2.N), and *A. mesenterica* (Dicks.) Pers. (Figure 2.O) belong to the order Auriculariales, characterized by gelatinous fruiting bodies that are generally stalkless or have short stalks (Wu et al. 2021). *Tremella fuciformis* Berk. (Figure 2.P), also known as the "snow fungus," has a jelly-like, white fruiting body (Kuo et al. 2023). *Tremella fuciformis* has been utilized as a food source, a medicinal ingredient, and a raw material for cosmetics (Ma et al. 2021) and *Dacrymyces spathularia* (Schwein.) G.W.Martin (Figure 2.Q) from Dacarymycatales order. The mushroom *D. spathularia* has fruiting bodies shaped like spatulas and features a jelly-like texture with yellow or orange coloration. Its surface texture is smooth and elastic, but as it ages, this mushroom becomes very fragile and easily disintegrates (Rahma et al. 2018).

Number of macrofungal Orders in the Banyak Mountain Forest Area

The exploration in this study revealed that fungi belonging to the order Polyporales (Figure 2.R-AG) were the most abundant, accounting for 48.5% of the total fungi identified (Figure 3). This indicates that the order Polyporales is well-adapted to the environmental conditions of the Banyak Mountain Forest. This finding aligns with the study by Arif (2020), which reported that Polyporales generally exhibit a high level of adaptability. Their adaptability is attributed to their robust and firm fruiting bodies, which make them more resistant to climate fluctuations and weather conditions. Polyporales are also the most frequently found macroscopic fungi in other forest areas in Indonesia, such as the Rawa Bento peat swamp forest (Sayuti et al. 2023), the lowland forest of Bukit Wangkang (Zulpitasari et al. 2019), and the highland forest of Lore Lindu National Park in Central Sulawesi (Yusran et al. 2021).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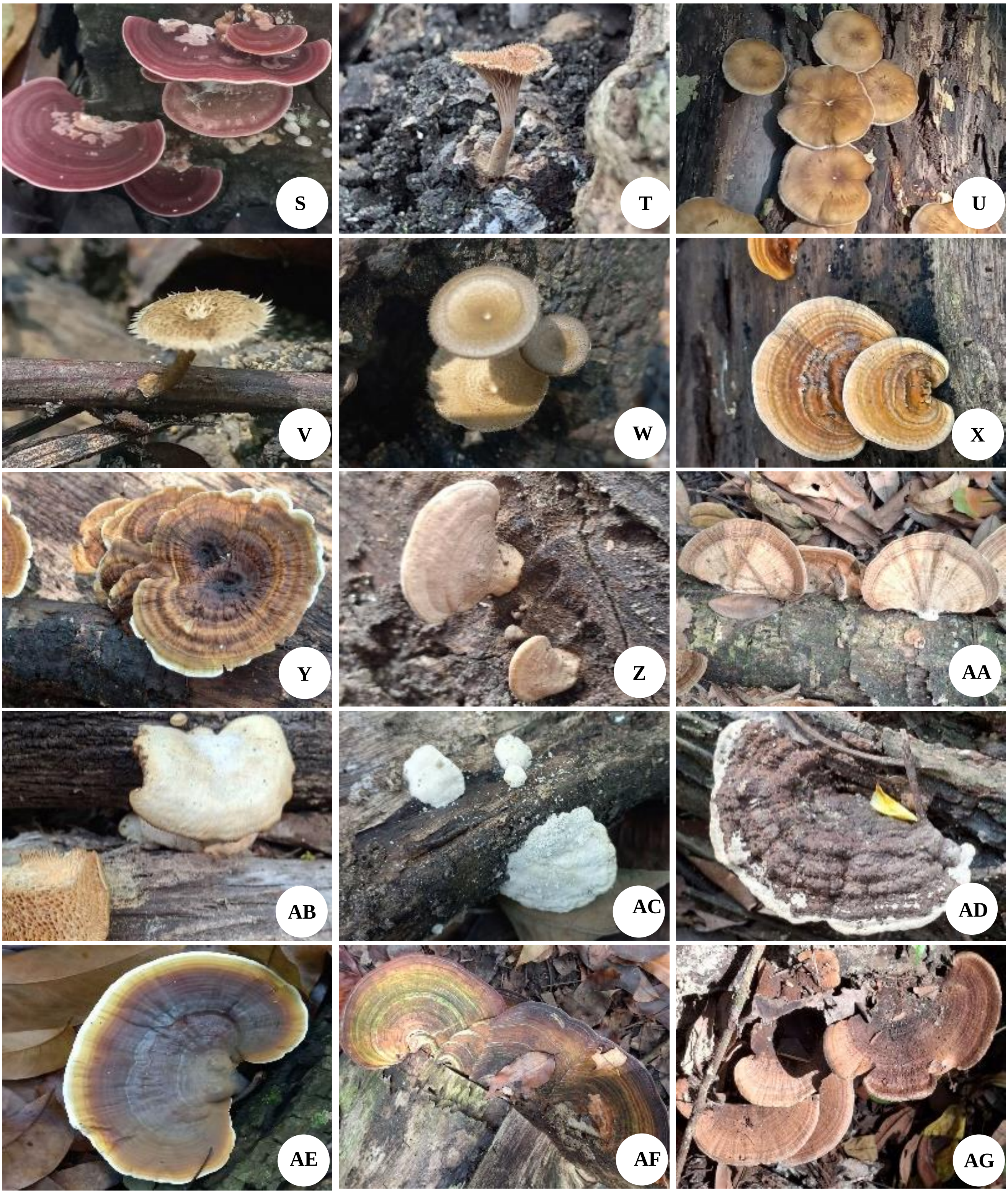

Figure 2. Macroscopic fungi identified in the Banyak Mountain Forest Area, Sragen District, Indonesia. A. *D. concentrica*; B. *X. primorskensis*; C. *X. apiculata*; D. *X. telfairii*; E. *H. haematostroma*; F. *H. griseobrunneum*; G. *C. disseminatus*; H. *P. plicatilis,* I. *C.* prunulus; J. *S. commune*; K. *Marasmius* sp.; L. *T. furfuracea*; M. *A. auricula*; N. *A. delicata*; O. *A. mesenterica*; P. *T. fuciformis*; Q. *D. spathularia*; R. *A. albida,* S. *F. feei*; T. *P. lecomtei*; U. *L. brumalis*; V. *L. crinitus*; W. *P. arcularius*; X. *T. hirsuta*; Y. *T. versicolor*; Z. *T. pubescens*; AA. *H. tenuis*; AB. *P. alveolaris*; AC. *T. chioneus*; AD. *E. scabrosa*; AE. *G. lucidum*; AF. *G. applanatum*; AG. *G. boninense*

Table 1. List of fungi found in the Banyak Mountain Forest Area, Sragen District, Indonesia

Divisio	Ordo	Familia	Species
Ascomycota	Xylariales	Xylariaceae	*Daldinia concentrica* (Bolton) Ces. & De Not.
			Xylaria primorskensis Y.M.Ju, H.M.Hsieh, Lar.N.Vassiljeva & Akulov
			Xylaria apiculata Cooke
			Xylaria telfairii (Berk.) Sacc.
			Hypoxylon haematostroma Mont.
			Hypoxylon griseobrunneum (B.S.Mehrotra) J.Fourn., Kuhnert & M.Stadler
Basidiomycota	Agaricales	Psathyrellaceae	*Coprinellus disseminatus* (Pers.) J.E.Lange
			Parasola plicatilis (Curtis) Redhead, Vilgalys & Hopple
		Schizophyllaceae	*Schizophyllum commune* Fr.
		Marasmiaceae	*Marasmius* sp. Fr.
		Entolomataceae	*Clitopilus prunulus* (Scop.) P.Kumm.
		Agaricomycetes	*Tubaria furfuracea* (Pers.) Gillet
	Auriculariales	Auriculariaceae	*Auricularia auricula* (L.) Underw.
			Auricularia delicata (Mont. ex Fr.) Henn.
			Auricularia mesenterica (Dicks.) Pers.
	Polyporales	Fomitopsidaceae	*Antrodia albida* (Fr.) Donk
			Fomitopsis feei (Fr.) Kreisel
		Polyporaceae	*Panus lecomtei* (Fr.) Corner
			Lentinus brumalis (Pers.) Zmitr
			Lentinus crinitus (L.) Fr.
			Polyporus arcularius (Batsch) Fr.
			Polyporus alveolaris (DC.) Bondartsev & Singer
			Trametes hirsuta (Wulfen) Lloyd
			Trametes versicolor (L.) Lloyd
			Trametes pubescens (Schumach.) Pilát
			Hexagonia tenuis (Fr.) Fr.
			Tyromyces chioneus (Fr.) P.Karst
			Earliella scabrosa (Pers.) Gilb. & Ryvarden
		Ganodermataceae	*Ganoderma lucidum* (Curtis) P.Karst.
			Ganoderma applanatum (Pers.) Pat.
			Ganoderma boninense Pat.
	Tremellales	Tremellaceae	*Tremella fuciformis* Berk.
	Dacrymycetales	Dacrymycetaceae	*Dacrymyces spathularia* (Schwein.) G.W.Martin

Fungi from the order Xylariales were the second most commonly found (Figure 2.A-F), comprising 18.2% of the total fungi and representing the only macroscopic fungal order from the division Ascomycota identified in Banyak Mountain. Members of the genus *Xylaria* are characterized by dense, finger- or club-shaped fruiting bodies. These fungi can be found on decaying wood, leaves, fruits, seeds, animal droppings, soil, and termite nests (Wangsawat et al. 2021). The secondary metabolites and bioactivities of fungi in the genus *Xylaria* continue to be explored due to their potential benefits, particularly in the field of health (Lin et al. 2016). The number of orders can be seen in Table 2.

The percentage of the number of macroscopic fungi species in each order in the Banyak Mountain Forest Area is presented in Figure 3.

Distribution of macrofungal species across families

The research findings revealed the presence of 33 species of fungi belonging to 12 families. The most dominant family was Polyporaceae, comprising 11 species, indicating that this family had the highest number of species in the research area. The fungi from the Polyporaceae family typically grow on wooden substrates, including both decayed/dead wood and living wood, classifying them as wood fungi.

Table 2. Number of Orders of macroscopic fungi in the Banyak Mountain Forest Area, Sragen District, Indonesia

Ordo	Number of families	Number of species
Agaricales	5	6
Auriculariales	1	3
Polyporales	3	16
Tremellales	1	1
Dacrymycetales	1	1
Xylariales	1	6
Grand total	12	33

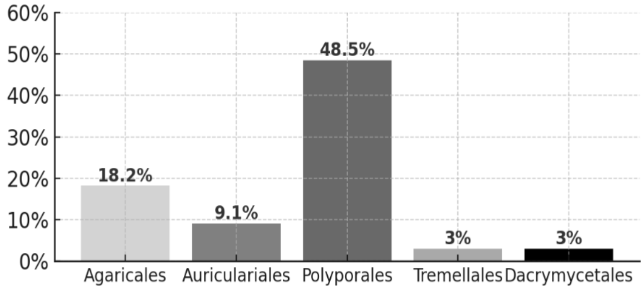

Figure 3. Percentage of the number of macroscopic fungi species in each order

The percentage of the number of macroscopic fungi species in each family in the Banyak Mountain Forest Area is presented in Figure 4.

The study found that the family Polyporaceae was the most diverse among all documented macroscopic fungi in the Banyak Mountain Forest Area, with a total of 11 species. This was followed by the family Xylariaceae with 6 species, Auriculariaceae and Ganodermataceae with 3 species each, Psathyrellaceae and Fomitopsidaceae with 2 species each, and 6 other families represented by only one species, as shown in Table 3. Among the identified macrofungal families, Xylariaceae was the only family classified under the division Ascomycota.

The family Polyporaceae, a frequent sight in diverse environments, including decaying wood and soil, is a subject of extensive research. The findings, as highlighted in the research by Lestari (2018), provide a wealth of knowledge about the prominent characteristics of Polyporaceae members, including their relatively large and robust fruiting bodies attached to decaying wood. These fungi's ability to survive in dry conditions, enabling them to flourish in environments with varying altitudes and moisture levels, is a testament to their adaptability. The prevalence of bracket and conk fungi, particularly those from the family Polyporaceae, in forest habitats further underscores their adaptability and resilience (Cababan et al. 2021).

Habitat distribution of macrofungi in the Banyak Mountain Forest

Climate is known to be a key factor in the formation of fruiting bodies, and seasonal changes have been linked to variations in the phenology, abundance, and distribution of fungal species (Sutjaritvorakul et al. 2017). The habitats of the macrofungi identified in this study are predominantly found in habitats such as moist soil and, notably, decayed wood (Figure 5). This latter habitat significantly influences the composition of macrofungal species in this survey. All species from the orders Polyporales, Auriculariales, and Tremellales were found exclusively on decayed wood, while some species from the order Agaricales and Xylariales were observed growing on soil. Decayed wood was identified as the primary habitat for macrofungi, harboring 29 species (88%), while 4 species (12%) were found on soil substrates. Deadwood, a rich source of nutrients and shelter, is a key habitat for saprophytic organisms, particularly macrofungi. Most macrofungi found in the Banyak Mountain Forest Area are decomposers of leaf litter and wood, contributing to the acceleration of the nutrient cycling process in forest ecosystems (Niego et al. 2023).

Macrofungi typically thrive on decayed wood and soil due to the substrates ability to provide an optimal environment for growth. Substrates serve as growth media and the primary source of nutrients for these fungi (Darmawanti et al. 2023). In the Banyak Mountain forest, the majority of Basidiomycota species identified were wood-decomposing fungi, as they flourish on rotting wood. This observation suggests that most fungal species identified in this study serve as decomposers within the forest ecosystem.

Table 3. Number of Families of macroscopic fungi in the Banyak Mountain Forest, Sragen District, Indonesia

Families	Number of species
Xylariaceae	6
Psathyrellaceae	2
Schizophyllaceae	1
Marasmiaceae	1
Entolomataceae	1
Agaricomycetes	1
Auriculariaceae	3
Fomitopsidaceae	2
Polyporaceae	11
Ganodermataceae	3
Tremellaceae	1
Dacrymycetaceae	1
Grand total	33

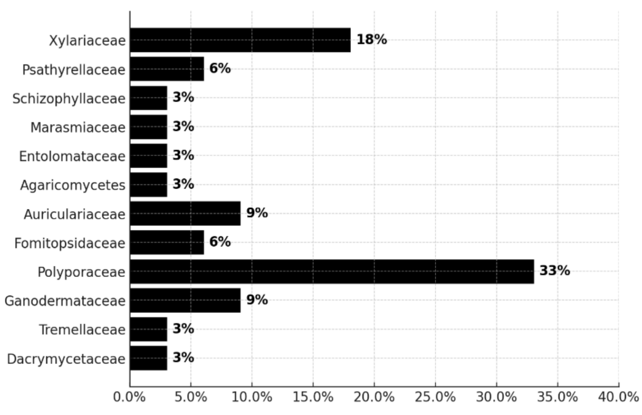

Figure 4. Percentage of the number of macroscopic fungi species based on families in the Banyak Mountain Forest, Sragen District, Indonesia

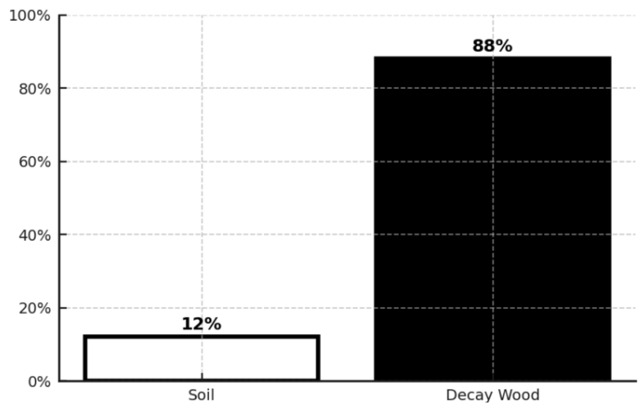

Figure 5. Distribution of macrofungal species across substrates

Fungi that inhabit decayed trunks and branches are classified as saprophytic fungi, playing a crucial role in recycling materials and nutrients within forest ecosystems. While most fungi can degrade and utilize carbohydrates such as cellulose, only wood-decomposing fungi can break down and utilize the carbohydrate complexes found in lignin-cellulose-hemicellulose structures within the cell walls of woody plants (Riley et al. 2014). Fungi growing on living plants are likely parasitic or pathogenic, whereas those on soil may belong to the mycorrhizal fungi group. These mycorrhizal fungi are microscopic and can colonize plant roots or live in the soil surrounding root systems (Rao et al. 2010). Most macrofungi in this study inhabited decayed wood, highlighting their predominantly saprophytic nature (Tadiosa et al. 2021). The role of macrofungi in nutrient recycling is significant, as evidenced by the species observed growing on soil substrates, including *Parasola plicatilis* (Curtis) Redhead, Vilgalys & Hopple, *Clitopilus prunulus*, *Tubaria furfuracea* (Pers.) Gillet, and *Xylaria telfairii* (Berk.) Sacc. Macrofungi can either live in colonies (clusters) or as solitary individuals. In the Banyak Mountain Forest Area, most of the identified macrofungal species were observed living in colonies.

Environmental parameters at Banyak Mountain

Environmental parameters were measured to assess the habitat conditions for mushroom growth in the Banyak Mountain forest Area. The data from the measurement of abiotic environmental parameters, including temperature, humidity, and soil pH, are presented in Table 4.

The environment influences the abundance of macroscopic mushrooms. The soil in the Banyak Mountain forest Area has humus characteristics, with soil acidity values ranging from 6.0 to 7.5. According to Gunawan (2001), mushrooms generally thrive well at pH levels between 5.5 and 7.5. This indicates that the soil pH in the Banyak Mountain Forest Area is suitable for mushroom growth. The pH of the substrate is one of the factors that can influence mushroom growth because pH affects enzymatic activity within the cells.

The humidity measurements ranged from 67 to 80%. According to Zabel et al. (2020), mushrooms prefer humidity levels between 40 and 80%, which are essential for maintaining water content and nutrient transport within cells. The measurement results show that the air humidity in the Banyak Mountain Forest Area is suitable for the survival of mushrooms. The moist forest conditions ensure an adequate supply of water, thus meeting the water and other nutrient needs for mushroom growth.

Table 4. Result of microclimate variable measurement

Environmental factors	Measurement range
Temperature (°C)	28-32°C
Air humidity (%)	67-80%
Soil pH	6.0-7.5

Based on observations, the air temperature in the Banyak Mountain forest area ranged from 28 to 32°C. This data provides an accurate figure of the thermal conditions in the Banyak Mountain Forest Area, which is crucial for understanding the local environmental dynamics. Generally, mushrooms can grow optimally at temperatures ranging from 15 to 40°C (Zabel et al. 2020). This means that the air temperature in the Banyak Mountain Forest Area is optimal for mushroom growth. This season is conducive to mushroom production as the temperature and relative humidity levels are adequate to facilitate the breakdown of organic matter by mushrooms (Wang et al. 2022).

The survival abilities of macroscopic mushrooms in nature vary. According to Putra et al. (2019), mushrooms are cosmopolitan organisms, making them easy to find in various types of habitats. However, their growth in nature is influenced by many factors that affect their distribution. Each type of mushroom has different adaptive abilities to a particular habitat. Additionally, the substrate found at all locations supports the growth of these mushroom species. Decayed wood is the dominant substrate at all locations.

Discussion

A wide variety of naturally occurring macrofungi can be found in Banyak Mountain due to its supportive environmental conditions. Macrofungal diversity varies significantly as a result of environmental factors such as decaying logs, moist soil, sandy soil, humus, and leaf litter (Vishwakarma et al. 2017). Variations in macrofungal distribution can also be attributed to multiple factors, including rainfall, the availability of suitable substrates, moisture-retaining wood with consistently high humidity, and forest type (Hu et al. 2022). Geographically, Indonesia is located in the tropics, situated along the equatorial latitude, with two distinct seasons and a unique climatic environment. The months of May to July experience high rainfall, which supports fungal growth.

Various macroscopic fungi that grow naturally can be found in the forest area of Banyak Mountain due to its supportive environmental conditions. These include fungi from the divisions Ascomycota and Basidiomycota. Given the abundance of dead trees and tree trunks in the Banyak Mountain Forest Area, along with the high humidity and water content, polypores tend to thrive on substrates rich in cellulose in this environment (Gilbert et al. 2002). Macroscopic fungi are found seasonally worldwide in various habitats such as humus-rich soil, decaying plant litter, and logs in forests, as well as in grasslands and even sandy soils and other types of soil. Macroscopic fungi that inhabit decayed wood or dead trees are commonly found due to their ability to survive at higher temperatures and the fact that their toughness deters herbivorous animals (Couceiro and Couceiro 2022).

This study revealed that the species composition and forest structure influence the microhabitat conditions (soil humidity, soil pH, and temperature) in each forest community, with air temperatures ranging from 28-32°C, humidity from 67 to 80%, and pH from 6.0 to 7.5. These conditions are highly conducive to the growth and development of fungi, particularly macroscopic fungi. The fungi found are known to play an important role as decomposers in the Banyak Mountain Forest Area, as they were predominantly found growing as saprophytes on decaying wood trunks and branches, with a smaller proportion associating with mycorrhizae.

In conclusion, 33 species of macroscopic fungi were found in the Banyak Mountain Forest Area, identified across 6 orders and 12 families, as reported in this study. The order Polyporales was the most dominant group at the study site. The Basidiomycota and Ascomycota fungi found generally live on decayed wood, with a smaller number living on soil. This study provides valuable information on the biodiversity of Basidiomycota and Ascomycota fungi in the Banyak Mountain Forest Area, Tangen Sub-district, Sragen District, Central Java, Indonesia.

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Fermented black soldier fly (*Hermetia illucens*) carcass meal as a substrate for its larva

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Abstract. *Riok PAH, Hariani N, Nugroho RA, Arif MF, Syafrizal, Aryani R, Manurung H, Rudianto. 2025. Fermented black soldier fly (*Hermetia illucens*) carcass meal as a substrate for its larva. Nusantara Bioscience 17: 49-56.* The black soldier fly (*Hermetia illucens* (Linnaeus, 1758)) is an insect commonly used as an organic waste decomposer. The Black Soldier Fly (BSF) has four life stages, and as an adult, it dies shortly after reproducing, which leads to it being an underutilized waste product. This study aimed to determine and compare the effects of the fermented BSF Carcass Meal (fBSFM) and Fermented Palm Kernel Meal (fPKM) as substrates on the growth rate and proximate composition of BSF Larvae (BSFL). The present research used a completely randomized design with four groups: a control group (100% fPKM) and three experimental groups with varying percentages of fBSFM (P1: 5% fBSFM, P2: 7.5% fBSFM, P3: 10% fBSFM). The results showed that substituting fBSFM at concentrations of 7.5- and 10% in fPKM increased growth rate, individual weight, biomass, and bioconversion, supporting their life cycle; the use of 15% fBSFM as BSFL substrate significantly impacted substrate reduction. The substrate reduction rate was measured at 55.18 ± 8.49%, suggesting that black soldier fly larvae can effectively reduce both fBSFM and fPKM. The different proportions of fPKM and fBSFM did not have a significant effect on the proximate values of the BSFL. Therefore, this research provides valuable insight for waste management and agriculture, suggesting that incorporating more than 5% of fermented BSF carcass meal into the substrate mixture with fPKM is advisable to enhance the growth indices of BSFL.

Keywords: Black soldier fly meal, *Hermetia illucens*, larval growth, proximate composition

INTRODUCTION

The Black Soldier Fly (BSF) was officially recognized as *Hermetia illucens* (Linnaeus, 1758) (Diptera: Stratiomyidae), has numerous nutritive value benefits for cattle and aquaculture (Rehman et al. 2023; Ferdousi et al. 2024). The BSF is commonly located in humid, nutrient-dense environments, marked by a significant accumulation of decaying organic matter from animals and plants (Diola et al. 2024; Lomonaco et al. 2024; Septiariva et al. 2024). The BSF Larvae (BSFL) possess several advantageous characteristics, such as a comparatively high protein and lipid value, the capacity to thrive on diverse waste materials unsuitable for human consumption, and their non-classification as a pest or nuisance species (Liu et al. 2022; Phaengphairee et al. 2023). The nutritional profile of BSF larvae can comprise as much as 40% protein (Ndotono et al. 2022) and 30% lipid (Lin et al. 2022), with variations contingent upon the substrate used for their growth (Newton et al. 2005; St-Hilaire et al. 2007). Various kinds of organic substrates influence the production and nutritive quality of BSFL (Lalander et al. 2019; Truzzi et al. 2020; Fischer and Romano 2021). The BSFL typically demonstrates accelerated development when provided with a diet containing a balanced ratio of easily digestible protein and carbohydrates (Cammack and Tomberlin 2017). Nevertheless, substrates with elevated protein levels may further promote larval growth, resulting in larger larvae (Nguyen et al. 2015; Tinder et al. 2017; Lalander et al. 2019).

The life cycle of BSF can be distinguished into four main steps: egg, larva, pupa, and adult. In larval steps, the tiny BSFL begin to feed on the organic matter around them after hatching. They are equipped with powerful mandibles that allow them to shred and consume various materials. As they feed, the larvae grow rapidly, shedding their exoskeletons several times as they molt. The larval step can last anywhere from 10 days to several weeks, depending on the availability of food and environmental conditions (Oliveira et al. 2016). Once they have reached their maximum size, the larvae enter a non-feeding stage called the prepupal stage, during which they seek out a dry, sheltered location to begin their transformation into pupae. During the pupal stage, the larvae transform into a non-feeding, immobile pupa. The pupal stage typically lasts about 1-2 weeks, during which time the pupa undergoes metamorphic changes to emerge as an adult fly (Muhayyat et al. 2016).

Finally, the adult BSF is a stout, shiny black insect with a distinctive pattern of white markings on its abdomen. Adults are strong fliers and are attracted to light. They feed on nectar, fruit juices, and other sweet substances. Males and females mate soon after emergence, and the female begins laying eggs within a few days. The adult stage typically lasts about 4-6 weeks, depending on environmental conditions. After mating, the male BSF died, followed by the female BSF soon after laying their eggs (Wardhana 2016). The dead body or carcass of both male and female BSF is usually thrown away and no longer used in the BSF farm. However, it's important to note the ecological significance of the adult BSF. Despite their short lifespan, these insects play a crucial role in waste recycling and resource recovery, particularly when integrated with palm oil plantation by-products.

Moreover, biomass that contains palm fronds and trunks is generated as a result of replanting operations in oil palm plantations. Palm oil mills produce various types of by-product waste while converting Fresh Fruit Bunches (FFB) into Crude Palm Oil (CPO). These include Empty Fruit Bunches (EFB), Mesocarp Fiber (MF), Palm Kernel Shell (PKS), Palm Kernel Meal (PKM), and Palm Oil Mill Effluent (POME). Indonesia has a significant abundance of these wastes, found in various locations across 22 provinces, including oil palm fields, FFB manufacture, and palm oil mills. These waste products provide economic worth as they may be converted into alternative fuels, fertilizers, and biochemical materials. Therefore, a pioneering study is essential to augment the value of PKM and foster sustainability in the oil palm sector (Hambali and Rivai 2017). Azizi et al. (2021) discovered that Palm Kernel Cake (PKC) has a Crude Protein (CP) content of around 14-18%, Crude Fiber (CF) content of 12-20%, Ether Extract (EE) content of 3-9%, and other minerals. Furthermore, Balandrán-Quintana et al. (2019) reported that PKM has a protein value between 14.4 and 20%, along with significant quantities of carbohydrates (50.3%) and crude fiber (16.7%).

Several studies have examined the growth of BSFL in various substrates (Spranghers et al. 2017; Shumo et al. 2019; Nugroho et al. 2024). However, no study has investigated the growth and nutritive value of BSFL raised in fermented Black Soldier Fly Carcass Meal (fBSFM) and fermented PKM (fPKM). This study aimed to examine the development and nutritional composition of the BSFL grown in a substrate containing fBSFM compared to fPKM. It represents the first report on the use of BSF carcasses as a potential substrate for their larvae.

MATERIALS AND METHODS

Study area

The present study was carried out at the laboratory of Animal Physiology, Development, and Molecular, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Mulawarman in Samarinda, East Kalimantan, Indonesia.

BSFL meal preparation

The BSF carcasses were dried using a dehydrator for 48 hours. The dried BSF carcass was ground into a meal using a food processor. The BSF meal (BSFM) was fermented for 5 days before being used as BSFL substrate. The fermented substrate was evaluated for proximate analysis. Table 1 shows a formula to make a fermented substrate for BSFL.

BSFL preparation and acclimatization

The BSFLs were reared from the egg phase, which was obtained from CV. Ahasa Larva Group, Samarinda, East Kalimantan, Indonesia. The eggs of BSF were randomly placed in four plastic boxes (35 length × 25 width × 12 height cm). Each box was filled with 3.25 g of BSF eggs. First instar BSFL hatchlings were given a mixture of fish pellets and water (100 g: 1 liter), 325 g/3 days for 7 days. The instar of BSFL was reared in a condition of temperature 27-29°C and humidity 60-80%. During the 1st instar of the BSFL rearing process, the substrate was sprayed with water daily to keep the substrate and larvae moist.

BSFL for proximate analysis

In the treatment stage, the BSFL rearing was divided into two purposes: proximate and growth analysis. For proximate analysis, an amount of 28.25 g of BSFL with an estimated number of ± 2,500 of seven seven-Day-Old Larvae (DOL) was put into the rearing tanks. Subsequently, the formulated fermented substrate was given for 10 days. Meanwhile, for growth analysis, a number of 30 individual larvae were weighed and placed into the rearing container (500 mL plastic bottle). The BSFL was given various fermented substrates for 10 days. Both BSFL for proximate and growth analysis were fed with fermented substrates following a previous study by Guo et al. (2021). All treatments of BSFL, either for proximate or growth analysis, had three replications. All plastic boxes and containers were placed in the room with a temperature of 27-29°C and humidity of 60-80% condition. On the 11th day, all larva was harvested for proximate and growth analysis. The harvested BSFL for proximate analysis was dried using a microwave. The dried BSFL was ground using a food processor to obtain a BSFL meal. The BSFL meal was weighed and stored in the freezer until it was used for proximate analysis.

Table 1. Black Soldier Fly Larvae (BSFL) substrate fermentation formula

Groups	Ingredient (g) (PKM:BSFM)	Molasses (g)	EM4 (mL)	Water (L)
fPKM	1000: 0	40	62	2
fBSFM 5%	950: 50	40	62	2
fBSFM 10%	925: 75	40	62	2
fBSFM 15%	900: 100	40	62	2

Note: fPKM: 100% Fermented Palm Kernel Meal; fPKM: various ratios of Palm Kernel Meal (PKM) with Black Soldier Fly Meal (BSFM). The addition of EM4 and water are based on the previous study (Nugroho et al. 2024; Nugroho et al. 2023)

Growth parameter analysis

The individual weight of the BSFL from the control and each treatment group was performed using an analytical balance (220 g × 0.0001 g). The individual weight of the larvae was calculated using the following formula (Romano 2022):

$$\text{Individual weight} = \text{Final individual weight} - \text{initial individual weight}$$

Biomass measurement was measured from either control or treatment groups of the BSFL fed various fermented substrates using an analytical balance and followed the previously used equation (Romano 2022):

$$\text{Biomass} = \text{Final population weight} - \text{initial population weight}$$

The growth rate of the BSFL was determined by dividing the average weight of an individual by the total treatment time. The growth rate was calculated using the following equation (Wevers et al. 2022):

$$GR = \frac{(W_f - W_i)}{T}$$

Where: GR: Growth Rate (gr/day); Wf: Average final weight of individuals BSFL (g); Wi: Average initial weight of individuals (g); T: Duration of the study (10 days).

Substrate Reduction (SR)

Substrate Reduction (SR) aims to determine the efficiency of the BSFL in reducing the fermented substrate or substrate given. The SR value was calculated by dividing the total weight of the fermented substrate consumed by the BSFL by the total weight of the fermented substrate. The SR was determined using the following formula (Diener et al. 2009):

$$SR = \frac{(FS_t - FS_r)}{FS_t} \times 100\%$$

Where: SR: Substrate Reduction (%); FS_t: Total weight of fermented substrate (54.2 g); FS_r: Weight of remaining fermented substrate (g).

Substrate bioconversion or Biomass Conversion Ratio (BCR) is the efficiency value of the BSFL in bioconverting fermented substrate into useful biomass. The BCR can be determined by dividing the BSFL biomass value by the total weight of the fermented substrate given. The BCR was evaluated following the equation used by Lindberg et al. (2022):

$$BCR = \frac{(\text{BSFL biomass final weight} - \text{BSFL biomass initial weight})}{FS_t} \times 100\%$$

Where: BCR: Bioconversion of Ratio (%); FS_t: Total weight of fermented substrate (54.2 g).

Survival

The BSFL survival percentage was determined by dividing the number of survival BSFL at the end of the study by the total BSFL population at the beginning of the study.

Proximate analysis

Proximate analysis was evaluated on the fermented substrate sample and black soldier fly larva meal. Each group of the BSFL was then processed for proximate evaluation, which included the assessment of crude protein, crude lipid/fat, fiber, ash, and moisture value. Crude protein analysis was conducted using the standard technique of the Association of Official Analytical Chemists (AOAC), AOAC International, 17th ed v.2, Gaithersburg, Md method (Horwitz 2000). Moisture, ash, and crude fiber content were determined using the gravimetric method as described by BSN (1992). Crude fat content was determined using BSN (1992) method, using the soxhlet extraction method. The proximate parameters content was determined following equations:

Crude protein

$$\%N = \frac{[(N_{acid})(mL_{acid}) - (mL_{bk})(N_{NaOH}) - (mL_{NaOH})(N_{NaOH})][1400{,}67]}{\text{mg sample}}$$

$$\%\text{ Protein} = \%N \times \text{Conversion factor}$$

Where: mL_{NaOH}: mL standard base needed to titrate sample; mL_{acid}: mL standard acid used for sample; mL_{bk}: mL standard base needed to titrate reagent blank; N_{acid}: Acid normality; N_{base}: Base normality; conversion factor: 6.25.

Crude fat

$$\%\text{ Crude fat} = \frac{W_1 - W_2}{W_0} \times 100\%$$

Where: W_0: Sample weight (g); W_1: Boiling flask weight (g); W_2: Boiling flask + fat weight (g).

Moisture content

$$\%\text{ Moisture} = \frac{W_1 - W_2}{W_0} \times 100\%$$

Where: W_0: Sample weight (g); W_1: Crucible + sample weight before drying (g); W_2: Crucible + sample weight after drying (g).

Ash content

$$\%\text{ Ash} = \frac{W_1 - W_2}{W_0} \times 100\%$$

Where: W_0: Sample weight (g); W_1: Crucible + ash sample weight (g); W_2: Crucible weight (g).

Crude fiber

$$\% \text{ Crude fiber} = \frac{W_1 - W_2}{W_0} \times 100\%$$

Where: W_0: Sample weight (g); W_1: Filter paper + fiber residue (g); W_2: Filter paper weight (g).

Data analysis

The data collected was analyzed utilizing SPSS version 25 (SPSS, Inc., USA). The Levene test was used to evaluate the normality of the acquired data. The statistical test results stated that the growth parameters data (individual weight, biomass, bioconversion, and growth rate) were normally distributed and homogeneous; the normally distributed and homogeneous data were analyzed using One Way ANOVA followed by Duncan's test with a 95% confidence level to determine the significance. Data that were not normally distributed and not homogeneous (substrate reduction, mortality, crude protein, crude fat, crude fiber, ash, and moisture) were analyzed with the Kruskal-Wallis non-parametric test.

RESULTS AND DISCUSSION

Based on the proximate analysis of various fermented substrates, it is shown that the fermentation process of BSFM, in combination with PKM, resulted in various proximate values (Table 2**)**. The protein content increases significantly by adding BSFM to the Palm Kernel Meal (PKM). The highest protein content was observed in the fBSFM 15% substrate, indicating that increasing the proportion of BSFM enhances the protein content. Fat content was relatively stable across all substrates, with only minor variations. This shows that the fermentation process has no real effect on fat content. Water content decreased as the proportion of BSFM increased. The lower water content in BSFM may cause this compared to PKM. Similar to moisture content, the ash content decreases slightly with the addition of BSFM. This might indicate a reduction in mineral content or changes in the mineral composition due to fermentation. Further, the fiber content slightly increases with higher proportions of BSFM. This could be due to the fibrous nature of BSFM.

As Table 3 shows, different ratio concentrations of fBSFM and fPKM varied the growth parameters values of BSFL. The fPKM group has the lowest individual weight (0.1195 ± 0.0012 g). Any levels of fBSFM improved individual weight and growth rate of BSFL compared to BSFL reared with fPKM. Various levels of the fBSFM 5-15% substrate groups significantly affected bioconversion and growth rate. The use of fBSFM higher than 5% enhanced the biomass of the BSFL. The highest percentage of substrate reduction (55.18 ± 8.49%) was found on the BSFL grown on fBSFM 15%. There was no significant difference in the BSFL survival which was reared using any substrate.

Various substrates given for BSFL did not significantly affect ($P>0.05$) all proximate parameters, including crude protein and crude fiber content. However, crude fat, moisture, and ash content may be considered significant based on the percentage form, which indicates a magnitude of more than 5% (Table 4).

Table 2. Proximate value of the various fermented substrates of the black soldier fly larvae

Parameters (%)	Groups			
	fPKM	**fBSFM 5%**	**fBSFM 10%**	**fBSFM 15%**
Crude protein	6.33	20.74	21.42	22.15
Crude fat	7.86	7.13	7.27	7.36
Moisture	33.14	13.81	13.56	13.24
Ash	5.27	4.36	4.51	4.70
Crude fiber	12.93	12.92	13.05	13.38

Noted: fPKM: 100% Fermented Palm Kernel Meal; fPKM: Fermented Various Ratios of Palm Kernel Meal (PKM) with Black Soldier Fly Meal (BSFM)

Table 3. The growth rate average value of BSFL fed various combinations of fermented black soldier fly meal and fermented palm kernel meal

Parameters	Groups			
	fPKM	**fBSFM 5%**	**fBSFM 10%**	**fBSFM 15%**
Individual weight (g)	0.1195 ± .0012[a]	0.1453 ± 0.0047[b]	0.1503 ± 0.0037[b]	0.1404 ± 0.005[b]
Biomassa (g)	3.07 ± 0.01[a]	3.41 ± 0.29[ab]	4.05 ± 0.15[b]	3.77 ± 0.23[b]
Substrate reduction (%)	42.73 ± 1.59[a]	52.88 ± 3.88[ab]	50.83 ± 0.71[ab]	55.18 ± 8.49[b]
Bioconversion ratio (%)	5.67 ± 0.01[a]	6.29 ± 0.55[ab]	7.47 ± 0.27[b]	6.95 ± 0.42[b]
Growth rate (g/day)	0.0119 ± .0001[a]	0.0145 ± 0.0005[b]	0.0150 ± 0.0004[b]	0.0141 ± 0.0005[b]
Survival (%)	95.56 ± 2.94[a]	100.00 ± 0.00[a]	95.55 ± 2.22[a]	94.44 ± 5.56[a]

Noted: fPKM: 100% fermented Palm Kernel Meal; fPKM: fermented various ratios of Palm Kernel Meal (PKM) with Black Soldier Fly Meal (BSFM). Mean ± SE followed by different superscripts (a, b) in the same row indicate significantly different between groups ($P<0.05$)

Table 4. Proximate average value of BSFL fed various combinations of fermented Palm Kernel Meat (fPKM) and fermented Black Soldier Fly Meal (fBSFM)

Parameters (%)	Groups			
	fPKM	fBSFM 5%	fBSFM 10%	fBSFM 15%
Crude protein	33.98 ± 0.002[a]	34.72 ± 0.007[a]	35.38 ± 0.005[a]	36.11 ± 0.002[a]
Crude fat	25.02 ± 2.05[a]	26.09 ± 0.22[a]	18.25 ± 0.66[a]	25.34 ± 3.74[a]
Moisture	7.40 ± 1.12[a]	5.35 ± 0.19[a]	7.01 ± 0.07[a]	6.44 ± 0.93[a]
Ash	6.75 ± 0.07[a]	7.42 ± 0.02[a]	7.93 ± 0.08[a]	7.09 ± 0.01[a]
Crude fibre	19.48 ± 0.86[a]	22.58 ± 1.02[a]	18.55 ± 1.24[a]	22.46 ± 2.44[a]

Note: C: Control (fermented 100% palm kernel meal); P1-P3: Treatment substrate groups, various ratio (g) fermented of PKM: BSF. P1: 950:50 (5%); P2: 925:75 (7.5%); P3: 900:100 (10%). Mean ± SE followed by different superscripts (a, b) in the same row indicates significant differences between groups (*P<0.05*)

Discussion

The utilization of Black Soldier Fly Larvae (BSFL) in organic waste valorization has garnered significant attention due to their ability to convert low-quality substrates into high-quality dietary protein (Kuznetsova et al. 2022). The BSFL can effectively consume various organic wastes, such as fermented Palm Kernel Meal (fPKM) (Nugroho et al. 2024). Previous research has also shown that black soldier fly larvae can be successfully reared on a diet consisting of palm kernel meal (Nugroho et al. 2023). Black soldier fly larvae can convert organic waste into valuable protein and fat, making them highly beneficial for the feed industry. The development of black soldier fly larvae with palm kernel meal can enhance the circular economy by repurposing agricultural waste and mitigating the environmental effects of trash disposal (Ganda et al. 2019).

This study, the first of its kind, explores the use of fermented Black Soldier Fly Carcass Meal (fBSFM) in combination with fPKM as a substrate for BSFL growth. The findings suggest that the fermentation process of the fPKM and fBSFM could lead to significant improvements in several nutritional parameters. The increase in crude protein with higher BSFM content indicates the potential of BSFM as a valuable protein source. The stable fat content suggests that the fermentation process does not significantly alter the lipid profile, which could be a promising finding. The reduction in moisture content with higher BSFM proportions could benefit storage and shelf-life. The slight decrease in ash content might indicate a change in mineral composition. Furthermore, the increase in crude fiber with higher BSFM content suggests that BSFM contributes to the fiber content of the substrate, which could have positive implications for the nutritional value of the substrate.

Fermentation, a crucial biological process, positively affects the proximate composition of various substrates used for cultivating BSFL. These larvae have emerged as a promising solution for efficiently converting organic waste into valuable biomass, with potential applications in animal feed (da-Silva et al. 2024) and biofuel production (Mohan et al. 2023). The proximate composition of the substrate, which includes moisture, protein, fat, fiber, and ash content, is a crucial factor in determining the nutritional value and suitability of the substrate for BSFL. Researchers have explored the use of diverse organic substrates, such as brewery wet grains, cattle dung, and rumen content, as viable options for BSFL production (Herlina et al. 2021).

Based on the growth data of BSFL maintained at the fPKM and various levels of fBSFM, it is known that individual weight and growth rate of BSFL showed a significant difference between BSFL maintained without fBSFM and with any levels of fBSFM. These findings are significant in the fields of entomology, agriculture, and waste management. The BSFL has a higher individual weight and growth rate in the substrate with the addition of fBSFM than without fBSFM. The addition of BSFM might increase the protein content of the substrate when compared to the control group without the addition of BSFM. The control group, which only had fPKM as a substrate for BSFL, had a low protein content of about 6.33%, resulting in a low larval growth rate (Table 2). The current finding is supported by Tschirner and Simon (2015), who revealed that BSFL grown on dry grain waste substrate with a protein content of 31.2% produced a greater individual weight of larvae (270.7 mg wet weight) compared to BSFL reared on dry beet waste substrate (8.5%), which only produced an individual weight of 34.8 mg. The protein content of the substrate also has a linear effect on larval weight, i.e., the higher the protein content of the black soldier fly larval substrate, the greater the larval weight produced (Anasya et al. 2022; Kieβling et al. 2023). Further, Joly (2018) also reported that the substrate in the form of a mixture of kitchen waste enhanced the growth of BSFL. Mixing different types of organic waste can increase heterogeneity and improve substrate quality due to improved nutrients, substrate structure, and substrate composition (Spranghers et al. 2017; Joly 2018).

Furthermore, the BSFL grown with fPKM and various levels substituting the fBSFM showed significantly better growth parameters. This indicates that substituting fPKM with fBSFM improved the individual weight, biomass, substrate reduction, bioconversion rate, and growth rate. The level substitution of fPKM with fBSFM at a level higher than 5% for BSFL growth enhances substrate nutrients, especially protein and fat, improving the rate of bioconversion and biomass in the BSFL. A similar study by Nash and Chapman (2014) revealed that Mediterranean fruit fly larvae (*Ceratitis capitata* (Wiedemann, 1824)) grown on a low-protein but high-carbohydrate substrate experienced significantly slower larval development. Based on the study by Singh et al. (2021) also mentioned that BSFL grown on substrate in the form of a mixture of organic waste had a higher substrate/waste reduction rate

(72%) compared to vegetable waste substrate without a mixture (61%).

Substituting fPKM using fBSFM in the form of BSFM may increase nutrients and energy content. The energy content of the BSFL's substrate affected the efficiency of the substrate reduction ability of the BSFL (Nguyen et al. (2015). In the fPKM substrate (Table 2), the crude protein content is relatively low, around 6.33%, due to the lack of available nutrients. This finding is supported by Gold et al. (2020), who mentioned that BSFL reared on cow dung waste substrate with a protein content of 11.1% had a lower substrate reduction percentage (12.7%) when compared to poultry feed substrate with a protein content of 19.1% and a substrate reduction value of 67.6%. Similarly, Naser El Deen et al. (2023) reported that BSFL reared on pig manure substrate with a mixture of grass silage (crude protein content of 8.22%) had a low substrate reduction index (1.08 g/day). This occurs due to the low availability of nutrients, especially protein, in livestock waste, which plays a significant role in the production of metabolic waste in livestock.

The survival rate of BSFL in all groups was not significantly different. The high survival rate is one parameter used to determine the feasibility of an organic material as a substrate for BSFL. Broeckx et al. (2021) stated that on a laboratory scale, the survivability and growth of BSFL can be influenced by the conditions during breeding, such as light, temperature, humidity, larval density, harvest time, substrate composition, and the physico-chemical content of the substrate used. Previous research has also indicated that BSFL reared on high-protein (22%) and high-fat (9.5%) substrates exhibited accelerated development and increased survivability (Oonincx et al. 2015).

This study showed that the proximate content of crude protein, crude fat, moisture content, ash content, and crude fiber in BSFL fed with the substitution of fBSFM to fPKM (5, 7.5, and 10%) substrate did not provide significant differences. This indicates that the addition of BSFM to the fPKM substrate did not affect the nutrient content of the BSFL and only affected the BSFL growth rate. This result is similar to the study by Eggink et al. (2022), who reported that BSFL reared on shrimp waste substrate (*Pandalus borealis* Krøyer, 1838) contains protein (36.2%) and fat (22.9%) levels did not show any significant difference with proximate of the BSFL. In addition, Nguyen et al. (2015), similarly reported that BSFL reared on vegetable, fruit (mixed), and fish (mixed) substrates had lower protein and fat contents, namely on vegetable and fruit substrates (12.9%; 2.22%) and on fish substrates (19.4%; 11.6%).

In conclusion, adding 5% BSFM has been shown to enhance both the growth rate and individual weight of the larvae. A concentration of 7.5% MSFM can further boost biomass and bioconversion rates. Furthermore, a 10% concentration enhances the substrate reduction ability, thereby making the processing of black soldier fly flour waste more efficient. The addition of BSFM can also support the life of black soldier fly larvae, as evidenced by a low mortality rate of larvae and no significant differences observed between treatments. The addition of BSFM with a concentration of 5-10% did not result in a significant difference in the mean values of nutritional components of BSFL, which were approximately 34.72-35.38% crude protein, and 18.55-22.58.7% crude fiber, but significant differences for 18.25-26.09% crude fat, 5.35-7.01% moisture, and 7.42-7.93% ash. Further, the protein content of 35.38% is potentially suitable for livestock feed, suggesting that utilizing adult BSF waste as a substrate mixture for BSFL is worth considering.

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Maximizing *Pleurotus ostreatus* yield with sustainable agricultural waste substrates in Arakan, Cotabato, Philippines

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Abstract. *Pedroso ML, Alagos ALC, Esgrina FJO, Habibun JF, Maun JN, Rodriguez RMM. 2025. Maximizing* Pleurotus ostreatus *yield with sustainable agricultural waste substrates in Arakan, Cotabato, Philippines. Nusantara Bioscience 17: 57-67.* Mushroom production supports a circular economy by transforming agricultural waste into valuable food resources while enhancing local agricultural economies. This study evaluates the potential of locally available agricultural waste substrates-chopped rice straw, aged rice hull, rough sawdust, and fine sawdust-for optimizing the production of oyster mushrooms (*Pleurotus ostreatus* (Jacq.) P.Kumm. in Arakan, Cotabato, Philippines. The effects of these substrates on key growth parameters, including mycelial growth, fruiting time, yield, and economic returns, were assessed. A Completely Randomized Design with five replicates per treatment was employed, using a substrate mixture of 89% agricultural waste, 10% molasses, and 1% lime. Statistical analysis revealed that fine sawdust facilitated faster mycelial growth and reduced fruiting time, whereas rough sawdust produced higher yields and larger mushrooms. Economic analysis indicated that rough sawdust yielded the highest return on investment at 190.21%, making it the most cost-effective substrate. These findings demonstrate the potential of agricultural waste as a sustainable substrate for oyster mushroom cultivation, promoting environmental sustainability and profitability for small-scale farmers. The study emphasizes the critical role of substrate selection in optimizing mushroom yield and provides practical insights for commercial mushroom production.

Keywords: Bioefficiency, mushroom substrate, oyster mushroom, sawdust, sustainable farming

INTRODUCTION

Mushrooms constitute a diverse group of fungi valued for their nutritional, medicinal, and ecological benefits. They are an important source of nutrition, particularly in regions facing food security challenges, due to their high protein content, low-fat levels, and bioactive compounds with health-promoting properties (Valverde et al. 2015; Hyde et al. 2019; Ślusarczyk et al. 2021). Among cultivated species, *Pleurotus ostreatus* (Jacq.) P.Kumm. (oyster mushroom) is particularly sought after for its rapid growth, adaptability to various organic waste substrates, and potential as a cost-effective protein alternative to meat (Khan et al. 2024; Pashaei et al. 2024). Additionally, oyster mushroom farming supports sustainable agriculture by utilizing agricultural waste, contributing to environmental conservation efforts (Akter et al. 2022; Aditya et al. 2024).

Despite these advantages, oyster mushrooms are highly perishable and require immediate consumption or preservation to maintain quality (Castellanos-Reyes et al. 2021; Dawadi et al. 2022). This perishability has driven efforts to enhance production efficiency, particularly for *P. ostreatus*, which thrives on low-cost agricultural waste such as rice straw, sawdust, and other organic by-products (Chauhan et al. 2024). The rapid colonization of substrates and minimal maintenance requirements make oyster mushrooms an attractive option for smallholder farmers seeking an additional income source while reducing waste (Dung et al. 2012; Jayaraman et al. 2024).

In the Philippines, oyster mushroom cultivation has gained prominence due to the country's favorable climate and abundant agricultural waste, which serve as suitable substrates (Chang et al. 2014; Alvarez and Bautista 2021). Converting agricultural waste into valuable food resources not only increases farmers' incomes but also reduces waste, contributing to environmental sustainability (Hoa et al. 2018; Riseh et al. 2024).

A critical factor influencing mushroom production is substrate selection. Substrate composition affects mycelial growth, fruiting, and overall yield. Various organic materials, including rice straw, sawdust, and sugarcane bagasse, have been evaluated for oyster mushroom cultivation, as they influence the carbon-to-nitrogen ratio, moisture retention, and aeration necessary for optimal growth (Hossain et al. 2005; Nithyatharani and Kavitha 2018; Bebarta et al. 2022). However, limited research has examined the comparative efficiency of locally available substrates under the specific environmental conditions of Arakan, Cotabato, Philippines. Arakan experiences warm temperatures year-round, ranging from 25 to 32°C (77 to 90°F), which exceeds the optimal fruiting range of 18 to 24°C for *P. ostreatus*. Consequently, this study implemented temperature regulation through shading and misting to optimize yields.

This study aims to evaluate the effects of four agricultural waste substrates- chopped rice straw, aged rice hull, rough sawdust, and fine sawdust on the growth and yield of *P. ostreatus* in Arakan, Cotabato, Philippines. Consistent with previous studies (Sanjel et al. 2021; Soriano and Mangune 2022; Latif et al. 2023), a controlled and uniform amount of molasses and lime was incorporated into the substrates. Molasses provides organic sugars essential for mycelial growth (Afify et al. 2012; Sughra et al. 2013) and enhances fungal colonization by supplying carbohydrates (Mangwanda et al. 2023), thereby improving substrate utilization efficiency. Lime was added primarily to increase the pH of the substrates, creating an alkaline environment that inhibits pathogenic growth while fostering optimal conditions for oyster mushrooms (Khan et al. 2013; Taylor et al. 2016).

The study assessed key growth parameters, including mycelial growth, fruiting time, and overall yield, to determine the most effective substrate for optimizing mushroom production in the region. Given that these substrates are locally available and often discarded, their utilization offers a cost-effective and sustainable solution for mushroom cultivation. Additionally, a financial analysis was conducted to calculate the Return On Investment (ROI) for each substrate, providing farmers and agribusinesses with valuable insights on maximizing profitability while promoting sustainable farming practices.

The findings contribute to the growing body of research on sustainable agriculture and mushroom cultivation, particularly within the Philippine agricultural context. Unlike broader studies that examine generic substrates, this research specifically investigates locally available agricultural waste in Arakan, Cotabato, offering region-specific recommendations for enhancing oyster mushroom production. The comparative analysis of rough and fine sawdust, combined with a financial ROI evaluation, provides new insights into optimizing yield and profitability. By refining substrate selection, this study aims to improve yields, promote environmentally responsible practices, and enhance the economic sustainability of mushroom farming. Furthermore, this approach aligns with the principles of a circular economy by reducing waste and repurposing agricultural by-products. In support of the United Nations Sustainable Development Goals (SDGs), this study contributes to SDG 12 (Responsible Consumption and Production) by promoting agricultural waste utilization, SDG 13 (Climate Action) by advocating sustainable farming practices, and SDG 8 (Decent Work and Economic Growth) by improving the economic prospects of smallholder farmers. Additionally, this study contributes to Sustainable Development Goal 2 (Zero Hunger) by enhancing food security through sustainable and locally-sourced protein production.

MATERIALS AND METHODS

Study area

This study was conducted in a controlled mushroom production facility at the College of Agriculture, Agribusiness, Forestry, and Food Sciences, Cotabato Foundation College of Science and Technology (CFCST), located in Doroluman, Arakan, Cotabato, Philippines (7°20,48”N, 125°05’38”E, 482.3 masl) (Figure 1). The mushroom house measured 5×10 m, with a designated growing area of 4×8 m, accommodating 500 fruiting bags arranged following a Completely Randomized Design (CRD). The effects of four agricultural waste substrates-chopped rice straw (T1, control), aged rice hull (T2), rough sawdust (T3), and fine sawdust (T4)-were evaluated, with each treatment replicated five times.

Experimental design

A Completely Randomized Design (CRD) was implemented to minimize bias and ensure statistical reliability. Each of the four substrate treatments was tested with five replications to account for variability and enhance result reproducibility.

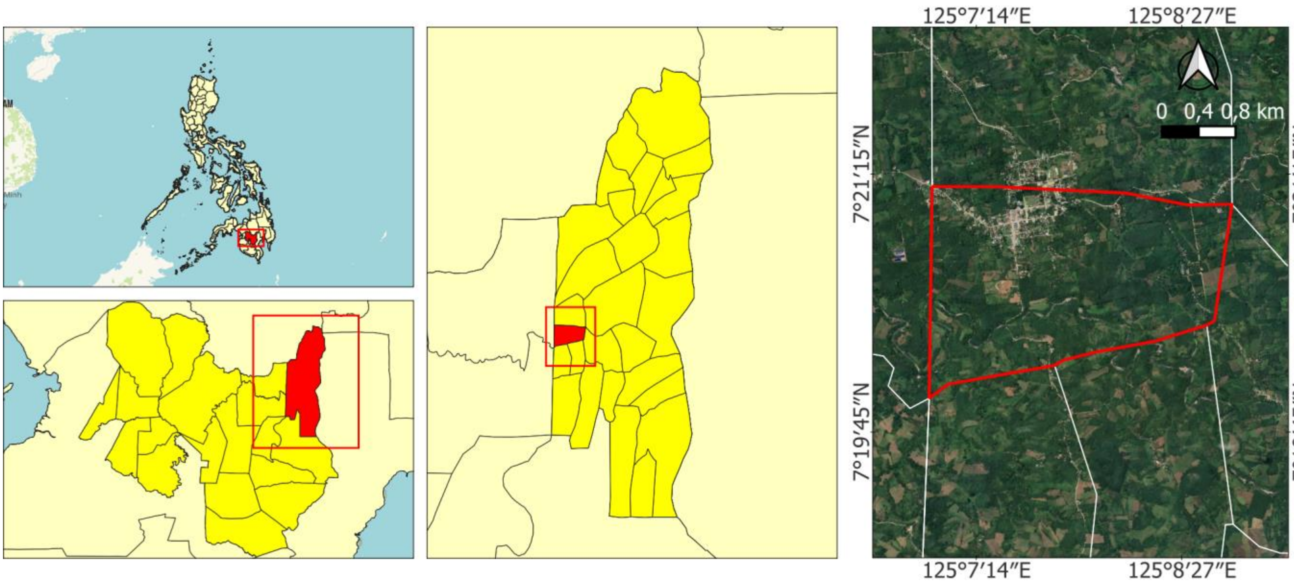

Figure 1. The location map of the experimental area at Doroluman, Arakan, North Cotabato, Philippines

Climatic conditions and cultivation

Oyster mushroom cultivation was conducted in a climate-controlled environment, where temperature, humidity, and irrigation were systematically regulated. The temperature ranged from 22 to 26°C, while relative humidity was maintained at 85-90% throughout the incubation and fruiting stages. Watering was performed twice daily to sustain optimal substrate moisture levels, ensuring successful mycelial growth and fruiting body development.

Procedures

Substrate preparation

Each 500 g fruiting bag was filled with a substrate mixture comprising 89% agricultural waste material (based on treatment), 10% molasses, and 1% lime (Figure 2.A). Molasses served as an organic sugar source, enhancing oyster mushroom colonization and substrate utilization efficiency. Lime increased substrate alkalinity, creating favorable conditions for oyster growth while inhibiting harmful pathogens. The substrates were dried to approximately 65% moisture content at 60-70°C to optimize water retention for fungal colonization. After drying, substrates underwent pasteurization and sterilization in an autoclave (121°C, 1 hour, 1.5 kg/cm² pressure) to eliminate microorganisms. Pasteurization aims to reduce the microbial load, while sterilization through autoclaving ensures the total elimination of microorganisms in the material.

Table 1 presents nutrient analyses on various substrates used for oyster mushroom cultivation, highlighting their suitability based on nutrient composition. Rice straw contains moderate amounts of nitrogen, phosphorus, and potassium, along with high silicon, which supports mycelial growth and fruiting. However, its low nitrogen content may require supplementation with nitrogen-rich materials to enhance growth. Rice husks, rich in carbohydrates and fiber, provide a good energy source for the mycelium, but their low crude protein and nitrogen make them less ideal unless supplemented. Tree sawdust, although not fully detailed in terms of nutrient composition, typically requires nitrogen supplementation to optimize growth due to its high lignin and cellulose content.

Sawdust of mahogany wood (*Swietenia macrophylla* G.King) offers high dry matter and energy content, but its low nitrogen means it also needs supplementation for healthy fungal growth. The crude fiber and nitrogen-free extract provide energy, while its mineral content is modest. Tamarind (*Tamarindus indica* L.), with high fiber but lower protein and nitrogen-free extract, is more suited for energy provision than protein, requiring nitrogen supplementation for improved growth efficiency. Sawdust of mango wood (*Mangifera indica* L.), offering higher crude protein and nitrogen-free extract, is a better substrate than tamarind for faster mycelial growth with less nitrogen supplementation. It also contains substantial fiber.

Therefore, substrates like rice straw and sawdust are energy-rich but low in nitrogen, requiring supplementation for optimal oyster mushroom growth. On the other hand, substrates like mango and tamarind have better nitrogen content, reducing the need for additional additives. Table 2 emphasizes the importance of nutrient balance and supplementation to achieve high yields in oyster mushroom cultivation. Thus, adding a uniform amount of molasses to different substrates would provide initial energy, helping substrates like rice husks and sawdust that are low in nitrogen. In comparison, an equal amount of lime would improve the pH balance of these different substrates, supporting healthier mycelial growth. Together, these additives can enhance the quality of the substrates, leading to more efficient colonization, higher yields, and better fruiting in oyster mushroom cultivation.

Grain spawn acquisition

The grain spawn for oyster mushrooms was obtained from the Department of Agriculture Regional Office XII, Balindog Research and Experiment Station in Kidapawan City, Cotabato. This government agency is known for producing high-quality oyster mushroom grain spawn, which is distributed to growers and farmers across surrounding municipalities and provinces in South-Central Mindanao, Philippines.

Inoculation

Following sterilization, fruiting bags were cooled for 48 hours (Figure 2.B). During this period, all inoculation tools (e.g., forceps, scalpels, trays) were sterilized using alcohol or flame treatment. Oyster mushroom grain spawn (20-30 grains per bag) was inoculated (Figure 2.C) using sterile forceps. The inoculated bags were sealed and transferred to a controlled incubation chamber, where temperature, humidity, and airflow were monitored to optimize mycelial growth.

Incubation and fruiting

The incubation period lasted 30-45 days (Figure 2.D). Bags were inspected regularly for contamination, discoloration, or stunted growth, while environmental conditions were carefully regulated. Once full mycelial colonization was observed, fruiting was initiated by adjusting humidity levels and spraying bags two to three times daily to maintain optimal moisture (Figure 2.E). Mushrooms were harvested daily by gently twisting and pulling the fruiting bodies without damaging the remaining mycelium. Early blooms were removed to prevent obstruction to developing fruiting bodies (Figure 2.F).

Preparation for marketing

Post-harvest, mushrooms were packaged into 100 g portions and sold in the open market at 35.00 Philippine Pesos per pack or three packs for 100.00 Philippine Pesos as part of a promotional pricing strategy.

Data collection

Key growth parameters were recorded throughout the harvesting period, with data collected from ten randomly selected samples per treatment for systematic tabulation and analysis.

Table 1. Nutrient analyses of the different substrates used for the production of oyster mushroom

Substrate type	Nutritional components	References
Rice straw	N (0.65%); P_2O_5 (0.225%); K_2O (1.68%); Ca (0.3%); Mg (0.2%); S (0.075%); Si (5.5%); Zn (0.003%); Fe (0.035%); Mn (0.045%); Cu (0.003%); Bo (0.001%)	Dobermann and Fairhurst (2000)
Rice hull/rice husk	carbohydrate (37.04%); crude protein (1.85%); moisture (7.93%); crude fat (3.76%); fiber (25.74%); ash content (23.39%); Ca (0.002949%); Mg (0.001932%); K (0.004828%); Na (0.000281%); Zn (0.000613%); Fe (0.001401%); Se (0.000125%); Co (0.000185%); Mn (0.001154%); Cu (0.000985%); P (0.000402%)	Nnadiukwu et al. (2023)
Tree sawdust		Hossain et al. (2012)
Swietenia macrophylla G.King	DM (95%); ME (1611.7 kcal/kg); CP (2.5%); CF (46%); NFE (44.6%); EE (1.2%); TA (0.7%)	*DM-Dry Matter *ME-Metabolized Energy
Tamarindus indica L.	DM (92.4%); ME (615 kcal/kg); CP (2.4%); CF (66%); NFE (15.8%); EE (0.6%); TA (7.6%)	*CP-Crude Protein *CF-Crude Fiber *NFE-Nitrogen Free Extract
Mangifera indica L.	DM (93%); ME (967.5 kcal/kg); CP (3.5%); CF (63%); NFE (24.5%); EE (1.0%); TA (1.0%)	*EE-Ether Extract *TA-Total Ask

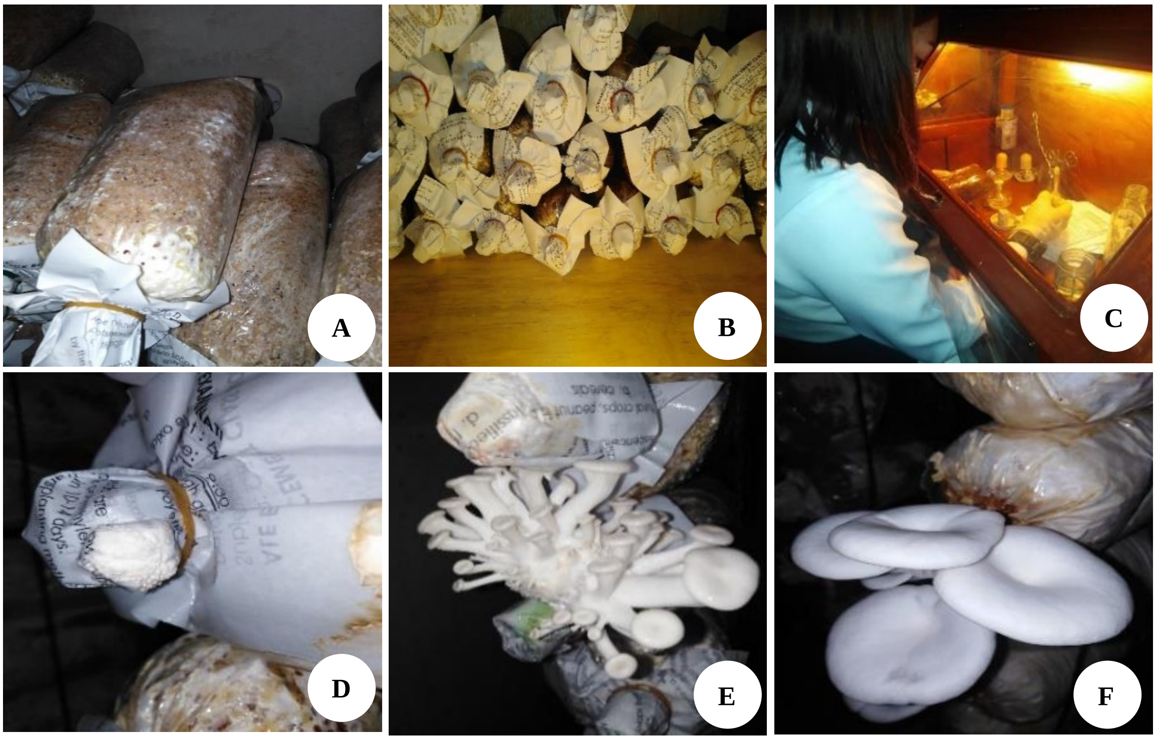

Figure 2. Overview of the substrate preparation, inoculation, and incubation process. A. Mixing of substrates; B. Inoculation of bags; C. Incubation phase; D. E. F. Fruiting initiation

Data analysis

Statistical analysis

Data were analyzed using the Statistical Tool for Agricultural Research (STAR-2.0.1) within a Completely Randomized Design (CRD) framework. The effects of different agricultural waste substrates on oyster mushroom yield and growth characteristics were assessed for statistical significance. A post hoc Least Significant Difference (LSD) test was performed to identify significant treatment differences.

Economic analysis

Next, to evaluate the financial viability of each substrate, the study calculated the Return on Investment (ROI) using the following formula:

$$\text{Return on Investment (ROI)} = \frac{\text{Net Income} \times 100}{\text{Production Cost}}$$

Where: Net Income = Total Sales - Total Production Costs; Production costs include all expenses related to substrate preparation, inoculation, environmental control, and labor.

The Return on Investment (ROI) was calculated by dividing the net income by the total production cost, using the formula: ROI (%) = (Net Income / Total Production Cost) × 100. Net income was determined by subtracting the total production cost from the total revenue. The total production cost included both direct and indirect expenses incurred during mushroom cultivation. Direct costs accounted for energy consumption, which was calculated based on electricity usage for pasteurization and control of the environment, along with water usage quantified by the total liters used for irrigation. Labor costs were estimated based on the number of working hours dedicated to substrate preparation, inoculation, incubation, and harvesting. Additionally, substrate preparation costs included the expenses for raw materials such as agricultural waste, molasses, and lime, as well as sterilization costs. This ROI calculation provided insights into the profitability and cost-effectiveness of each substrate, helping to identify the most sustainable and economically viable method for oyster mushroom cultivation.

The economic analysis such as; Break-Even Point (BEP), Payback Period, Net Present Value (NPV), and Internal Rate of Return (IRR), were used to determine the profitability of the oyster mushroom production. The data were analyzed using manual formulas for NPV and IRR. Additionally, spreadsheet software like Microsoft Excel was used to compute the IRR using the built-in IRR function and NPV with different discount rates. Here's a detailed breakdown:

Break-Even Point (BEP): This calculates the number of units (or monetary value) needed to cover the total expenses. It was determined using the formula:

$$\text{BEP (Units)} = \frac{\text{Total Expenses}}{\text{Price per Unit}}$$

Payback Period: It is the time taken for the initial investment to be recouped through the net profits. It is computed by dividing the initial investment by the monthly profit:

$$\text{Payback Period (Months)} = \frac{\text{Initial Investment}}{\text{Monthly Profit}}$$

Net Present Value (NPV): Net Present Value (NPV) is a financial metric used to determine the value of a series of future cash flows (profits or revenues) in today's terms, taking into account the time value of money. It is essential for evaluating the profitability of investments. This was calculated by discounting future cash flows at a rate of 10% per month. The formula used was:

$$NPV = \sum \left(\frac{CFt}{(1+\mathrm{r})t}\right) - I$$

Where: $\boldsymbol{CF_t}$ is the cash flow at time **t**, **r** is the discount rate, and $\boldsymbol{I}$ is the initial investment.

A positive NPV indicates a profitable investment, where the returns exceed the cost of capital. While, a negative NPV would indicate that the investment will not recover the cost and will result in a loss.

Internal Rate of Return (IRR): This was calculated using an iterative process, where the NPV is set to zero and the discount rate that satisfies this condition is found. The IRR formula is:

$$0 = \sum \left(\frac{CFt}{(1+IRR)t}\right) - I$$

If the IRR is greater than the discount rate (e.g., 10%), the investment is profitable because it exceeds the required rate of return. However, if the IRR is lower than the discount rate, the investment is not profitable as it does not generate enough return to justify the costs.

RESULTS AND DISCUSSION

Oyster mushroom growth characteristics and yield performance

The growth characteristics and yield performance of oyster mushrooms cultivated on different agricultural waste substrates are summarized in Tables 2 and 3. Key parameters measured included mycelial growth length after planting, the number of flushes per batch and per bag, the number of flushes post-planting, fruit weight (g), mushroom cap circumference (cm), stalk length (cm), and overall yield performance. Each substrate formulation consisted of 89% agricultural waste, 10% molasses, and 1% lime, ensuring a balanced nutrient composition for optimal mycelial development and fruiting body formation. The variation in these growth parameters across different substrates highlights the influence of substrate composition on mushroom productivity and quality.

Mycelial growth in 1st and 2nd week after planting

Significant differences in mycelial growth were observed across the different substrates (Tables 2 and 3). At one and two weeks after planting, the fine sawdust substrate (T4) produced the longest mycelial growth, with mean lengths of 3.47 and 7.46 cm, respectively. In contrast, the rice hull substrate (T2) exhibited the shortest mycelial growth, with mean lengths of 2.35 and 5.47 cm, respectively. The control substrate, rice straw (T1), showed moderate performance. Using fine sawdust increased colonization time by 15.66% at two weeks and 18.84% at one week. This suggests that fine sawdust promotes faster and more extensive mycelial expansion (Miah et al. 2017), which is important for subsequent mushroom development. A well-established mycelial network enhances nutrient absorption and accelerates the transition to fruiting stages (Stanley and Awi-Waadu 2010; Ufitinema et al. 2023), with fine sawdust likely contributing to optimal fungal colonization due to its texture and aeration properties (Abiodun et al. 2022; Hultberg and Golovko 2024).

Number of days to flush after planting

The time required for the first flush varied significantly among substrates (Tables 2 and 3). The fine sawdust substrate (T4) resulted in the shortest flush time, averaging 37.92 days, while the rice straw substrate (T1) had the longest flush time at 50.86 days. The 25.44% reduction in flush time with fine sawdust highlights its efficiency in initiating fruiting, allowing shorter production cycles and more frequent harvests. This may be attributed to superior moisture retention and microbial composition in fine sawdust, which creates an optimal environment for primordia formation (Horisawa et al. 1999). Shorter flush times also reduce labor and operational costs, making fine sawdust a practical choice for commercial production (Ejigu et al. 2022; Argaw et al. 2023).

Number of flushes of fruitbodies per bag

The number of flushes per bag, a key indicator of productivity, varied significantly (Tables 2 and 3). The rough sawdust substrate (T3) had the highest flushes per bag, with a mean of 10.60, whereas aged rice hull (T2) produced the fewest at 4.46. Compared to the control (T1: rice straw), rough sawdust, supplemented with 10% molasses and 1% lime, increased fruit production by 107.03%. This demonstrates that wood-based substrates enhance fruiting potential (Sofi et al. 2014; Tarko and Sirna 2018; Nwaogu et al. 2024). Higher flush numbers sustain market supply and increase profitability, likely due to improved aeration and substrate stability in rough sawdust (Jackson 2018; Oyebanji et al. 2021).

Length of stalk (cm)

Mushroom stalk length influences consumer preference and marketability. Tables 2 and 3 indicate that the rough sawdust substrate (T3) produced the longest stalks, averaging 6.13 cm, while rice straw (T1) yielded the shortest. Using rough sawdust increased stalk length by 29.32%, enhancing visual appeal and handling ease. Longer stalks often correlate with improved nutrient uptake and substrate utilization (Kinge et al. 2016; Onyeka et al. 2018), supporting higher biomass accumulation and yield potential (Megersa and Tolessa 2024).

Circumference of fruit (cm)

The study measured cap circumference to assess the effect of different substrates on fruit size. Tables 2 and 3 show that the widest caps were produced on the fine sawdust substrate (T4), with a mean circumference of 26.08 cm, whereas the smallest were observed in mushrooms grown on rice straw (T1) at 18.30 cm. This 42.51% increase in cap size with fine sawdust highlights its effectiveness in promoting larger fruit bodies, which enhance marketability (De Cianni et al. 2023). Larger caps are preferred due to their visual appeal, cooking versatility, and potential nutritional benefits (Das et al. 2021). The improved cap development in fine sawdust may be linked to superior aeration, moisture retention, and nutrient availability (Marinou et al. 2013; Kinge et al. 2016).

Table 2. Oyster mushroom agronomic characteristics and yield performance using different agricultural waste materials (89% substrate, 10% molasses, and 1% lime) from a CRD experiment with 4 (T) Treatments and 5 (R) Replications

Treatments	Length of mycelia (cm)		No. of days to flush	No. of flushes/bag	Length of stalk (cm)	Circumference of cap (cm)	Fruit weight (cm)	Total yield (g)
	1 week	2 weeks						
T1-Rice straw	2.92[b]	6.45[b]	50.86[a]	5.12[bc]	4.74[c]	18.30[c]	13.64[b]	136.38[b]
T2-Aged rice hull	2.35[c]	5.47[c]	48.26[b]	4.46[c]	5.31[bc]	19.54[c]	16.28[b]	158.54[b]
T3-Rough sawdust	2.95[b]	5.58[c]	40.86[c]	10.60[a]	6.13[a]	23.03[b]	36.49[a]	364.94[a]
T4-Fine sawdust	3.47[a]	7.46[a]	37.92[d]	7.02[b]	5.59[ab]	26.08[a]	35.79[a]	357.92[a]
F-Test value	**500.65**	**253.38**	**456.05**	**16.58**	**6.96**	**15.01**	**35.61**	**36.60**
F-Tab value	**5.95**	**5.95**	**5.95**	**5.95**	**5.95**	**5.95**	**5.95**	**5.95**
CV (%)	**1.57**	**2.27**	**1.43**	**22.26**	**9.02**	**9.35**	**18.01**	**18.00**
F-Test > F-Tab	**	**	**	**	**	**	**	**

Note: The means in a column with the same letter superscripts are not significantly different based on the Least Significant Difference (LSD) test at the 1% level. The coefficient of variation (C.V.) expresses the degree of variation relative to the mean for each growth parameter, providing insights into the consistency of the data. A highly significant (**) difference was observed between treatments, as indicated by the F-test, where the computed F-value exceeded the F-tabulated value at a 1% significance level. The high F-coefficients further confirm that the differences among substrate treatments significantly influenced oyster mushroom growth characteristics and yield performance.

Table 3. Significance testing of growth and yield parameters in oyster mushrooms under different substrate treatments

Parameters	F-test Value	F-tab Value	Comparison	Conclusion
Mycelial growth (1 week)	500.65	5.95	F-test > F-tab	Significant difference
Mycelial growth (2 weeks)	253.38	5.95	F-test > F-tab	Significant difference
Days to flush	456.05	5.95	F-test > F-tab	Significant difference
Number of flushes per bag	16.58	5.95	F-test > F-tab	Significant difference
Stalk length	6.96	5.95	F-test > F-tab	Significant difference
Cap circumference	15.01	5.95	F-test > F-tab	Significant difference
Fruit weight	35.61	5.95	F-test > F-tab	Significant difference
Total yield	36.6	5.95	F-test > F-tab	Significant difference

Fruit weight (g) per bag

Fruit weight per bag is a vital metric for assessing yield efficiency. Tables 2 and 3 reveal that rough sawdust (T3) resulted in the heaviest fruits, with a mean weight of 36.49 g, followed closely by fine sawdust (T4) at 35.79 g. Both significantly outperformed rice straw (T1), which yielded the lightest mushrooms. Mushrooms grown on rough sawdust were 167.57% heavier than those on rice straw, demonstrating the benefits of sawdust-based substrates for enhancing fruit weight. This aligns with studies showing that lignocellulosic substrates provide a stable, nutrient-rich environment for mushroom growth (Kumla et al. 2020; Boadu et al. 2023; Sour et al. 2024).

Total yield (g)

Total yield is a key measure of productivity. According to Tables 2 and 3, the rough sawdust substrate (T3) produced the highest total yield at 364.94 g per bag, significantly higher than the 136.38 g per bag recorded for rice straw (T1). This 167.60% increase highlights the superior efficiency of rough sawdust in oyster mushroom production. The high yield potential of rough sawdust may be attributed to its high cellulose and hemicellulose content, which serve as primary energy sources for fungal growth (Ahmed and Mohesien 2020; Kaoke et al. 2024). Its coarser texture also facilitates better air circulation, reducing compaction and supporting sustained fruiting. These findings suggest that selecting the appropriate substrate is critical for optimizing yield and improving the overall efficiency of oyster mushroom farming.

Return on Investment (ROI)

Table 4 summarizes the return on investment (ROI) analysis for oyster mushroom production across different substrates. This table presents a detailed income statement for 1,000 fruiting bags over a three-month production cycle, offering insights into the financial viability of each substrate. ROI serves as a crucial performance indicator, assessing the profitability and cost-effectiveness of various cultivation methods. Understanding these financial dynamics helps growers optimize production strategies for maximum economic returns.

Among the tested substrates, rough sawdust (T3) demonstrated the highest financial viability, yielding a net income of 17,939.25 pesos and an impressive ROI of 190.21%. This result highlights the efficiency and profitability of rough sawdust as a substrate for oyster mushroom cultivation. Closely following, fine sawdust (T4) generated a net income of 17,412.75 pesos, achieving an ROI of 184.63%. These high returns highlight the economic advantage of using sawdust, whether rough or fine-textured, as an optimal substrate for commercial production (Chen et al. 2020; Adams et al. 2022).

In contrast, the aged rice hull substrate (T2) produced a significantly lower net income of 2,709.25 pesos, resulting in an ROI of 29.51% (Table 4). While less efficient than sawdust, aged rice hulls still provided a positive return, suggesting they can serve as an alternative substrate, albeit with limitations. The lower profitability of rice hulls may be attributed to their reduced structural stability, lower organic matter content, and limited capacity for sustained fruiting across multiple flushes (Okigbo et al. 2021; Costa et al. 2023; Kordi et al. 2024).

The control substrate, chopped rice straw (T1), yielded the lowest net income at 1,547.25 pesos, with an ROI of 17.82% (Table 4). This modest return highlights the limited economic feasibility of using rice straw compared to other tested substrates. The lower performance of rice straw may be linked to its lower nutrient availability, rapid decomposition rate, and reduced moisture retention capacity, all of which can negatively impact mushroom yield, growth, and overall profitability (Sitaula et al. 2018; Muswati et al. 2021; Subedi et al. 2023).

Discussion

Effect of substrate composition on mushroom growth and yield

The findings of this study demonstrate that substrate composition significantly influences the growth performance and yield of oyster mushrooms (*P. ostreatus*). Statistical analyses confirmed that all treatments exhibited highly significant effects, with P-values of 0.00-well below the 0.01 significance threshold. The high F-test values further support this conclusion, particularly for mycelial length after one week (F = 500.65) and two weeks (F = 253.38), number of days to flush (F = 456.05), number of flushes per bag (F = 16.58), stalk length (F = 6.96), cap circumference (F = 15.01), fruit weight (F = 35.61), and overall yield (F = 36.60). These results highlight the pivotal role of substrate composition in optimizing oyster mushroom cultivation.

Table 4. Income statement of one thousand (1,000) fruiting bags of oyster mushroom production for three months

Particulars	T1-chopped rice straw (control)	T2-aged rice hull	T3-rough sawdust	T4-fine sawdust
Gross income	10, 228.50	11, 890.50	27, 370.50	26, 844.00
Total expenses	8,681.25	9, 181.25	9, 431.25	9, 431.75
Net income	1, 547.25	2, 709.25	17, 939.25	17, 412.75
ROI (%)	17.82%	29.51%	190.21%	184.63%

Note: Assumption = Suggested retail price is 35.00 Philippine pesos/100 g fruiting bag/3 months production

These findings align with previous research emphasizing the importance of substrate selection. Naeem et al. (2014) reported that sawdust-based substrates enhance growth rates and increase stalk heights in *P. ostreatus*, supporting this study's observations. Similarly, Islam et al. (2009), Orngu et al. (2021), and Hultberg et al. (2023), identified sawdust as the most effective medium for mycelial colonization and fruit body development. The direct influence of substrate composition on both mycelial expansion and fruiting body development is well established, reinforcing the current study's results. Additionally, Buah et al. (2010), Getachew et al. (2019), and Grimm et al. (2024) reported that oyster mushrooms typically begin fruiting within three to eight weeks, depending on the substrate used-consistent with the flush cycles observed in this study. These findings collectively suggest that substrate selection is crucial not only for maximizing yield but also for ensuring predictable and efficient production cycles.

Comparison of substrates: Sawdust vs. rice straw and rice hull

The study results indicate that oyster mushrooms cultivated on sawdust-based substrates, particularly rough sawdust, exhibited superior growth and yield compared to other tested substrates. Argaw et al. (2023) and Naeem et al. (2014) previously observed that *P. ostreatus* grown on sawdust yielded the highest number of fruiting bodies, a trend corroborated by the present study. Sawdust appears to provide an optimal environment for mycelial colonization and fruiting, as also noted by Shah et al. (2004), Bhattacharjya et al. (2014), Adams et al. (2022), and who reported biological efficiency values as high as 65.22% when using sawdust substrates.

Furthermore, Bhattacharjya et al. (2014), Besufekad et al. (2019), and Pedroso et al. (2024) found that sawdust substrates produced larger cap circumferences compared to straw-based substrates, which typically yielded smaller fruiting bodies (Rambey et al. 2020). These observations reinforce the idea that sawdust not only enhances growth rates but also promotes the development of larger, more commercially viable mushrooms. Studies by Pardeep et al. (2002) and Varghese and Amritkumar (2020) further established sawdust as the most effective substrate for maximizing mushroom production. Shah et al. (2004) and Pathmashini et al. (2008) also emphasized the superior bioefficiency of sawdust compared to other agricultural waste materials. These consistent findings highlight the advantages of sawdust-based substrates in terms of both productivity and economic return.

Economic implications: Return on Investment (ROI)

Beyond its biological advantages, sawdust-based substrates also demonstrated significant economic benefits. This study found that rough sawdust yielded the highest net income and ROI, representing a 172.39% increase compared to the control substrate. These findings emphasize the importance of selecting high-yielding substrates to enhance both production efficiency and financial viability in mushroom farming (Dulay et al. 2021; Suwannarach et al. 2022). The economic advantages of rough sawdust make it a highly attractive option for both small-scale and commercial mushroom growers seeking to maximize profitability.

The economic potential of oyster mushroom farming is particularly relevant within the Philippine agricultural sector. Historical production challenges, including high input costs and inconsistent cultivation practices, have led to declines in national mushroom production (Chang et al. 2014). However, adopting high-yielding substrates such as rough sawdust may help reverse these trends by increasing yield potential while reducing production costs. The widespread use of cost-effective substrates could support industry revitalization, benefiting both smallholder farmers and larger agribusinesses by ensuring sustainable production and improved financial returns.

Sustainability and waste reduction

Utilizing agricultural waste materials as mushroom substrates presents an opportunity for both economic and environmental sustainability. This study demonstrates that repurposing sawdust and rice hulls for mushroom cultivation can significantly improve yield and profitability while reducing organic waste accumulation. By adopting sawdust-based substrates, mushroom farmers can lower production costs and increase financial returns while simultaneously contributing to sustainable farming practices.

The use of agricultural by-products in mushroom cultivation aligns with the principles of circular economy and resource efficiency. Repurposing these materials helps mitigate environmental impact by diverting organic waste from landfills, promoting a more sustainable agricultural model. Additionally, sawdust and rice hull substrates are readily available and cost-effective, making them viable options for mushroom growers in regions with limited access to traditional cultivation materials. These findings highlight the potential for integrating sustainability into commercial mushroom farming, supporting both economic development and environmental conservation.

In conclusion, this study highlights the critical role of substrate selection in optimizing oyster mushroom yield and profitability. Rough sawdust emerged as the most effective substrate, offering the highest yield and ROI, making it particularly suitable for commercial production. Fine sawdust also demonstrated potential for enhancing mycelial growth, providing an alternative for growers seeking a faster production cycle. Although aged rice hulls and rice straw yielded lower returns, they remain viable alternatives, particularly in areas where sawdust is less accessible. By utilizing agricultural waste materials, this study supports the United Nations Sustainable Development Goals (SDGs), particularly SDG 12 (Responsible Consumption and Production) and SDG 13 (Climate Action), by promoting sustainable agricultural practices and waste reduction. Sawdust-based substrates not only enhance economic returns but also contribute to environmental sustenance. Future research should explore additional alternative substrates, such as corn cobs and coffee grounds, to further improve sustainability and profitability in mushroom farming. Expanding the scope of substrate testing could provide valuable insights into optimizing mushroom production while advancing resource-efficient agricultural practices.

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Metal concentration and environmental risk assessment of roadside soils in highly urbanized areas of Bukidnon, Philippines

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Abstract. *Labajo JRN, Cristobal JU, Pabiona MG, Tan RS, Gutierrez-Suson J, Ebare-Tiongco L. 2025. Metal concentration and environmental risk assessment of roadside soils in highly urbanized areas of Bukidnon, Philippines. Nusantara Bioscience 17: 68-83.* Five urbanized localities in Bukidnon were selected based on population density and vehicular activities. Soil samples were collected, and metal concentration was determined using AAS, sediment quality guidelines, and the contamination index. The study assessed the metal concentration and environmental risk assessment of soil along the road from five localities in the Province of Bukidnon: It was observed that the highest total concentration for Mn (229.46±21.26), Fe (199.87±51.78), Co (38.13±16.18), Zn (32.43±19.13), Cu (18.17±4.52), Cr (17.95±1.76), and Cd (0.901±0.063) mg kg^{-1} respectively, Mn, exhibited the highest (lgeo), of "moderately contaminated" level. Co was the highest CF value corresponding to "moderate contamination." The Pollution Load Index (PLI) value of all sampling sites was "perfection or low pollution index." Mn has the highest EFs of "moderately severe enrichment." The Potential Ecological Risk Index (PERI) revealed that metals are "low risk" and "low." For a multi-element factor, there is "no potential ecological risk" for all study sites. The Toxic Risk Index (TRI) shows "no toxic risk" observed by the metal(iod)s. While Cr has the highest mHQ, with "low severity of contamination." The study underscores the need for practical measures to mitigate metal contamination in agricultural areas, recommending that policymakers, local government officials, and stakeholders develop mitigation approaches to protect the environment and public health.

Keywords: Bukidnon, environmental risk assessment, geo-accumulation index, metal concentration, soil contamination

Abbreviations: Cfi: Contamination Factor, EF: Enrichment Factor, ERL: Effect Range Low, Igeo: Geoaccumulation, mHQ: Modified Hazard Quotient, MPI: Modified Pollution Index, PEL: Probable Effect Level, PERI: Potential Ecological Risk Index, PLI: Pollution Load Index, SEL: Severe Effect Level, SQGs: Sediment Quality Guidelines, TEL: Threshold Effect Level, TRI: Toxic Risk Index

INTRODUCTION

Bukidnon is a province situated in the Northern Mindanao region of the Philippines. It is regarded as one of the country's major agricultural regions and has a large land area. The region's rich soil supports the growth of crops such as rice, corn, sugarcane, bananas, and vegetables (Giles et al. 2019). Due to industrialization, bioaccumulation through food chains, metals potentially threaten ecological and agricultural systems and human health. With the increasing industrialization and growing amount of vehicle activities, recent studies show that the metal concentration in soil, water, air, or biological organisms exceeds the background levels of the respective element. Heavy metals may be released into the environment by human activities such as industrial processes, mining, and transportation, resulting in soil, water, and air contamination. Heavy metals hazardous effects on human health and the environment have motivated investigations into their occurrence, distribution, and accumulation in the atmosphere. Heavy metals in soil and vegetation can lead to bioaccumulation, where these metals are absorbed by plants and animals, ultimately leading to human exposure through the food chain (Jaishankar et al. 2014). Heavy metal exposure can result in serious health concerns such as cancer, neurological diseases, and organ damage (Mahurpawar 2015). The heavy metal pollution in Philippine urban centers, critical gaps remain in understanding contamination dynamics in unique agro-urban interfaces like Bukidnon. Urbanization and vehicular activities in Bukidnon contribute to elevated metal concentrations in roadside soils, posing ecological risks measurable through integrated pollution indices.

Sediment Quality Guidelines (SQGs) are instruments used in environmental regulation to evaluate sediment quality in bodies of water. Next, to protect aquatic organisms and public health, SQGs are created based on the concentration of toxic substances in sediment. Sediment toxicity tests, bioaccumulation studies, and ecological risk assessments are just a few sources from which the SQGs are derived. The SQGs are expressed as contaminant concentrations in sediment (Gao et al. 2016). The potential risk to aquatic organisms and public health is assessed by comparing these concentrations to those observed in the environment.

The Sediment Quality Triad is one of the SQGs frequently used and is more useful than global sediment quality (Lison et al. 2020). Three elements comprise the triad: chemical characterization of sediment contaminants, toxicity testing with benthic organisms, and assessment of the benthic community. Another commonly utilized set of SQGs is Canada's Sediment Quality Guidelines for the Protection of Aquatic Life (Canadian Council of Ministers of the Environment 2018). These recommendations provide threshold concentrations for various contaminants in sediment, such as metals, pesticides, and Polycyclic Aromatic Hydrocarbons (PAHs). SQG development is a continuous process as more knowledge about the toxicity of contaminants and their effects on aquatic life and human health becomes available.

The term "pollution assessment" in the context of heavy metals refers to the assessment of environmental contamination with heavy metals, which can result from various anthropogenic activities, including industrial processes, mining, and agriculture. The roadside soil contamination in Bukidnon, Philippines, was assessed employing six indices: Geo-accumulation (Igeo), Contamination Factor (CF), Pollution Load Index (PLI), Enrichment Factor (EF), Potential Ecological Risk Index (PERI), and Toxic Risk Index (TRI). By synthesizing these tools, the study bridges a critical gap in multi-index applications for agricultural-urban interfaces, offering a replicable model for similar regions in ASEAN. Heavy metal concentrations in soil, water, sediment, and biota are measured to assess the degree of pollution and identify potential risks to people's and ecosystems health. The technique has also been widely applied in numerous studies on the evaluation of heavy metal pollution, including the Index of Geoaccumulation (Igeo) (Muller 1969), Pollution Load Index (PLI) (Tomlinson et al. 1980), Enrichment Factor (EF) (Mokhtarzadeh et al. 2020), Potential Ecological Risk Index (PERI) (Hakanson 1980; Hu et al. 2019). A contamination factor, usually soil or sediment, is a measurement of the level of heavy metal contamination in that sample compared to a reference sample from an uncontaminated area. The contamination factor is frequently used in environmental research to evaluate the level of heavy metal pollution and the potential risks to the environment and human health. The work of Muller (1969), who created a method for assessing heavy metal pollution in sediment using several pollution indices, including the contamination factor, contains one of the earliest references to the contamination factor. Previous studies in the Philippines focused on single metals or regions, neglecting Bukidnon's unique agro-urban dynamics. This study addresses this by combining geochemical and ecological risk analyses. Heavy metal accumulation in Bukidnon's roadside soils threatens food safety and ecosystem health, yet comprehensive risk assessments are lacking.

This research assessed the metal concentration in the roadside soil of the five highly urbanized localities in Bukidnon, Philippines. Specifically, the study aimed to: (i) Determine the concentration of metals (e.g., cadmium, chromium, copper, cobalt, Iron, manganese, and Zinc) in soil samples collected from roadside areas Malaybalay, Valencia, Maramag, Don Carlos, and Quezon, Bukidnon; (ii) Evaluate the potential risks related with metal contamination in the roadside soil of the study area; and (iii) Compare the five localities metal concentrations in soil samples and identify any significant differences.

MATERIALS AND METHODS

Sampling sites, sample collection, and analysis

A 5-10 cm surface soil/topsoil was collected from each selected sampling site from five localities in the Province of Bukidnon along the Sayre Highway, shown in Table 1. Samples were collected 1 meter from roads to capture deposition from vehicular emissions, avoiding direct runoff. Composite samples (10-15 per site) ensured spatial representativeness. The collection was done during the dry season from April to May 2023. Twenty-four soil sediments were collected from five localities along the Sayre Highway from Cagayan to Davao Road (Figure 1). Soil samples from each location were homogenized and air-dried in circulating air at 30°C, sieved through a 0.2 mm mesh, placed in labeled ziplock bags, and analyzed in Soil and Plant Analysis Laboratory (SPAL), Central Mindanao University (CMU), Maramag, Bukidnon, Philippines. Metals were analyzed via Flame AAS (Agilent 240FS) after the soil was digested with H_2SO_4-H_2O_2 digestion method (Sanders 2012). Quality control included reference material. Cd, Cr, Cu, Co, Fe, Mn, and Zn were prioritized due to their prevalence in vehicular emissions (e.g., tire wear, lubricants). As and Pb were excluded due to equipment constraints but flagged for future work.

Sediment quality guidelines

Several experts have accepted Sediment Quality Guidelines (SQGs) for the toxicological evaluation of sediment-related metals, promoting soil condition monitoring and biota safety, and implementing ecological and environmental policies and guidelines. SQGs, such as Probable Effect Level (PEL), Severe Effect Level (SEL), Threshold Effect Level (TEL), Effect Range Low (ERL), Effect Range Medium (ERM), and Lowest Effect Level (LEL), were used to assess the potential biotic influence of metal(oid)s estimated in soil samples (Rahman et al. 2014).

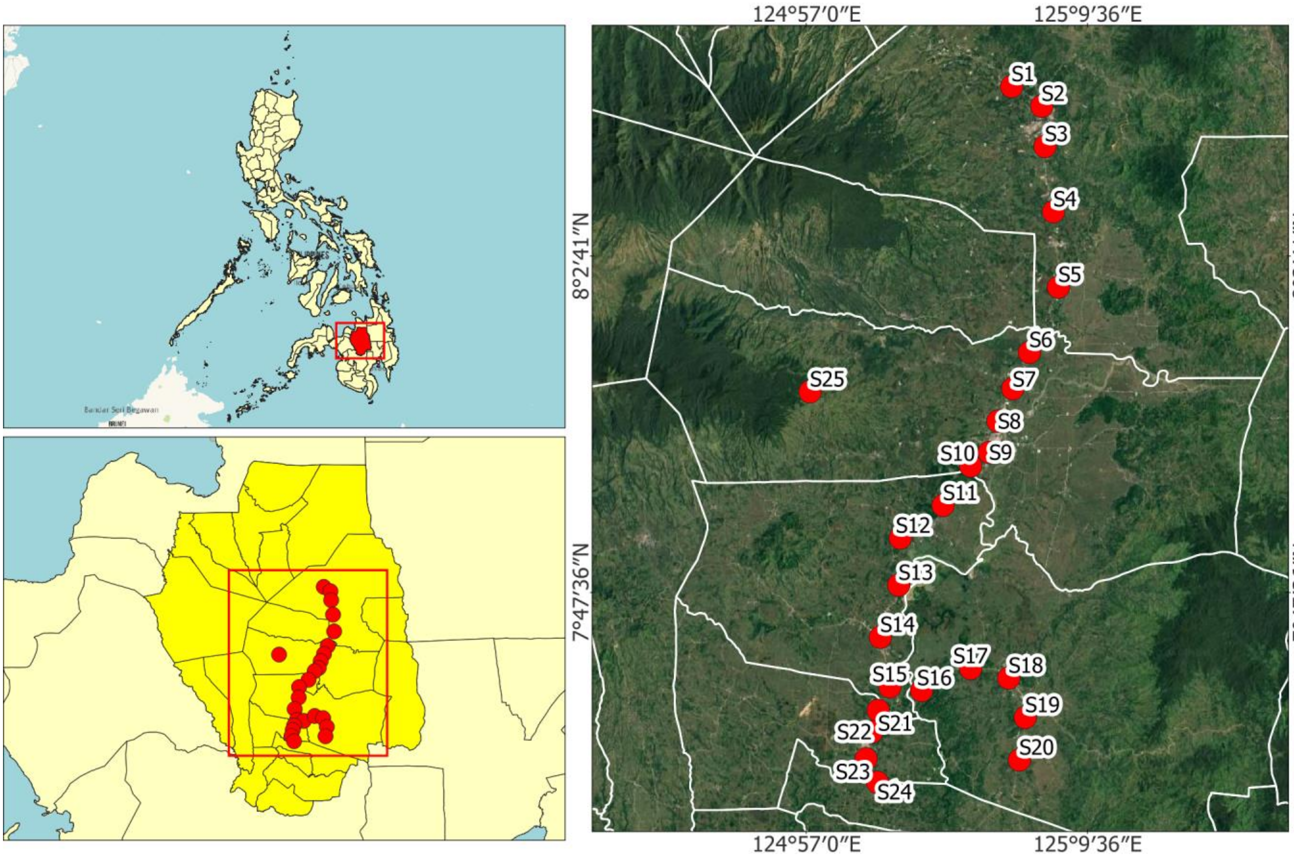

Figure 1. Location map of the study sites of the five localities in Bukidnon, Philippines

Table 1. Location of the five localities in Bukidnon along the Sayre Highway and sampling sites of the study

Location/localities	Barangay	Site	GPS Coordinates	
			Latitude	Longitude
Malaybalay City	Kalasungay-Sumpong, Sayre Highway	S1	8.171441°	125.103377°
	Malaybalay City Proper, Sayre Highway	S2	8.156778°	125.125834°
	Casisang-San Jose, Sayre Highway	S3	8.126646°	125.128267°
	Laguitas-Aglayan, Sayre Highway	S4	8.077913°	125.134625°
	Cabangahan-Bangcud, Sayre Highway	S5	8.021518°	125.138337°
Valencia City	Colonia-Dabong-dabong, Sayre Highway	S6	7.972782°	125.116646°
	Bagontaas, Sayre Highway	S7	7.945641°	125.104181°
	Hagkol, Valencia City, Sayre Highway	S8	7.921394°	125.093103°
	Poblacion, Valencia City, Sayre Highway	S9	7.898098°	125.086565°
	Lumbo, Sayre Highway	S10	7.887847°	125.072786°
Maramag	Musuan-Dologon, Sayre Highway	S11	7.858441°	125.052152°
	Tubigon-Bayabason, Sayre Highway	S12	7.834034°	125.020144°
	Panadtalan, Sayre Highway	S13	7.798834°	125.018918°
	Poblacion, Maramag, Sayre Highway	S14	7.759908°	125.005250°
	Camp 1, Sayre Highway (Quezon and Don Carlos)	S15	7.722809°	125.012364°
Quezon	Blue Water-Zubiri Village, Sayre Highway	S16	7.720066°	125.036069°
	San Jose, Sayre Highway	S17	7.736545°	125.072693°
	Poblacion, Quezon, Sayre Highway	S18	7.729403°	125.101166°
	Salawagan, Sayre Highway	S19	7.699946°	125.113549°
	Mibantang, Sayre Highway	S20	7.668192°	125.109175°
Don Carlos	Sinanguyan, Sayre Highway	S21	7.705232°	125.003633°
	Poblacion1, Don Carlos, Sayre Highway	S22	7.689238°	124.998808°
	Poblacion 2, Don Carlos, Sayre Highway	S23	7.669020°	124.994597°
	Pinamaloy, Sayre Highway	S24	7.651526°	125.003027°
Background soil	Mt. Kalatungan, Mt. Nebo Valencia City	S25	7.943360°	124.952730°

Metal pollution assessment

Six major indices were used in this study to assess pollution based on toxic metal concentrations in soil and samples: Geo-accumulation index (Igeo), Contamination factor (Cfi), Pollution Load Index (PLI), Enrichment Factors (EFs), Potential Ecological Risk Index (PERI), Modified Pollution Index (MPI), and degree of contamination (Cd). These indexes have the potential to be effective in comprehensively assessing the degree of soil pollution (Mazurek et al. 2017).

Geo-accumulation Index (lgeo)

Muller (1969) presented the geo-accumulation index (Igeo). This index quantifies the degree or level of metal pollution in the soil samples presented in Table 2. The following formula was used to determine the geo-accumulation index (Igeo) for sediment samples:

$$I_{geo} = \log_2\left[\frac{C_n}{1.5B_n}\right]$$

Where: "Cn" is the metal concentration measured in sediment samples in the study area. Bn is a natural compositional and mineralogical concentration of any specific metal in an undisturbed or parent source, and 1.5 is the background matrix correction due to lithological effects.

Contamination Load Index (CF)

The contamination factor for the same metal (c) was calculated by comparing the pre-industrial reference value with the same metal concentration in soil samples. The total of all contamination factors determines the degree of contamination, and the following is the formula used to calculate the Contamination Factor (Cfi):

$$C_f^i = \frac{C^i}{C_n^i}$$

Where: Cfi is the measured concentration of metals in soil samples, and Cni is the standard pre-industrial reference level Table 3. The contamination levels may be classified based on their intensities on a scale ranging from 0 to 6, "0" none, "1" none to medium, "2" moderate, "3" moderately to strong, "4" strongly polluted, "5" strong to very strong, "6" very strong.

Pollution Load Index (PLI)

The Pollution Load Index (PLI) was computed using the Contamination Factors (Cfi). The Cfi is the quotient calculated by dividing the concentration of each metal. By taking the n-root from the n-CFs that were obtained for each metal, the PLI of the site was determined. The Pollution Load Index (PLI) is often displayed as follows, according to Tomlinson et al. (1980):

$$PLI = \sqrt[n]{C_{f1} x C_{f2} x C_{f3} x \ldots\ldots\ldots\ldots x C_{fn}}$$

Where: "n" is the quantity of the studied metals, previously mentioned in a prior condition, and "Cf" is the contamination factor. The PLI supplies a simple yet reasonable intent to evaluate a site's quality where an estimation is made (Tomlinson et al. 1980). PLI less than one indicates perfection; PLI more than one indicates a decline in site quality; and PLI = 1 indicates that only baseline contamination levels are available.

Table 2. Geo-accumulation index values

Index class	Lgeo value	Level of contamination classification
0	$I_{geo} < 0$	Uncontaminated
1	$0 < I_{geo} < 1$	Uncontaminated to moderate contamination
2	$1 < I_{geo} < 2$	Moderately contaminated
3	$2 < I_{geo} < 3$	Moderately to heavily (strongly) contaminated
4	$3 < I_{geo} < 4$	Heavily (strongly) contaminated
5	$4 < I_{geo} < 5$	Heavily (strongly) extremely contaminated
6	$I_{geo} \geq 5$	Extremely contaminated

Note: In most cases, obtaining the background value of any particular metal of interest from the pre-industrial period is practically impossible. Due to this reason, researchers have adopted different approaches for acquiring the (B_n) Background Value of any metal of interest to perform the I_{geo} calculation. The B_n is obtained using the standard permissible limit prescribed (WHO 1995, 2010, 2016). The geo-accumulation index was proposed by Muller in 1969 and is based on the seven grades or classes shown in Table 3

Table 3. Background levels of the metal

Metal	Background (mg kg^{-1})	Pre-industrial reference level	FAO/WHO maximum permissible values soil (mg kg^{-1})	Toxic response factor
Cadmium	1.1	1	0.800	30
Chromium	31	90	100	2
Cobalt	36	36	0.1-50	5
Copper	25	50	36	5
Iron	3.12	7000	7000-50000	--
Manganese	400	850	2000	1
Zinc	65	175	50	1

Enrichment Factor (EFs)

The level of contamination is measured using the enrichment factors and was determined using the formula based on Mokhtarzadeh et al. 2020:

$$EF = \frac{\left(\frac{C_i}{C_{ref}}\right)_{Sample}}{\left(\frac{C_i}{C_{ref}}\right)_{background}}$$

Where: "Ci" is the target element's concentration, and "C_{ref}" is the reference element's concentration. The reference element used was an undisturbed soil sample for each site because of its low concentration (Mokhtarzadeh et al. 2020). The classification of Enrichment Factors (EFs) using the following indicators: EF < 1 indicates no enrichment, EF < 3 is minor enrichment, EF = 3-5 is moderate enrichment, EF = 5-10 is moderately severe enrichment, EF = 10-25 is severe enrichment, EF = 25-50 is very severe enrichment, and EF > 50 extremely severe enrichment.

Potential Ecological Risk Index (PERI)

With the increasing amount of heavy metals in sediments that could harm ecological health, an ecological risk assessment has been used as a diagnostic tool for water pollution prevention for heavy metals (Hu et al. 2019). A technique for evaluating a potential ecological risk index was created by Hakanson in 1980. This method considers the potential Ecological risk factor (Eri) of a single element, and the following formulas can be used to calculate the PERI of a multi-element (Rahman et al. 2014):

$$E_r^i = T_r^i / C_f^i$$

$$RI = \sum_{i=1}^{n} E_r^i$$

Where: Cfi is the contamination factor for the element of "i"; "Tri" is the toxic response factor for the given element of "i," which accounts for the toxic and sensitivity requirements. Using the potential ecological risk assessment. Then, to assess the RI, the following terminology was used: RI < 150 is low risk, 150 ≤ RI < 300 is moderate, 300 ≤ RI < 600 considerable, and RI ≥ 600 is very high. Moreover, to describe the (ERF), the following terminology was used: Er < 40, low, 40 ≤ Er < 80, moderate, 80 ≤ Er < 160, considerable, 160 ≤ Er < 320, high, and Er ≥ 320, very high.

Toxic Risk Index (TRI)

Zhang et al. 2020 developed the toxic risk index, which was used to give a more thorough assessment of environmental biota danger. The following formula was utilized to determine TRI using two threshold values for SQGs (TEL and PEL standard):

$$TRI_i = \sqrt{\frac{(C_i/TEL_i)^2/(C_i/PEL_i)^2}{2}}$$

Where: The concentration of ith metal is Ci, and the probable effect level is "PEL," and threshold effect levels are "TEL" for the ith metals. To interpret the TRI using the following values: TRI ≤ 5 indicates no toxic risk, 5 < TRI ≤ 10, low toxic risk, 10 < TRI ≤ 15, moderate toxic risk, 15 < TRI ≤ 20, considerable toxic risk, and TRI > 20, very high toxic risk.

Modified Hazard Quotient (mHQ)

The modified hazard quotient is a novel approach to evaluating the level of risk that each metal poses to living organisms in an area (Barjoee et al. 2021). Its reliability, validity, and accuracy have been established by (Benson et al. 2018). This method detects contamination by comparing metal concentrations in sediment (mg kg^{-1}) to adverse ecological impact distributions at slightly different Threshold Effect Levels (TEL), Probable Effect Levels (PEL), and Severe Effect Levels (SEL), as previously described. The distributions are shown in Table 4. The mHQ index is determined using the following formula:

$$HQ = \sqrt[2]{\left[C_i\left(\frac{1}{TEL_i} + \frac{1}{PEL_i} + \frac{1}{CEL_i}\right)\right]}$$

Where: Ci is the analyzed sample's determined metal concentration. Eight categories of contamination were identified from the mHQ values: mHQ > 3.5 (extreme severity), 3.0 ≤ mHQ < 3.5 (very high severity), 2.5 ≤ mHQ < 3.0 (high severity), 2.0 ≤ mHQ < 2.5 (considerable severity), 1.5 ≤ mHQ < 2.0 (moderate severity), 1.0 ≤ mHQ < 1.5 (low severity), 0.5 ≤ mHQ < 1.0 (very low severity), and mHQ < 0.5 (nil to very low severity).

Data analysis

Microsoft Excel 2012 was used to perform statistical analysis on the mean and standard deviation. The associations between metal concentration were assessed using the Pearson correlation coefficient and stepwise multiple regression analysis using STAR IRRI Nebula Software.

Table 4. Recommended sediment quality guideline values for metal and associated levels of concern are to be used in assessing sediment quality

Metal	TEL, mg kg^{-1}	SEL, mg kg^{-1}	PEL, mg kg^{-1}
Cadmium	0.99	2	1.2
Chromium	43.4	110	26
Cobalt	--	--	--
Copper	31.6	110	16
Iron	--	--	--
Manganese	--	--	--
Zinc	121	820	163

Sources: MacDonald et al. (2000a, b); de Deckere et al. (2011)

RESULTS AND DISCUSSION

Total metal concentration

The total Cd concentration in soil samples taken from the study sites was 0.901 ± 0.063 mg $kg^{-1,}$ ranging from 0.772 to 1.034 mg kg^{-1} (Figure 2.A). The average Cd concentration in the sampling sites was found to be greater than the FAO's allowable limit value of 0.800 mg kg^{-1}, except for site 3, with the lowest Cd concentration of 0.772 mg kg^{-1}, and site 24, which shows the highest Cd concentration value of 1.034 mg kg^{-1}. Higher than the background value of 0.596 mg kg^{-1} from an undisturbed soil sample. A similar result was observed in roadside soils in the City of Cagayan de Oro, which had a Cd concentration ranging from 0.07 to 0.51 mg kg^{-1} (Olarte and Besagas 2019). Cd contamination is particularly concerning in urban areas, where vehicular emissions and other anthropogenic activities have been identified as significant contributors. Roadside soils are particularly vulnerable to cadmium contamination, which can contaminate crops and groundwater, pose significant health risks to humans and animals, and negatively impact soil quality and nutrient cycling, leading to ecological damage (Zhang et al. 2016). Cd exposure can have adverse effects on human health and the environment, including reduced growth and survival of plants, changes in soil microorganisms, and increased bioaccumulation of metals in the food chain (WHO 2010; Abbas et al. 2018).

The average total Cr concentration in soil sampling sites from five localities along the Sayre Highway was 17.95 ± 1.76 mg $kg^{-1,}$ ranging from 16.233 to 24.693 mg kg^{-1}, as shown in Figure 2.B. The mean Cr concentration of the studied soil samples was higher than or equal to the background soil value of 16.77 mg kg^{-1} and lower than the FAO/WHO maximum allowed value of 100 mg kg^{-1}. It also shows that the highest concentration of Cr among the 24 sampling locations was observed in site 2, which has a Cr concentration value of 24.693 mg kg^{-1}. In contrast, site 5, site 12, and site 23 showed the lowest Cr concentration value of 16.233 mg kg^{-1}. A similar result was observed in many places in the Philippines and other countries: Iloilo, Philippines (Sarinas et al. 2007), in Romblon Province (Senoro et al. 2022), lower than the soil quality standard of 64 mg kg^{-1} (Canadian Council of Ministers of the Environment 2022). Chromium contamination in roadside environments is a significant concern due to its potential environmental impact on human health. This contamination could result from chromium leaching from metal-plated car parts, brake linings, and other industrial sources (Bulska 2011; Balaji et al. 2023). Several studies have investigated the levels of chromium in roadside soils and plants, with many findings elevated concentrations of the toxic hexavalent form (Kaur et al. 2021; Prasad et al. 2021). Heavy metals contaminate areas through industrial effluents, sewage and sludge, and vehicular emissions. As a result, vegetables cultivated on such grounds are likely unfit for human consumption due to heavy metal contamination (Hembrom et al. 2020; Proshad et al. 2020).

The average concentration of total Co in the soil samples from sampling sites along the Sayre Highway was 38.130 ± 16.183 mg $kg^{-1,}$ ranging from 8.852 to 70.191 mg kg^{-1} (Figure 2.C). The data shows that some of the sites have higher Co concertation samples from S1, S5, S7, S8, S10, S15, S17, S18, S22, and S24 having the highest recorded Co concentration values ranging from 37.580 to 70.191 mg kg^{-1} higher than the FAO/WHO maximum permissible limit of 36 mg kg^{-1} (WHO 1995), and higher than the background soil value of 32.15 mg kg^{-1}. Similar results were found in a study in Tacloban City, Philippines (Pacle-Decena et al. 2018). It shows that soil contamination of Co is anthropogenic, mainly due to activities like burning coal and oil or industrial waste and emissions. On the other hand, high doses may result in polycythemia, anemia, and congestive heart failure (Lison 2022). Cobalt plays a crucial role in the fixation of N2, which gives crops the macronutrient N (Hu et al. 2021). Co is part of the composition of proteins and enzymes in plant metabolism.

The soil samples had the second-highest concentration of total iron among all the elements assessed in the study. The concentration in the soil samples was 105.583 to 298.113 mg kg^{-1}, shown in (Figure 2.D), with an average value of 229.72 ± 21.46 mg kg^{-1}. The results in different sampling sites show a lower Fe concentration value than the FAO/WHO maximum permissible limit of 7,000 to 50,000 mg kg^{-1}. The study also shows an accumulation of Fe due to its higher concentration than the background soil concentration value of 58.191 mg kg^{-1}, indicating that the Fe source might be mainly anthropogenic. Most Bukidnon soil is predominantly Adtuyon soil series, which is clay loam derived from weathering basalts, andesites, and other igneous rock (Carating et al. 2014). In the Philippines, 6,367 mg kg^{-1} was observed in the Romblon Province (Senoro et al. 2022). Human activities such as industrial emissions, wastewater, and solid waste are the most significant anthropogenic sources of heavy metal soil pollution (Sodango et al. 2018). Iron can also negatively affect human health when it is present in high environmental concentrations. Exposure to iron-rich particulate matter from traffic was connected to a higher risk of lung cancer in urban populations (Fussell et al. 2022; Hammond et al. 2022).

This total Mn concentration in soil samples was 229.720 ± 21.458 mg kg^{-1}, with the highest element concentration among the other elements in the study (Figure 2.E). Soil samples have Mn concentrations ranging from 193.094 to 295.719 mg kg^{-1}. The sampling site 23 has the highest Mn concentration. However, the average Mn concentration value was higher than the background soil value of 35.490 mg kg^{-1} and lower than the FAO/WHO maximum allowable value of 2000 mg kg^{-1}. This indicates that Mn accumulation is present in these areas and that the primary sources of Mn in the study sites may be anthropogenic and natural. Senoro et al. 2022 also reported a 14.93 mg kg^{-1} Mn concentration in Romblon Province, Philippines, lower than the Mn concentrations of the present study. Mn is essential for proper neurological function, bone development, and wound healing. However, excessive intake or exposure to high Mn concentrations can negatively affect human health. Chronic exposure to elevated Mn levels can harm the central nervous system, liver, and kidneys, and neurotoxic effects, particularly a condition known as manganism (Miah et al. 2020).

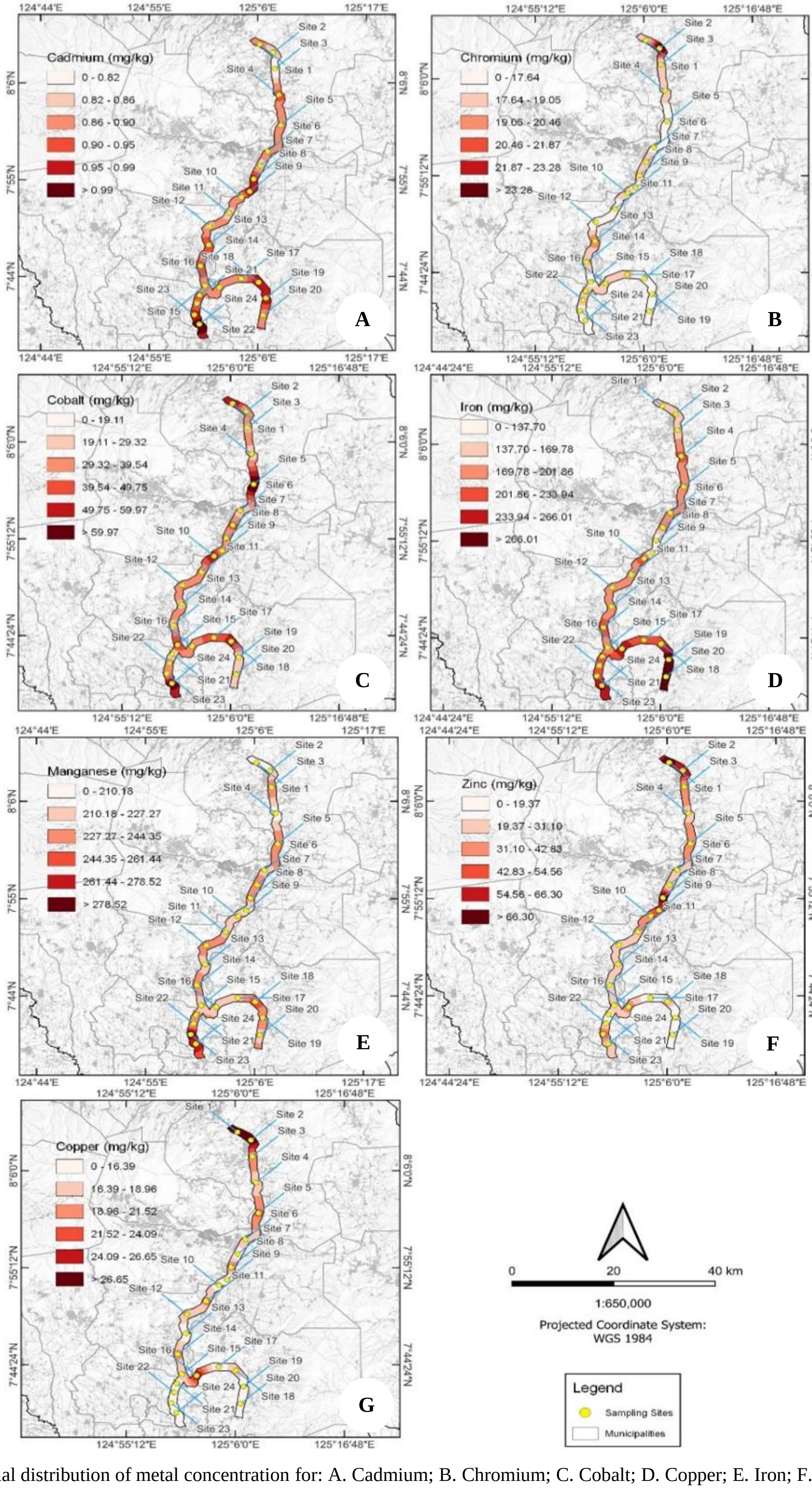

Figure 2. Spatial distribution of metal concentration for: A. Cadmium; B. Chromium; C. Cobalt; D. Copper; E. Iron; F. Manganese; and G. Zinc in roadside soil of five highly urbanized localities in the Province of Bukidnon, Philippines

The soil sampling sites had a mean total Zn concentration of 32.427 ± 19.133 mg kg^{-1}, ranging from 7.623 to 69.283 mg kg^{-1}. This study showed that the zinc concentration in soil samples from 24 sampling locations along the Sayre Highway is less than 50 mg kg^{-1} FAO/WHO maximum permissible value. However, some sampling sites that exceed the allowable limit, such as S1, S2, S9, and S23, are shown in (Figure 2.F). Zinc is also commonly used in industrial and consumer applications, such as galvanizing steel and manufacturing batteries. Excessive exposure to Zinc can adversely affect human health and the environment. Zinc contamination along roadsides can occur from various sources, including traffic-related emissions, road dust, and runoff from galvanized steel and other zinc-containing materials. Elevated zinc levels in roadside soils and plants can adversely affect soil quality and plant growth and pose the risk of human exposure (Alengebawy et al. 2021; Angon et al. 2024).

The collected soil samples have a mean total Cu concentration of 18.173±4.516 mg $kg^{-1,}$ ranging from 13.073 to 29.220 mg kg^{-1}. The highest Cu concentration was at sampling site 1, while the lowest Cu concentration was found to be at S20, as shown in Figure 2.G. Subsequently, the study findings were lower than the Cu concentration in soil/sediments of FAO/WHO maximum permissible value/limit of 50 mg kg^{-1} (Table 3). It also revealed that soil samples from different sampling sites were higher than the background soil sample of 12.765 mg kg^{-1} Cu, which showed an accumulation of Cu in the soil. All soil samples were lower than the WHO permissible limit, indicating a low impact of Cu on ecology and suggesting enough supply of Cu for plant uptake. A study by Senoro et al. 2022, shows 233.10 mg kg^{-1} of Cu in the Romblon Province, higher than the Cu permissible limit. Copper can enter the environment through natural processes and human activities, such as mining, smelting, and waste disposal (Nyiramigisha, 2021). Elevated levels of copper in soils and water bodies can adversely affect human health and the environment (Xu et al. 2024; Chowdhary et al. 2020). Copper contamination along roadsides can occur from various sources, including traffic-related emissions, road dust, and runoff from copper-containing materials, such as brake pads and wires. Ingestion of high levels of copper has been linked to the development of Wilson's disease. This genetic disorder leads to copper accumulation in the body (Barber et al. 2021; Lucena-Valera et al. 2023). Elevated levels of copper in roadside soils and plants can adversely affect soil quality and plant growth and pose a risk of exposure to humans and wildlife. The pressing issue of heavy metal accumulation in roadside soils can have serious adverse effects on the environment and human health. These results show the critical need for pollution management strategies, sustainable road infrastructure development, and continuous monitoring to reduce heavy metal contamination in urban and rural settings.

Soil total metal concentration in five localities

Table 5 shows the metal concentration in the roadside soil at the five studied localities along the Sayre Highway. The mean and standard deviation values show the average analysis of the results, which shows the decreasing order of metal accumulation in soil samples mg kg^{-1} that was Mn (229.46±21.26) > Fe (199.87±51.78) > Co (38.13±16.18) > Zn (32.43±19.13) > Cu (18.17±4.52) > Cr (17.95±1.76) > Cd (0.901±0.063), respectively. The result showed that the metal concentrations at the sites in the study are below the maximum allowable values or limits for soil and sediments set by the WHO (World Health Organization). According to metal analysis, the soil samples had the highest Mn concentration and the least accumulated cadmium. These metals have accumulated, and as a result, their levels are significantly higher than those of the background soil sample. Additionally, it demonstrates that the average metal concentrations shown were below the standard allowable limit, indicating that the metal concentrations in the roadside soil along the Sayre High in Don Carlos, Malaybalay City, Valencia City, Maramag, and Quezon, Bukidnon, Philippines, are low.

Heavy metal and toxic element accumulation in topsoil horizons in urban areas is a significant environmental issue since it may long-term affect human health, well-being, and soils (Nikolov et al. 2020). The data revealed that Don Carlos Bukidnon, Philippines, has the highest Cd with a mean concentration value of 0.951 ± 0.064 mg kg^{-1}. Higher Cr concentration was found in Malaybalay and Valencia with Cr values of 18.95 ± 3.03 mg kg^{-1} and 18.03 ± 0.951 mg $kg^{-1,}$ respectively. Malaybalay City has the highest average Cu, Co, and Zn concentrations of 23.22 ± 5.28 mg kg^{-1}, 43.32 ± 20.63 mg $kg^{-1,}$ and 46.41 ± 18.59 mg kg^{-1}, respectively. The locality of Quezon, Bukidnon, has the highest concentration in the study, with an average Fe concentration value of 262.568 ± 34.92 mg kg^{-1}. Mn concentration in the soil samples collected from Don Carlos, Bukidnon, with the Mn of 253.57 ± 33.76 mg $kg^{-1,}$ has the highest observed concentration.

Table 5. Metal concentration of soil samples from five different localities along the Sayre Highway in Bukidnon, Philippines (mg kg^{-1})

Area/sites	Total Cd	Total Cr	Total Cu	Total Co	Total Fe	Total Mn	Total Zn
Malaybalay	0.852±0.055	18.95±3.30	23.22±5.28	43.32±20.63	173.12±28.21	218.02±22.17	46.41±18.59
Valencia	0.907±0.070	18.03±0.951	16.85±2.71	38.05±14.16	161.27±55.83	222.54±12.81	43.61±24.89
Maramag	0.891±0.047	17.54±1.53	17.81±2.06	35.10±11.97	179.04±19.13	227.91±10.45	26.27±5.20
Quezon	0.913±0.058	17.41±0.730	17.44±5.65	36.18±14.30	262.57±34.92	231.34±14.71	17.90±11.91
Don Carlos	0.951±0.064	17.758±1.25	14.89±0.571	37.97±25.35	229.25±33.55	253.57±33.76	26.82±17.42
Permissible limit	0.800	100	0.1-50	36	7000-50000	2000	50
Background soil	0.596	16.77	12.77	32.15	58.19	35.49	5.75

The trends of metal concentration of roadside soil in Sayre Highway of five localities in the Province of Bukidnon, Philippines, are shown in Table 6. Mn and Fe are the most common elements found in the roadside soils along the Sayre Highway, where heavy metals are present and accumulate. The elevated amounts of metals relative to background soil values suggest continuous buildup, which may result in long-term environmental deterioration and possible health hazards, even while metal concentrations are still below regulatory limits. Hence, to reduce the dangers of heavy metal pollution, it is crucial to have tighter emission restrictions and ongoing monitoring of metal accumulation.

Pollution assessment

Geo-accumulation Index (lgeo)

The geo-accumulation index (lgeo) has been used widely to assess the degree of pollution. Comparing the differences between pre-industrial and current concentrations makes it possible to evaluate environmental contamination (Li et al. 2014). The average of lgeo values from different sampling sites was found to be Mn (2.104 ± 0.130 mg kg^{-1}), Zn (1.668 ± 0.871 mg kg^{-1}), Fe (1.146 ± 0.395 mg kg^{-1}), Cd (0.008 ± 0.100 mg kg^{-1}), Cu (0.114 ± 0.326 mg kg^{-1}), Co (-0.476 ± 0.668 mg kg^{-1}), Cr (-0.493 ± 0.129 mg kg^{-1}). The detailed data for each metal sampling site can be found in Figure 3. This study revealed that the lgeo for Mn in S1, S2, S4, and S9 were 1.85, 1.98, 1.93, and 1.91, respectively, belonging to index class 2 and indicating "moderately contaminated" Table 2. The rest of the sampling site revealed that the lgeo for Mn, ranging from 2.02 to 2.47, belongs to the index class 3, indicating that the soil samples from this site are "moderately heavily (strongly) contaminated." In S1 and S8 samples, the lgeo for Zn was 3.00 and 3.17, which belong to index class 4, which indicates that there is "heavily (strongly) contaminated," S2, S5, S9, S10, S16, and S23 has lgeo value ranging from 2.11 to 2.88, which belongs to index class 3 which indicates that soil samples are "moderate heavily (strongly) contaminated," S19, S20, S21, and S17 belongs to index class of 1 and 0 respectively which indicates soil samples is "no contamination or uncontaminated to moderately contaminated" samples. While S3, S4, S6, S7, S11, S12, S13, S14, S15, S20, and S24 have a lgeo value ranging from 1.02 to 2.00 belongs to index class 2, which indicates that there is "moderate contamination" among all samples.

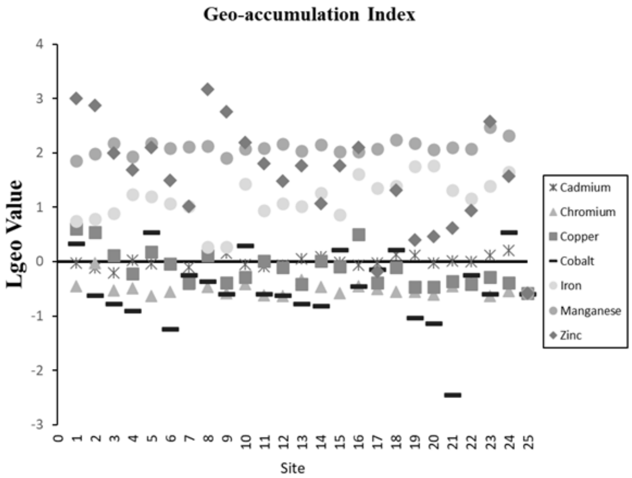

Figure 3. Metal geo-accumulation index (Igeo) of soil in five localities along the Sayre Highway

Table 6. The trend of metal concentrations in the roadside soil in Sayre Highway Bukidnon, Philippines

Areas/sites	Metals in roadside soil
Malaybalay	Mn > Fe > Zn > Co > Cu > Cr > Cd
Valencia	Mn > Fe > Zn > Co > Cr > Cu > Cd
Maramag	Mn > Fe > Co > Zn > Cu > Cr > Cd
Quezon	Fe > Mn > Co > Zn > Cu > Cr > Cd
Don Carlos	Mn > Fe > Co > Zn > Cr > Cu > Cd

Table 7. Comparison of metal concentrations (mg kg^{-1}) in roadside soils with International Guidelines and Regional Studies

Metal	This study (Bukidnon, Philippines)	WHO/FAO permissible limit	EPA RSL (residential soil)	Previous studies in the Philippines	Other regional studies (Asia)
Cd	0.901 ± 0.063	0.800	0.78	0.07-0.51 (Cagayan de Oro)*	0.2-2.2 (Chiang Mai & Lamphun, Thailand)**
Cr	17.95 ± 1.76	100	210	14.93 (Romblon)†	7.84-18.94 (Central Java, Indonesia)‡
Cu	18.17 ± 4.52	50	3100	233.10 (Romblon)†	153.7 (Southern Vietnam)§
Co	38.13 ± 16.18	50	23	10.5 (Tacloban)¶	8.3 (Hanoi, Vietnam)‖
Fe	199.87 ± 51.78	7000-50,000	N/A	6367 (Romblon)†	14,410-30,590 (Dhaka, Bangladesh)**
Mn	229.46 ± 21.26	2000	1800	14.93 (Romblon)†	573.32 (China)††
Zn	32.43 ± 19.13	50	23,000	25-70 (Cagayan de Oro)*	70.8-164.2 (Singapore)‡‡

Note: WHO/FAO: World Health Organization/Food and Agriculture Organization. EPA RSL: U.S. Environmental Protection Agency Regional Screening Level (US EPA 2024). * Olarte and Besagas (2019); ** Somsunun et al. (2023); Senoro et al. (2022); Dewi et al. (2021); § Dat et al. (2021); ¶ Pacle-Decena et al. (2018); ‖ Nguyen et al. (2021); ** Khan and Shah (2023); †† Kun et al. (2023); ‡‡ Goh et al. (2022)

The Fe lgeo in the sampling site shows that "contamination" exists. S1, S2, S3, S8, S9, S11, and S15 values ranging from 0.27 to 0.89 belong to index class 1, which indicates that there is "uncontaminated to moderate contamination." While the rest of the sampling sites belong to index class 2 with the lgeo values ranging from 1.01 to 1.77, which indicates that soil samples from these sites are "moderately contaminated." lgeo for Cd in sampling sites S4, S8, S9, S13, S14, S18, S19, S21, S22, S23, and S24 belong to index class 1, which indicates that the soil samples from these following sites are "uncontaminated to moderate contamination." In contrast, the remaining samples from different areas are "uncontaminated." The Cd lgeo values from soil sampling sites S4, S8, S9, S13, S14, S18, S19, S21, S22, S23, and S24 are falling to index class 1, or "uncontaminated to moderately contaminated." In contrast, other sample sites have belonged to "uncontaminated." lgeo values for Cu show that sampling sites from S1, S2, S3, S5, S8, S11, S14, and S16 indicating that there are "uncontaminated to moderately contaminated" in these sampling sites are class 1; other samples were found to be "uncontaminated," the lgeo of Cr were less than zero indicating that the soil samples were not polluted, detailed data of the lgeo values are presented in (Figure 3). While Cd, Cu, Co, and Cr mostly stay within the uncontaminated to moderately contaminated range, Mn and Zn exhibit the highest contamination levels across the sampling sites, according to the geo-accumulation index (Igeo) analysis. These results show possible environmental hazards that could impact soil quality, plant growth, and the general health of the ecosystem, especially in sites with greater contamination levels.

Contamination Factor (CF)

(Cf) was used to quantify the level of contamination concerning measured background values from geologically similar and uncontaminated places or the average crustal composition of a particular metal (Ladigbolu and Balogun 2011), while PLI was used to evaluate the degree to which the soil sediment associated with heavy metals might impact the microflora and macrofauna of the soil (Yadav and Yadav 2018), these two essential indices were proposed to measure the metal pollution in the sediment sample (Hakanson 1980). The Cf of all of the samples from sampling sites of the five localities along the Sayre Highway had the following order: Co > Cd > Cu > Mn > Cr > Zn > Fe with the Cf mean ± SD values of 1.059±0.450, 0.901±0.063, 0.363± 0.090, 0.270±0.025, 0.199±0.019, 0.185±0.109, 0.029±0.007 respectively, falling into class 1 "none to moderate contamination"; detailed information on Cf values for each sampling site is presented in Table 8. Based on the Contamination Factor (Cf) analysis, the highest contamination levels are found in Co and Cd, and all sampled sites along the Sayre Highway fall into the "none to moderate contamination." Although the current level of heavy metal deposition is not yet dangerous, continuous monitoring is necessary to avoid ecological hazards since extended exposure may impact microbial populations, soil health, and general environmental quality. The constant human activities, including industrial discharge, agricultural practices, and vehicle emissions, may eventually lead to an increase in the buildup of heavy metals.

Table 8. Soil quality guidelines for metal concentration for Contamination Factor (CF), Pollution Load Index (PLI)

Area/sites	Contamination factor							Pollution load index	
	Cd	**Cr**	**Cu**	**Co**	**Fe**	**Mn**	**Zn**	**PLI_{value}**	**PL_{Iindex}**
S 1	0.881	0.204	0.584	1.691	0.021	0.227	0.396	0.319	Perfection
S 2	0.823	0.274	0.558	0.871	0.021	0.247	0.362	0.297	Perfection
S 3	0.772	0.195	0.415	0.785	0.023	0.284	0.197	0.251	Perfection
S 4	0.913	0.199	0.330	0.720	0.029	0.240	0.159	0.241	Perfection
S 5	0.870	0.180	0.436	1.950	0.029	0.284	0.212	0.303	Perfection
S 6	0.871	0.191	0.372	0.569	0.026	0.266	0.140	0.230	Perfection
S 7	0.836	0.210	0.292	1.130	0.025	0.272	0.100	0.235	Perfection
S 8	0.967	0.203	0.415	1.044	0.015	0.272	0.446	0.286	Perfection
S 9	0.996	0.188	0.292	0.893	0.015	0.236	0.335	0.248	Perfection
S 10	0.864	0.210	0.314	1.648	0.034	0.263	0.225	0.294	Perfection
S 11	0.842	0.183	0.388	0.893	0.024	0.267	0.173	0.249	Perfection
S 12	0.853	0.180	0.356	0.871	0.026	0.282	0.138	0.242	Perfection
S 13	0.927	0.221	0.287	0.785	0.025	0.257	0.168	0.243	Perfection
S 14	0.950	0.203	0.388	0.764	0.030	0.280	0.103	0.243	Perfection
S 15	0.882	0.188	0.361	1.562	0.023	0.255	0.168	0.265	Perfection
S 16	0.854	0.205	0.542	0.979	0.038	0.255	0.212	0.294	Perfection
S 17	0.880	0.197	0.292	1.216	0.032	0.263	0.044	0.217	Perfection
S 18	0.977	0.191	0.356	1.562	0.033	0.297	0.123	0.277	Perfection
S 19	0.973	0.190	0.277	0.656	0.042	0.283	0.065	0.221	Perfection
S 20	0.881	0.184	0.277	0.613	0.043	0.262	0.068	0.215	Perfection
S 21	0.906	0.205	0.298	0.246	0.031	0.270	0.076	0.189	Perfection
S 22	0.895	0.211	0.287	1.130	0.028	0.263	0.095	0.238	Perfection
S 23	0.970	0.180	0.314	0.893	0.033	0.348	0.296	0.289	Perfection
S 24	1.034	0.192	0.292	1.950	0.039	0.313	0.147	0.297	Perfection
Mean±SD	0.901±0.063	0.199±0.019	0.363±0.090	1.059±0.450	0.029±0.007	0.270±0.025	0.185±0.109	0.258±0.034	Perfection

Pollution Load Index (PLI)

The pollution in the particular study areas was determined using the pollution load index (PLI) (Tomlinson et al. 1980). This index is a convenient tool for comparing the pollution levels of various sites. The list provides a crucial and pertinent approach when rating a place or an area class. A score of one indicates that only benchmark levels of contaminants are present, whereas zero indicates perfection. On the other hand, some attributes would indicate dynamic site and quality degradation. This study found that the average PLI value for 24 sampling sites was 0.258 ± 0.034, ranging from 0.189 to 0.319, as shown in Table 8. All soil samples pollution load index values were PLI < 1, indicating "perfection or low pollution" status. However, Rahman et al. (2021) and Unsal et al. (2024) suggested that continual observation should be required to measure the pollutant load index more precisely, owing to the contamination of heavy metals in indoor dust samples. In the Pollution Load Index (PLI) analysis, every location sampled has a PLI of less than 1, which denotes a "perfection or low pollution" classification. This implies that no severe heavy metal contamination has occurred in the research locations. However, measurable pollutant levels emphasize the necessity of ongoing monitoring because anthropogenic activities and long-term environmental conditions may lead to progressive accumulation.

Enrichment Factor (EF)

The enrichment factor (EF) of the metal indicator in Table 9 is used to identify the presence and extent of anthropogenic pollution deposition on the topsoil. The possible contamination index is determined by the normalization of one metal's presence in topsoil concerning the presence of another element. An exceptionally stable component of the soil, devoid of vertical movement and degradation events, is referred to as a reference element. Reference elements are selected based on their association with finer particles and their resistance to anthropogenic alterations, ensuring that their concentrations reflect natural processes rather than human activities (Barbieri 2016). Al, Fe, Mn, and Rb are commonly used as reference elements in geochemical investigations, as they aid in identifying geochemical anomalies and normalizing heavy metal concentrations in sediment studies (Sun et al. 2018; Dominech et al. 2022).

Iron (Fe) was chosen as the reference element for determining the enrichment factor in a study by Vineethkumar et al. (2020), pointing out that many researchers studying marine and estuarine ecosystems have used this method. The results of EF of metals in the study are shown in Table 9. The EF ranges from (1.295 to 1.735) with an average of (1.511 ± 0.105) for Cd, (0.968 to 1.472) with an average EF value of (1.070 ± 0.105) for Cr, (1.083 to 2.288) an average of (1.423 ± 0.354) for Cu, (0.275 to 2.183) with an average of (1.186 ± 0.503) for Co, (1.814 to 4.716) for Fe with an average of (3.435 ± 0.890), (5.441 to 8.332) for Mn with an average of (6.473 ± 0.605), and (1.325 to 13.576) with an average EF value of (5.639 ± 3.327) for Zinc respectively.

Table 9. Soil quality guidelines for enrichment factor (*EF*) of the soil samples from five different localities along the Sayre Highway

Area/sites	**Enrichment factor**						
	Cd	**Cr**	**Cu**	**Co**	**Fe**	**Mn**	**Zn**
S 1	1.478	1.095	2.288	1.893	2.527	5.441	12.049
S 2	1.381	1.472	2.184	0.976	2.578	5.923	11.009
S 3	1.295	1.045	1.623	0.879	2.782	6.801	6.005
S 4	1.532	1.070	1.291	0.807	3.545	5.751	4.835
S 5	1.460	0.968	1.706	2.183	3.443	6.801	6.459
S 6	1.461	1.026	1.457	0.638	3.138	6.370	4.250
S 7	1.403	1.128	1.145	1.266	3.036	6.508	3.049
S 8	1.622	1.089	1.623	1.169	1.814	6.525	13.576
S 9	1.671	1.007	1.145	1.000	1.814	5.647	10.197
S 10	1.450	1.128	1.228	1.845	4.054	6.301	6.850
S 11	1.413	0.981	1.519	1.000	2.883	6.387	5.257
S 12	1.431	0.968	1.395	0.976	3.138	6.749	4.186
S 13	1.555	1.185	1.125	0.879	3.036	6.164	5.127
S 14	1.594	1.089	1.519	0.855	3.596	6.697	3.146
S 15	1.480	1.007	1.415	1.749	2.731	6.112	5.127
S 16	1.433	1.102	2.122	1.096	4.563	6.112	6.459
S 17	1.477	1.057	1.145	1.362	3.850	6.301	1.325
S 18	1.639	1.026	1.395	1.749	3.952	7.110	3.730
S 19	1.633	1.019	1.083	0.734	5.072	6.783	1.976
S 20	1.478	0.987	1.083	0.686	5.123	6.284	2.073
S 21	1.520	1.102	1.166	0.275	3.749	6.456	2.301
S 22	1.502	1.134	1.125	1.266	3.341	6.301	2.885
S 23	1.628	0.968	1.228	1.000	3.952	8.332	8.995
S 24	1.735	1.032	1.145	2.183	4.716	7.489	4.478
Cn	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Mean±SD	1.511±0.105	1.070±0.105	1.423±0.503	1.186±0.503	3.435±0.890	6.473±0.605	5.639±3.327

This study revealed that 100% of Cd EF values are EF<3, "minor enrichment," Cr shows 12.5% of the samples are EF<1, which indicates "no enrichment," and 87.5% of the soil samples had Cr values in the EF<3 range which means "minor enrichment." 100% of Cu EF values were EF<3, or "minor enrichment" was observed. On the other hand, Co EF values were identified as 50% EF<1 and 50% EF<3 of the sampling sites falling into "no enrichments" and "minor enrichment," respectively. The Fe EF values showed that there are 8.33, 29.17, and 62.5% are classified as EF<3 "minor enrichment," EF=3-5 "moderate enrichment," and EF=5-10 "moderately severe enrichment," respectively. Mn EF values are classified as "severe enrichment," with 100% of the sampling site being EF=5-10. While Zn EF values indicate that there is a "minor enrichment," "moderate enrichment," "moderately severe enrichment," and "severe enrichment" were observed with 20.88% EF<3, 29.17% EF=3-5, 29.17% EF=5-10, and 16.67% EF=10-25 respectively with a soil sample from S1 has the highest EF value for Zn at 12.05 (Mokhtarzadeh et al. 2020). The result shows that some sampling sites have higher EF values than 1. The presence of these elements in the sampling sites has increased relative to the average natural abundance because of human activity and pollution (Bern et al. 2019). The results suggest that the elevated levels of these metals in the soil result from human activities such as industrial emissions, agricultural inputs, and vehicle pollution. The necessity for focused pollution management measures is highlighted by the high EF values for Mn and Zn in several locations, which show significant deviations from natural background levels.

Potential ecological risk

The results of the assessment of the Ecological Risk Factor (ERF) and Ecological Risk Index (ERI) are shown in Table 10. This study shows that the potential ecological risk factor is in the following decreasing order: Cd > Zn > Mn > Cu > Cr > Co, ranging from (29.014 to 38.860), (2.525 to 22.727), (2.874 to 4.405), (8.562 to 18.051), (7.299 to 11.111) and (2.564 to 20.325) respectively with mean ± SD values of (33.460 ± 2.307) for Cd, (7.598 ± 4.896) for Zn, (3.729 ± 0.328) for Mn, (14.445 ± 2.960) for Cu, (10.110 ± 0.833) for Cr, and (5.824 ± 3.549) for Co. The ERF for all elements in soil sampling sites was observed to be lower than RI<150. The results indicate "low" that soil samples collected in Malaybalay, Valencia, Maramag, Quezon, and Don Carlos, Bukidnon pose a minimal potential ecological risk. The result of ERF shows that 100% of the soil sampling sites in five localities along the Sayre Highway had an average value of (2.053 ± 0.039) ranging from 1.99 to 2.12, with S17 showing the highest ERF value and S1 as the lowest ERF value, this also shows ERF is lower than 40, which indicates that the potential ecological risk index for soil sampling sites is low. In the Ecological Risk Index (ERI) and potential Ecological Risk Factor (ERF) analyses, all soil sampling locations demonstrate low ecological risk, with Cd having the highest risk potential of all the metals examined. All elements ERF values are below the 40 thresholds, indicating little environmental concern. These results suggest that although there is some metal contamination, the environment is not currently seriously threatened. However, as growing anthropogenic activities may increase ecological risk, continuous monitoring is necessary to identify any changes.

Table 10. Potential Ecological Risk Index (PERI) of soil samples from the five localities along the Sayre Highway

Area/sites	Ecological risk index						Ecological risk factor	
	Cd	**Cr**	**Cu**	**Co**	**Mn**	**Zn**	**ER_{value}**	**ER_{index}**
S 1	34.052	9.804	8.562	2.957	4.405	2.525	1.991	Low
S 2	36.452	7.299	8.961	5.741	4.049	2.762	2.007	Low
S 3	38.860	10.256	12.048	6.369	3.521	5.076	2.059	Low
S 4	32.859	10.050	15.152	6.944	4.167	6.289	2.056	Low
S 5	34.483	11.111	11.468	2.564	3.521	4.717	2.020	Low
S 6	34.443	10.471	13.441	8.787	3.759	7.143	2.067	Low
S 7	35.885	9.524	17.123	4.425	3.676	10.000	2.079	Low
S 8	31.024	9.852	12.048	4.789	3.676	2.242	1.998	Low
S 9	30.120	10.638	17.123	5.599	4.237	2.985	2.033	Low
S 10	34.722	9.524	15.924	3.034	3.802	4.444	2.037	Low
S 11	35.629	10.929	12.887	5.599	3.745	5.780	2.052	Low
S 12	35.170	11.111	14.045	5.741	3.546	7.246	2.062	Low
S 13	32.362	9.050	17.422	6.369	3.891	5.952	2.054	Low
S 14	31.579	9.852	12.887	6.545	3.571	9.709	2.050	Low
S 15	34.014	10.638	13.850	3.201	3.922	5.952	2.038	Low
S 16	35.129	9.756	9.225	5.107	3.922	4.717	2.020	Low
S 17	34.091	10.152	17.123	4.112	3.802	22.727	2.125	Low
S 18	30.706	10.471	14.045	3.201	3.367	8.130	2.030	Low
S 19	30.832	10.526	18.051	7.622	3.534	15.385	2.101	Low
S 20	34.052	10.870	18.051	8.157	3.817	14.706	2.116	Low
S 21	33.113	9.756	16.779	20.325	3.704	13.158	2.143	Low
S 22	33.520	9.479	17.422	4.425	3.802	10.526	2.072	Low
S 23	30.928	11.111	15.924	5.599	2.874	3.378	2.029	Low
S 24	29.014	10.417	17.123	2.564	3.195	6.803	2.026	Low
Means±SD	33.460±2.307	10.110±0.833	14.445±2.960	5.824±3.549	3.729±0.328	7.598±4.896	2.053±0.039	Low

Toxic Risk Index (TRI)

The (TRI) alternative computation offers a more detailed evaluation of the possible toxicity of the specific metal(oid) in the ecosystem illustrated in Table 11. The TRI value of Cd, Cr, Cu, and Zn was calculated using the Threshold Effect Levels (TEL), Probable Effect Levels (PEL), and Severe Effect Levels (SEL) values shown in Table 4. Based on the result of this study, the average TRI value in decreasing order: Zn > Cd > Cr > Cu was 0.953, 0.857, 0.424, and 0.038. The study's average value suggests that metal(iod)s provide no risk, and the TRI value was less than 5 (TRI < 5; no toxic risk) (Zhang et al. 2016). This suggests that soil health and ecological stability may not be at risk by the presence of these metals in the study locations along the Sayre Highway.

Modified Hazard Quotient (mHQ)

Figure 4 shows a pollution index that measures the degree of contamination. Benson et al. (2018) established the Modified Hazard Quotient (mHQ), which indicates each metal concentration used to evaluate the degree of pollution in soil samples and sediments, as well as the threshold edge for harmful environmental dispersions such as TEL, PEL, and SEL. The evaluation of mHQ is essential since it assesses the risk of a specific metal(oid) to the aquatic environment and biota (Emenike et al. 2020). The Cr levels in the soil samples revealed that 100% of the sampling sites are 1.0≤ mHQ < 1.5 "low severity of contamination" with an average Cr mHQ value of (1.50 ± 0.021), same with Cr and Cu shows 100% of the sampling sites are classified as 0.5 ≤ mHQ < 1.0 "very low severity of contamination," with an average mHQ value of (0.678 ± 0.029) and (0.800 ± 0.084) with values ranging from (0.649 to 0.785) and (0.714 to 0.9980 respectively. Zn has an average mHQ value of (1.277 ± 0.169) with sampling sites S8 and S9 shown to be 0.5 ≤ mHQ < 1.0 "very low severity of contamination" with mHQ values of (0.938). At the same time, 91.67% of the sample belongs to 1.0 ≤ mHQ < 1.5 "low severity of contamination," with a value ranging from (1.106 to 1.572) for Zn. Therefore, it has been suggested that the study sites were at "very low severity of contamination" to "low severity of contamination" and indicated no concern for ecological and environmental risk (Benson et al. 2018).

Relationship of metal concentration between metals in the soil

Correlation between metals in soil: Analyzing the correlation between metal concentrations can explain the migration and transportation rules of different forms of metals and be used for soil remediation and mitigations. Table 12 shows the r-values for the correlation between soil Cd concentration and Cr, Cu, Co, Fe, Mn, and Zn, which were -0.255, -0.405, 0.133, 0.195, 0.340, and 0.052, respectively. The data shows that Cr and Cu concentrations have a negative, non-significant correlation with Cd concentrations. Co, Fe, Mn, and Zn positively correlate with Cd concentration, indicating that Co, Fe, Mn, and Zn in the soil increase with the increase of soil Cd concentration. There was a negative correlation between soil Co, Fe, and Mn with r-values of -0.108, -0.227, and -0.315, respectively, with the Cr concentration. This implies that when Cr concentration in the soil decreases, the amount of Co, Fe, and Mn increases.

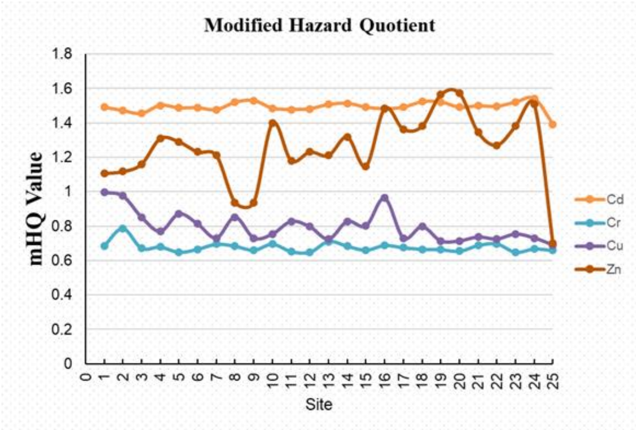

Figure 4. Modified Hazard Quotient (mHQ) values of soil samples from the five localities of the study along the Sayre

Table 11. Toxic risk index of metals along the study site of Sayre Highway

Metal(oid)s	TRI_{values}	TRI_{factor}
Cadmium	0.857	TRI ≤ 5' is considered as no toxic risk
Chromium	0.425	TRI ≤ 5' is considered as no toxic risk
Copper	0.358	TRI ≤ 5' is considered as no toxic risk
Zinc	0.952	TRI ≤ 5' is considered as no toxic risk

Table 12. Pearson's multi-linear correlation analysis of metal concentration

	Zn_p	Cd_s	Cr_s	Cu_s	Co_s	Fe_s	Mn_s	Zn_s
Cd_s	-0.183	1						
Cr_s	0.111	-0.255	1					
Cu_s	0.404	-0.405	0.382	1				
Co_s	0.030	0.133	0-.108	0.198	1			
Fe_s	-0.351	0.195	-0.227	-0.326	-0.002	1		
Mn	-0.601**	0.340	-0.375	-0.315	0.106	0.402	1	
Zn_s	0.386	0.052	0.281	0.604**	0.219	-0.623**	-0.180	1

Note: *: Correlation is significant at the 0.05 level; **: Correlation is significant at the 0.01 level

Cu and Zn positively correlated with Cr concentration with r-values of 0.382 and 0.281, respectively. The Cu concentration has a positive, highly significant correlation with Zn with an r-value of 0.604** and a positive correlation in Co with an r-value of 0.198. In contrast, Fe and Mn had a negative, non-significant correlation with r-values of -0.108 and -0.227, respectively. The Co concentration shows a positive correlation with Mn and Zn and a negative non-significant correlation with Fe with r-values of 0.106, 0.219, and -0.002, respectively. Lastly, the correlation between Mn and Zn had a negative, non-significant correlation with each other, with an r-value of -0.180.

In conclusion, the result of this study on metal concentrations in roadside soil shows the possible risks it would cause to human health and the environment. The concentrations in the soil complied with the FAO/WHO maximum permissible limits, suggesting a potential transfer of these heavy metals from the soil to the plants. The metal concentrations in roadside soil samples offer valuable understanding of metals distribution and accumulation patterns in the studied localities. These results can serve as a basis for developing targeted mitigation strategies to reduce human exposure to dust and the consumption of plants along highways. The indices used in this study, such as the (lgeo), (CF), (ERI), (PLI), (EF), and (TRI), helped assess the contamination levels, ecological risks, and possible toxicity associated with heavy metals in the soil. All of these indices show a low risk of ecological damage. However, it also highlights the importance of continuous monitoring to ensure the long-term sustainability and health of the ecosystem. Sampling was limited to the dry season, potentially underestimating wet-season leaching. Preventive measures must be taken to reduce metal contamination in agricultural areas and ensure the safety of food production systems. Implementing green barriers along highways and enforcing emission standards could reduce metal deposition.

Future research should focus on identifying the source, doing long-term monitoring, and developing sustainable agricultural practices that lessen the amount of heavy metals that plants absorb. Adopt phytoremediation using native plant species to immobilize metals. While this study focuses on 7 metals, future studies should include Pb, As, and Hg. More studies on the possible health effects of long-term exposure to heavy metals are needed to protect the community's well-being and advance safe and sustainable farming practices.

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Diversity of plants as a food supplement and medicine for livestock in cattleman communities of Wonosobo, Central Java, Indonesia

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Abstract. *Aprilia FN, Ardhyanti FAK, Paramesti H, Prabowo GAS, Safira RN, Nisyawati, Setyawan AD. 2025. Diversity of plants as a food supplement and medicine for livestock in cattleman communities of Wonosobo, Central Java, Indonesia. Nusantara Bioscience 17: 84-95.* The Wonosobo community in Central Java, Indonesia mostly participates in agriculture and animal husbandry. Individuals engaged in agriculture and animal husbandry exhibit a significant reliance on local resources for the management of food production and animals. This study aimed to find plant diversity that serves as animal feed and supplements for cattle in Wonosobo. The investigation was conducted in October 2024 in the Kalibawang Sub-district of Wonosobo, Central Java, Indonesia, utilizing data gathered from four villages: Dempel, Kalialang, Karangsambung, and Mergolangu. This study included direct surveys and comprehensive interviews with 126 livestock farmers to collect data. The study documented 74 plant species from 37 families utilized as animal feed. The predominant plant families were Fabaceae, Euphorbiaceae, Asteraceae, Poaceae, and Moraceae, encompassing several plants with considerable potential for application in feed, medicine, and animal supplements, presenting a promising perspective for the future of agriculture in Wonosobo. Of the 78 species, 15 were utilized by the inhabitants of Kalibawang Sub-district for medicinal applications, including *wora-wari* (*Hibiscus rosa-sinensis*), *sengon laut* (*Paraserianthes falcataria*), *brotowali* (*Tinospora cordifolia*), coconut (*Cocos nucifera*), papaya (*Carica papaya*), jackfruit (*Artocarpus heterophyllus*), *Gliricidia* (*Gliricidia sepium*), *sulanjana* (*Pennisetum purpureum*), cassava (*Manihot esculenta*), jenu (*Derris elliptica*), banana (*Musa x paradisiaca*), ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), candlenut (*Aleurites moluccanus*), and mahogany (*Swietenia mahagoni*). The highest UV index was recorded in *Artocarpus heterophyllus* Lam and *Carica papaya* L., with a value of 0.023. The highest indices for RFC (0.37) is *Calliandra calothyrsus* Meissn and IAR (Informant Agreement Ratio) (0.94) is *Artocarpus heterophyllus* Lam; however, the highest ICF (Informant Consensus Factor) index was associated with the diarrhea (0.66).

Keywords: Animal feed, cattleman, diversity plant, food supplement, medicinal plant

INTRODUCTION

Livestock development is an integral component of country development. Livestock commodities contribute to the improvement of human resource quality by providing meat, milk, and eggs. Livestock farming helps to support household lives by providing monetary revenue and products. Indonesia has enormous potential in the livestock sector if it continues to expand, as demand for livestock products grows (Tenrisanna and Kasim 2021). Central Java is essential for Indonesia's livestock production, aiming to be a global food supplier by 2045. Demand for livestock-based foods in emerging nations is projected to quadruple by 2050 (Agus and Widi 2018).

Farmers in rural areas often rear between two and six cattle for assets and savings. One example may be located in Magelang District, Central Java, Indonesia. Magelang District has 87,750 goats, 92,100 sheep, 78,286 cows, and 5,978 buffalo (Widarni et al. 2020). Forage, which typically consists of grass and legumes, is used to provide the feed requirements of cattle. Calliandra is one of the plants that are often used as animal feed. Calliandra is a popular feed for a variety of animals, including cattle, goats, and sheep. Its relatively high protein and mineral content make it an acceptable partial feed alternative for cattle. Furthermore, feeding Calliandra to livestock has little effect on their health or production (Aishwaryalakshm et al. 2025).

Wonosobo, in Central Java, serves as the research site. The majority of the population of Wonosobo, Central Java, is employed as farmers and livestock breeders. Livestock management in Wonosobo is done traditionally and according to the cold highland terrain and available resources. Farmers in Wonosobo must pay more attention to the amount and quality of feed offered to their cattle. They do not feed the livestock based on their physiological state or body weight. The feed offered is mostly grass vegetation, with extra supplements provided just when needed (Hakim et al. 2019).

When coping with ailments, the community typically turns to medicinal herbs. Protozoan parasites frequently cause livestock illnesses. Parasitic illnesses have an impact on animal health and productivity. Protozoan parasites can induce fever, anorexia, anemia, and mortality in acute infections (Maharana et al. 2016). Plants have long been utilized for health and medicinal reasons, including animal care. Plant extracts, also known as phytobiotics or phytogenics, are gaining popularity because to their bioactive properties that can benefit cattle health. These phytobiotics are frequently employed in traditional and alternative veterinary care, particularly among small-scale farmers with limited resources (Kuralkar and Kuralkar 2021). In recent years, scientists and animal feed practitioners have been increasingly interested in phytobiotics as safe and effective natural additives (Bagno et al. 2018). Because of its accessibility and cost, herbal medicine continues to be the major treatment technique for small farmers with little resources (Mirzaei-Aghsaghali 2012). This illustrates that phytobiotics are essential in sustainable animal husbandry, both as a therapy and as an alternative to conventional antibiotics. Furthermore, the utilization of nearby flora for livestock feed has left many communities reliant on forest resources. The agricultural community, for example, is reliant on trees. Agricultural communities depend on forests, so effective livestock feed management is essential (Njurumana et al. 2020).

This research is necessary since there has been no previous research on the diversity of plants utilized as food supplements and veterinary treatment in Wonosobo's livestock community. In reality, Wonosobo's natural resources offer great promise as feed additives and veterinary medications, helping to improve feed quality, improve livestock health, and boost long-term livestock productivity.

MATERIALS AND METHODS

Study area

The study was carried out in four villages in Kalibawang Sub-district, Wonosobo District, Central Java, Indonesia. Dempel is the first village, with coordinates of 7°30'49"S and 109°55'51"E. Karangsambung is the second village, with coordinates of 7°29'39"S and 109°55'26"E. Kalialang is the third village, with coordinates of 7°29'11"S and 109°55'51"E. Mergolangu is the fourth village, with coordinates 7°30'52"S and 109°53'11"E (Figures 1 and 2).

Wonosobo has 98,468 hectares and has a wide range of elevations, which affects temperature and rainfall. 33.33% of its area is at an altitude of 250-500 m asl., 50% at 500-1,000 m asl., and 16.67% above 1,000 m asl. (Nurlaely 2022). The area has a tropical climate with two seasons: dry and wet, with typical daily temperatures ranging from 24 to 30°C and dropping to 20°C overnight. In July and August, nighttime temperatures can reach 12-15°C, while daytime temperatures vary from 15-20°C (Hardiko et al. 2016).

In terms of rainfall, the average number of wet days in Wonosobo between 2020 and 2022 varies by sub-district, with Wonosobo having the highest average of 247 days, followed by Kalikajar and Watumalang with 235 and 236 days, respectively. In contrast, Wadaslintang had the lowest average of 180 rainy days. From 2020 to 2022, the Kalibawang receives an average of 166 rainy days. Kalibawang receives less rain than other Sub-districts in Wonosobo on average. This data shows considerable changes in rainfall patterns between sub-districts, which are impacted by local altitude and climate (Central Bureau of Statistics Wonosobo District 2023). According to the reference, Wonosobo has an annual rainfall of 3,500-4,000 mm, which is appropriate for *Calliandra calothyrsus* growth. This species flourishes in locations with considerable rainfall, generally exceeding 1,500 mm per year. Its capacity to fix nitrogen increases soil fertility, making it advantageous to agriculture and cattle. Calliandra can also assist to avoid soil erosion in Wonosobo's steep terrain, which is prone to strong rains.

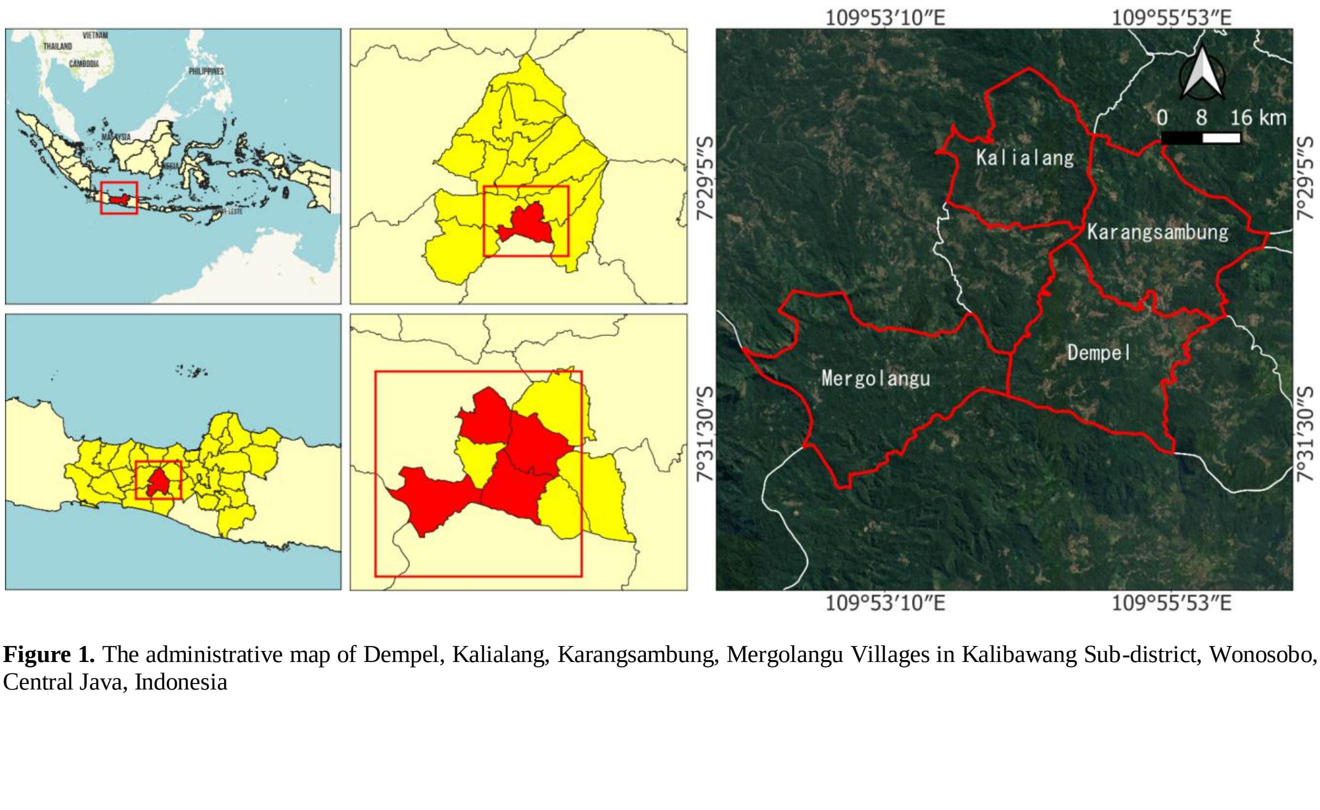

Figure 1. The administrative map of Dempel, Kalialang, Karangsambung, Mergolangu Villages in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

Figure 2. Livestock farming in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia: A. Dempel Village; B. Karangsambung Village; C. Kalialang Village; D. Mergolangu Village

Procedures

Ethnobotanical data collection

This study was done using direct surveys in Kalibawang Sub-district, which was derived into four villages. The survey was conducted in the villages of Dempel, Karangsambung, Kalialang, and Mergolangu. The survey sought to personally examine the plants utilized by local communities for livestock uses such as feed, medication, and supplementation. Furthermore, field observations were carried out by strolling around the study areas to directly document the sorts of plants utilized by local inhabitants for livestock purposes, ensuring a thorough understanding of their use in natural environments. Direct field observations and conversations with villagers revealed specific information about the plant used (Amalyadi 2024). The data from the direct survey was recorded onto a tally sheet for data recap once the field survey was done. The Kalibawang Sub-district has 9,829 households, and the questionnaire was designed for the heads of families who belong to the forest farmer group (*Kelompok Tani Hutan*) (Herdananta et al. 2024). Because of the population's homogenous features, the sample number is calculated using the Slovin formula and a basic random sampling procedure with a margin of error of 0.1. Throughout the interviews, this study distinguishes between owners and laborers. We determined whether respondents were livestock owners or laborers by asking particular questions at the start of the interview. However, the vast majority of those interviewed were cattle owners. The formula used is as follows:

$$n = N/(1+Ne^2)$$

Where: n: sample size; N: Population size; e: Acceptable margin of error.

The approach employed was in-depth interviews with 126 participants. Respondents were chosen from among those who own animals, such as cows, goats, and sheep, as well as those who work as livestock herders. This strategy guarantees that participants have meaningful experience procuring feed for their livestock. This was accomplished by systematic interview, with one person asking the questions and another serving as the informant. Data were also collected in a semi-structured approach, with follow-up questions asking respondents to elaborate on their responses (Knott et al. 2022). The questionnaire began with generic inquiry, and gradually included questions concerning:

i. Name
ii. Age
iii. How many livestock do you own?
iv. What types of livestock do you raise?
v. What are the local names of plants commonly used as animal feed?
vi. Are there variations in plant names across different areas?
vii. What plants are commonly used as animal feed?
viii. What are the benefits of these plants for animal health?
ix. Are there any plants specifically used to treat livestock diseases?
x. Which parts of the plants are typically used?
xi. How are these plant parts processed before use?
xii. What are the common methods of processing plants before feeding them to livestock?
xiii. Are these plants processed into beverages, used topically, or incorporated as dietary supplements?
xiv. What diseases commonly affect livestock in your area?
xv. Are there any specific plants used to treat these diseases?
xvi. How are these plants administered for treating livestock diseases?

All the information collected was then processed through data analysis to conclude.

Specimen identification

The identification of plant species is influenced by the community's local knowledge, particularly the use of local names for species that do not yet have scientific names. Identification keys are used to document specific characteristics such as leaves, fruits, flowers, and wood/stem properties. Following that, taxonomic identification is done using the Global Biodiversity Information Facility (GBIF) website (https://www.gbif.org/species/search).

Data analysis

This study employed a qualitative technique combined with an ethnographic approach to gain a full understanding of social phenomena from the views of participants. This technique was chosen because of its capacity to thoroughly investigate and evaluate certain social phenomena within the framework of community everyday life. The research's implementation focused on monitoring general behaviors that indicate patterns of social acts and interactions within the community as the first component investigated. The second component is investigating personal and communal experiences that influence knowledge and meaning in certain social situations (Mau and Naibili 2022).

According to Chaachouay et al. (2019), Use Value (UV) is a quantitative measure for determining the value of a plant species in a community based on how frequently informants utilize it. It is calculated with the following formula:

$$UV = \frac{\Sigma Ui}{N}$$

Where: Ui: Total number of uses reported by an informant for a specific species; N: Total number of informants mentioning the species.

Trotter and Logan (2019) first introduced the Informant Consensus Factor (ICF) to measure the uniformity of local knowledge. The ICF value ranges from 0 to 1. The index is calculated using the formula:

$$ICF = \frac{[Nur - Ns]}{[Nur - 1]}$$

Where: Nur: Number of use reports for each category; Ns: Number of species used for a specific category by all informants.

The Relative Frequency of Citation (RFC), proposed by Tardío and Pardo-de-Santayana (2008), serves to identify the local importance of a species. RFC has a value ranging from 0 to 1. The index is calculated using the formula:

$$RFC = \frac{Fc}{N}$$

Where: Fc: Number of informants mentioning the species use; N: Total number of informants.

The Informant Agreement Ratio (IAR) is a quantitative index used to measure the level of agreement among informants regarding the use of specific species in a particular category, such as for medicinal or other purposes (Chaachouay et al. 2019). The value for this factor ranges from 0 to 1. The IAR for each use category is calculated using the following formula:

$$IAR = \frac{Nur - Nt}{Nur - 1}$$

Where: Nur: Number of mentions in each category; N: Number of taxa used in each category.

RESULTS AND DISCUSSION

Socio-economics

Based on the data from the respondents' socio-economics characteristics (Table 1), which reveals males (64.29%) as the primary sources of information about plants for livestock, we can draw a link to the issue of gender inequality that persists in the livestock sector. Gender disparity in access to and control over resources and benefits continues to disproportionately affect males (Sumarti and Fuah 2015). This evidence supports this notion, as males outnumber women in terms of knowledge for cattle. Furthermore, Nadhira and Sumarti (2017) note that there is still a gender bias in livestock production, particularly in the dairy farming industry, where women are less likely to engage in training. This might explain why only 35.71% of women became resource persons, as women's access to education and training in this profession needs further expansion.

Furthermore, Kurniawati and Trisna (2019) found that education level, number of livestock industries, and position in the family economy all had an impact on gender equality in livestock business. Women's poor education and ownership of livestock resources may have contributed to their decreased participation in providing information to cattle. This data demonstrates that these challenges remain significant, and that women's participation in the cattle sector must be promoted in order to achieve more gender equality. However, after conducting the interviews, it was shown that male respondents were often better knowledgeable about cattle and pharmaceuticals. This is not to say that female respondents lacked knowledge in these areas; some women displayed considerable competence, but their numbers were comparatively low compared to males.

Table 1. Social and economic characteristics of respondents

Parameter	Specification	Frequency	Percentage (%)
Gender	Male	81	64.29
	Female	45	35.71
Age	<20	5	3.97
	21 - 30	15	11.90
	31 - 40	19	15.08
	41 - 50	22	17.46
	51 - 60	35	27.78
	>60	30	23.81
Education	Elementary school	102	80.95
	Junior high school	12	9.52
	Senior/vocational high school	10	7.94
	Non-formal	2	1.59
Occupation	Laborer	23	18.25
	Farmer	75	59.52
	Self-employed	9	7.14
	Housewife	16	12.70
	Student	2	1.59

Based on the age classification data in Table 1, which shows the dominance of the age group above 50 years in providing knowledge about plants for livestock, there is a strong correlation with Nurdiyansah et al. (2020), who state that farmers in the productive age range (15-56 years) have better physical abilities to run livestock businesses actively and effectively. Despite the physical advantages of the productive age group, this data reveals that categories E (51-60 years) and F (>60 years) had the biggest resource groups, with percentages of 27.78 and 23.81%, respectively.

This can be explained by the age-related drop in physical engagement in cattle activities, according to Nurdiyansah et al. (2020). As physical capacities decline, older farmers may be more useful in imparting information and experience, but their physical participation in livestock management reduces. Even if their physical engagement in the cattle sector is waning, older farmers have extensive knowledge and many years of experience, making them valuable resource persons (Indey et al. 2021).

Furthermore, May et al. (2019) suggest that younger farmers are more active in business management and development than older farmers, as seen by the comparatively low percentage of respondents in group B (21-30 years), which is just 11.90%. Although younger farmers are more physically active, older farmers' experience and skills are still valued in this livestock business. Therefore they continue to be the primary source of information.

According to the informants' education data, the vast majority (80.95%) only had elementary education, with a significantly lower fraction having secondary education (Junior High School 9.52%, Senior High School 7.94%) and very few having a non-formal education background (1.59%). This is comparable to the study made by Indey et al. (2021), who argue that education level is an essential predictor of farmers' capacity to utilize technology and innovation in livestock farming. This data demonstrates a significant obstacle in the deployment of technology among farmers with low education.

Farmers with higher education are more receptive to innovation and can better run their businesses. However, because the majority of the respondents had a poor level of education, it is likely that the implementation of contemporary technologies or innovations in the livestock industry in the Kalibawang area need further improvement. Farmers with a low education may depend more on conventional methods of conducting their livestock businesses, limiting the adoption of new technology that might boost efficiency and productivity. As a result, it is critical to expand farmers' access to non-formal education or training in the area so that they are better prepared to embrace and apply technology and innovations in their livestock activities, as suggested by Indey et al. (2021). Thus, enhanced education may play an essential role in promoting the growth of a more modern and profitable cattle industry.

According to data on the occupations of resource individuals who contributed information about plants for cattle, the majority were farmers or breeders, accounting for 75 people (59.52%). This demonstrates that knowledge of plants for livestock is mostly learnt by those who are actively involved in agricultural and livestock activities. Farmers and breeders have extensive firsthand experience, thus they play a significant role as resource individuals in this setting. The labor group is the second largest, with 23 members or 18.25%. Although laborers are not directly active in agricultural or animal husbandry, they may assist these industries and so have important knowledge of the crops used for livestock.

Housewives accounted for 16 persons, or 12.70% of those interviewed. Housewives may play an essential role in maintaining household land that supports cattle or family farming businesses, even if they do not have formal agricultural or animal husbandry credentials. The self-employed category, with nine members, or 7.14%, demonstrates that, while small, there are individuals who operate independent businesses other than animal husbandry and have expertise of livestock crops. Meanwhile, students numbered just two, or 1.59%, indicating a relatively low level of engagement by young people in giving information on livestock crops, possibly due to a lack of practical experience in the activity. Overall, this data suggests that farmers and breeders are the most knowledgeable about livestock crops, followed by other groups who play supporting roles or are indirectly connected in the livestock business.

Plant diversity

Table 2 reveals that 37 plant families are dispersed among four villages in Kalibawang Sub-district: Dempel, Karangsambung, Kalialang, and Mergolangu, each with a different number of species. The two largest families with eight species are Fabaceae and Poaceae, represented by *Albizia chinensis, Arachis hypogaea, C. calothyrsus, Erythrina variegata, Falcataria moluccana, Gliricidia sepium, Indigofera tinctoria, Leucaena leucocephala* for the Fabaceae family, and the Poaceae family, which is represented by ten species namely *Cenchrus purpureus, Digitaria ciliaris, Echinochloa colonum, Ischaemum rugosum, Oplismenus hirtellus, Oryza sativa, Paspalum conjugatum, Saccharum officinarum, Zea mays,* and *Pennisetum purpureum* followed by the Asteraceae family, which includes seven species: *Ageratum conyzoides, Acmella paniculata, Cosmos caudatus, Cyanthillium cinereum, Chromolaena odorata, Eclipta alba, Eclipta prostrata*. Furthermore, the Euphorbiaceae family is represented by six species: *Manihot esculenta* for local names containing *singkong* and *bodin, Aleurites moluccanus, Manihot glaziovii* for local names containing *singkong taun* and *bodin karet*, and *Sauropus androgynus* (Figure 3). Figure 4 depicts some plant species utilized as food components and animal supplements.

Table 2. Plant species diversity for livestock in the villages of Dempel, Karangsambung, Kalialang, and Mergolangu of Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

Family	Species name	Local name	Used part	Processed	Benefit				UV	RFC
					1	2	3	4		
Acanthaceae	*Asystasia gangetica* (L.) T. Anders.	*Krompolan*	Leaves	Direct				●	0.007	0.015
Acanthaceae	*Clinacanthus nutans* Lindau	*Lingser*	Leaves	Direct				●	0.007	0.015
Acoraceae	*Acorus calamus* var. americanus L.	*Sringu*	Leaves	Direct				●	0.007	0.015
Anacardiaceae	*Mangifera indica* L.	*Pelem*	Leaves	Direct			●		0.007	0.015
Apiaceae	*Centella asiatica* (L.) Urb.	*Godong rendeng*	Leaves	Direct				●	0.007	0.015
Araucariaceae	*Agathis dammara* (Lamb.) Rich.	*Damar*	Leaves	Direct			●		0.007	0.007
Arecaceae	*Cocos nucifera* L.	*Kelapa*	Water	Direct		●			0.007	0.007
Arecaceae	*Johannesteijsmannia altifrons* (Rchb.f. & Zoll.) H.E.Moore	*Pajong*	Leaves	Direct				●	0.007	0.007
Arecaceae	*Arenga pinnata* Merr	*Aren*	Leaves	Direct			●		0.007	0.007
Asteraceae	*Ageratum conyzoides* L.	*Bandotan*	Leaves	Direct			●		0.007	0.253
Asteraceae	*Acmella paniculata* (Wall. ex DC.) R.K.Jansen	*Jotang*	Leaves	Direct			●		0.007	0.111
Asteraceae	*Eclipta prostrata* (L.)	*Arong*	Leaves	Direct				●	0.007	0.007
Asteraceae	*Cyanthillium cinereum* (L.) H. Rob.	*Sawi langit*	Leaves	Direct				●	0.007	0.007
Asteraceae	*Cosmos caudatus* Kunth.	*Kenikir*	Leaves	Direct				●	0.007	0.007
Asteraceae	*Eclipta alba* (L.) L.	*Urang-aring*	Leaves	Direct				●	0.007	0.015
Asteraceae	*Chromolaena odorata* (L.) King & H.E. Robins	*Kirinyuh*	Leaves	Direct			●		0.007	0.023
Bombacaceae	*Durio zibethinus* Murray	*Durian/duren*	Leaves	Direct				●	0.007	0.007
Bombacaceae	*Ceiba pentandra* (L.) Gaertn.	*Randu*	Leaves	Direct			●		0.007	0.023
Capparaceae	*Cleome rutidosperma* DC.	*Ijon*	Leaves	Direct				●	0.007	0.023
Caricaceae	*Carica papaya* L.	*Pepaya*	Leaves	Direct		●			0.023	0.134
			Leaves	Direct			●			
			Leaves	Direct		●				
Cornaceae	*Aucuba japonica* Thunb.	*Porin*	Leaves	Direct				●	0.007	0.015
Cucurbitaceae	*Momordica charantia* L.	*Pare*	Leaves	Direct				●	0.007	0.007
Cucurbitaceae	*Citrullus lanatus* (Thunb.) Matsum.	*Semangka*	Leaves	Direct				●	0.007	0.031
Cyatheaceae	*Alsophila glauca* (Bl.) J.Sm	*Pakis pohon*	Leaves	Direct			●		0.007	0.007
Cyperaceae	*Cyperus kyllingia* Endl.	*Ijon*	Leaves	Direct			●		0.007	0.095
Euphorbiaceae	*Manihot glaziovii* Mull. Arg.	*Bodin karet*	Leaves	Direct			●		0.007	0.365
Euphorbiaceae	*Manihot esculenta* Crantz	*Bodin*	Leaves	Direct			●		0.015	0.388
			Fruit peel	Dried		●				
Euphorbiaceae	*Sauropus androgynus* (L.) Merr.	*Katuk*	Leaves	Direct			●		0.007	0.023
Euphorbiaceae	*Manihot glaziovii* Mull. Arg.	*Bodin karet*	Leaves	Direct				●	0.007	0.055
Euphorbiaceae	*Aleurites moluccanus* (L.) Willd	*Miri/kemiri*	Leaves	Direct				●	0.007	0.007
Fabaceae	*Calliandra calothyrsus* Meissn	*Andra/andro*	Leaves	Direct			●		0.007	0.515
Fabaceae	*Gliricidia sepium* (Jacq.) Kunth ex Walp.	*Laraside*	Leaves	Direct			●		0.015	0.182
			Leaves	Direct		●				
Fabaceae	*Albizia chinensis* (Osbeck) Merr.	*Albasiyah*	Leaves	Direct			●		0.007	0.166
Fabaceae	*Arachis hypogaea* L.	*Kacang*	Leaves	Direct				●	0.007	0.007
Fabaceae	*Leucaena leucocephala* (Lam.) de Wit	*Mlanding*	Leaves	Direct				●	0.007	0.015
Fabaceae	*Indigofera tinctoria* L.	*Tarum*	Leaves	Direct				●	0.007	0.007
Fabaceae	*Erythrina variegata* L.	*Dadap*	Leaves	Direct				●	0.007	0.039
Fabaceae	*Falcataria moluccana* (Miq.) Barneby & Grimes	*Sengon laut*	Leaves	Direct		●			0.007	0.023
Gnetaceae	*Gnetum gnemon* L.	*Melinjo*	Leaves	Direct				●	0.007	0.031
Liliaceae	*Curculigo latifolia* Dryand. ex W.T.Aiton	*Ijon*	Leaves	Direct				●	0.007	0.015
Malvaceae	*Hibiscus rosa-sinensis* L.	*Wora wari*	Leaves	Direct		●			0.007	0.023
Meliaceae	*Swietenia mahagoni* (L.) Jacq.	*Mahoni*	Leaves	Direct			●		0.007	0.063
			Leaves	Direct		●				
Menispermaceae	*Tinospora cordifolia* (Willd.) Hook.f.	*Brotowali*	Leaves	Direct			●		0.015	0.071
			Leaves	Direct		●				
Moraceae	*Ficus lyrata* Warb.	*Wiledah*	Leaves	Direct			●		0.007	0.269
Moraceae	*Artocarpus heterophyllus* Lam	*Nangka*	Fruit	Direct				●	0.023	0.452
			Leaves	Direct		●				
			Leaves	Direct			●			
Moraceae	*Artocarpus altilis* (Parkinson) Fosberg	*Sukun*	Leaves	Direct			●		0.007	0.015
Moringaceae	*Moringa oleifera* Lam.	*Kelor*	Leaves	Direct				●	0.007	0.023
Musaceae	*Musa x paradisiaca* L.	*Pisang*	Leaves	Direct		●			0.015	0.214
			Leaves	Direct				●		
Myrtaceae	*Psidium guajava* L.	*Jambu kluthuk*	Leaves	Direct				●	0.007	0.031
Papilionaceae	*Derris elliptica* (Wall.) Benth.	*Jenu*	Leaves	Direct		●			0.007	0.015
Poaceae	*Zea mays* L.	*Jagung*	Leaves	Direct				●	0.015	0.023
			Cob	Dried				●		

Poaceae	*Cenchrus purpureus* (Schumach.) Morrone	*Paspa*	Leaves	Direct		●			0.015	0.325
			Leaves	Direct	●					
Poaceae	*Ischaemum rugosum var. segetum* Salisb.	*Blembem*	Leaves	Direct				●	0.007	0.015
Poaceae	*Oryza sativa* L.	*Damen*	Leaves	Direct				●	0.007	0.047
Poaceae	*Digitaria ciliaris* Retz.	*Suket*	Leaves	Direct				●	0.007	0.007
Poaceae	*Echinochloa colonum* (L.) Link	*Ijon*	Leaves	Direct				●	0.007	0.007
Poaceae	*Saccharum officinarum* L.	*Tebu*	Leaves	Direct		●			0.007	0.007
Poaceae	*Pennisetum purpureum* Schumach	*Sulanjana*	Leaves	Direct				●	0.007	0.007
Poaceae	*Paspalum conjugatum* P.J.Bergius	*Ijon*	Leaves	Direct				●	0.007	0.079
Poaceae	*Oplismenus hirtellus* (L.) P. Beauv	*Rumput keranjang*	Leaves	Direct			●		0.007	0.015
Polygonaceae	*Polygonum chinense* L.	*Madhusoleng*	Leaves	Direct			●		0.007	0.007
Polypodiaceae	*Diplazium esculentum* Swartz	*Pakis*	Leaves	Direct				●	0.007	0.015
Rubiaceae	*Coffea arabica* L.	*Kopi/sampang*	Leaves	Direct				●	0.007	0.023
Rubiaceae	*Diodia virginiana* L.	*Ijon*	Leaves	Direct				●	0.007	0.007
Rutaceae	*Citrus sinensis* (L.) Osbeck	*Jeruk*	Leaves	Direct				●	0.007	0.023
Sapotaceae	*Manilkara zapota* (L.) P. Royen	*Sawo*	Leaves	Direct				●	0.007	0.015
			Fruit	Direct				●		
Tectariaceae	*Tectaria dissecta* (G.Forst.) Lellinger	*Pakis*	Leaves	Direct				●	0.007	0.007
Thelypteridaceae	*Thelypteris* sp.	*Pakis*	Leaves	Direct				●	0.007	0.015
Urticaceae	*Pipturus albidus* (Hook. & Arn.) A. Gray	*Terudang*	Leaves	Direct			●		0.007	0.007
Urticaceae	*Pilea melastomoides* (Poir.) Bl.	*Poh-pohan*	Leaves	Direct			●		0.007	0.023
Verbenaceae	*Tectona grandis* L.f.	*Kebek*	Leaves	Direct			●		0.007	0.023
			Fruit	Direct				●	0.007	0.023
Verbenaceae	*Stachytarpheta jamaicensis* (L.) Vahl	*Pecut kuda*	Leaves	Direct			●		0.007	0.007
Zingiberaceae	*Zingiber officinale* Rosc.	*Jahe*	Fruit	Direct		●			0.007	0.007
Zingiberaceae	*Curcuma longa* L.	*Kunyit*	Fruit	Direct		●			0.007	0.007

Note: The benefits of livestock feed are: 1: Supplement, 2: Medicine, 3: Food, 4: Mixture. ●: Present

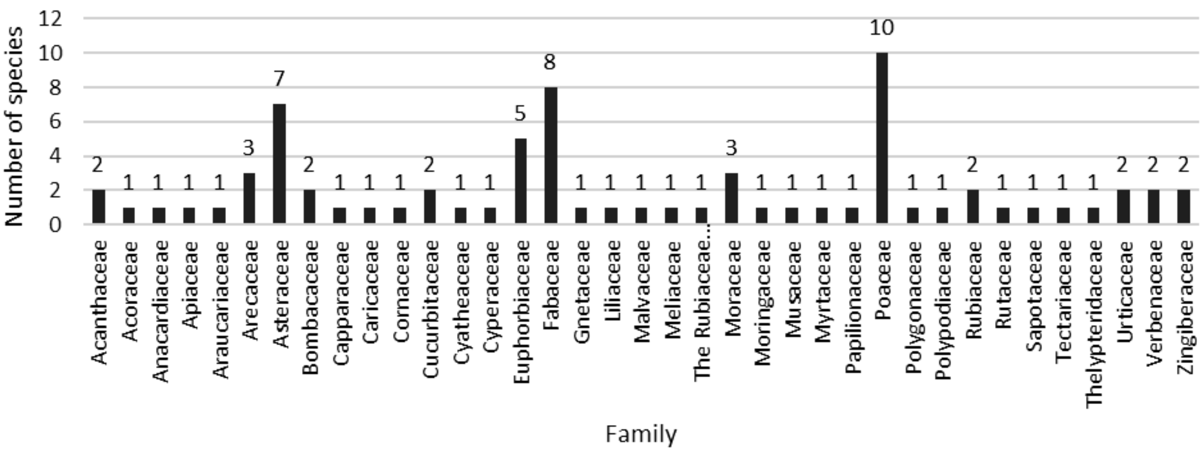

Figure 3. Number of species per family of animal feed plants in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

The Moraceae family includes three species namely *Artocarpus altilis, Artocarpus heterophyllus,* and *Ficus lyrata*. The Arecaceae family include three species namely *Agathis dammara, C. nucifera,* and *Johannesteijsmannia altifrons*. There are seven families represented by two species, the The Rubiaceae family includes two species namely *Coffea arabica* (also known as kopi/sampang) and *Diodia virginiana*. The Acanthaceae family includes the species *Asystasia gangetica* and *Clinacanthus nutans*. The Bombacaceae family includes *Ceiba pentandra* and *Durio zibethinus*. The Cucurbitaceae family includes *Citrullus lanatus* and *Momordica charantia*. The species *Curcuma longa* and *Zingiber officinale* belong to the Zingiberaceae family. The Urticaceae family includes *Pipturus albidus* and *Pilea melastomoides*. The final family represented by two species is the Verbenaceae family, which includes *Stachytarpheta jamaicensis* and *Tectona grandis*.

The data indicates that the Fabaceae and Poaceae families are the most prevalent in livestock feed. The Fabaceae family, commonly referred to as Leguminosae, is acknowledged as the legume family of plants. The Fabaceae family is positioned third in terms of plant species diversity, encompassing around 751 genera and 19,000 species. The Fabaceae family is abundant in protein and micronutrients (Asfaw and Abebe 2021). When utilized appropriately, plants from the Fabaceae family can provide a significant protein source for animals (Mabeza et al. 2018).

Figure 4. Some livestock feed plants in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia. A. *Blembem* leaves (*Ischaemum rugosum* var. *segetum*); B. *Krompolan* leaves (*Asystasia gangetica*); C. *Wiledah* leaves (*Ficus lyrata*); D. *Kebek* leaves (*Tectona grandis*); E. *Melinjo* leaves (*Gnetum gnemon*); F. Calliandra leaves (*Calliandra calothyrsus*); G. *Sulanjana* leaves (*Pennisetum purpureum*); H. Cassava peel (*Manihot esculenta*)

The Poaceae family is positioned fifth in terms of species count. It comprises around 10,000 species that can be employed for various economic and medicinal applications in humans (Fatima et al. 2018). The family occupies a significant role among plant families owing to its diverse applications for humans, including food, feed, medicine, and industrial uses. Significant crops for human consumption in this family encompass soft wheat, corn, and rice, among various others. The significance of plants from the Poaceae family in livestock feed is well-documented (Baranovsky et al. 2019).

Utilizing plant species diversity data, UV (Use Value) and RFC (Relative Frequency of Citation) values offer significant insights into the utility and societal recognition of specific plants. This study indicates that *G. sepium* possesses a UV value of 0.015 and an RFC of 0.126. This indicates that this plant serves multiple purposes within the community and enjoys significant recognition. *M. esculenta* exhibited comparable UV and RFC values of 0.015 and 0.269, respectively. This suggests that cassava/*bodin* is a versatile plant with numerous applications, frequently referenced in the daily lives of local communities. This underscores the significance of cassava in both practical applications and indigenous understanding.

Conversely, *C. arabica* exhibited marginally lower values, recording a UV of 0.007 and an RFC of 0.023. While coffee is a widely recognized and utilized crop, its comparatively lower UV value indicates that its applications may not be as varied as those of other crops with higher UV values. A comparable observation can be noted for *C. nucifera*, which likewise exhibits a UV value of 0.007. Nonetheless, coconut demonstrates more specialized applications in comparison to other crops, even though it is broadly acknowledged and employed.

In general, plants exhibiting elevated UV and RFC values, such as *G. sepium* and *M. esculenta*, are associated with a greater range of applications and are well-regarded within the community. In the meantime, species like *C. arabica* and *C. nucifera* are frequently referenced as sources, although their application tends to be more restricted within local contexts. A diagram illustrating a comparison of these values can effectively demonstrate the significance of these plants in communities' lives.

Plant for medicinal purposes on cattle

Fifteen species of plants have been identified as having medicinal properties for the treatment of diseases in livestock (Table 3). In Indonesia, the Arecaceae family has been extensively utilized by local communities, as nearly all parts of the plant serve distinct purposes, including applications in traditional medicine. The Zingiberaceae family includes over 1,300 species that originate from tropical Asia and are commonly found in Indonesia, thanks to the region's favorable climate. Plants from this family are well-known for their unique aromatic characteristics and significant application in traditional medicine (Teusiit et al. 2024).

The Malvaceae family includes 78 genera and over 1,000 species, predominantly found in South America, with a presence in nearly all regions except for extremely cold areas. Members of this family are categorized as herbs, distinguished by their alternate leaf arrangement, palmate venation, and the presence of stipules. Fabaceae, also referred to as Leguminosae, is a flowering plant family that holds considerable economic importance and possesses medicinal properties advantageous for the health of both humans and livestock (Erarslan and Koçyiğit 2019).

Moraceae is found in tropical and subtropical regions and holds significant economic value as a source of food, timber, and medicine. A bibliographic review indicates that this family encompasses a variety of secondary metabolites, including flavonoids, tannins, phytosterols, and alkaloids, which exhibit properties associated with anti-inflammatory, antimicrobial, and antioxidant effects (Basnett et al. 2023). The Caricaceae family, which includes 30 species across four genera, is cultivated not only for its fruits but also possesses therapeutic potential for addressing digestive disorders, skin issues, and inflammatory conditions (Yadav et al. 2022).

Poaceae, commonly known as the grass family, ranks as the fifth-largest flowering plant family, encompassing around 50 sub-family 660 genera, and 10,000 species. A number of species from this family are employed in the treatment of hypertension, diabetes, inflammation, helminthic infections, and gastric ulcers, and they function as diuretics and antioxidants. The Euphorbiaceae family, commonly referred to as the spurge family, exhibits a broad distribution and holds considerable promise for medicinal uses in both tropical and non-tropical areas (Fatima et al. 2018).

Papilionaceae, a deciduous rainforest tree, and Musaceae, both have a longstanding history in traditional medicine, utilized since ancient times. Different plant-parts of Papilionaceae, such as leaves, wood, bark, and seeds, along with its flour, have been utilized to tackle a variety of health concerns. Musaceae has become a focal point for the pharmaceutical industry owing to its wide range of health benefits. These encompass anti-diabetic, anti-ulcer, antioxidant, and wound healing properties, along with several additional benefits. Phytochemical compounds including alkaloids, steroids, glycosides, and flavonoids are essential to its pharmacological activities (Tchamadeu et al. 2017).

The Meliaceae family, consisting of 740 species across 58 genera, has shown considerable promise in the health sector due to its rich array of active compounds, which include alkaloids, diterpenoids, flavaglines, lignans, limonoids, sesquiterpenoids, and triterpenoids. These compounds demonstrate a range of advantageous biological activities, including anti-cancer, anti-inflammatory, antiviral, antimicrobial, and antidiabetic effects. The Meliaceae family presents a valuable opportunity for the advancement of innovative pharmaceuticals, fostering optimism within the industry (Riyadi et al. 2023).

Table 3. Utilization of plants for medicine and IAR value

Family	Local name	Species name	Treating a disease	Part	Processed	IAR
Arecaceae	*Coconut*	*Cocos nucifera* L.	Diarrhea	Leaves	Drunk	0
Caricaceae	*Pepaya*	*Carica papaya* L.	Diarrhea	Leaves	For mixing	0.81
			Skin	Leaves	Rubbed	
			Tongue bumps	Leaves	Rubbed	
Euphorbiaceae	*Singkong/budin*	*Manihot esculenta* Crantz	Appetite	Leaves	For mixing	0.93
Fabaceae	*Sengon laut*	*Falcataria moluccana* (Miq.) Barneby & Grimes	Skin	Leaves	Rubbed	0
Fabaceae	*Laraside*	*Gliricidia sepium* (Jacq.) Kunth ex Walp.	Skin	Leaves	Rubbed	0.86
Malvaceae	*Wora-wari*	*Hibiscus rosa-sinensis* L.	Skin	Leaves	Rubbed	0
Meliaceae	*Mahoni*	*Swietenia mahagoni* (L.) Jacq.	Tongue bumps	Leaves	Rubbed	0.57
Menispermaceae	*Brotowali*	*Tinospora cordifolia* (Willd.) Hook.f.	Skin	Leaves	Rubbed	0.62
Moraceae	*Nangka*	*Artocarpus heterophyllus* Lam	Skin	Leaves	Rubbed	0.94
			Diarrhea	Leaves	For mixing	
Musaceae	*Pisang*	*Musa x paradisiaca* L.	Appetite	Leaves	For mixing	0.88
Papilionaceae	*Jenu*	*Derris elliptica* (Wall.) Benth.	Skin	Leaves	Rubbed	0
Poaceae	*Paspa*	*Cenchrus purpureus* (Schumach.) Morrone	Skin	Leaves	Rubbed	0.92
Zingiberaceae	*Tebu*	*Saccharum officinarum* L.	Appetite	Fruit	For mixing	0
Zingiberaceae	*Jahe*	*Zingiber officinale* Rosc.	Appetite	Fruit	For mixing	0
Zingiberaceae	*Kunyit*	*Curcuma longa* L.	Appetite	Leaves	For mixing	0

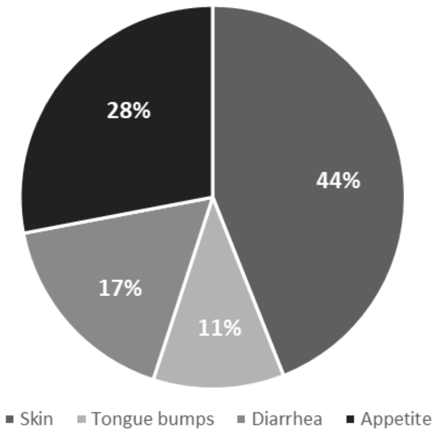

Figure 5. Number of species and their utilization

Disease cure

In our study, livestock feed plant are categorized into four groups according to their advantages. Initially, the feed utilized as a supplement serves to enhance the nutritional quality of livestock. Secondly, food that serves a medicinal purpose includes bioactive compounds that can aid in the prevention or treatment of diseases and has been utilized by local communities as a form of traditional medicine for an extended period. Third, this feed serves solely as a staple food without providing any additional benefits. Ultimately, there exists a feed that serves as a mixture, specifically a combination of various types of feed to achieve more optimal benefits. Ultimately, there exists a feed that serves as a mixture, specifically a combination of various types of feed to achieve more optimal benefits. We identified a total of 12 families exhibiting significant pharmacological potential. In the classification of diseases, four primary categories were commonly observed in livestock, with the following percentage distribution: skin diseases at 44%, appetite issues at 28%, diarrhea at 17%, and tongue nodules at 11% (Figure 5). The primary application of medicinal plants by residents is for the treatment of skin diseases in livestock, accounting for 44%. This includes families such as Caricaceae, Fabaceae, Malvaceae, Menispermaceae, Moraceae, Papilionaceae, Poaceae, which are utilized in addressing skin ailments in livestock. The presence of bioactive compounds in these families, which act as antimicrobial and anti-inflammatory agents, as well as stimulants for skin cell regeneration, indicates their promising potential in pharmacological applications (Khan et al. 2017).

Furthermore, 28% of medicinal plants are utilized by residents for first aid in cases of bloating or appetite loss in their livestock. The combination of antimicrobial, anti-inflammatory, and carminative properties of the plants is essential in addressing bloating in animals. Bioactive compounds derived from plants in the Euphorbiaceae, Musaceae, and Zingiberaceae families function by alleviating inflammation, safeguarding the mucous membrane, regulating intestinal motility, and minimizing gas, thus aiding in the normalization of digestive function and expediting recovery in animals suffering from bloating (Prabowo et al. 2023).

Residents utilize approximately 17% of medicinal plants for the treatment of diarrhea in livestock, particularly those from the Arecaceae, Caricaceae, and Moraceae families, due to their bioactive compounds that promote digestive health. Coconut leaves from the Arecaceae family possess astringent properties that assist in reducing excessive intestinal secretions while simultaneously balancing electrolyte levels, which is crucial for preventing dehydration resulting from diarrhea. Papaya leaves, belonging to the Caricaceae family, are known to contain the enzyme papain, which exhibits anti-inflammatory properties and aids in digestion. As a result, they are effective in alleviating diarrhea by normalizing intestinal activity. Furthermore, jackfruit leaves belonging to the Moraceae family contain natural antimicrobial compounds, including flavonoids, which are effective in addressing pathogens responsible for intestinal infections while also promoting the regeneration of damaged digestive tissues (Ermis et al. 2021).

Additionally, 11% of the utilization of medicinal plants is focused on treating tongue nodules in livestock. Papaya leaves from the Caricaceae family and mahogany leaves from the Meliaceae family have shown effective therapeutic properties for this condition. Among these medicinal plants, papaya leaves are known to contain papain enzymes, which have the ability to combat bacterial infections and promote tissue regeneration, thus alleviating inflammation and swelling of the tongue (Nasri et al. 2022). Mahogany leaves contain a high concentration of flavonoids and alkaloids, which possess antimicrobial and anti-inflammatory properties. These compounds contribute to the inhibition of pathogen growth and the alleviation of irritation in affected regions. Incorporating these leaves into livestock feed or engaging in direct contact with the source of the disease, such as by rubbing the affected area, is essential for farmers when their animals exhibit unusual symptoms.

IAR of medicinal plant for cattle

The calculation results indicate that the species exhibiting the highest Informant Agreement Ratio (IAR) is *A. heterophyllus* (Jackfruit), which has a value of 0.94 (Table 3). This value demonstrates that jackfruit offers medicinal advantages for livestock. A value of the IAR (Informant Agreement Ratio) that approaches 1 indicates a greater level of consensus within the community. The medicinal component of the Jackfruit is derived from its leaves. Many people assert that Jackfruit has the potential to address diarrhea and skin ailments in livestock. The prevalence of jackfruit trees in the area is a contributing factor to the utilization of this plant in medicinal applications.

Conversely, there are seven species that hold a value of 0. This value reflects a discrepancy in the responses provided by the informants. The species listed are *C. longa, C. nucifera, D. elliptica, F. moluccana, Hibiscus rosa-sinensis, S. officinarum, Z. officinale*. The use of these plants as feed for livestock is limited, with merely 1 to 2 people among 126 informants identifying them as medicinal for livestock.

Table 4. Disease categories index by ICF (Informant Consensus Factor)

Type of disease	ICF
Skin	0.416
Tongue bumps	0
Diarrhea	0.666
Appetite	0.2

ICF of the plant using on disease treatment by cattle community

The data presented in Table 4 indicates that the ICF (Informant Consensus Factor) value, utilized to assess the consistency of local knowledge, is highest for diarrhea, recorded at 0.666. This indicates a strong consensus among informants concerning the utilization of plant species for the treatment of diarrhea. The strong consensus suggests that the plant species utilized have been traditionally recognized for their effectiveness in treating diarrhea, as multiple informants referenced the same plant. The lowest ICF (Informant Consensus Factor) value recorded was 0 for tongue pustule disease, signifying that informants uniformly reported no plant species for the treatment of this ailment. This may result from various factors, including insufficient knowledge or experience regarding effective treatments for tongue pustules, or it is possible that traditional medicine does not frequently address this ailment. Another ailment that exhibits a low Informant Consensus Factor (ICF) value is appetite, recorded at 0.2. This indicates that there is considerable variation in the application of plants for addressing appetite issues, accompanied by a relatively low level of agreement among informants. In summary, elevated ICF (Informant Consensus Factor) values indicate a greater consensus on the application of specific plant species for particular medical conditions. Conversely, low values indicate a deficiency in consensus or potentially greater variability in traditional treatment methods.

In conclusion, the 90 species of plants documented in Kalibawang Sub-district, Wonosobo, Central Java, were derived from 37 families. Fabaceae, Euphorbiaceae, Asteraceae, Poaceae, and Moraceae are among the most frequently encountered families. These families contain numerous species that have the potential to be used as combinations of feed, medication, and livestock supplements. The highest UV and RFC values were exhibited by *G. sepium* and *M. esculenta*, which suggests that they are highly popular among the community and have a diverse utility, according to the analysis. *Artocarpus heterophyllus* demonstrated the maximum IAR (Informant Agreement Ratio) value of 0.94, indicating a robust consensus among community members regarding its medicinal advantages for livestock. The gastroenteritis treatment exhibited the highest ICF (Informant Consensus Factor) value, with a value of 0.666, indicating a firm consensus regarding the efficacy of plants in treating this condition. Seven plant species with IAR (Informant Agreement Ratio) values of 0 and tongue nodule disease with an ICF (Informant Consensus Factor) of 0 suggest that informants have minimal levels of agreement regarding the medicinal use of these plants.

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Physicochemical properties and antibacterial activity of essential oil fractions obtained from fresh *Litsea angulata* leaves

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Abstract. *Putri AS, Nur M, Kusuma IW, Egra S, Kuspradini H. 2025. Physicochemical properties and antibacterial activity of essential oil fractions obtained from fresh* Litsea angulata *leaves. Nusantara Bioscience 17: 96-102. Litsea angulata* Blume (Lauraceae) is widely grown in Kalimantan Island and is traditionally used as a medicinal plant. This work aimed to analyze the characteristics of the physicochemical from *L. angulata* oil fractions and the potency of its antibacterial activity. *Litsea angulata* fresh leaves were distilled, and their oil fractions were obtained by simple fractional distillation using water and steam distillation methods. The fraction of essential oil is based on the different distillation times. In this experiment, the following distillation periods were tested: 0-60 min, 60-120 min, and 120-180 min. The yield and color determined the physicochemical properties, while the chemical properties, as their compositions, were analyzed by GC-MS. The agar diffusion method was employed to assess antibacterial activity. Two kinds of synthetic standards, including chlorhexidine and chloramphenicol, were used to compare their antibacterial properties. *Streptococcus sobrinus, Streptococcus mutans* and *Staphylococcus aureus* were the microorganisms used in this investigation. The results showed fractions yield (range 0.09-1.66%) reached a maximum in 0-60 min, and color clearness. All oil fractions have various colors and chemical components according to their treatments. The diameter inhibition against selected bacteria (range 10.22-15.11 mm) was highest at fraction 0-60 min. This essential oil is potentially developed in the pharmaceutical industry as a natural alternative product.

Keywords: GCMS, *Litsea agulata*, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus sobrinus*

INTRODUCTION

Essential oils are high-priced hydrocarbons generated solely by certain plant species. Their applications have a lengthy history in traditional herbal medicine, which lacks empirical validation. In recent decades, there has been a worldwide increase in interest in essential oils. A significant rise in global awareness regarding research-driven technologies that facilitate and sustain health condition management has been seen. As a consequence, the topic of essential oils has garnered increased interest and relevance within the fields of academic research and industrial applications (Oliveira et al. 2021). The global essential oil market is highly promising, with target markets including Japan, the United States, the United Kingdom, and several other European nations. The oils in demand comprise citronella oil, clove-leaf oil, ylang-ylang oil, vetiver oil, sandalwood oil, and patchouli oil. These oils will then be refined to create items suitable for use as perfumes, pharmaceuticals, dental pastes, confections, or flavoring ingredients for food (Sastrohamidjojo 2021).

Essential oils, fragrant essences produced from aromatic herbs, have been utilized in several ways, i.e., aromatherapy, food preservation and flavoring, perfumery, pharmaceutical, and beauty care products (Naeem et al. 2018; Herman et al. 2019). These organic compounds have a variety of medicinal effects and biotechnological uses (Oliveira et al. 2014). Oil-secreting glands can be observed in several plant structures, including roots, flowers, wood, fruits, barks, seeds, and leaves, with occasional distribution over the entire plant (Naeem et al. 2018). Numerous studies have demonstrated the presence of essential oils' antiviral, antibacterial, antifungal, anti-inflammatory, anticarcinogenic, antimutagenic, and antioxidant properties, among other things. The lactone group in the sesquiterpenoid compound group is known as an active molecule that is toxic to pathogenic bacteria (Shaaban et al. 2012). Currently, around 3000 essential oils have been identified. Among other things, peppermint oil has properties that can cure various ailments, such as relieving headaches, flu, cough, sinusitis, nasal congestion, and other digestive problems (Nayak et al. 2020). Lemongrass oil can reduce anxiety, while also acting as an antibacterial, anti-inflammatory, and antifungal (Gaba et al. 2020). Lavender oil can relieve insomnia, muscle pain, stress, and poor sleep quality. Tea tree and chamomile oils relieve fungal skin infections, dandruff, and head lice (Sahu et al. 2025). Chamomile oil also relieves anxiety and improves sleep quality (Kazemi et al. 2024). Clove oil helps with acne, strengthens hair roots, improves scalp circulation, and reduces dandruff (Haro-González et al. 2021). Jasmine oil helps with irregular heartbeat, insomnia, and stress (Shah et al. 2022). Nutmeg oil helps stimulate digestion, invigorate the skin, and boost energy (Vuković et al. 2022). The majority of aromatic plant species are members of the following families: Myrtaceae, Lauraceae, Umbelliferae, Labiatae, and Asteraceae (Hassaballa and Alsiddig 2022).

The genus *Litsea* Lam., a member of the family Lauraceae, exhibits a notable prevalence in tropical and subtropical climates. This plant is an important tropical forest vegetation and is known for its essential oil (Jose et al. 2015; Kamle et al. 2019). Litsea has various characteristic aromas and is potentially used as a medicinal plant (Li et al. 2024). *Litsea angulata* Blume, belonging to the genus *Litsea* (seed part), is used by Kalimantan local people to treat boils (Kuspradini et al. 2019) examined various components of this species, including bark, branches, and leaves, as possible sources of natural antioxidants and antibacterial agents. This study is limited to the essential oil of *L. angulata*; thus, the purpose of the current research was to evaluate the physicochemical characteristics and antibacterial properties of the essential oil fraction derived from fresh leaves using simple fractional distillation.

MATERIALS AND METHODS

Plant material and research location

The study's raw material was *L. angulata* leaves from Botanical Garden of Universitas Mulawarman (-0.450032°N, 117.216505°E), Samarinda, East Kalimantan, Indonesia (Figure 1). The work experiment was carried out at the Forest Product Chemistry and Renewable Energy Laboratory, Faculty of Forestry, Universitas Mulawarman, using 4,250 g of fresh materials. The collected material was prepared for distillation without any treatment or drying process.

Fractional distillation

The fractional distillation procedure used in this study was simple, using water and steam distillation techniques. The essential oils were fractionated based on different distillation times, as described by the method of Zheljazkov's research (Zheljazkov et al. 2014) with minor adjustments. This experiment treated the distillation periods of 0-60 min (fraction 1), 60-120 min (fraction 2), and 120-180 min (fraction 3). The essential oil fractions were recovered directly and dehydrated using anhydrous sodium sulfate (Na_2SO_4) and stored at room temperature in firmly closed dark vials prior to analysis. Essential oil yield is expressed in percent oil (%v/v).

Physicochemical parameters

Color, refractive index, and chemical composition determined the profile of the physicochemical properties of the achieved fractions.

Color

In this study, the color of the oil fraction was observed by the method of Shabbir et al. (2009).

Refractive index

The refractive index value was determined according to Patty and Loupatty 2016, and measurements were made with the help of a hand refractometer (Atago, Japan).

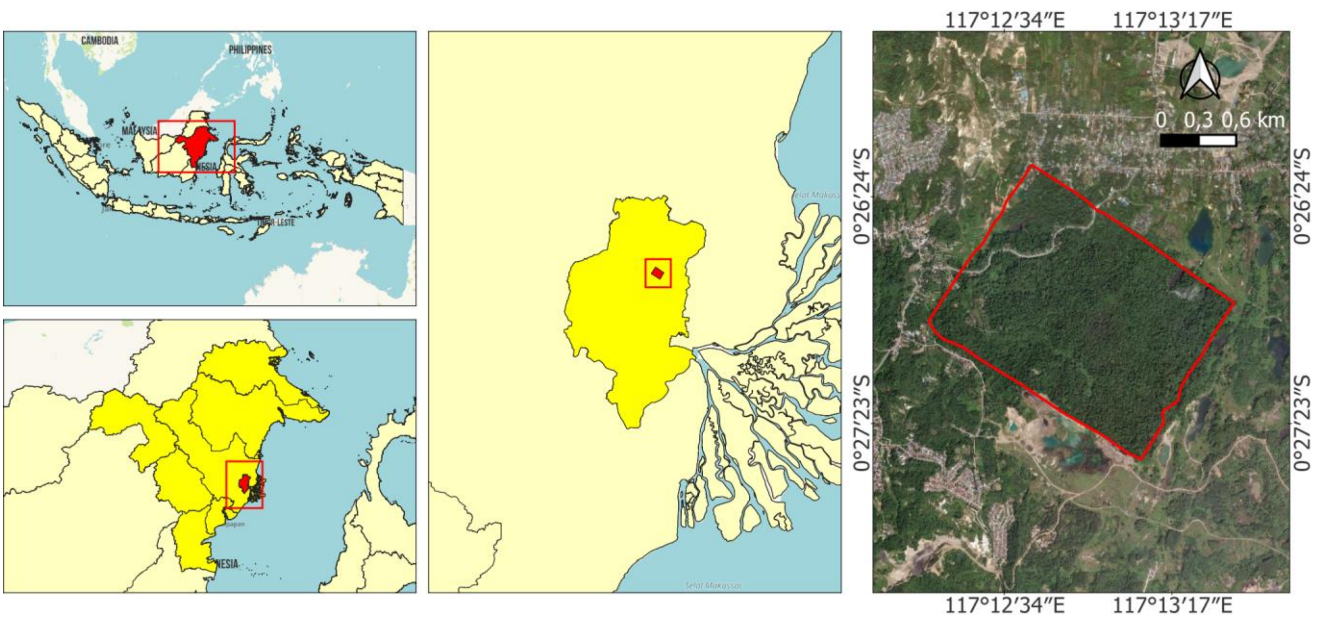

Figure 1. Research sites: Botanical Garden of Universitas Mulawarman, Samarinda, East Kalimantan, Indonesia

Gas Chromatography-Mass Spectrometry (GC-MS)

The identification of essential oil fraction chemical compositions was determined with the application of the GC-MS technique by the modification procedure of Iordache (Iordache et al. 2011). In our studies, we used an RTX-5MS capillary column (Ultra Shimadzu-QP-2010) 30 m × 0.25 mm × 0.25 µm. The following settings were used to collect GC-MS spectra: The injector and detector temperatures were 250 and 280°C; the inlet pressure was 93.5 kpa; split ratio was 200; column flow was 1.44 mL.minute^{-1}; the linear velocity was 49.3 cm.second^{-1}; carrier gas was helium and purge flow 3.0 mL.minute^{-1}. The Kovats retention indices were performed on the basis of a range of alkane standard solutions (C8 to C40) using the measurement of van Den Dool and Kratz 1963. The identities of the compositions were assigned by comparing their mass spectra with those available in the NIST database, and the retention indices, taken from the literature and our own data, confirmed this.

Antibacterial assay

The activity of antibacterial from essential oil fractions was evaluated by the agar well diffusion method (Kuspradini et al. 2018). The bacteria used in this study include *Streptococcus mutans* Clarke, 1924, *Streptococcus sobrinus*, and *Staphylococcus aureus* Rosenbach, 1884. Briefly, the agar plates were inoculated with bacterial suspension, and 20 µL of the samples were loaded into wells of 7 mm diameter. The bacterial incubation time was 18-24 h at 37°C (Su et al. 2015). Chloramphenicol and chlorhexidine were used as standard antibiotics for positive control. A solution containing 40% ethanol was included as a negative control. The essential oil's concentration was measured and converted to mg (mg/20 µL). The test was repeated in triplicate.

Data analysis

GC-MS analyzed the presence of chemical constituents. The chromatographic peak area was used to calculate the percentage components of oil and given as the average of three duplicate analyses. The inhibition zone diameter was measured in mm by a ruler in each replication, and the average values were calculated.

RESULTS AND DISCUSSION

In this work, we meticulously investigated the leaves of *L. angulata*, identifying and obtaining three essential oil fractions through a comprehensive research process that involved simple fractional distillation using water and steam distillation methods. The yields of these oil fractions, a result of our thorough research, are detailed in Table 1. We further conducted a rigorous physicochemical characterization of these oils, including their color, refractive index, and chemical composition. The physico-properties, such as color and refractive index, are also presented in Table 1. In comparison, Table 2 provides a comprehensive chemical profile of the extracts, instilling confidence in our readers about the chemical constituents of all extracts.

In this case, essential oil yield typically ranged from 0.09 to 1.66%, and fraction 1 gave higher yields than other fractions with a clear color. Distillation time influenced the yield of essential oil produced. The oil was optimally distilled at a distillation time of 0-120 min, while the yield produced was very low at 120-180 min of distillation time. This is also related to the color of essential oils. At the beginning of the distillation time, the color of the essential oil obtained was clear, and after 60 to 180 min was more concentrated.

The refractive index of *L. angulata* ranges from 1.407 to 1.427, with a highest value of 1.427, which was detected in fraction 3. These results are in contrast with the physical characteristics of the essential oil produced by the different distillation periods. The essential oil's refractive index is closely linked to its composition, while the constituents are dependent on the soil nutrients. The longer the chained compositions (sesquiterpenes and oxygenated compounds) that are distilled, the greater the essential oil medium density, making it hard for light to be refracted and resulting in a higher essential oil's refractive index (Arpi et al. 2011).

GC-MS analysis investigated the ingredients of three fractions of *L. angulata* leaves essential oil. More than 15 compounds were identified in each fraction. Table 2 summarizes the appearance of chemical components in samples.

Fraction 1 is composed of essential oil samples extracted from 0-60 min of distillation time, which are characterized by a higher percentage of camphor (62.38%) and followed by α-phellandrene (26.07%). Fraction 2 consists of oils extracted from the distillation periods of 60-120 m. These fraction oils were found to contain camphor, α-phellandrene, *trans*-caryophyllene, and α-humulene, which were detected at 37.14, 23.81, 10.06, and 10.06%, respectively. Fraction 3 is composed of essential oils isolated from 120-180 min of distillation time, which is characterized by a higher content of sesquiterpenes group in the total of 68.76% compounds. In comparison, trans-caryophyllene was a dominant component (28.53%) and had a lower content in the monoterpenes group (23.03%). However, in the *L. angulata* oil obtained after 120 min of distillation, α-phellandrene was detected at about 10.21%.

Table 1. The physicochemical profiles of *Litsea angulata* leave essential oil fractions

Samples	Yields (%)	Color	Refractive index
Fraction 1	1.66	Clear	1.407
Fraction 2	0.41	Yellowish	1.414
Fraction 3	0.09	Yellow pale	1.427

Table 2. Chemical constituents of *L. angulata* leave essential oil fractions

Chemical components	Percentage (%)			RI^{exp}			RI^{lit}
	Frc 1	Frc 2	Frc 3	Frc 1	Frc 2	Frc 3	
β-Pinene	0.92 ± 0.03	0.87 ± 0.04	0.36 ± 0.01	952	953	953	952
β-Myrcene	0.45 ± 0.01	0.39 ± 0.01	-	962	963	-	962
α-Phellandrene	26.07 ± 0.38	23.81 ± 0.56	10.21 ± 0.28	1002	1003	1002	1001
α-Terpinene	0.60 ± 0.11	-	-	1013	-	-	1014
m-Cymene	2.45 ± 0.10	4.97 ± 0.09	2.84 ± 0.05	1021	1021	1021	-
β-Phellandrene	-	2.33 ± 0.04	1.37 ± 0.04	-	1028	1028	1032
(-)-Limonene	1.77 ± 0.11	-	-	1025	-	-	-
γ-Terpinene	0.39 ± 0.01	0.48 ± 0.01	-	1054	1054	-	-
α-Terpinolene	1.14 ± 0.04	1.61 ± 0.02	-	1084	1084	-	1089
Camphor	62.38 ± 0.64	37.14 ± 0.40	6.69 ± 0.08	1150	1149	1147	1144
(-)-Borneol	0.54 ± 0.07	0.44 ± 0.01	-	1168	1168	-	-
Decanal	-	0.65 ± 0.01	1.14 ± 0.06	-	1192	1192	-
cis-Sabinol	-	-	0.44 ± 0.01	-	-	1200	-
6,6-Dimethyl-2-(3-oxo-butyl)-bicyclo[3.1.1]heptan-3-one	0.51 ± 0.02	1.48 ± 0.01	1.37 ± 0.02	1243	1293	1243	-
Acetic acid 1,3,3-trimethyl-2-oxa-bicyclo[2.2.2]oct-6-yl ester	-	1.89 ± 0.10	1.40 ± 0.04	-	1315	1315	1431
2,6-Heptanedione, 3-methylene-	-	0.89 ± 0.04	0.74 ± 0.00	-	1323	1323	-
α-Copaene	0.44 ± 0.01	1.97 ± 0.04	6.17 ± 0.09	1375	1703	1376	1376
trans-Caryophyllene	1.11 ± 0.03	10.06 ± 0.28	28.53 ± 0.30	1579	1212	1471	-
α-Humulene	-	10.06 ± 0.28	3.59 ± 0.05	-	1832	1766	-
Alloaromadendrene	-		0.35 ± 0.00	-	-	1883	-
δ-Cadinene	-		1.19 ± 0.04	-	-	1923	1523
(-)-Germacrene A	-	1.35 ± 0.01	7.00 ± 0.10	-	2048	2051	-
β-Bisabolene	-	2.23 ± 0.04	6.62 ± 0.04	-	2088	2091	-
Germacrene D	-		0.83 ± 0.03	-	-	1477	-
δ-Cadinene	-	2.15 ± 0.05	4.26 ± 0.01	-	1478	1477	-
Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	-	-	0.84 ± 0.01	-	-	1465	-
Nerolidol b (cis or trans)	-	-	0.39 ± 0.01	-	-	1455	-
(-)-Caryophyllene oxide	-	0.77 ± 0.02	3.20 ± 0.13	-	1408	1414	-
Torreyol	-	0.55 ± 0.01	-	-	1558	-	-
α-Cedrol	-	-	0.81 ± 0.03	-	-	1576	-
δ-Cadinol	-	-	2.07 ± 0.00	-	-	1563	-
α-Cadinol	-	-	0.86 ± 0.33	-	-	1550	-
α-Bisabolol	-	-	0.62 ± 0.13	-	-	1523	-
d-Nerolidol	-	-	0.54 ± 0.06	-	-	1970	-
Monoterpenes	96.69	72.65	23.03				
Sesquiterpenes	1.55	20.28	68.76				
Other	0.55	4.26	3.50				

Note: [a]Frc: fraction; [b]RI^{exp}: retention indices of the experiment; [c]RI^{lit}: retention indices of literature

In this case, distillation time affects the chemical constituents and value of the main components identified from the samples. The high and low chemical constituent appearance percentages from monoterpenes and sesquiterpenes groups may be influenced by molecular rearrangement and hydrolysis, which may develop during the distillation process (Wesołowska et al. 2010); hence, to release their active compounds, essential oils should be distilled at the correct time (Schmidt 2020). Gamma terpinene has a boiling point of 181 to 182°C and a KI of about 1085. In the work of Olmedo et al. (2014), where essential oil was fractionated with short-path molecular distillation, it was determined that from this approximate value of KI, a heavier fraction of higher boiling point was constituted, which is what is observed: the increase of fraction 3 from approximately said KI (Olmedo et al. 2014).

A new source of camphor and α-phellandrene was found in this aromatic plant. In our previous project results (Kuspradini et al. 2020), camphor and α-phellandrene were not detected in *L. angulata* leaves crude oil. The quality of chemical compounds found in plants may be affected by environmental circumstances at the time of sampling and growing parameters such as soil type. Ilić et al. (2022) mentioned that the concentration of essential oil depends on the population variations and the climate factors. Based on the results of Goyal et al. 2021, there is a significant difference in the percentage of oil between geographical regions and cultivation altitude. Consequently, more research into environmental or climatic conditions is required for this species.

Camphor and α-phellandrene were two dominant volatile components with the highest concentrations detected in the

L. angulata fresh leaves essential oil. Camphor hydrazone derivatives have anticancer, antitussive, and insecticide activities, and they may be used to treat Leishmaniasis disease (da Silva et al. 2020). Camphor also has various negative side effects when used in high doses, including nausea, vomiting, headache, dizziness, tremors, seizures to death (Santos and Cabot 2015). α-phellandrene is commonly used in fragrances and has a characteristic, slightly green, terpenic, citrus, black pepper-like aroma. Only a few papers reported on the α-phellandrene biological activity, and it has been discovered that it is ineffective as a hypercholesterolemic, hyperlipidemic agent, antibacterial, and antifungal (Işcan et al. 2012). Thangaleela et al. (2022) assessed the function of α-phellandrene across a range of healthcare sectors.

Table 3 demonstrates the antibacterial activity of *L. angulata* essential oil fractions. All fractions could inhibit the growth of the tested microorganisms (*S. mutans*, *S. sobrinus*, and *S. aureus*). This result showed that fraction 1 was active in inhibiting the growth of *S. sobrinus* and *S. aureus* (15.11 and 16.89 mm, respectively). At the same time, the highest diameter against *S. mutans* was found in fraction 2. The strength of antibacterial activity on the oil fractions is presented in Table 3.

From the results presented above, all samples have the bacterial strains' inhibitory activities except the lowest concentration for all fractions against *S. sobrinus*. Fraction 1 showed the best inhibition towards *S. sobrinus* and *S. aureus* at the highest concentration; however, the greatest activity was shown by fraction 2 against *S. mutans*. The role assures the activity indicated by fractions 1 and 2 of the chemical content in the oil, which is rich in monoterpenes. Sesquiterpenes also have a role in bacterial inhibition, as seen in the activity possessed by fraction 3; however, they have not shown the highest activity. The chemical components and the quantity of main compounds in the extracts can affect the inhibitory activity (Qin et al. 2024). The presence of major chemical ingredients in the extract influenced their biological activities.

Literature surveys revealed that various essential oils from a number of plants contained camphor as a main component, demonstrating antifungal properties (Sobhy et al. 2023). Bouazama et al. (2017) investigated *Lavandula pedunculata* (Mill.) Cav. and *Lavandula dentata* L. from South Morocco, which contained 53.1 and 50.3% of camphor possessing inhibitory activity toward *Pseudomonas aeruginosa* A, *Escherichia coli* E, *S. aureus*, and *Rhodococcus fascians*. *Cinnamomum camphora* (L.) J.Presl, known as a camphor-producing plant, has been assayed as an antibacterial agent, potentially. In line with the research of Chaudhari et al. (2020), cinnamon oil fraction from *C. camphora* showed the inhibition toward *S. mutans* of about 12.51 mm. Previous work stated *C. camphora* oil exhibits its antibacterial potential against *Bacillus subtilis*, *P. aeruginosa*, *E. coli*, *Salmonella typhimurium*, *S. aureus*, and *Chromobacterium violaceum* (Hu et al. 2019; Wang et al. 2019). Moreover, based on Chen et al. (2020) statement that the volatile oil derived from *C. camphora* leaves was tested to inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) (the value of MIC 0.8 mg/mL).

As mentioned by İşcan (2017), the monoterpene α-phellandrene showed moderate to good inhibitory efficacy against various bacteria and Candida species. According to the findings of Zhang et al. (2017), α-Phellandrene and Nonanal might be effectively employed as biological fungicides to combat *Penicillium cyclopium* in postharvest tomato fruits. These volatile compositions were capable of penetrating cell lipid structures and damaging the fungal cell membrane's integrity. Thangaleela et al. (2022) reviewed that α-Phellandrene exhibited in vitro antimicrobial activities against bacterial strains *Salmonella choleraesuis*, *B. subtilis*, *E. coli*, *Rhodococcus rhodochorus*, *Micrococcus luteus*, *S. aureus*, *Arthrobacter protophormiae*, and fungus *Aspergillus flavus*.

In the case of fraction 3, the appearance of trans-caryophyllene as a sesquiterpenes group also supports, to a considerable degree, the inspected activity. The compound trans-caryophyllene has pharmacological effects as an antibacterial and antifungal. The *trans*-caryophyllene analgesic property extracted from the volatile oil of *Cordia verbenacea* showed good anti-inflammatory and antiplasmodic efficacy (Fernandes et al. 2007; Leonhardt et al. 2010; Astani et al. 2011).

Table 3. Antibacterial properties of *Litsea angulata* leaf essential oil fractions

Samples	**Concentrations (mg/20 μL)**	**Diameter of inhibition (mm)**		
		Streptococcus mutans	***Streptococcus sobrinus***	***Staphylococcus aureus***
Fraction 1	21.50	20.33 ± 1.45	15.11 ± 1.02	16.89 ± 1.02
	10.75	11.33 ± 1.53	9.22 ± 0.19	16.78 ± 1.09
	5.38	8.33 ± 0.88	0.00 ± 0.00	14.11 ± 0.51
Fraction 2	19.30	22.44 ± 1.35	14.33 ± 2.19	16.33 ± 2.33
	9.65	17.67 ± 1.00	8.33 ± 0.58	16.33 ± 1.45
	4.83	8.56 ± 0.51	0.00 ± 0.00	13.67 ± 1.15
Fraction 3	19.10	12.89 ± 1.84	10.22 ± 0.69	13.11 ± 1.92
	9.55	9.44 ± 0.69	8.11 ± 0.51	12.89 ± 0.19
	4.78	7.89 ± 0.19	0.00 ± 0.00	11.67 ± 0.88
K(+)[1]	0.01	23.89 ± 0.38	27.89 ± 0.38	19.22 ± 3.72
K(+)[2]	0.01	15.67 ± 0.58	15.67 ± 0.33	15.78 ± 0.19

Note: [a]K(+)[1]: chloramphenicol; [b]K(+)[2]: chlorhexidine

In conclusion, the results reported on the essential oil fraction yield and chemical constituents of *L. angulata* at different distillation periods. We consider that for producing the highest quantity and quality of *L. angulata* essential oil, it is judicious to distill for 0-120 min to gain the optimal and maximum percentage of camphor. This plant was potentially a new source of camphor and α-phellandrene as a monoterpenes group. As shown in the antibacterial properties, the components from the monoterpenes group played a role in this work. It is summarized that the essential oil of *L. angulata*, rich in monoterpenes group components, can be used as a natural alternative product as a medicinal plant to inhibit the growth of *S. mutans*, *S. sobrinus*, and *S. aureus*, offering hope for new treatments.

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Bird identification and conservation in Semarang Traditional Markets, Indonesia

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Abstract. *Nur 'Azizah HP, Nabila I, Damayanti K, Khawarizmi IA, Sholiqin M, Indrawan M, Wahyuni T, Iskandar J, Setyawan AD. 2025. Bird identification and conservation in Semarang Traditional Markets, Indonesia. Nusantara Bioscience 17: 103-117.* Birds are one of the wildlife species that humans widely utilize as food, pets, economic resources, and for their aesthetic value. Bird markets in Indonesia have become an integral part of people's lives, but this can also be a threat if not properly controlled. The objectives of this study were to identify bird species traded in bird markets, assess their selling prices, analyze the conservation status and endemicity of traded species, and evaluate the sustainability of bird markets. This research was conducted in three traditional markets in Central Java Province, Indonesia, i.e., (i) Banyuputih Market in Salatiga City; (ii) *Pasar Pon* Ambarawa in Semarang District; and (iii) Kartini Market in Semarang City, using a descriptive qualitative method with a survey and observation approach. The collected data were grouped based on several categories, such as the type of bird species, conservation status, and the location where the species was found. The results of this study showed 46 families with 136 bird species, of which 18 species are non-endemic and 118 species are endemic. Based on the conservation status, according to IUCN, several species are rare (23 species), namely endangered (9 species), vulnerable (3 species) and near threatened (11 species); as well as 13 species are included in Appendix II of CITES; and 14 species are nationally protected according to Permen LHK No.P.20/2018. Most birds come from wild hunting (106 sp.) instead of breeding (30 sp.). Two endemic bird species, including rare according to the three institutions, are *Pycnonotus cyaniventris* (*cucak kelabu*) and *P. zeylanicus* (*cucak rawa*). Suppliers and traders need to pay attention to the sustainability of the bird trade through biological conservation by providing more breeding birds. Efforts are needed to apply the principles of sustainable development that include economic, social, and environmental aspects in the bird trade in bird markets.

Keywords: Birds, conservation, diversity, trade, traditional market

INTRODUCTION

Birds are a group of animals that can be found all over the world, and their species can live in all kinds of places, including urban areas that have no natural counterparts (Lees et al. 2022). The presence of birds serves as a balance in the ecosystem, as they act as apex predators, seed decomposers, pollinators, and pest controllers (Adelina et al. 2016). According to Kartono et al. 2020, the number of bird species in Indonesia increased from 1,771 species in 2018 to 1,777 species in 2019; 168 species were declared endangered, and 30 species are in crisis status by the IUCN. Keeping birds is an option that is often chosen by some Indonesians because it is believed that its cultivation is relatively easy compared to other animals (Putranto et al. 2024). Interest in keeping birds arises from various factors, such as attractive colors and patterns or melodious sounds. In addition, the Indonesian tradition of keeping birds has been around for a long time (Fikriansyah and Wismarini 2023).

In urban areas, biodiversity is an important resource that serves as an environmental buffer and balancer, which is influenced by the characteristics of the ecosystem (Wuisang 2015). Semarang City has varied geographical conditions, including coastal areas, flat urban areas, and hilly areas, contributing to the city's high biodiversity (Suwarso et al. 2019). Birds are one of the many species of animals that can be found in Semarang City. Some common bird species traded in Semarang City include *Lonchura punctulata* (Linnaeus, 1758), *Columba livia* f. *domestica*, and *Serinus canaria* subsp. *domestica* (Ghifari et al. 2016). The economic value of birds can be seen from the bird trade that takes place in various regions, especially in big cities. Various bird species are traded, both for hobby keeping and contests, creating markets and bird stalls (Delfiah et al. 2024). Along with the increasing demand for keeping birds, bird markets have emerged in many parts of Indonesia (Iskandar et al. 2020). As such, bird markets symbolize a particular culture or society by reflecting, on a small scale, the diversity of cultures and birds present in an area (Albuquerque et al. 2014).

Bird markets in Indonesia play a crucial role in the lives of many, particularly in cities like Semarang, where they serve as hubs for social interaction and community bonding

(Iskandar et al. 2019). These markets also host chirping competitions, attracting enthusiasts and celebrating the beauty of bird sounds. However, the popularity of these markets brings challenges such as overexploitation and poaching, which threaten wild bird populations (Humaero et al. 2023). For instance, *Garrulax leucolophus* (Hardwicke, 1816), an endemic species from Sumatra, is nearing extinction due to uncontrolled trade (Bušina et al. 2018). It is essential to enforce regulations that protect bird populations and promote ethical trade practices (Damara et al. 2022). Researchers can utilize direct observation methods and qualitative data collection to assess bird diversity without relying on ecological analyses like biodiversity indices, researchers can utilize direct observation methods and qualitative data collection. Qualitative research is a research approach that produces data in the form of descriptive data sourced from observations in written, oral or behavioral form from research subjects.

This approach involves recording bird species, their behaviors, and interactions within their environments. Studies indicate that factors such as habitat complexity, food availability, and predator presence significantly influence bird diversity. For example, urban bird diversity is closely linked to the quality and quantity of green spaces (Thompson et al.2022). Furthermore, direct observations can reveal how species adapt to environmental changes (Alba et al. 2022). Conservation efforts must be prioritized due to the increasing threat to bird diversity in Indonesia (Iskandar et al. 2021), conservation efforts must be prioritized. Collaborative actions among government bodies, communities, and individuals are vital to maintaining bird biodiversity, which offers ecological, economic, and cultural benefits for future generations (Setiawan et al. 2024). Birds represent not only Indonesia's identity and heritage but also symbolize a commitment to nature conservation (Yapsenang et al. 2022). The research that focuses on bird species diversity and conservation status is crucial for understanding market sustainability. Hence, using observation methods like point counting, researchers can document species presence and behavior while also examining vegetation variations that affect bird habitats (Ridwan et al. 2015; Kurnia et al. 2021). This research aims to contribute significantly to bird conservation efforts and support local ecosystem preservation.

MATERIALS AND METHODS

Study area

This research was conducted in September 2024 at three markets in Salatiga and Semarang, Central Java Province, Indonesia (Figure 1), i.e., (i) Banyuputih Market located in Sidorejo Sub-district, Salatiga City; (ii) *Pasar Pon* Ambarawa in Ambarawa Sub-district, Semarang District; and (iii) Kartini Market in East Semarang Sub-district, Semarang City. Geographically, Banyuputih Market is located at 7°18'50.367 S, and 110°29'5.233 E. Then *Pasar Pon* Ambarawa is located at 7°14'55.348 S and 110°25'15.593 E; Kartini Market is located at 6°59'5.638 S and 110°26'16.026 E. Based on the data available in the Central Bureau of Statistics (CBS Semarang Regency 2024), Banyuputih Market has an area of about 2,586 m^2, *Pasar Pon* Ambarawa has an area of about 240,000 m^2, and Kartini Market has an area of about 12,288 m^2.

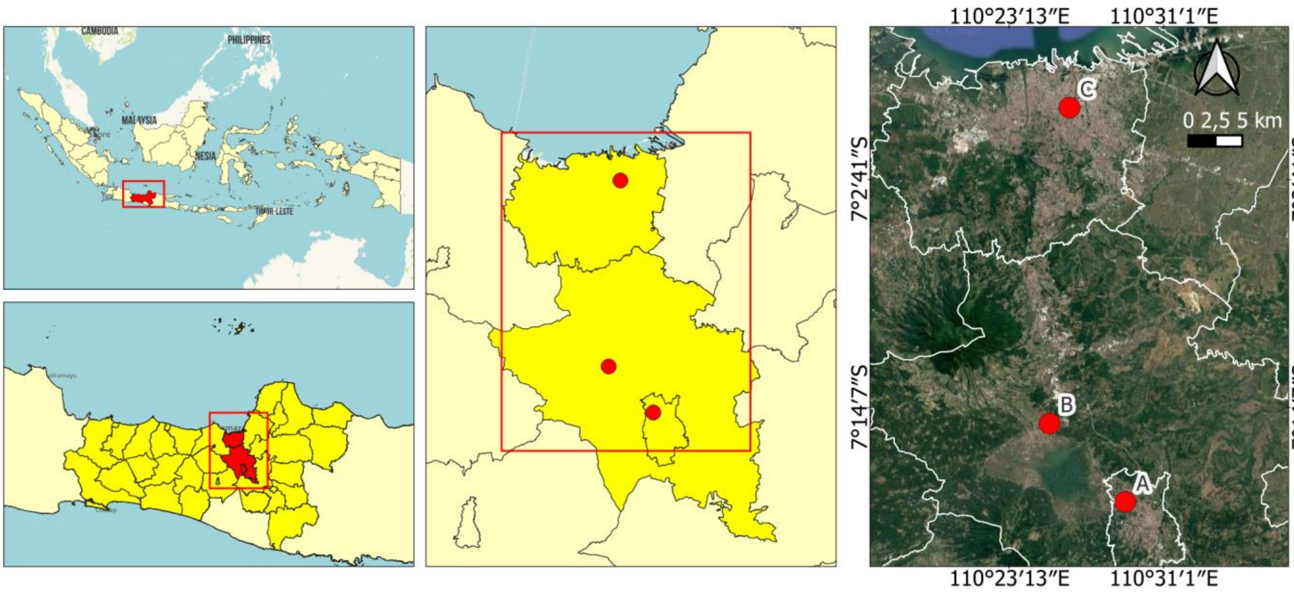

Figure 1. Research locations in Semarang Traditional Markets, Central Java, Indonesia: A. Banyuputih Market in Salatiga City; B. *Pasar Pon* Ambarawa in Semarang District; C. Kartini Market in Semarang City

Procedures

This research was conducted using a descriptive qualitative method with a survey and observation approach. In each market, the types of birds traded were recorded and identification of bird species in each market was also carried out. The data collection process was carried out using the interview method and direct observation in the field. Interviews were conducted with 150 informants with 50 informants at each market. The respondents ranged in age from 22 to 81 years old. The frequency of the study was carried out for 3 consecutive days. Each market gets a duration of 1 day for observation. Observations were carried out from 07.00 to 14.00. This is related to the market productivity scheme that applies in the three locations. The study was conducted at a time related to increasing market productivity, especially on Saturday and Sunday.

The questions asked to the informants included the types of birds sold, where the birds were obtained from, and the market price of the birds. Furthermore, bird morphology observations were conducted to determine bird characteristics such as color and shape as well as market conditions such as the number of stalls, market conditions, and market layout, Indonesia name of bird, selling price, bird sources, and population of dominate.

Prior to the research, we conducted a preliminary survey. At the *Pasar Pon*, it was deliberately carried out to coincide with the Javanese date, namely the *Pasar Pon* or called a special day for buying and selling livestock, one of which is birds at *Pasar Pon* Ambarawa. So that the study directly relates to the peak of the day where the traders sell all their birds in large quantities. Based on the results of a direct survey in the field, Banyuputih Market consists of 21 stalls, while *Pasar Pon* Ambarawa has 52 stalls, and Kartini Market consists of 40 stalls. Banyuputih Market and Kartini Market are markets that specialize in selling various types of birds. At the same time, *Pasar Pon* Ambarawa sells not only birds but also other types of animals, such as rabbits, goats, cows, clothes, medicines, and tools. In the Banyuputih Market, some traders' stalls have been converted into warehouses, but many traders continue to sell in this market. *Pasar Pon* Ambarawa itself is a market that sells a variety of animals, but bird traders are located in the same location. This market has a large number of bird traders who sell with fixed building stalls or not. Kartini Market is a specialized market for bird trading, but now, some of the stalls have closed and are not operating. Bird species data obtained totaled 136 species spread across three markets.

Data analysis

In this study, primary data were obtained from interviews and observations at Banyuputih Market, *Pasar Pon* Ambarawa, and Kartini Market. Researchers made direct observations and also took photos of several bird species as visual data to support the observation results. Then, the results of the interviews from several different informants were compared. Then, the collected data were grouped based on several categories, such as the type of bird species, conservation status, and the location where the species was found. The conservation status of each bird species refers to the Minister of Environment and Forestry Regulation (Permen LHK No. P.20/MENLHK/setjen/Kum.1/6/2018) and SKJB field guidebook for national level status (MacKinnon 2010), then to the IUCN Red List (Kasambe 2014; IUCN 2024) and CITES Appendix II for international level status. The data were then presented in the form of tables, photos, and descriptive analysis to describe the diversity of bird species distributed in the three markets.

RESULTS AND DISCUSSION

Bird species traded

Based on direct observation/survey and interviews conducted with the bird traders in Banyuputih Market, Salatiga, a total of 35 bird species representing 20 families were found. On the other hand, *Pasar Pon* has the highest number of species, with 84 species representing 35 families. Kartini Market also shows a high number of bird species, with 63 species representing 34 families. With an overall total of 136 bird species representing 46 families, it shows that *Pasar Pon* is superior in terms of the number of bird species. In comparison, Kartini Market is superior in terms of the number of families, as shown in Table 1 and some bird images are shown in Figure 2. The most commonly traded bird species in Banyuputih Market are from the families Cisticolidae (4 species), Columbidae (4 species), and Pycnonotidae (3 species). Meanwhile, in *Pasar Pon*, the most commonly traded bird species are from the families Cisticolidae (5 species), Phasianidae (6 species), Pycnonotidae (7 species), Timaliidae (6 species), and Zosteropidae (8 species). Whereas in Kartini Market, the most commonly traded bird species are from the families Pycnonotidae (6 species) and Sturnidae (5 species). Of the total families in the three markets, the families Timaliidae and Zosteropidae were most commonly traded, with 10 bird species each.

In addition, the ratio of dominant and non-dominant bird species showed that non-dominant species outnumbered the dominant species. Dominant and non-dominant species were determined based on their abundance in the three markets. Some of the bird species in the three markets are protected birds. It can be concluded that even though some bird species in Indonesia have protected status, it cannot guarantee that these bird species are not traded freely. Indonesia is an archipelagic country known for its diversity of animals protected by the government, as well as being a habitat for endemic animals that have characteristics in each region and are not found in other regions (Hardjasoemantri 2009). This is supported by research by Laksono and Ucuk (2023) that although the government protects many species, it is inversely proportional to the amount of illegal trade and crime of protected animals. In 2021, the Biodiversity Conservation Officer noted that 179 bird species were categorized as endangered in Indonesia. This is an alarming number, especially given the rampant illegal trade of protected bird species in international markets. This poses a major threat to the sustainability of these bird species, with many of them at risk of extinction if the situation continues (Al Fasha et al. 2023).

Table 1. Bird species found in Banyuputih Market, *Pasar Pon* Ambarawa, and Kartini Market

Family	Species	Indonesian name	Common name	Selling price (x Rp.1000)	Bird sources	Conservation status: IUCN	App. II of CITES	Permen LHK No.P.20/2018	Endemicity	Population	Market location
Aegithinidae	*Aegithina tiphia* (Linnaeus, 1758)	*Sirtu*	Common iora	25-300	Wh	LC	●	-	●	D	PPA
Alaudidae	*Mirafra javanica* Horsfield, 1821	*Branjangan*	Mirafra javanica	1-5	Wh	LC	●	-	●	ND	BM, KM
Artamidae	*Artamus leucorhynchos*	*Kekep babi*	The white-breasted wood swallow	250-700	Wh	LC	-	-	●	ND	KM
Campephagidae	*Lalage nigra* (J.R.Forster, 1781)	*Kapasan kemiri*	Pied triller	280-380	Wh	LC	-	-	●	ND	KM
Campephagidae	*Pericrocotus speciosus* (Latham, 1790)	*Mantenan*	Scarlet minivet	35-145	Wh	VU	-	-	●	ND	PPA
Chloropseidae	*Chloropsis cochinchinensis* (Gmelin, 1789)	*Cucak ranting*	Blue-winged leafbird	250-400	Wh	LC	-	-	●	ND	KM
Chloropseidae	*Chloropsis hardwickii* Jardine & Selby, 1830	*Cungkok*	Chloropsis hardwickii	2-3400	Wh	LC	-	-	●	ND	KM
Chloropseidae	*Chloropsis sonnerati* Jardine & Selby, 1827	*Cucak ijo meranti*	Large leaf cica	250 -400	Wh	LC	-	-	●	ND	BM, PPA, KM
Chloropseidae	*Chloropsis venusta* (Bonaparte, 1850)	*Kinoi*	Blue-masked leafbird	250	Wh	NT	-	-	●	ND	PPA
Cisticolidae	*Orthotomus ruficeps* (Lesson, 1830)	*Prenjak kepala merah*	Ashy tailorbird	110-246	Wh	LC	-	-	●	D	BM, KM
Cisticolidae	*Orthotomus sutorius* (Pennant, 1769)	*Prenjak lumut*	Common tailorbird	15-30	Wh	LC	-	-	●	ND	BM, PPA, KM
Cisticolidae	*Prinia atrogularis* (Moore, 1854)	*Ciblek gunung*	Eurasian tree cigun	60-1	Wh	LC	-	-	●	ND	BM, PPA
Cisticolidae	*Prinia familiaris* (Horsfield,1821)	*Ciblek kristal*	Bar-winged prinia	200-1	Br	NT	-	-	●	ND	PPA
Cisticolidae	*Prinia flaviventris* (Delessert, 1840)	*Ciblek tebu*	Prinia flaviventris	60-85	Wh	LC	-	-	●	ND	BM, PPA
Cisticolidae	*Prinia inornata* Sykes, 1832	*Ciblek sawah*	White-browed wren-warbler	60 -100	Wh	LC	-	-	●	ND	BM, PPA, KM
Cisticolidae	*Prinia polychroa* (Temminck, 1828)	*Ciblek kebun*	Brown prinia	85-250	Wh	LC	-	-	●	ND	BM, PPA
Columbidae	*Columba livia* J.F.Gmelin, 1789	*Merpati*	Domestic pigeon	35-100	Br	LC	-	-	-	D	BM, KM
Columbidae	*Ducula badia* (Raffles, 1822)	*Pergam gunung*	Mountain imperial pigoen	60-80	Br	LC	-	-	●	D	PPA
Columbidae	*Geopelia striata* (Linnaeus, 1766)	*Perkutut*	Zebra dove	180-500	Br	LC	-	-	●	D	BM, PPA
Columbidae	*Streptopelia bitorquata* (Temminck,1809*)*	*Tekukur*	Island collared dove	20-50	Wh	LC	-	-	●	D	PPA
Columbidae	*Streptopelia risoria* (Linnaeus, 1758)	*Puter*	Zebra dove	170-600	Br	LC	-	-	●	D	BM, KM
Columbidae	*Streptopeliachinensis* (Scopoli, 1786)	*Derkuku*	Spotted dove	25-30	Wh	LC	-	-	●	D	BM, KM
Columbidae	*Treron vernans* (Linnaeus, 1771)	*Punai*	Green pigeon	50	Wh	LC	●	-	●	ND	PPA
Corvidae	*Cissa thalassina* (Temminck, 1826)	*Engkek keling*	Pioden werdd gynffonfer	500	Wh	EN	-	●	●	ND	PPA
Corvidae	*Corvus corone* Linnaeus, 1758	*Gagak*	Carrion crow	850	Wh	LC	-	-	●	ND	PPA
Corvidae	*Platylophus galericulatus* (Cuvier, 1816)	*Cililin*	Crested jayshrike	2	Wh	NT	-	-	●	ND	KM
Cuculidae	*Cacomantis sepulcralis*	*Kedasih*	Plaintive cuckoo	160-250	Wh	LC	●	-	●	ND	KM
Dicaeidae	*Dicaeum cruentatum* (Limnaeus, 1758)	*Kemladean merah*	Scarlet-backed flowerpecker	125	Wh	LC	-	-	●	ND	PPA, KM
Dicaeidae	*Dicaeum trochileum* (Sparrman, 1789)	*Bangsit*	Scarlet-headed flowerpecker	100-300	Wh	LC	-	-	●	ND	PPA
Dicruridae	*Dicrurus macrocercus* Vieillot, 1817	*Sri gunting*	Black drongo	300	Wh	LC	-	-	●	ND	PPA

Estrididae	*Erythrura gouldiae* (Gould, 1844)	*Gould amadine*	Gouldian finch	1	Br	NT	-	-	-	ND	KM
Estrildidae	*Lonchura malacca* (Linnaesu, 1766)	*Red Papua*	Black headed munia	85	Wh	LC	-	-	●	ND	PPA
Estrildidae	*Lonchura oryzivora* (Linnaeus, 1758)	*Gelatik belong*	Java sparrow	85-125	Br	EN	-	●	●	ND	PPA
Estrildidae	*Lonchura punctulata* (Linnaeus, 1758)	*Pipit*	Scaly-breasted munia	3-20	Wh	LC	●	-	●	D	PPA, KM
Estrildidae	*Neochmia phaeton* (Hombron & Jacquinot, 1841)	*Finch Papua*	Crimson finch	350-600	Br	LC	-	-	●	D	BM
Fringillidae	*Carduelis cucullata* (Swainson,1820)	*Red siskin*	The red siskin gynffonfer	1	Br	EN	-	-	-	ND	PPA
Fringillidae	*Crithagra leucopygia* Sundevall, 1850	*Sanger*	Yellow-rumped seedeater	550-1200	Br	LC	-	-	-	ND	KM
Fringillidae	*Eophona personata* (Temminck & Schlegel, 1848)	*Pipit Jepang*	Japanese grosbeak	150	Br	LC	-	-	-	D	KM
Fringillidae	*Serinus atrogularis* (A.Smith,1836)	*Black throat*	Serinus atrogularis	100-2300	Br	LC	-	-	-	ND	KM
Fringillidae	*Serinus canaria* (Linnaeus, 1758) subsp. *domestica*	*Kenari lokal*	American singer canary	500-2500	Br	LC	-	-	-	D	KM
Laniidae	*Lanius schach* Linnaeus, 1758	*Cendet*	The long-tailed shrike	160-2	Wh	LC	-	-	●	ND	PPA, KM
Leiothrichidae	*Garrulax canorus* (Linnaeus, 1758)	*Hwamei*	Chinese hwamei	3	Wh	LC	●	-	●	ND	KM
Leiothrichidae	*Garrulax chinensis* (Scopoli, 1786)	*Poksay hongkong*	Chestnut-backed laughing thrush	200-500	Wh	LC	-	-	-	ND	PPA
Leiothrichidae	*Garrulax leucolophus* (Hardwicke, 1816)	*Poksay jambul*	White-crested laughingthrush	1100	Wh	LC	-	-	●	ND	PPA, KM
Leiothrichidae	*Garrulax mitratus* (S.Muller,1836)	*Poksay genting*	Chestnut- capped laughingthrush	200-700	Wh	NT	-	-	●	ND	PPA, KM
Leiothrichidae	*Garrulax palliatus* (Bonaparte, 1850)	*Poksai mantel*	Sunda laughingthrush	500	Wh	NT	-	-	●	ND	BM
Leiothrichidae	*Garullax lugubris* (S.Muller, 1836)	*Poksay rambu*	Black laughingthrush	350	Wh	LC	-	-	●	ND	PPA
Leiothrichidae	*Leiothrix lutea* (Scopoli, 1786)	*Robinhun*	Red-billed leiothrix	125	Wh	LC	-	-	●	ND	PPA
Leiothrichidaes	*Heterophasia pulchella* (Godwin-Austen, 1874)	*Murai air*	Beautiful sibia	150	Wh	LC	-	-	●	ND	PPA
Locustellidae	*Megalurus palustris* Horsfield, 1821	*Dempyak*	Striated grassbird	400	Wh	LC	-	-	●	ND	PPA
Meliphagidae	*Lichmera limbata* (S. Muller,1843)	*Cucak kombo*	Brown honeyeater	135-1100	Wh	LC	-	-	●	ND	PPA
Meropidae	*Merops leschenaulti* Vieillot, 1817	*Kerik kerik senja*	Blue-tailed bee-easter	130	Wh	LC	-	-	●	ND	PPA
Muscicapidae	*Copsychus malabaricus* (Scopoli, 1786)	*Murai batu*	White-rumped shama	2-10	Br	LC	●	-	●	ND	BM, KM
Muscicapidae	*Copsychus saularis* (Linnaeus, 1758)	*Kacer*	Oriental magpie-robin	600	Wh	LC	-	-	●	ND	KM
Muscicapidae	*Copsychus saularis* (Linnaeus, 1758)	*Kacer poci*	Javan grey-magpie robin	350-3	Wh	LC	-	-	●	ND	BM
Muscicapidae	*Copsychus sechellarum* A.Newton, 1865	*Kacer ireng*	Robin frith y seychelles	500-2500	Wh	EN	●	-	●	ND	BM, PPA
Muscicapidae	*Cyanoptila cyanomelana* (Temminck, 1829)	*Sulingan*	Brown-chested the blue-and-white flycatcher	190-540	Wh	LC	-	-	●	ND	PPA
Muscicapidae	*Cyornis herioti* R.G.W.Ramsay, 1886	*Sulingan gunung*	Blue-breasted blue flycatcher	1	Wh	LC	-	-	●	ND	KM
Muscicapidae	*Cyornis sanfordi* Stresemann, 1931	*Srikatan*	Brown-chested jungle-matinan blue flycatcher	150	Wh	EN	-	●	●	ND	PPA
Muscicapidae	*Rhinomyias olivaceus* (Hume, 1877)	*Sikatan rimba dada coklat*	Blue-breasted blue flycatcher	430-575	Wh	LC	-	-	●	ND	KM
Nectariniidae	*Aethopyga temminckii* (S.Muller,1843)	*Kolibri ekor merah*	Temminck's sunbird	21-116	Wh	LC	-	-	●	D	KM
Nectariniidae	*Anthreptes malacensis* (Scopoli, 1786)	*Kolibri manggar*	Brown-throated sunbird	30-50	Wh	LC	-	-	●	ND	BM, PPA, KM
Nectariniidae	*Anthreptes singalensis* (Scopoli, 1786)	*Kolibri muncang*	Ruby-cheeked sunbird	150-500	Wh	LC	-	-	●	ND	PPA
Nectariniidae	*Arachnothera longirostra* (Latham, 1790)	*Pijantung pisang*	Little spiderhunter	50	Wh	LC	-	-	●	ND	PPA
Nectariniidae	*Cinnyris jugularis* (Linnaeus, 1766)	*Kolibri canting*	Garden sunbird	25-150	Wh	LC	-	-	●	ND	BM
Nectariniidae	*Leptocoma calcostetha* (Jardine, 1843)	*King konin*	Copper-throated sunbird	350-2	Wh	LC	-	-	●	ND	BM, PPA

Oriolidae	*Oriolus chinensis* Linnaeus, 1766	*Podang emas*	Black naped oriele	500	Wh	LC	-	-	●	ND	KM
Oriolidae	*Oriolus xanthornus* (Linnaeus, 1758)	*Kepodang kerudung hitam*	Black hoodedoriele	300-1600	Wh	LC	-	-	●	ND	PPA
Pachycphalidae	*Pitohui dichrous* (Bonaparte, 1850)	*Cucak Papua*	Todirhamphus sanctus	30-35	Wh	LC	-	-	●	ND	BM
Paridae	*Melanochlora sultanea* (Hodgson, 1837)	*Gelatik batu sultan*	Sultan tit	400	Br	LC	-	-	●	ND	KM
Paridae	*Parus major* Linnaeus, 1758	*Gelatik batu*	Great tit	35-300	Wh	EN	-	-	●	ND	PPB, PPA
Passeridae	*Passer domesticus* (Linnaeus, 1758)	*Burung gereja*	Eurasian tree sparrow	60	Wh	LC	-	-	●	D	PPB, KM
Pellorneidae	*Alcippe pyrrhoptera* (Bonaparte, 1850)	*Flamboyan*	Javan fulvetta	35-40	Wh	LC	-	-	●	ND	BM
Pellorneidae	*Malacocincla sepiaria* (Horsfield, 1821)	*Salakan*	Horsfield's babbler	100	Wh	LC	-	-	●	ND	BM
Phasianidae	*Chrysolophus pictus* (Linnaeus, 1758)	*Ayam golden pheason*	Chinese pheasant	9400	Wh	LC	-	-	-	ND	KM
Phasianidae	*Coturnix ypsilophora* (Bosc,1792)	*Puyuh lokal*	Coturnix coturnix	15-50	Br	LC	-	-	●	ND	PPA
Phasianidae	*Excalfactoria chinensis* (Linnaeus,1766)	*Puyuh Canada*	Blue-breasted quail	200-500	Br	LC	-	-	-	ND	PPA
Phasianidae	*Gallus domesticus* Linnaeus, 1758 *var ketawa*	*Ayam ketawa*	Laughing chiken	105-950	Br	LC	-	-	●	ND	PPA
Phasianidae	*Gallus domesticus* Linnaeus, 1758 *var pelung*	*Ayam pelung*	Pelung long crower	100- 2	Br	LC	-	-	●	ND	PPA, KM
Phasianidae	*Gallus gallus* (Linnaeus, 1758)	*American silkie*	Silkie	170-315	Br	LC	-	-	-	ND	KM
Phasianidae	*Gallus gallus* (Linnaeus, 1758) f. *domesticus*	*Ayam serama*	Malaya serama	35-400	Br	LC	-	-	●	ND	PPA
Phasianidae	*Gallus varius* (Shaw, 1798)	*Ayam hutan*	Red partridge	75-150	Wh	LC	-	-	●	ND	PPA
Phylloscopidae	*Phylloscopus inornatus (Blyth, 1842)*	*Cikrak polos*	Inornate wabler	40-50	Wh	LC	-	-	●	ND	BM
Phylloscopidae	*Phylloscopus trivirgatus* Strickland, 1849	*Blereng*	Mountain leaf-warbler	60	Wh	LC	-	-	●	ND	PPA
Picidae	*Dendrocopos macei* (Vieillot, 1818)	*Pelatuk sampit*	Fulvous-breasted woodpecker	85	Wh	LC	-	-	●	ND	PPA
Picidae	*Dinopium javanense* (Ljungh, 1797)	*Pelatuk bawang*	Common flame back	290-779	Wh	LC	-	-	●	ND	PPA
Picidae	*Picus puniceus* (Horsfield, 1821*)*	*Pelatuk sampit*	Crismon wingned woodpecker	150	Wh	LC	-	-	●	ND	KM
Psittacidae	*Aratinga solstitialis* (Linnaeus, 1758)	*Sun conure*	Sun parakeet	3	Br	EN	-	-	-	ND	KM
Psittacidae	*Nymphicus hollandicus* (Kerr, 1792)	*Falk*	Cockatiel	600	Br	LC	-	-	-	ND	PPA
Psittacidae	*Psittacula krameri* subsp. *manillentis* (Bechstein, 1800)	*Indian ringneck*	Rose-ringed parakeet	4	Br	LC	-	-	-	ND	KM
Psittacidae	*Pyrrhura molinae* (Massena & Souance, 1854)	Green cheek conure	Green-cheeked parakeet	1250	Br	LC	-	●	-	ND	KM
Psittacidae	*Pyrrhura rupicola* (Tschudi, 1844)	Cinnamon conure	Conure	1250	Wh	LC	-	-	-	ND	KM
Psittacidae	*Trichoglossus rubritorquis* (Vigors & Horsfield, 1827)	*Perkici leher merah*	Red collared lorikeet	300	Br	LC	-	-	-	ND	KM
Psittaculidae	*Agapornis* sp.	*Love bird*	Love bird	125-2	Br	LC	-	-	-	ND	BM, PPA, KM
Psittaculidae	*Loriculus galgulus* (Linnaeus, 1758)	*Srindit*	Blue-crowned hanging parrot	100	Wh	LC	-	●	●	ND	PPA
Psittaculidae.	*Melopsittacus undulatus* (Shaw, 1805)	*Parkit*	Budgerigar	65-80	Wh	LC	●	-	●	D	PPA
Pycnonotidae	*Alophoixus ochraceus* (Moore, 1854)	*Cucak jenggot*	Grey-cheeked bulbul	400-680	Wh	LC	-	-	●	ND	PPA, KM
Pycnonotidae	*Alophoxius bres* (Lesson, 1831)	*Jenggot mini*	Brown-cheeked bulbul	40	Wh	LC	-	-	●	ND	BM
Pycnonotidae	*Pycnonotus aurigaster* (Vieillot, 1818)	*Kutilang*	Sooty-headed bulbul	10-65	Wh	LC	-	-	●	ND	BM, PPA, KM
Pycnonotidae	*Pycnonotus bimaculatus* (Horsfield, 1821)	*Rengganis*	Orange-spotted bulbul	60-80	Wh	LC	-	-	●	ND	PPA
Pycnonotidae	*Pycnonotus cyaniventris* Blyth, 1842	*Cucak kelabu*	Ixodia cyaniventris	100	Wh	NT	●	●	●	ND	PPA
Pycnonotidae	*Pycnonotus flavescens* Blyth, 1845	*Merbah*	Flavescent bulbul	1050	Wh	LC	-	-	●	ND	PPA
Pycnonotidae	*Pycnonotus goiavier* (Scopoli, 1786)	*Terucuk*	Yellow-vented bulbul	30-60	Wh	LC	-	-	●	D	BM, PPA, KM
Pycnonotidae	*Pycnonotus melanicterus* (Gmelin, 1789)	*Kutilang emas*	Black-capped bulbul	40-50	Wh	LC	-	-	●	ND	BM, KM
Pycnonotidae	*Pycnonotus plumosus* Blyth, 1845	*Kapas tembak*	Olive-winged bulbul	500-1	Wh	LC	-	-	●	ND	PPA, KM
Pycnonotidae	*Pycnonotus zeylanicus* (Gmelin, 1789)	*Cucak rawa*	Straw-headed bulbul	7	Br	VU	●	●	●	ND	PPA, KM

Rallidae	*Amaurornis phoenicurus* (Pennant, 1769)	*Ruak ruak*	White-breasted waterhen	50	Wh	LC	-	-	●	ND	KM
Renidae	*Irena puella* (Latham, 1790)	*Cucak biru*	Asian fairy bluebird	300	Wh	LC	-	-	●	ND	PPA
Rhipiduridae	*Rhipidura javanica* (Sparrman, 1788)	*Srikatan kipas*	Pield fantail	180-300	Wh	LC	-	●	●	ND	PPA
Sittidae	*Sitta frontalis* Swainson, 1820	*Rambatan doraemon*	Black-crested bulbul	150	Wh	LC	-	-	●	ND	PPA
Sturnidae	*Acridotheres javanicus* Cabanis, 1851	*Jalak kebo*	Javan myna	60	Wh	VU	-	-	●	ND	KM
Sturnidae	*Acridotheres melanopterus* (Daudin, 1800)	*Jalak putih*	Black-winged myna	300-1100	Wh	EN	-	●	●	ND	PPA, KM
Sturnidae	*Acridotheres tristis* (Linnaeus, 1766)	*Jalak nias*	Common myna	350	Wh	LC	-	-	●	ND	PPA
Sturnidae	*Aplonis panayensis* (Scopoli, 1786)	*Cucak keling*	Asian glossy starling	100	Wh	LC	-	-	●	ND	PPA
Sturnidae	*Leucopsar rothschildi* Stresemann, 1912	*Jalak bali*	Bali myna	2	Br	EN	-	●	●	ND	KM
Sturnidae	*Scissirostrum dubium* (Latham, 1802)	*Rio rio*	Grosbeak starling-grosbeak myna	350	Wh	LC	-	-	●	ND	PPA
Sturnidae	*Sturnus contra* (Linnaeus, 1758)	*Jalak suren*	Asian pied starling	500-600	Br	LC	-	●	●	ND	BM, KM
Sturnidae	*Sturnus sinensis*(Gmelin,1788)	*Jalak kapasan*	White shouldered starling	100-650	Wh	LC	-	-	●	ND	KM
Timaliidae	*Pomatorhinus montanus* Horsfield, 1821	*Cucak kopi*	Sunda cica-kopi	125	Wh	LC	-	-	●	ND	PPA
Timaliidae	*Stachyris grammiceps* (Temminck, 1828)	*Sniper*	White-breasted babbler	125	Wh	NT	-	●	●	ND	PPA
Timaliidae	*Timalia pileata* Horsfield, 1821	*Kaso-kaso*	Chestnut-capped babbler	85-290	Wh	LC	-	-	●	ND	KM
Trochilidae	*Arachnothera affinis* (Horsfield, 1821)	*Pijantung gunung*	Grey breasted spiderhunter	20-340	Wh	NT	●	-	●	ND	KM
Turdidae	*Geokichla citrina* (Latham, 1790)	*Anis merah*	Geokichla citrina	600- 6	Wh	LC	-	-	●	ND	BM, PPA, KM
Turdidae	*Geokichla interpres* (Temminck, 1828)	*Anis kembang*	Zoothera interpres	600	Wh	NT	●	-	●	ND	BM, PPA, KM
Turdidae	*Geokichla sibirica* (Pallas, 1776)	*Anis siberia*	Geokichla sibirica davisoni	100-150	Wh	LC	-	-	●	ND	PPA
Tytonidae	*Tyto alba* (Scopoli, 1769)	*Burung hantu serak sulawesi*	Barn owl	50	Wh	LC	-	-	●	ND	PPA, KM
Zosteropidae	*Heleia javanica* (Horsfield, 1821)	*Opior Jawa*	Javan grey-throated white-eye	50	Wh	LC	-	●	●	ND	PPA
Zosteropidae	*Lophozosterops javanicus* (Horsfield, 1821)	*Pia-pia*	Javan grey-throated white-eye	50	Wh	LC	-	-	●	ND	KM
Zosteropidae	*Zosterops chloris* Bonaparte, 1850	*Pleci lombok*	Lemon-bellied white-eye	50-150	Wh	LC	-	-	●	ND	PPA
Zosteropidae	*Zosterops flavus* (Horsfield, 1821)	*Pleci lokal*	Javan white-eye	250-1	Wh	NT	-	●	●	ND	PPA
Zosteropidae	*Zosterops japonicus* Temminck & Schlegel, 1845	*Pleci bustomi*	Warbling white-eye	100-150	Wh	LC	-	-	●	ND	BM
Zosteropidae	*Zosterops melanurus* Hartlaub, 1865	*Pleci*	Warbling white-eye	150	Wh	LC	-	-	●	ND	PPA, KM
Zosteropidae	*Zosterops montanus* Bonaparte, 1850	*Pleci monti*	Mountain white-eye	80-500	Wh	LC	-	-	●	ND	PPA
Zosteropidae	*Zosterops novaeguineae* Salvadori, 1878	*Pleci Papua*	Papuan white-eye	80-250	Wh	LC	-	-	●	ND	PPA
Zosteropidae	*Zosterops palpebrosus* subsp. *auriventer* Hume, 1878	*Pleci auriventer*	Indian white-eye	75- 350	Wh	LC	-	-	●	ND	PPA
Zosteropidae	*Zosterops wallacei* Finsch, 1901	*Pleci wallacea*	Yellow-ringed white-eye	157-690	Wh	LC	-	-	●	ND	PPA

Note: Common name: MacKinnon (2010). Bird sources: Wh: Wild hunting, Br: Breeding. Conservation status: IUCN: EN: Endangered, VU: Vulnerable, NT: Near Threatened, LC: Least Concern. App. II of CITES, Permen LHK No.P.20/2018, Endemicity: ●: present/listed, -: absent. Population: Dominant (> 100 individuals); Non-dominant (< 100 individuals). Market location: BM: Banyuputih Market; PPA: Pasar Pon Ambarawa; KM: Kartini Market.

Figure 2. The bird species found include. A. *Pycnonotus zeylanicus*; B. *Agapornis* sp.; C. *Geopelia striata*; D. *Cissa thalassina*; E. *Serinus canaria*; F.*Garrulax mitratus* ; G. *Lonchura oryzivora*; H. *Columba livia* f. *domestica*; I. *Aratinga solstitialis*; J. *Nymphicus hollandicus*; K. *Geokichla interpres*; L. *Pyrrhura molinae*

The high demand for birds from consumers encourages traders and bird catchers to use various methods to obtain supplies of bird species, often without regard to conservation principles (Pratiwi et al. 2021). Often, these methods are carried out without regard to conservation principles that should guide natural resource management. This practice not only harms threatened species but can also disrupt the ecosystem as a whole. Illegal bird capture and uncontrolled trade can cause imbalances in bird populations, which in turn disrupt food chains and other ecological interactions (Hughes et al. 2023). Government regulations play an important role in reducing exploitation and poaching, starting from the aspects of trade rules, species protection, and consequences of violations to more effective law enforcement. Therefore, government regulations serve as the main foundation for conservation efforts. Structured policies and consistent enforcement will support bird conservation efforts in the long term and create a more sustainable and responsible market.

Sales price

Based on Table 1, in Kartini Market, prices can reach up to Rp600,000 for *Geokichla citrina* (Latham, 1790) and around Rp170,000-Rp315,000 for *Gallus gallus* (Linnaeus, 1758). At Banyuputih Market, *G. citrina* is also sold at Rp600,000, while *Prinia atrogularis* (Moore, 1854) can be purchased from around Rp60,000 to Rp1,000,000. At *Pasar Pon*, *G. citrina* is also offered at Rp600,000, and *Gallus domesticus* are sold from Rp100,000 to Rp2,000,000. There are significant variations in bird prices across the three markets depending on the species and its conservation status. For example, *Chloropsis sonnerati* Jardine & Selby, 1827 in Kartini Market and Banyuputih Market are marketed at Rp250,000-Rp400,000, while in *Pasar Pon*, they are both around Rp250,000-Rp400,000. In addition, *Chrysolophus pictus* (Linnaeus, 1758) is only available in Kartini Market at Rp9,400,000; there are no records of sales in the other two markets. Overall, Kartini Market offered a wider range of prices for a variety of birds, including *G. gallus* and *G. citrina*. However, Banyuputih Market and *Pasar Pon* also provide competitive pricing options for some bird species, such as *P. atrogularis* and *C. sonnerati*. Therefore, market selection should be based on individual preferences regarding the type of bird you want to buy and your budget.

Bird sources

Based on the source of birds from this study, they are divided into species from the wild and breeding. The number of species originating from wild captures is 106 species and the results of breeding are 30 species of birds. The media used in the capture include nets, sticky rice, and snares. While breeding focuses on breeding in large numbers. Bird traders from the three markets admitted that they received a lot of bird supplies from hunters both from Java and outside Java. The types of wild-caught birds sold are mostly local species whose populations are still widely found in nature. While the breeding species are more dominant in types of birds originating from abroad such as *Serinus canaria* subsp. *domestica*, *Agapornis* sp., *Melopsittacus undulatu*s and other imported bird species. However, in Indonesia, breeding of bird species that are starting to become rare has begun. The types of birds that are bred are *Pycnonotus zeylanicus* and *Leucopsar rothschildi*. These two species have indeed begun to be bred because they are very difficult to find in nature. The cucak rowo species has been successfully bred by breeders in Indonesia with the aim of preserving and the value of its chirping. The difficult breeding character is still a slight obstacle to breeding this species (Lestari et al. 2017). Meanwhile, the Bali starling species is also carried out in Bali and all breeders in Indonesia who have received certification from the BKSDA. The Bali starling is still a vulnerable and sensitive species to breed because of its easily stressed character and the need for a comfortable environment (Milati 2024). However, many breeders continue to try to develop the potential existence of the *P. zeylanicus* and *L. rothschildi* species to prevent extinction. Market demand has indeed increased for these two types of birds. However, the number of species from breeding has not been able to meet the level of demand from traders in the market. Meanwhile, the type of wild-caught bird whose population is decreasing, namely *Zosterops flavus*, has indeed experienced a drastic decline due to the very high demand by consumers. The type of colony bird makes this species easy to catch in large numbers. So that its existence is getting thinner and harder to find, especially on the island of Java. Even this type of bird has begun to be bred because it is difficult to find. So, it can be predicted that the bird market will start to have difficulty getting a supply of birds that are of high interest, but availability in nature is decreasing, so breeding birds becomes one of the answers to balance these problems and conservation efforts.

Conservation status and endemicity of traded birds

In the three markets, 136 species were found to be traded and belonged to 46 families. The 136 bird species were then identified using IUCN, Appendix II of CITES, and Minister of Environment and Forestry Regulation No. P.20/MENLHK/setjen/Kum.1/6/2018. IUCN (International Union for Conservation of Nature) itself is a provider of assessment and analysis of comprehensive conservation data (Osipova et al. 2020). Meanwhile, CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) itself is an international trade convention that regulates wildlife trade, which was established to prevent species extinction (Sollund 2022). The Minister of Environment and Forestry Regulation No. P.20/MENLHK/ setjen/Kum.1/6/2018 contains the determination of which plant and animal species are protected.

IUCN red list

The conservation status of a species is an important issue that will determine how the species will survive. The determination of conservation status is not only based on the number of remaining populations but also the increase or decrease in population size in a certain period, the rate of breeding success, threats, and others (Yapsenang et al. 2022). After classification according to IUCN, 9 species were categorized as red or endangered, including *engkek keling* (*Cissa thalassina* (Temminck, 1826)), *gelatik batu* (*Parus major* Linnaeus, 1758*)*, gelatik belong (*Lonchura oryzivora* (Linnaeus, 1758)), jalak bali (*L. rothschildi* Stresemann, 1912) jalak putih (*Acridotheres melanopterus* (Daudin, 1800)), kacer ireng (*Copsychus sechellarum* A.Newton, 1865), red siskin (*Carduelis cucullata*), Srikatan (*Cyornis sanfordi* Stresemann, 1931), and Sun Conure (*Aratinga solstitialis* (Linnaeus, 1758)). Next, 3 species fall into the vulnerable category, including cucak rawa (*P. zeylanicus* (Gmelin, 1789)), jalak kebo (*Acridotheres javanicus* Cabanis, 1851), and mantenan (*Pericrocotus speciosus* (Latham, 1790)). Further, 11 species fall into the near-threatened category, including anis kembang (*G. interpres*), ciblek kristal (*P. familiaris* Horsfield), cililin (*P. galericulatus*), cucak kelabu (*P. cyaniventris*), gould amadine (*E. gouldiae*), kinoi (*C. venusta*), kolibri (*Arachnothera affinis*), pleci lokal (*Zosterops flavus*), poksai mantel (*G. palliatus*), poksay genting (*G. mitratus*), and sniper (*S. grammiceps*). At the same time, other species fall into the least concern category.

This study found 9 endangered bird species that are still traded. At least 7 of the 9 species were found at *Pasar Pon* Ambarawa. Although these 9 species are endangered, 4 species are not included in the list of protected animals according to the Minister of Environment and Forestry Regulation No. P.20/2018. The four species are gelatik batu (*P. major*), kacer ireng (*C. sechellarum*), red siskin (*C. cucullata*), and sun parakeet (*A. solstitialis*). Policymakers must consider including these species in the list of protected animals to prevent further exploitation. In addition, the prices of these endangered species are not all classified as expensive; the cheapest is at Rp35,000, and the most expensive is at Rp3,000,000. The low selling price of these species can be caused by successful reproduction in captivity and also local economic conditions. The next category below endangered is vulnerable. In the three markets, three species were found in the vulnerable category. Of the four species, one of them is a protected animal, namely the cucak rawa (*P. zeylanicus*). In addition, cucak rawa also has a high selling value, reaching Rp7,000,000. In Indonesia, this bird has quite a lot of enthusiasts despite its high price (Yong et al. 2018). One of the strong reasons why this species has a high price is because of its melodious chirping, which is always a consideration for bird enthusiasts; the other three species have selling prices in the range of IDR 35,000-250,000. In addition, it is known that the four bird species are endemic birds. Then, under the vulnerable category is the Near Threatened category, where 11 bird species were found in the three markets. The price value of these birds is in the price range of IDR 20,000-2,000,000. Of the 11 bird species, only 5 are on the protected list, including *Platylophus galericulatus, P. cyaniventris, Chloropsis venusta, Z. flavus*, and *Stachyris grammiceps*. In all three markets, many traders sell bird species categorized as least concern conservation. This means that there are around 116 bird species that are safe to trade. This category refers to species that have stable populations and are not subject to threats that could disrupt their survival. Species in this category can survive due to their wide geographical distribution, active breeding, and good survival skills. The wide geographical distribution of these species is a reason for optimism about their survival.

Appendix II CITES

The identification results showed that 13 bird species were included in Appendix II of CITES[LT1]. These birds include *Aegithina tiphia, Mirafra javanica, Treron vernans, L. rothschildi* , *Lonchura punctulata, Garrulax canorus, Copsychus malabaricus, Copsychus sechellarum, Melopsittacus undulatus, P. cyaniventris, P. zeylanicus, A. affinis,* and also *G. interpres.* CITES Appendix II is a list of species that are not threatened with extinction but could be endangered if trade continues without regulation (Shivambu 2024). In other words, the list of species in this appendix indicates that trade permits can be granted if only they are not detrimental to the survival of the species in the wild. The species *M. undulatus* (Parakeet) is known to be the dominant bird in the *Pasar Pon* Ambarawa, which may indicate that the demand for this species is high in the local market.

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Around the world, wild-caught animals are traded in wildlife markets, but it's hard to tell what's legal and what's not (Nijman et al. 2022). Indonesia has a serious problem with illegal trade in wild plants and animals (Shepherd et al. 2020). This problem not only threatens the existence of various species but also disrupts the balance of ecosystems that are very important for the survival of life on Earth. This regulation functions as a legal basis to reduce the number of poaching and illegal trade. Based on the Minister's regulation, 14 bird species were found to be protected species in all three markets. The birds include *C. thalassina, L. oryzivora, C. sanfordi, Loriculus galgulus, P. cyaniventris, P. zeylanicus, Pyrrhura molinae, Rhipidura javanica, A. melanopterus, Gracupica contra, L. rothschildi, S. grammiceps, Z. flavus,* and *Heleia javanica.* However, despite strict regulations, observations show that these birds are still widely traded. In fact, two species were found in both markets, *P. zeylanicus* (*cucak rawa*) and *G. contra* (*jalak suren*), which is an indicator of the high demand for these two species.

Endemicity

The majority of animals found in Indonesia are endemic to Indonesia, but there are also many non-endemic animals from various countries (Latifah et al. 2021). Endemic animals themselves are animal species that inhabit a region or area that make the region or area distinctive because of the presence of these species in it, and these animals are not found in other places (Hadi et al. 2024). In this study, 18 species were found to be non-endemic species, and 118 species were endemic. The non-endemic species include *G. gallus, C. pictus, Serinus atrogularis, Pyrrhura rupicola, Nymphicus hollandicus* (Kerr, 1792), *Erythrura gouldiae* (Gould, 1844), *P. molinae, Psittacula krameri* subsp. *manillensis* (Bechstein, 1800), *S. canaria, Agapornis* sp., *C. livia, N. hollandicus, Eophona personata* (Temminck & Schlegel, 1848), *Garrulax chinensis* (Scopoli, 1786), *Excalfactoria chinensis, C. cucullata, Crithagra leucopygia* Sundevall, 1850, and *A. solstitialis.* These bird species come from various countries such as South America, Vietnam, Cambodia, Japan, Australia, the Netherlands, Central America, Africa, India, and China. These non-endemic species can enter Indonesia for several reasons, such as legal international trade and also through natural migration. Regarding foreign bird species or imported birds developed in Indonesia, it can be said that they are not invasive if the species are released into the wild. This is because birds that come from abroad are mostly finch and hook-billed seed-eating species from breeding. When these breeding birds are released in Indonesia, they will have difficulty getting food in the wild, because this type of species is not used to the Indonesian environment and has been growing in cages for a long time (Yuliana et al. 2021). Indirectly, they will die if released into the wild without a food supply from humans.

Discussion

Market sustainability

Based on the results obtained, it can be seen in Table 2 that wild-caught birds rather than farmed birds dominate the source of birds marketed. This condition is quite threatening to the sustainability of trade activities, considering that the number of wild catches cannot be ascertained. Wild capture is also a threat to the extinction of bird species in the wild. The supply of birds from farms can be increased again to maintain the sustainability of bird trade in the market. With livestock, the market's need for birds can be met, while birds in the wild will be maintained. Hence, it will result in the sustainability of bird trade in the market.

Based on the results obtained, it can also be seen that trade still happens with birds that are protected according to IUCN, Appendix II CITES, and Regulation of the Minister of Environment and Forestry No. P.20/MENLHK/setjen/Kum.1/6/2018. These birds were obtained from wild catches, but some also come from farms. It is quite unfortunate that many protected bird species come from wild catches more than from farms. Wild-caught birds that are protected according to Regulation of the Minister of Environment and Forestry No. P.20/MENLHK/setjen/Kum.1/6/2018 include *P. galericulatus, P. cyaniventris, C. thalassina, Acridotheres tristis* (Linnaeus, 1766), *A. melanopterus, C. venusta, Z. flavus, S. grammiceps, R. javanica, L. galgulus.* Meanwhile, farmed birds include *G. gallus, P. zeylanicus, L. oryzivora, L. rothschildi,* and *G. contra.* The dominant bird species in the three markets is *Pycnonotus goiavier* (Scopoli, 1786). In the future, there could be an increase in the demand for birds on the market, which could potentially encourage illegal trade, especially when regulations are not enforced. The supply of birds is very important and has quite an influence on the continuity of trade in the bird market. Dependence on poaching to obtain birds can be an obstacle; this is because the population of birds in the wild, which is the main source, continues to decline. Therefore, it is necessary to increase the source of birds from farms. It is important to apply the principle of sustainable development, which includes economic, social, and environmental aspects in trade in the bird market (Iskandar et al. 2019).

Specific steps need to be taken with a regulatory, economic, and collaborative approach to ensure the sustainability of the bird market in Indonesia (Nijman et al. 2022). The first thing that can be done is to encourage sustainable bird breeding. The government needs to encourage legal and responsible bird breeding as a source of supply for birds in the public market. Then, the certification of captive-bred birds and its regulations are also made to ensure the welfare of birds and prevent exploitation practices. The second thing that can be done is to raise awareness of the importance of maintaining the sustainability of the bird market, and the third thing that can be done is to enforce the law against illegal trade. It is necessary to carry out regular supervision both in offline traditional markets and in online stores.

Table 2. The types of birds that are traded and take part in singing contests

Species	**Selling price (x Rp. 1000)**	**IUCN conservation status**
Geokichla citrina	60-600	LC
Mirafra javanica	100-500	LC
Lanius schach	16-200	LC
Copsychus sechellarum	50-250	EN
Copsychus saularis	35-300	LC
Serinus canaria subsp. *domestica*	50-250	LC
Leptocoma calcostetha	35-200	LC
Cinnyris jugularis	25-15	LC
Agapornis sp.	125000-200	LC
Copsychus malabaricus	200-1000	LC
Zosterops japonicus	Oct-15	LC
Orthotomus ruficeps	11-246	LC

Note: LC: Least concern, EN: Endangered

The government needs to increase supervision to detect and stop unlawful bird trade. Furthermore, it is necessary to collaborate between agencies such as the conservation center with the community and also traders. These steps, if carried out consistently and collaboratively, will be able to maintain the sustainability of the bird market in Indonesia and of course, preserve existing birds. When compared to the international scene, Indonesia is indeed considered to have a very diverse number of bird species. One country that imports birds is China, this bird trade is a step to develop bird variations based on the beauty of feather color, voice and physique and is associated with several countries including southern China, Myanmar, India and the Southeast Asian region (Ptak 2012). With the increasing trade in songbirds, this has led to the extinction of birds and the yellow-breasted bunting species can be used as a leading species in East Asia (Heim et al. 2021). Meanwhile, in the international arena, the Beo bird species has become a very hot issue because of the rampant trade, especially in West Asia, Southeast Asia, South Africa and Europe, which are the largest suppliers of this almost extinct species (Chan et al. 2021). So, the international world has set rules that ensnare beo caught by liars who must go through an accreditation system so that absolute extinction does not occur (Fiennes et al. 2024).

Naming and classification of birds

The scientific study of the interactions between birds and a particular society, both past and present, is known as ethnoornithology, which is a subdiscipline of ethnobiology (Thomsen et al. 2024). Banyuputih Market, *Pasar Pon*, and Kartini Market are located in Central Java, a region where the Javanese culture plays a significant role in bird naming. In society, the recognition or naming of bird species is generally influenced by the visual characteristics and sounds produced. Therefore, bird identification is often carried out at the genus level. However, there are also several types of birds whose names are not based on observable characteristics but rather come from fairy tales or folklore, such as the Lampung Java sparrow (*Cacomantis* *merulinus* (Scopoli, 1786)) (Muhammad et al. 2020). The influence of Javanese culture on bird naming is profound, as it shapes the local languages, cultures, and traditions of the community. One species can have several different names in different regions, a result of the unique interactions and communication between local residents and immigrants, as well as the influence of bird sellers. The naming of bird species is generally based on the bird's vocalizations, feather color, morphological characteristics, habitat, and distinctive behavior (Putri et al. 2021).

Based on its morphology, *Oriolus chinensis* Linnaeus, 1766 is known as the golden oriole because it has a body dominated by bright yellow, similar to the color of gold. This bird also has black wings and a black stripe on the head that passes through the eyes, giving it a striking appearance. The name 'golden oriole' is significant in many cultures, symbolizing wealth and prosperity. In addition, *Streptopelia chinensis* is called a turtle dove because it has a descending tone that is repeated over and over again, which sounds like "*te-kukkuurrr*," which is elongated. The name 'turtle dove' is often associated with peace and love in various regions. This sound is one of the important identification characteristics of this species, in addition to its physical characteristics. Naming based on morphology and vocalization helps recognize and distinguish the two bird species. Based on its vocalization, *Orthotomus ruficeps* (Lesson, 1830) is called a ciblek because the bird's voice can be heard in various vibrations, such as "*trrriii-yip*" and "*trrrri*," which have a high tone. In addition, this bird also produces sounds similar to "*ciiii-blék, ciii-blék*" and the nasal sound "*cicicici*," which further adds to the uniqueness of its vocalization. In comparison, *L. punctulata* is called emprit because it is known for its distinctive vocalization, producing the sound "*priit ... priitt*" (Muhammad et al. 2020). This sound is an important identification for this species and is often a characteristic that bird watchers easily recognize. Based on its habitat, *Cyornis* herioti R.G.W.Ramsay, 1886 is called the mountain flute because it is often found in mountainous areas or highlands. In addition, *L. rothschildi* is called the Bali starling because the bird's native habitat is the tropical forests of Bali and the bird species is closely related to its original habitat. Some birds are classified based on their behavior and habits. For example, *Passer montanus* (Linnaeus, 1758) is commonly called the sparrow bird because the bird is often found in places of worship, especially on church roofs. Because it is often found around churches, many people call it a sparrow. This bird has physical characteristics such as a brick-red head, a black throat with a white neck edge, and a grayish-white belly. Female birds have a slightly paler color than males. People also believe that sparrows bring good news regarding fortune, work, and soulmates (Fatimah and Muhammad 2023). *Tyto alba* (Scopoli, 1769) are called owls because these birds are often active at night. They generally perch on trees and are often used to guard agricultural land or rice fields by farmers to eradicate pests. *Tyto alba* have unique physical characteristics, such as large eyes that allow good vision at night, a flat disc-shaped face, and soft and thick feathers that support quiet flight. Their beak is small and sharp, while their large, sharp claws function to catch prey. Their feather colors vary, generally consisting of brown, gray, and white, which helps in camouflage (Sangster et al. 2013). The ciblek species belong to the same family but have differences in some physical colors and sound characteristics. In Banyuputih Market and *Pasar Pon* Ambarawa, two similar bird species were found, namely ciblek kebun (*Prinia familiaris*) and the ciblek kristal (*P. familiaris*). The ciblek kebun bird species has a physical color with yellow in the rectum to the chest, the sound characteristics possessed by the ciblek kebun are relatively dominant in the sound of reason. While the ciblek kristal is dominated by relatively dark feathers on the black and white patterns on the wings and does not have a yellow color in the rectum to the chest, but a pure white color. The eye color of the ciblek kristal is dominantly red with a typical long shot chirping sound. However, both species have the same posture, fighting style, and solitary nature.

Market chain systems and conservation implications

The bird market chain system in Indonesia involves various factors that play an important role in the bird trade, from breeders to traders and consumers. The majority of birds sold in the market are farm products (Mulyadi and Dede 2020). Banyuputih Market, *Pasar Pon*, and Kartini Market are not only places for transactions but also centers of social interaction where knowledge about birds and care practices is shared. The dynamics of supply and demand in these markets can affect the price and sustainability of certain species. Not only that, the threat of an outbreak or disease is also a factor that can affect price spikes and the availability of species in the market (Ferlito and Respatiadi 2019).

Unmanaged bird trade can threaten bird populations in the wild, especially for endangered species. Many of the birds traded come from wild catches, which has the potential to reduce their populations significantly. Therefore, the application of sustainable development principles in the bird trade is very important. This includes strict monitoring of traded species, as well as enforcement of regulations that protect species protected by IUCN (Panigrahi and Jins 2018). As such, the bird trade must be conducted ethically and responsibly to ensure no species are threatened with extinction due to overexploitation. This conservation effort should utilize a community-based approach in Indonesia. This collaborative approach not only creates a sense of ownership and responsibility for bird conservation but also allows for solutions to conservation violations. With this approach, conservation strategies can be designed to remain in line with community customs without compromising animal conservation principles. Things that can be done to improve animal conservation include educating bird traders and buyers, enforcing regulations in bird markets, enforcing laws in the community, and developing ecotourism or nature-based activities without disturbing bird habitats (Marshall et al. 2020). As the public gains a better understanding of the impacts of the bird trade and the importance of protecting species and their habitats, there is hope for conservation awareness. This hope lies in the potential for the community to play an active role in conservation and support sustainable trade practices. These practices can transform birds from mere cultural symbols to powerful testaments of our commitment to nature conservation. Collaborative efforts between the government, communities, and individuals are needed to ensure that bird biodiversity in Indonesia is maintained.

Sustainability of economic, social and environmental aspects in the bird market

From an economic perspective, bird trading activities in traditional markets, including Ambarawa Market, Pon Market and Kartini Market, are considered very good. The supply of birds from the wild is considered economically good by traders. This is because the purchase price from hunters is cheap and can be resold at a high price to general consumers. However, when compared to birds from breeding, it is indeed far, this is because birds from breeding will be sold at a fairly high price on the grounds that the results of official captivity are based on the breeder's ring (Delfiah et al. 2024). So that the sale is considered to have a small profit. However, for bird species with protected status, it will be more difficult to find because the selling value is very high and requires certification from the BKSDA institution. Sometimes traders also hesitate to sell protected birds because they violate applicable laws. So that especially rare birds will provide a high selling value or profit if they do come from official breeding. In terms of the economy, it has now entered the mode of buying birds from breeders that have rings because they are considered to have more economic value and are legally safe.

Socially, people will indeed be interested in wild-caught birds because they are considered cheaper than breeding birds. The market will be even more crowded if the variety of types of birds traded is quite diverse. However, wild-caught birds also have a risk of dying easily due to many factors, including: more sensitive endurance, easy to stress, difficult to adapt and takes a long time to make a sound (Setiawan et al. 2022). So some people also choose birds from breeding because they are easier to adapt and faster to make a sound, especially among bird chirping enthusiasts for competitions. Socially, the bird market will also apply warnings to traders and suppliers regarding protected bird species that cannot be traded. This is done so that traders and markets can be more compliant with the legality of the laws governing the trade in animals. Indirectly, traders will also pay attention to the rules so that social activities in the bird market are more conducive and safe.

The environmental aspect is also highly emphasized for traders and consumers themselves, especially on the principle of conservation. From now on, in general, the public has realized the importance of preserving the sustainability of the bird species in demand. Breeders have begun to emerge to compete to carry out breeding in order to reduce the purchase of wild-caught birds (Iskandar et al. 2015). In addition to high-value breeding results, it also has a positive impact on the sustainability of nature. Starting from the endemic or local bird species native to Indonesia that are starting to become extinct or imported birds. Sometimes breeders or bird lover communities also release birds into the wild so that their numbers increase. This is done so that sustainability in nature improves and minimizes the number of extinctions.

Based on research that has been conducted at Banyuputih Market, *Pasar Pon* Ambarawa, and Kartini Market, it was found that 46 families with 136 species of birds were traded at varying prices. On average, the birds are endemic animals, namely 118 species. Then, based on data analysis, it is known that 116 bird species are safe to trade, but there are still 11 species with near-threatened status, 3 species with vulnerable status, and 9 species are endangered. Therefore, from the source, traders need to pay attention to how the bird trade continues. Efforts are needed to implement the principles of sustainable development that include economic, social, and environmental aspects in trade in the bird market.

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The potential of *Hibiscus sabdariffa* seed extract and rice straw as coagulants-adsorbents for processing batik liquid waste

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Abstract. *Rahmani AS, Masykuri M, Setyono P. 2025. The potential of* Hibiscus sabdariffa *seed extract and rice straw as coagulants-adsorbents for processing batik liquid waste. Nusantara Bioscience 17: 118-128.* One of the efforts to reduce the content of heavy metals in this batik liquid waste is by applying the process of a combination of coagulation and adsorption. This process involves the use of the coagulant of Rosella Seeds Extracts (RSE) to neutralize the charge of the heavy metal ions, making them more likely to bind to the adsorbents from rice straw biomass. The adsorption then occurs, where the neutralized heavy metal ions are physically or chemically bound to the surface of the adsorbents, effectively removing them from the liquid waste. This study aims to determine the decrease in chromium metal levels (Cr^{6+}) in artificial batik liquid waste using this coagulation-adsorption process with biocoagulants of rosella seed extracts combined with straw rice adsorbents. $K_2Cr_2O_7$ solution was used as an artificial solution for batik liquid waste. The coagulation process was repeated 3 times to determine the optimum RSE biocoagulant, both doses, pH, and contact time. The first coagulation was carried out by mixing biocoagulant RSE into a 10 ppm $K_2Cr_2O_7$ solution. The coagulation process was carried out in the same way at pH 2, 4, 5, 6, 8, and 10 using the optimal dose of biocoagulant. The result showed that the optimum dose of biocoagulant RSE was 30 × 103 ppm, with an absorbance value, and the percentage of chromium metal (Cr6+) was, respectively, 0.623 and 68.69%. Meanwhile, the optimum pH for RSE biocoagulant was at pH 2 (acid condition), with absorbance and the percentage of chromium metal (Cr6+) values being, respectively, 0.617 and 69.02%. These findings have potential applications in the field of environmental science and waste management, particularly in the development of effective and sustainable methods for treating industrial waste.

Keywords: Adsorben, batik liquid waste, biocoagulant, chromium, rice straw, rosella

INTRODUCTION

Waste from the batik business, dyes, are often non-degradable organic chemicals that pollute the environment. Only about 5% of this coloring material is used, with the remaining 95% being discharged into the atmosphere. Dyes such as CrCl3 (chromium chloride), $K_2Cr_2O_7$ (potassium dichromate), and mordants (dye binders) such as $Cr(NO_3)_2$ (chromium nitrate) are sources of the dangerous heavy metal chromium (Cr^{6+}) (Hastuti et al. 2016; Pirkarami and Olya 2017; Koosdaryani et al. 2019). Chromium metal (Cr^{6+}) is difficult to decompose in the environment and can ultimately accumulate in the human body through the food chain. Continuous exposure to high doses of chromium metal causes cancer of the human lungs and digestive organs (Saputro et al. 2016; Zarkasi et al. 2018). The Indonesian batik business has grown significantly. According to data from the Ministry of Industry of the Republic of Indonesia, 47,755 Small and Medium Batik Companies (SMEs) were established in Indonesia in 2015 (Pinasti and Adawiyah 2016). However, problems such as environmental degradation caused by batik waste are losses that must be minimized. According to Amutha (2017) and Budiyanto et al. (2018), waste disposal into the rivers and land is the primary source of water pollution and soil contamination. Diniyati (2012) reported that the quality and quantity of Pete River Water in the Masaran Sub-district, Sragen District, Central Java, had the levels of color, odor, pH, nitrate, nitrite, phosphate, COD, and COD levels, highlighting the severity of contamination. One promising method to address these issues is coagulation-adsorption.

Environmental pollution caused by the disposal of liquid batik waste requires wastewater treatment. One method of wastewater treatment is coagulation-adsorption. Coagulation is the process of mixing coagulants (chemicals) or precipitants into raw liquid waste at a high rotation speed in a short time. One of the coagulants that can be used to process liquid waste is the seeds of the roselle seed extract biocoagulant. The high glutamic acid and arginine content in seed extract biocoagulant protein can coagulate (Tounkara et al. 2013). These amino acids will provide the overall charge to the protein, depending on its isoelectric point. This cationic protein can neutralize the negative charge of dye particles in liquid waste (Yong and Ismail 2016).

Adsorption is the accumulation of several molecules, ions, or atoms at the boundary between two phases. The phenomenon occurs due to unbalanced forces at the boundary between the surfaces of two phases, which causes changes in the concentration of molecules, ions, or atoms between the phases (Santoso et al. 2014). The goal is to absorb the targeted substance in liquid, solid, or semisolid form. The absorbed substance is called an adsorbate/solute, and the adsorbent is called an adsorbent. Agricultural waste in the form of rice straw can be used as an adsorbent in waste processing (Liu et al. 2013). Chemically, the content of rice straw consists of lignin (22%), cellulose (38%), hemicellulose (35%), and several other nutrients (5%) (Gummert et al. 2019; Taufik et al. 2021). In addition, rice straw can remove heavy metal pollution, dyes, and phenolic compounds (Mahamad et al. 2015; Li et al. 2017).

This research combined these two methods using natural ingredients, namely roselle (*Hibiscus sabdariffa* L.) seeds as a coagulant and rice straw as an adsorbent. This study aimed to test the effectiveness of the combination of *H. sabdariffa* seed coagulant with rice straw adsorbent in processing liquid waste from the batik industry and determine the perception of batik business actors regarding innovations in processing batik liquid waste.

MATERIALS AND METHODS

Equipment and materials

Equipment used in this study included UV-Vis spectrophotometry, measuring cups, beakers, ovens, magnetic stirrers, 100 mesh sieves, grinders, blenders, timers, scales, measuring flasks, pH meters, and measuring pipettes. Meanwhile, the materials utilized in this study were roselle seed extract (*H. sabdariffa*) or RSE, rice straw, distilled water, filter paper, synthetic $K_2Cr_2O_7$ solution, NaOH solution, and H_2SO_4 solution.

Preparation of biocoagulant

The method used to create a biocoagulant from Roselle Seed Extract (RSE) was adapted from Yong and Ismail (2016). The RSE was washed using distilled water and then dried in the oven for 120 minutes at 60°C to let it evaporate. The dried seeds were then processed through a 100 mesh screen to ensure that the size is consistent before being ground using a grinder to produce powder. To extract the coagulant, 5 g of RSE powder was combined with 100 mL of 0.5 M NaCl solvent. This extraction was completed in 2 minutes using a blender. The resulting mixture was then filtered using a filter paper to remove impurities, and the cationic proteins served as a biocoagulant. The biocoagulant was immediately used to prevent bacterial digestion of the organic components, allowing for more effective coagulation results.

Preparation of adsorbent

The adsorbent used in this research is rice straw waste obtained from agricultural land. The preparation of the rice straw adsorbent began with thorough washing to remove dirt and impurities, followed by drying in an oven at 105°C until a stable weight was achieved, ensuring the complete removal of moisture. The dried rice straw was then ground using a mechanical grinder, which was calibrated to produce a consistent particle size suitable for adsorption. To achieve uniformity, the coarse ground material was passed through a 100-mesh sieve, standardizing the particle size to approximately 150 µm. Any particles that did not conform to the desired size were reprocessed to ensure consistency. The resulting fine rice straw powder was then stored in a sealed, desiccated container to prevent contamination and preserve its adsorptive properties. This approach ensures a uniform adsorbent with optimal surface area, leading to consistent and reliable adsorption performance in subsequent experiments.

Preparation of batik liquid waste

The artificial batik liquid waste used for this study was an aqueous $K_2Cr_2O_7$ solution. It was a simplified model of real batik wastewater, focusing specifically on chromium (Cr6+) contamination, which is a significant concern in the actual batik effluents. The K2Cr2O7 in the artificial waste isolates Cr6+ as the primary contaminant, allowing targeted evaluation of the coagulation-adsorption treatment for chromium reduction (Birgani et al. 2016). Its production was based on the National Standardization Agency's process for producing $K_2Cr_2O_7$ 500 ppm. In a 100 mL volumetric flask, 141.4 mg of oven-dried $K_2Cr_2O_7$ was dissolved in distilled water. Then, a dilution of 10 ppm was performed.

FTIR (Fourier Transform Infra-Red) analysis method

The FTIR method used in this study aimed to identify organic compounds and analyze the functional groups present in the roselle seed coagulant and rice straw adsorbent. FTIR characterization was performed before and after interaction with the heavy metal chromium (CrVI), allowing observation of spectral changes that indicate interactions or bonding between roselle seed coagulant and rice straw adsorbent. The BTR and JP samples were dried and ground into fine powders. A small amount of each sample was then mixed with potassium bromide (KBr) in a ratio of approximately 1:100 (sample:KBr) to form pellets. These pellets were subjected to FTIR analysis using an FTIR spectrophotometer, within a scanning range of 4000-400 cm^{-1}. FTIR detects the infrared spectrum generated by the vibrational bonds within an organic compound, which provides information about the types of functional groups present in the material. By comparing the spectra before and after exposure to Cr(VI), changes in peak positions, intensities, or the disappearance of peaks were used to infer the involvement of certain functional groups in metal binding. The FTIR research results revealed that some functional groups remained present before and after contact with Cr(VI), whereas absorption changes or disappearances were also seen at specific wavenumber. This demonstrates the development of bonds between the tested materials and Cr(VI). For example, in roselle seeds, active groups such as alkyl isothiocyanate (N=C=S) shifted after reacting with Cr(VI). Meanwhile, the O-H, C-H, C-C, and C-O groups found in rice straw helped with heavy metal adsorption.

Determination of biocoagulant dose and optimal pH for lowering chromium metal levels (Cr^{6+})

Two coagulation processes were performed in this study. The first coagulation was performed to establish the optimal biocoagulant dosage, and the second was to establish the optimal biocoagulant pH. Biocoagulant dosages of 0, 10, 20, 30, 40, and 50 mL were added to 1000 mL of synthetic $K_2Cr_2O_7$ solution at 10 ppm, and agitated for 90 minutes. The solution mixture was then separated from the precipitates using filter paper before the absorbance value was determined using UV-Vis spectrophotometry at a wavelength of 540 nm by SNI 6989.71:2009. The ideal dosage of the biocoagulant was established based on the absorbance value; the standard outlines the spectrophotometric method for measuring hexavalent chromium (Cr-VI) concentrations in water and wastewater samples within the range of 0.1 to 1.0 mg L^{-1} at wavelengths of 530 or 540 nm (BSN 2009). The second coagulation followed the same procedure as the first one, creating several solutions with pH values of 2, 4, 5, 6, 8, and 10.

Determination of the optimal condition of the coagulation process

At this stage, the prepared coagulant and adsorbent were added to a 10 ppm sample solution. The mixture was stirred using the optimal contact time determined for the coagulation process. To evaluate the effect of contact time on the adsorption process, the mixture was tested with time variations of 30, 60, 90, 120, 150, and 180 minutes using the optimal coagulant-to-adsorbent composition ratio.

Determining the appropriate parameters for the coagulation process is an important step in determining the efficacy of a coagulation method in treating wastewater, particularly in terms of reducing the concentration of contaminants in the sample. The produced adsorbent and coagulant were combined with the sample solution at a 10-ppm concentration. In addition to determining the most efficient contact duration for the coagulation process, this mixing procedure seeks to ascertain the ideal composition ratio between the coagulant and adsorbent.

In order to determine the ideal duration for the coagulant and adsorbent to bind efficiently and precipitate particles in the sample solution, changes in contact time were applied, specifically at 30, 60, 90, 120, 150, and 180 minutes. Important variables like the degree of turbidity, the effectiveness of sedimentation, and the decrease in pollutant concentration were routinely tracked during this process. The outcomes of this phase offer important information on the best circumstances for increasing coagulation efficiency in wastewater treatment, which will ultimately result in safer effluent that satisfies environmental regulations.

RESULTS AND DISCUSSION

FTIR-based biocoagulant and adsorbent characterization

The results of FTIR analysis of roselle seeds before and after contact with (Cr6+), as shown in Table 1, revealed several shifts in the absorption bands. For instance, the C-H deformation of the aromatic ring shifted from 722.37 to 723.34 cm^{-1}. The interaction likely causes a redistribution of electron density within the aromatic system, altering the bond stiffness and, consequently, the vibrational frequency. The O-H stretching associated with alcohols, ethers, carboxylic acids, and esters shifted from 1,165.05 to 1,166.98 cm^{-1}. The shift indicates that Cr6+ forms bonds or interactions, such as electrostatic attraction or coordination, with hydroxyl groups. This interaction weakens the hydrogen bonding within the functional groups, causing a minor shift in the vibrational frequency. Similarly, the C-H bending of alkanes showed a slight shift from 1,462.11 to 1,464.03 cm^{-1}. These shifts indicate an interaction between the functional groups of roselle seeds and (Cr6+), suggesting that roselle seeds are effective in binding and reducing (Cr6+) through coagulation.

Similar shifts were observed for rice straw as summarized in Table 2. The C-H stretching of alkenes shifted from 789.88 to 792.78 cm^{-1} after contact with (Cr6+), and the NO_2 stretching showed shifts from 1,316.47 to 1,319.37 cm^{-1}, as well as from 1,509.36 to 1,511.29 cm^{-1}. These shifts indicate the interaction between the nitro compounds and (Cr6+), confirming that rice straw can also effectively adsorb (Cr6+). Additionally, changes in C=C stretching vibrations from 2,156.51 to 2,135.29 cm^{-1} further support the adsorption of (Cr6+) by rice straw.

Impact of doses on the coagulation process

Several doses of the RSE biocoagulant were used to generate the absorbance ratio and adsorption concentration of chromium metal in Table 3. The 10 × 10^3 ppm dosage group was found to have the highest absorbance value (0.708) and the lowest adsorbed concentration of chromium metal (Cr^{6+}) (1.902) among the tested dosages. The contact duration employed was 90 minutes. The lowest chromium metal (Cr^{6+}) absorbance value was created by a dosage of 30 × 10^3 ppm, in contrast, and the highest chromium metal (Cr^{6+}) adsorbed concentration was obtained by this dose (5.54). Adsorption concentration refers to the amount of a substance, Cr6+, that is adsorbed onto the surface of the adsorbent (roselle seeds) per unit weight or area. It is typically expressed in units such as milligrams of adsorbate (Cr6+) per gram of adsorbent.

The absorbance value decreases when RSE biocoagulant is applied up to 30 × 103 ppm and increases again at higher doses. However, while utilizing RSE, the adsorption concentration of chromium metal was limited to 30 × 103 ppm and will decrease when the dose is raised.

RSE biocoagulant has a pattern of increasing the percentage of adsorbed chromium metal (Cr6+) at doses up to 30 × 10^3 ppm. Still, it decreased if the dose was increased, which was due to the results of the adsorbed concentration of chromium metal (Cr6+) in Figure 1. According to these findings, 68.69% of the chromium metal (Cr6+) was adsorbed at the dosage of 30 × 10^3 ppm, which was the highest among the doses.

The pH effect on the coagulation process

Following that, a 90-minute contact duration with a dosage of 30 × 10^3 ppm was examined to identify the ideal pH for employing RSE biocoagulant. Table 4 shows the absorbance values that were obtained. The absorbance value for pH 2 was 0.617, the lowest absorption value among the pHs tested. When the pH went up to 4, the absorbance value climbed to 0.704, and the absorbance value continued to rise when the pH was raised, especially at pH 4, 5, 6, 8, and 10.

Table 1. IR spectrum variations between roselle seeds (biocoagulant) before and following contact with (Cr6+)

Seeds oselle	Roselle seeds+(Cr6+)	Range (cm^{-1})	Intensity	Functional group	Kind of vibration
722.37	723.34	690-900	Solid	C-H deformation	Aromatic ring
1165.05	1166.98	1050-1300	Solid	O-H stretching	Alcohol, ether, carboxylic acid, ester
1462.11	1464.03	1340-1470	Solid	C-H bending	Alkane
1662.71	1662.71	1610-1680	Inconsistent	C-H bending	Alkene
1744.69	1744.69	1690-1760	Solid	C=O stretching	Aldehyde, ketones, carboxylic acid, ester
2854.77	2854.77	2850-2970	Solid	C-H stretching	Alkane
2926.14	2925.17	2850-2970	Solid	C-H stretching	Alkane
3380.4	3380.4	3300-3500	Mid	O-H stretching	Amine, amide

Table 2. IR spectrum variations between rice straw (adsorbent) before and following contact with (Cr6+)

Rice straw	Rice straw+(Cr6+)	Range (cm^{-1})	Intensity	Functional group	Kind of vibration
789.88	792.78	675-995	Solid	C-H stretching	Alkene
1098.51	1097.54	1050-1300	Solid	C-O bending	Alcohol, eter, acid, carboxylate, ester
1316.47	1319.37	1300-1370	Solid	NO_2 stretching	Nitro compound
1371.45	1372.41	1300-1370	Solid	NO_2 stretching	Nitro compound
1509.36	1511.29	1500-1570	Solid	NO_2 stretching	Nitro compound
1623.17	1628.95	1610-1680	Inconsistent	C=C stretching	Alkene
2156.51	2135.29	2100-2260	Inconsistent	C=C stretching	Alkyne
2920.35	2902.03	2850-2970	Solid	C-H stretching	Alkane

Table3. Optimization of RSE biocoagulants depending on doses

Dosage (10^3 ppm)	The absorbance value on average	Adsorption concentration (ppm)
0 (control)	1.981	0
10	0.708	1.902
20	0.668	1.961
30	0.623	2.028
40	0.644	1.997
50	0.668	1.959

Table 4. pH-based RSE biocoagulant optimization (dosage = 30 × 10^3 ppm)

pH	The absorbance value on average	Adsorbed concentration (ppm)
2	0.617	2.038
4	0.704	1.907
5	0.675	1.950
6	0.678	1.947
8	0.671	1.956
10	0.664	1.967

Optimization of coagulant adsorbent combination

This optimization was carried out based on comparing the composition and contact time. Optimization results (Table 5) were obtained based on the composition of the RSE coagulants and rice straw adsorbent. The ratio of 1:0.5 has the lowest absorbance value compared to other comparisons, which is 0.565 mL L^{-1}. In addition, the comparison produces the highest concentration of chromium metal (Cr^{6+}) metal compared to other comparisons, which is 2.115 ppm. Based on Table 6, the concentration of adsorbed substances increased with increasing contact time from 1.552 ppm at the 30th minute to 1.632 ppm at the 150th minute. After the 150th minute, the adsorbed concentration did not increase anymore, even returning to the initial value at the 180th minute.

Figure 2 shows that the percentage of chromium metal (Cr6+) adsorption is dependent on the solution's pH conditions. pH 2 gives the highest adsorption rate (69.02%). Figure 3 revealed that the lowest adsorption percentage is at the extremes 1:0 = 68.70% and 0:1 = 67.49%, where only one component was used, either coagulant or adsorbent alone. This suggests that neither coagulant nor adsorbent alone is highly effective in adsorbing Cr^{6+}. The maximum adsorption percentage, 71.63%, occurs at the ratio 1:0.5, where the coagulant was mixed with half the proportion of adsorbent. This indicates that a balanced combination of coagulant and adsorbent significantly enhances Cr^{6+} adsorption efficiency.

Based on Figure 4, the optimal contact time for Cr^{6+} adsorption is 150 minutes, where the percentage reaches its peak at 55.30%. Beyond 150 minutes, the adsorption efficiency decreases due to Cr^{6+} ions are released back into the solution over time. This trend shows that the adsorption efficiency improves with longer contact time, as the coagulant and adsorbent have more time to interact with Cr^{6+} ions.

Table 5. Optimization of RSE biocoagulant and rice straw (RS) adsorbent combinations based on composition ratio (reference contact time = 90 minutes)

Comparison (RSE:RS)	Compositions of average absorbance values (mL L^{-1})	Adsorbed concentration (ppm)
1:0	0.623	2.029
1:0.25	0.599	2.065
1:0.5	0.565	2.115
1:0.75	0.588	2.081
1:1	0.594	2.072
0.75:1	0.576	2.099
0.5:1	0.584	2.087
0.25:1	0.588	2.081
0:1	0.647	1.993

Table 6. Optimization of coagulant and adsorbent combinations based on contact time

Contact time (minutes)	Average absorbance value (mL L^{-1})	Adsorbed concentration (ppm)
30	0.572	1.552
60	0.561	1.568
90	0.539	1.601
120	0.530	1.615
150	0.518	1.632
180	0.572	1.552

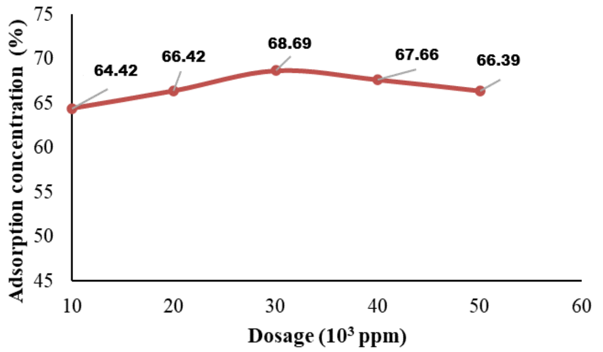

Figure 1. The chromium metal (Cr6+) adsorbed percentage depends on the doses of the RSE biocoagulant

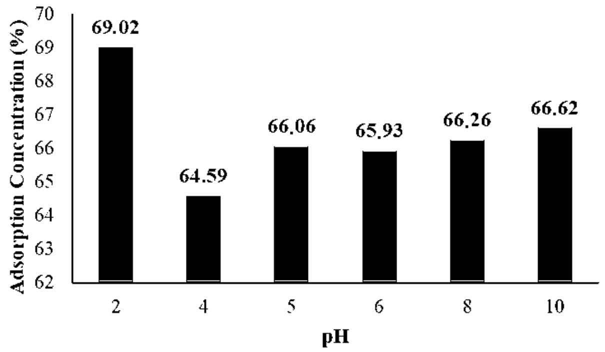

Figure 2. Adsorption of chromium metal (Cr) as a percentage of pH

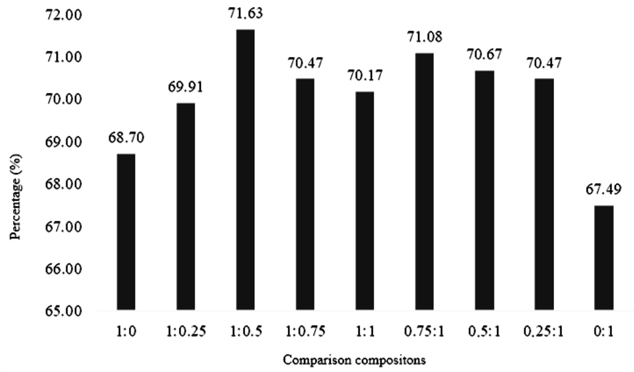

Figure 3. Percentage of chromium metal adsorption (Cr6+) based on the comparison of coagulant and adsorbent compositions

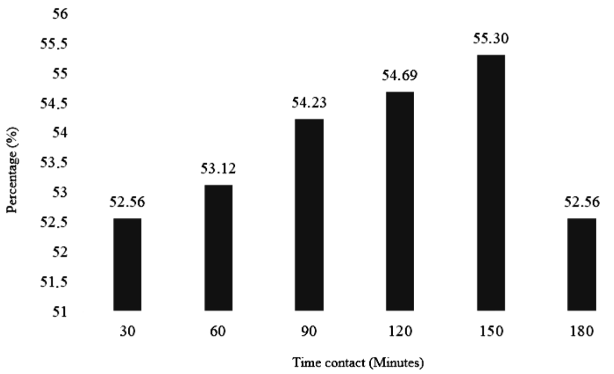

Figure 4. Percentage of chromium metal adsorption (Cr6+) based on contact time with coagulant composition and adsorbent 1: 0.5

Langmuir and Freundlich isotherm

Adsorption equilibrium can be interpreted as a mathematical translation of a particular isotherm in the adsorbent used. In general, overall adsorption predictions can be made by modeling isotherm data with linear analysis as a mathematical approach. The isotherm model is commonly used in determining the equilibrium model of adsorption in various studies, namely the Langmuir isotherm and the Freundlich isotherm.

Figure 5 shows the Langmuir isotherm pattern with a determinant coefficient of 0.998. The Langmuir isotherm accounts for the surface heterogeneity and multilayer adsorption, making it a more accurate representation of the adsorption process for rice straw. Thus, the Langmuir model effectively captures the adsorption mechanism of Cr^{6+} onto rice straw, highlighting its suitability for describing adsorption on natural heterogeneous adsorbents.

The resulting Freundlich isotherm graph (Figure 6) shows that the line formed is linear with intercept = log k and slope = 1/n. It can be seen that the (Cr^{6+}) adsorption research by rice straw follows the Freundlich isotherm pattern at a determinant coefficient of 0.9997. This behavior is attributed to the heterogeneous nature of the rice straw surface, which provides a variety of adsorption sites with differing affinities for Cr^{6+} ions. Rice straw, as a natural and porous material, contains cellulose, hemicellulose, lignin, and silica, which contribute functional groups such as hydroxyl and carboxyl.

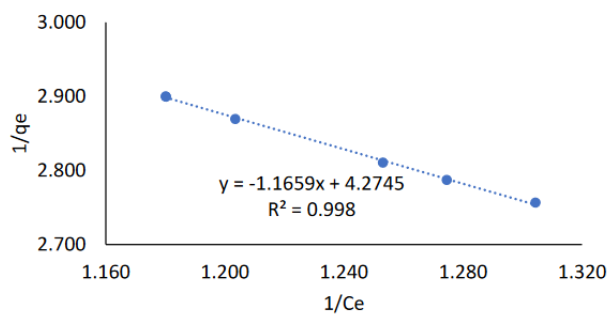

Figure 5. Graph of the Langmuir isotherm

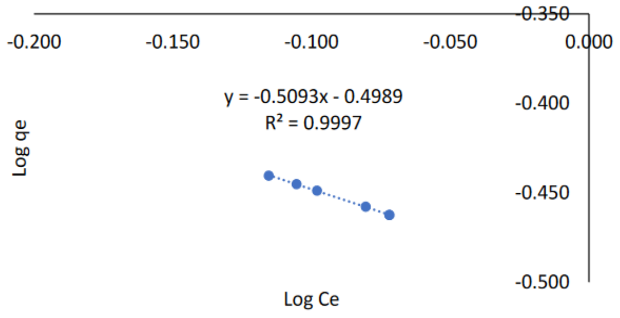

Figure 6. Graph of isotherm Freundlich

Discussion

The updated FTIR analysis, supported by the data from Table 1, shows a clear shift in the IR spectra of roselle seeds before and after contact with ($Cr6^{+}$). The absorption band for C-H deformation of the aromatic ring shifted slightly from 722.37 to 723.34 cm^{-1}. Similarly, the O-H stretching associated with alcohol, ether, carboxylic acid, and ester moved from 1,165.05 to 1,166.98 cm^{-1}, indicating interaction with (Cr6+). The C-H bending of alkane also showed a minor shift from 1,462.11 to 1,464.03 cm^{-1}, further supporting the occurrence of bonding between roselle seeds and (Cr6+). The shifts in wave numbers reflect chemical interactions, particularly with alkyl isothiocyanate, which is responsible for the coagulation process and leads to the reduction of (Cr6+).

In contrast, Table 2 shows the changes in the IR spectrum of rice straw. The absorption band for C-H stretching in alkenes shifted from 789.88 to 792.78 cm^{-1} after contact with (Cr6+), suggesting an interaction between the rice straw and (Cr6+) ions. A similar shift was observed in the NO_2 stretching, where wave numbers changed from 1,316.47 to 1,319.37 cm^{-1} and from 1,509.36 to 1,511.29 cm^{-1}. This interaction indicates that nitro compounds in the rice straw played a role in the adsorption process. The changes in the C=C stretching vibrations from 2,156.51 to 2,135.29 cm^{-1} further support the conclusion that multiple chemical bonds within the rice straw structure interact with (Cr6+), leading to higher adsorption capacity.

According to Table 1, most of the chemical compounds in roselle seeds before and after contact with (Cr^{6+}) are similar. However, the uptake in roselle seeds after interaction with (Cr^{6+}) shifted, and some has been lost because there was an interaction and bond between roselle seeds and (Cr^{6+}), such as a shift and loss of the wave number of the active chemical compounds of roselle seeds' alkyl isothiocyanate (N=C=S) after making contact with (Cr^{6+}). Based on measurements of the IR spectrum, it may be assumed that the active chemical particles, 4-alpha-4-rhamonsiloxy-benzyl-isothiocyanate, were responsible for the drop in (Cr^{6+}) levels. These chemically active substances mix with roselle seed particles to create bigger clumps that settle (adsorption between the positively charged roselle seed protein and oxo anions (HCrO4-). A study by Bahrodin et al. (2021) emphasizes the impact caused by chemical coagulants, which include chemical residues, toxic sludge, and health diseases, upon prolonged consumption. Thus, there is an emerging trend of transferring from chemical to natural coagulants. According to Hoong and Ismail (2018), rosella seeds can be chemical coagulants in processing industrial liquid waste since they are safer, biodegradable, and ecologically friendly. Zheng et al. (2021) argue that *H. sabdariffa* can be used as a natural coagulant because of the high protein content in its seeds. The protein content in *H. sabdariffa* is positively charged, while the dye waste is negatively charged. Coagulation occurs between the protein and dye waste, causing this mixture to become heavier and denser. This is what causes dyes to be removed from wastewater through sedimentation.

Table 2 shows that most of the functional groups found in rice straw adsorbents, before and after being interacted with (Cr^{6+}), tended to be the same; the difference is seen in absorption after being interacted with (Cr6+), which experiences a magnification, and some are missing. This is caused by the interaction and bond between rice straw and (Cr^{6+}), such as shifting. It is assumed that the formation of complexes between (Cr^{6+}) and certain functional groups in rice straw can cause a greater absorption shift, but does not always change the structure of the functional group permanently. (Cr^{6+}) can affect the saturation level of adsorption sites in rice straw and the interaction type. The type and structure of functional groups in rice straw influence the strength of interaction with (Cr^{6+}). Functional groups with a more complex structure and larger surface area have a higher adsorption capacity for (Cr^{6+}). Ponzoni et al. (2015) argue that high concentrations of (Cr^{6+}) can cause competition between functional groups to bind (Cr^{6+}) ions (Jahanmahin et al. 2016). This can induce an absorption shift, where (Cr^{6+}) binds to functional groups with higher affinity, although these functional groups do not always change their structure permanently. Hasan et al. (2009) stated that the interaction between (Cr^{6+}) and rice straw is not limited to electrostatic attraction. Other mechanisms, such as complexation, ion exchange, and physical adsorption, may also occur.

The absorbance esteem, adsorption fixation, and representation of the level of adsorbed chromium metal (Cr^{6+}) in Figure 1, the utilization of RSE biocoagulant at a concentration of up to 30 × 10^3 ppm, shows that the coagulation cycle is moving toward the balance point. In contrast, the equilibrium point happens at a concentration of more than 30×10^3 ppm, where there is an expansion in the absorbance value and a reduction in the adsorption capacity of chromium metal (Cr^{6+}). Meanwhile, the decrease in the percentage of adsorbed chromium metal (Cr^{6+}) was not excessive and tended to be consistent because a dense layer of biocoagulant particles surrounds the effluent particles. The surface of the effluent particles is saturated in this form, and therefore, the probability of the two interacting is relatively low. Apart from that, the precipitated chromium metal (Cr^{6+}) will dissolve again. As a result, there was no more coagulation process, which is no longer efficient (Yuniyarti and Isbandi 2018).

The formation of flocks (sediments) during the coagulation process is highly reliant on the coagulant dose utilized. The coagulant charge neutralizes liquid waste particles and encourages them to cling together. Because low-liquid-waste particles have low energy and form weak and tiny precipitates, a minimal dose quantity will neutralize them. Excessive dosing of liquid waste can potentially result in charge reversal, resulting in poor and inefficient coagulation activity (Patel and Vashi 2012). This biocoagulant may bind chromium metal (Cr6+) present in liquid batik waste colors due to its high amount of cationic peptides such as glutamate and arginine. Subsequently, coagulation between the protein and the color occurs, causing it to grow heavier and denser. Due to this, dyes are eliminated from wastewater through sedimentation (Alsukaibi 2022). Aside from that, pH is another aspect that affects the effective utilization of RSE in the coagulation process.

In Figure 2, we found the percentage of chromium metal (Cr6+) adsorption owing to the pH conditions of the solution. According to the results from the absorbance value, adsorption concentration, and visualization of the percentage of chromium metal (Cr6+) adsorption in Figure 2, pH is one of the affecting elements. Significantly impacts the stability of the proteins present in the seeds throughout the coagulation process (Jones 2016). Based on Figure 3, the optimal composition for removing Cr^{6+} using a coagulant and adsorbent mixture is at a ratio of 1:0.5. This balance ensures the best performance, leveraging the combined effects of both components. Using only one component or deviating too far from this ratio reduces the adsorption efficiency due to incomplete removal mechanisms. When the proportion of either coagulant or adsorbent dominates, the adsorption efficiency decreases, suggesting that both components are essential and work synergistically to maximize chromium removal. The slight decrease in efficiency beyond the 1:0.5 ratio indicates diminishing returns or saturation effects in the adsorption process.

Figure 4 shows that the optimal contact time for Cr^{6+} adsorption using a coagulant-to-adsorbent ratio of 1:0.5 is 150 minutes, achieving the highest efficiency of 55.30%. The adsorption efficiency decreases due to Cr^{6+} ions are released back into the solution over time. According to Yong and Ismail (2016), the coagulation process employing RSE is very pH-dependent. The negative charges in the artificial sample solution will attach to the cationic amino acids found in RSE, such as glutamic acid and aspartic acid (Jones 2016). Acidic circumstances enhance the density of positive charges (H+) surrounding the coagulant hydrolysate and improve the ability of cationic amino acid groups to behave as coagulants. Cationic coagulant proteins will neutralize dissociated organic components in liquid waste and promote particle adhesion (Cao et al. 2010). As a result, chromium metal adsorption effectiveness increases in acidic conditions, particularly at pH 2. The application of RSE effectively reduced turbidity and Chemical Oxygen Demand (COD) in liquid waste, supporting the findings obtained in this study. Consequently, RSE has the most excellent efficacy in eliminating turbidity and COD in acidic circumstances (pH 2) during coagulation, while its efficacy decreases in alkaline conditions (8-11). In acidic circumstances (pH 2), RSE removes turbidity and COD with 90.69 and 54.38% efficiency, respectively. Other research indicates that RSE works successfully in acidic circumstances during the coagulation process (Sibartie and Ismail 2018).

According to Yong and Ismail (2016), RSE can remove up to 95.1% of dyes. Furthermore, using RSE as a biocoagulant with other approaches, such as adsorption, would boost its efficiency. According to Hoong and Ismail (2018), the use of RSE as a biocoagulant and activated carbon as an adsorbent at coagulant and adsorbent dosages of 209 and 150 mg L^{-1}, respectively, at pH 2, was able to remove up to 96.67% of dyes.

The combination of RSE and rice straw adsorbent coagulants with a ratio of 1: 0.5 is the most optimal to reduce chromium metal levels (Cr^{6+}) in a sample solution. Thus, the most optimum combination of RSE and rice straw adsorbents was found in a ratio of 1: 0.5 (Table 5). In line with the results of the concentration of chromium metal (Cr^{6+}), the percentage of chromium metal (Cr^{6+}) is adsorbed in a sample solution using several comparisons of RSE coagulants and rice straw adsorbents showing that the ratio of 1: 0.5 produces the highest percentage, which is 71.63% (Figure 3). The lowest rate is produced by the coagulant RSE and rice straw adsorbent in a ratio of 0: 1, which is 67.49%. A ratio of 1:0.5 probably produces the optimal combination between the amount of RSE coagulant and the adsorption capacity of rice straw. At a ratio of 1:0.5, this interaction may reach an optimal balance, allowing efficient (Cr^{6+}) adsorption and minimal interference from other compounds. Other ratios may result in less optimal interactions, such as competition between compounds for adsorption sites or the formation of unstable complexes. According to Jian et al. (2016), rice straw has complex surface properties, with various functional groups that can bind (Cr^{6+}). According to Zhang et al. (2018), the dominant type of interaction influences the saturation level of adsorption sites and the stability of the (Cr^{6+}) rice straw complex. Mhlarhi et al. (2023) argue that (Cr^{6+}) binds to functional groups with higher affinity, although these functional groups do not always change its structure permanently.

The trend (Figure 4) is that the percentage increase in adsorbed by chromium metal (Cr^{6+}) occurs at contact times up to 150 minutes, but there is a decrease in percentage at a longer contact time of 180 minutes. The percentage is adsorbed by chromium metal (Cr^{6+}) due to the highest combination of RSE and Adsorbent rice straw coagulants occurring during contact for 150 minutes, which is 55.30%.

Thus, the optimal condition of the coagulant combination of RSE and rice straw adsorbents occurs in a ratio of 1:0.5 with a contact time of 150 minutes in the sample solution. At a contact time of 150 minutes, a balance was reached between the adsorption and desorption rates of (Cr^{6+}). At a contact time of 180 minutes, (Cr^{6+}) desorption began to dominate, causing a decrease in the percentage of (Cr^{6+}) adsorbed. Over time, the adsorption rate slows due to the reduction in available adsorption sites and the increase in the activation energy required to bind (Cr^{6+}). Cr($^{6+}$) is present in ionic form (CrO_4^{2-}), which has a negative charge (Tian et al. 2023). Rice straw contains functional groups such as carboxyl (COOH) and hydroxyl (OH), which have negative charges (Ahmed et al. 2023). Electrostatic attraction occurs between (Cr^{6+}) and negatively charged functional groups, causing (Cr^{6+}) to be adsorbed on the surface of rice straw.

Langmuir isotherm indicates (Figure 5) that the adsorption process is chemical, and a single layer of (Cr6+) is above the surface of the rice straw adsorbent at a constant temperature. The appropriate area variation and the porosity of the adsorbent can be tolerated with the Qm constant, which shows that the higher maximum adsorption capacity can be produced from the surface area and volume of large pores. The greater the Qm value in the Langmuir equation shows that the greater the adsorption capacity. It can be seen that the Cr6+ adsorption research by rice straw follows the Freundlich isotherm (Figure 5) pattern due to the porous structure of rice straw, which comprises micro- and mesopores, increasing the available surface area for adsorption and supporting multilayer adsorption, particularly at higher Cr^{6+} concentrations. Research conducted by Purnamasari et al. (2017) obtained an R2 value of 1 for the equation of Langmuir and Freundlich. However, the cost of b is lower than Kf; this means that this isotherm controller is a Langmuir rather than a Freundlich one because it is strengthened by results that refer to the price of b and Kf. At specific pressures, the level of adsorption is determined by the value of b, which depends on the temperature and adsorption enthalpy. The value of b is increased if there is a decrease in system temperature.

One of the considerations for a person who will adopt an innovation is whether the innovation benefits the adopter. Based on the results above, most batik businesses are optimistic that this innovation can provide benefits, even though a small portion of them judge that this is not the case (2%). This is inseparable from the costs incurred for this innovation, which is relatively cheaper than the conventional materials they used previously in the processing of batik liquid waste. This innovation offers a more cost-effective solution than traditional liquid waste processing methods. Cheaper raw material costs and more efficient processes can increase batik business profits. This is an essential consideration for batik entrepreneurs, especially for those who have limited capital. In addition, batik businesses assess this innovation by their conditions and habits in processing liquid waste (compatible). Business actors also realize that this innovation aligns with their expectations to preserve the environment. Batik entrepreneurs recognize the importance of protecting the environment. This innovation offers an environmentally friendly solution to reducing water pollution from liquid batik waste. This aligns with the expectations of batik entrepreneurs to run their business sustainably and responsibly.

The complexity of applying innovation also has an effect. The majority of business actors assess positive, which shows that the innovation of the combination of RSE coagulants and rice straw adsorbents has a low level of complexity, so it can be easily applied in processing batik liquid waste. This means that most business people assess this innovation as having a low level of complexity. The wastewater treatment process using a combination of RSE coagulant and rice straw adsorbent is easy to implement and understand. This convenience allows businesses to adopt innovations without making significant changes to existing infrastructure or expertise. Another critical factor that determines whether an innovation can be adopted which can be trialability and observability. They judge very positive (90%) and positive (10%) that this innovation can be tried first in a limited manner, and they judge very positively (89%) and positive (11%) can be directly observed in the processing of batik liquid waste. By offering a low-risk approach and clear visibility of benefits, trialability and observability play a significant role in why batik businesses show such positive sentiment towards this wastewater treatment innovation.

This study demonstrated the potential of *H. sabdariffa* seed extract and rice straw as effective natural coagulant-adsorbents for reducing chromium (Cr^{6+}) levels in artificial batik liquid waste. The optimal coagulation condition using RSE was achieved at a dosage of 30×10^3 ppm and pH 2, resulting in a Cr^{6+} reduction of 68.69 and 61.02%, respectively. The efficiency of RSE is influenced by its protein content, with both underdosing and overdosing leading to reduced coagulation performance due to insufficient charge neutralization or charge reversal effects. Additionally, RSE showed greater coagulation efficiency under acidic conditions, with performance declining at higher pH values.

The positive response from batik business actors toward the innovation of combining RSE and rice straw suggests practical potential for its application in small-scale industries. However, further research is recommended to evaluate floc volume, sedimentation time, and its performance under actual batik wastewater conditions, considering the complexity and variability of real waste characteristics.

Based on the FTIR analysis and data presented in Table 1, significant shifts in the IR spectrum of roselle seeds before and after contact with Cr^{6+} were observed. The absorption band for C-H deformation in the aromatic ring slightly shifted from 722.37 to 723.34 cm^{-1}. Similarly, the O-H stretching associated with alcohols, ethers, carboxylic acids, and esters shifted from 1,165.05 to 1,166.98 cm^{-1}, indicating an interaction with Cr^{6+}. The C-H bending in alkanes also experienced a minor shift from 1,462.11 to 1,464.03 cm^{-1}, further supporting the formation of bonds between roselle seeds and Cr^{6+}. These wavenumber shifts reflect chemical interactions, particularly with alkyl isothiocyanates, which play a role in the coagulation process and lead to Cr^{6+} reduction. The chemical compounds interacting with roselle seeds before and after contact with Cr^{6+} remained relatively consistent. However, after interaction with Cr^{6+}, certain shifts and the disappearance of specific compounds were observed due to bond formation between roselle seeds and Cr^{6+}, such as the shift and disappearance of the active alkyl isothiocyanate (N=C=S) wavenumber in roselle seeds. Based on IR spectrum measurements, it can be assumed that the active chemical particle, 4-alpha-4-rhamnosyloxy-benzyl-isothiocyanate, is responsible for Cr^{6+} reduction. This active compound interacts with roselle seed particles to form larger flocs, which settle through an adsorption process between the positively charged roselle seed proteins and the oxide anion ($HCrO_4^-$).

Analysis of Table 2, shows changes in the IR spectrum of rice straw, where the absorption band for C-H stretching in alkenes shifted from 789.88 to 792.78 cm^{-1} after contact with Cr^{6+}, indicating an interaction between rice straw and Cr^{6+} ions. A similar shift was observed in NO_2 stretching, where the wave numbers changed from 1,316.47 to 1,319.37 cm^{-1} and from 1,509.36 to 1,511.29 cm^{-1}. This interaction suggests that nitro compounds in rice straw play a role in the adsorption process. Changes in C=C stretching vibrations from 2,156.51 to 2,135.29 cm^{-1} further support the conclusion that some chemical bonds in the rice straw structure interact with Cr^{6+}, leading to increased adsorption capacity. The measurements presented in Table 2 indicate that most functional groups in rice straw remained unchanged before and after interaction with Cr^{6+}. However, after interacting with Cr^{6+}, some absorption bands intensified while others disappeared. This phenomenon is attributed to interactions and bonding between rice straw and Cr^{6+}, such as spectral shifts. High Cr^{6+} concentrations also caused competition among ion-binding sites, leading to absorption shifts (Ponzoni et al. 2015). The interaction between Cr^{6+} and rice straw can result in complexation, ion exchange, and physical adsorption, rather than solely electrostatic attraction (Hasan et al. 2009).

Absorbance values, adsorption concentration, and the percentage of adsorbed chromium (Cr^{6+}) can be observed in Figure 1. The use of RSE bio-coagulant at doses up to 30 × 10^3 ppm showed that the coagulation process approached equilibrium. However, at doses exceeding 30 × 10^3 ppm, absorbance values increased while Cr^{6+} adsorption concentration decreased. This decline in adsorption percentage occurs because a dense bio-coagulant layer surrounds the waste particles, reducing interaction and allowing previously precipitated chromium to redissolve, making coagulation ineffective (Yuniyarti and Isbandi 2018). Positively charged coagulants neutralize liquid waste particles, enabling them to aggregate. However, excessive coagulant doses may cause charge reversal, reducing coagulation efficiency (Patel and Vashi 2012).

Roselle seeds function as a bio-coagulant by binding Cr^{6+} in batik wastewater due to their high content of cationic peptides, such as glutamate and arginine. The coagulation process between proteins and dye compounds increases the weight and density of the mixture, facilitating the removal of dyes from wastewater via sedimentation (Alsukaibi 2022). Additionally, pH influences the effectiveness of roselle seeds in coagulation. A previous study by Sibartie and Ismail (2018) demonstrated that using roselle seeds as a bio-coagulant in wastewater treatment was successful. According to Yong and Ismail (2016), RSE can remove up to 95.1% of dyes. Furthermore, using RSE as a bio-coagulant in combination with other methods, such as adsorption, can enhance its efficiency. Hoong and Ismail (2018) reported that a combination of RSE as a bio-coagulant and activated carbon as an adsorbent, at doses of 209 and 150 mg L^{-1}, respectively, at pH 2, could remove up to 96.67% of dyes. The optimal combination of roselle seed coagulant and rice straw adsorbent was found at a 1:0.5 ratio, which was most effective in reducing Cr^{6+} concentrations in the sample solution. Consistent with the Cr^{6+} concentration results, the percentage of Cr^{6+} adsorbed in the solution using various RSE-to-rice-straw ratios showed that a 1:0.5 ratio achieved the highest adsorption percentage of 71.63%. This ratio allows for optimal equilibrium, facilitating efficient Cr^{6+} adsorption with minimal interference from other compounds. Other ratios may result in less effective interactions, such as competition for adsorption sites or the formation of unstable complexes. According to Jian et al. (2016), rice straw has a complex surface with various functional groups capable of binding Cr^{6+}. Zhang et al. (2018) stated that the dominant type of interaction influences the saturation level of adsorption sites and the stability of the Cr^{6+} rice straw complex. Mhlarhi et al. (2023) suggested that Cr^{6+} binds to functional groups with higher affinity, though these functional groups do not always undergo permanent structural changes.

One of the factors influencing innovation adoption is the perceived benefits. Based on the findings, most batik entrepreneurs positively assessed the innovation, with only a small percentage (2%) expressing reservations. The lower cost compared to conventional materials made this innovation more attractive. Additionally, its eco-friendliness aligns with batik entrepreneurs' aspirations for sustainable and responsible business practices. The complexity of implementation also plays a role. Most entrepreneurs found that the combination of RSE coagulant and rice straw absorbent had low complexity, making it easy to apply in batik wastewater treatment. With a low-risk approach and clear benefits, this innovation has been well-received by batik entrepreneurs.

This study demonstrates the effectiveness and cost-effectiveness of the combination of Roselle Seed Extract (RSE) and rice straw effectively reduces hexavalent chromium (Cr^{6+}) levels in synthetic batik wastewater. The optimization results indicate that the optimal dose of RSE bio-coagulant is 30 × 10^3 ppm, with a Cr^{6+} adsorption efficiency of 68.69%. Coagulation was most effective at pH 2, achieving a Cr^{6+} reduction efficiency of 69.02%. The optimal combination of RSE coagulant and rice straw adsorbent was a 1:0.5 ratio, yielding the highest adsorption efficiency of 71.63%. Additionally, the optimal contact time for maximum efficiency was 150 minutes, beyond which Cr^{6+} ions began to desorb back into the solution. The combination of RSE and rice straw as a coagulant-adsorbent offers an environmentally friendly and cost-effective alternative to conventional chemical coagulants. This study confirms the potential of RSE as a natural, biodegradable, and effective coagulant for batik wastewater treatment. Implementing this method can help mitigate the environmental impact of the batik industry while providing a sustainable wastewater treatment alternative for small and medium enterprises. Further research is recommended to explore the efficiency of this method under various wastewater parameters and its impact on floc volume and sedimentation time.

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Development of anti-obesity herbal drink from butterfly pea flower (*Clitoria ternatea*) extract and lemon (*Citrus limon*) juice

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Abstract. *Anugrahani AD, Indarto D, Pamungkasari EP, Wijayanti L, Utami F. 2025. Development of anti-obesity herbal drink from butterfly pea flower (*Clitoria ternatea*) extract and lemon (*Citrus limon*) juice. Nusantara Bioscience 17: 129-136.* Obesity is a multifactorial disease with a high global prevalence and higher risk for cardiometabolic diseases. Obesity management comprises diet, physical exercise, and pharmacotherapy. Some obese patients have low compliance with diet and exercise therapy, while long-term use of anti-obesity drugs causes adverse side effects. Herbal drinks have also been used for obesity treatment, but their efficacy remains debatable. Herbal drink products have been developed, including butterfly pea flower extract and lemon juice, which contain flavonoids and anthocyanins that have anti-obesity activity. The purpose of this study was to create a herbal drink for obesity treatment and analyze acceptability, microbiological contamination, and nutritional and phytochemical contents. An organoleptic test assessed the acceptability of the herbal drink. Analysis of macronutrient content used proximate methods. Vitamin C and microbiological tests were performed using spectrophotometric and Total Plate Count (TPC) methods. Delphinidin and hesperidin levels were measured using a High-Performance Liquid Chromatography (HPLC) method. Statistical analysis was performed using Kruskal-Wallis and followed by the post-hoc Mann-Whitney with $p<0.05$ to determine the best formula of the four formulas (F1-F4) in this study. Organoleptic test results showed that formulation F3 of the herbal drink was the most liked and acceptable by the panelists. The herbal drink (F3) contained 0.82% fat, 0.34% protein, 0.099% carbohydrate, 3,527.25 mg/100 g vitamin C, 21.66 ppm delphinidin, and 525.68 ppm hesperidin. In conclusion, herbal drink has the potential to become an alternative anti-obesity treatment. Further research is needed to test the effectiveness of herbal drinks on weight loss and lipid profile improvement in obese patients.

Keywords: Butterfly pea flower, delphinidin, herbal drink, hesperidin, lemon, obesity

INTRODUCTION

Obesity is a multifactorial disease characterized by excessive fat accumulation in the adipose tissues, which increases the risk of cardiometabolic diseases and other non-communicable diseases (Powell-Wiley et al. 2021). Research in Australia states that obese adults had a higher risk of developing type 2 diabetes (OR:12.76, 95% CI: 8.88-18.36), arthritis (OR: 2.25, 95% CI: 1.90-2.68), heart disease (OR: 2.05, 95% CI: 1.54-2.74), asthma (OR: 1.97, 95% CI: 1.49-2.62) and depression (OR:1.96, 95% CI: 1.56-2.48) compared to normal weight adults (Keramat et al. 2021). Globally, adult obesity contributes to 60% of premature deaths (Sultana et al. 2021). Determinants of obesity include high consumption of sweet beverages, lack of exercise, endocrine hormone disruption, decreased energy metabolism, and genetic factors such as MC4R, PPARG, and TCF7L2 gene polymorphisms. In the digitalization era, many people worldwide tend to have several bad habits, such as consuming more fast food than healthy diet food, drinking beverages with higher energy, and having low physical activity (Valeeva et al. 2022).

Current global obesity management focuses on diet and physical exercise, but there is no single diet can be universally applied to everyone with obesity (Roman et al. 2024). A restricted calorie diet, consisting of low carbohydrate and fat, may not be effective in the long term due to poor compliance. A study in a health care center in Guadalajara (Spain), of 209 obese adult patients, showed that 67.5% of patients were not compliant with diet and exercise for weight loss programs. The obstacles experienced by obese patients included 27.8% of patients not having a healthy diet prescription, 17.7% of patients experiencing joint pain, 14.8% of patients being bored with dietary patterns, and 11.5% of patients being lazy (Trujillo-Garrido and Santi-Cano 2022). The second popular diet for obesity is the ketogenic diet, which is characterized by high fat intake (60%) and low carbohydrate intake (10%). The results showed that the effectiveness of the ketogenic diet on weight loss was not better than other types of diets. Not everyone can adopt a ketogenic diet, such as obese patients with complications of type 1 diabetes, renal failure, and heart disease patients because they have to limit their daily fat intake (Kim 2021). Personalized nutrition is recommended for body weight management of obese people because they are based on the individual specific health condition, gender and age-related needs, food preference, and appropriate motivational support (Roman et al. 2024). Anti-obesity drug therapy is the last alternative if diet and exercise alone do not work or in cases of obesity accompanied by complications of other diseases (Brandfon et al. 2023). Administration of anti-obesity chemical drugs such as phentermine, liraglutide, bupropion, and orlistat can have a weight loss effect. Still, their long-term use can cause side effects that are detrimental to health (Tak and Lee 2021). Therefore, alternative herbal treatments from natural ingredients are needed (Sayed et al. 2023). Anti-obesity herbs that are already popular include green coffee, green tea, ginger, and lemon-garlic. However, their effectiveness is still low (Alfaifi et al. 2020); for example, to provide a weight loss effect of 1.78 kg and a decrease in waist circumference of 2.06 cm, it is necessary to consume 500 mg/day of green tea extract for 12 weeks (Lin et al. 2020).

In our previous study, we made three formulations of butterfly pea flower and lemon combination drink (E1-E3) in the ratio of 75:25%, 80:20%, and 85:15%. The laboratory test results in our study showed that the herbal drink, a combination of butterfly pea flower and lemon, contains flavonoids and anthocyanins that have anti-obesity properties (Indarto and Utami 2023). The most abundant flavonoid in lemon is hesperidin, which can increase leptin sensitivity and inhibit lipogenesis (Xiong 2019). The primary anthocyanin in butterfly pea flowers is delphinidin, which has anti-obesity properties due to its ability to inhibit adipogenesis (Chayaratanasin et al. 2019). Administering a combination of 80:20% and 85:15% butterfly pea flower extract and lemon juice to obese male rats for 21 days reduced body weight, as well as improved lipid profiles (total cholesterol, LDL cholesterol, and triglycerides) and increased HDL cholesterol (Indarto and Utami 2023). However, no data exist on the herbal drink's safety and effectiveness as a human obesity treatment. Therefore, this study aimed to develop an herbal drink for this purpose by analyzing acceptability, microbiological contamination, and nutritional and phytochemical contents.

MATERIALS AND METHODS

Research design

An experimental laboratory was initially conducted to reformulate the herbal drink, followed by a sensory analysis by panelists that evaluated its color, taste, viscosity, and flavor. Collected data were then analyzed statistically. Finally, exploratory research was conducted on the nutritional and phytochemical content of the best formula.

Figure 1. The process of making an herbal drink

Preparation for making an herbal drink

Making an herbal drink refers to the previous study conducted by Indarto and Utami (2023) with a slight modification, locally, this product is called *Litrusia*. Briefly, F1-F4 formulas of herbal drink contained 1% butterfly pea flower extract and were added to lemon juice with an 80:20% ratio (F1 and F2) and 85:15% (F3 and F4. After that, F1 and F3 herbal drinks were added with 3.25% liquid stevia sweetener (Daylistz, CV. Sarsy Sinar Stevia, Subang, West Java, Indonesia), while F2 and F4 herbal drinks were added with 4.87% liquid stevia sweetener. The process of making these herbal drinks has complied with the HACCP (Hazard analysis and critical control points) system procedure, which is a food safety management system to prevent the occurrence of food-borne diseases (Awuchi 2023). The process of making herbal drinks can be seen in Figure 1.

Acceptability analysis of herbal drink

The sensory evaluation of the herbal drink, including color, flavor, texture, and taste, was assessed by 10 expert panelists who worked as lecturers in Nutrition Sciences, Department of Nutrition, State Health Polytechnic Malang, East Java (Djekic et al. 2021). Each panelist was given a glass of mineral water before and after F1-F4 assessments. The acceptability test was adopted from the five hedonic scales: 1: Extremely dislike, 2: Dislike, 3: Somewhat dislike, 4: Like, and 5: Extremely like.

Microbiological contamination, anthocyanin content, macro and micronutrient levels in the herbal drink

A microbial contamination-free test was conducted using a total plate count method. Determination of anthocyanin content of herbal drink was performed using a differential pH method, pH 1 and pH 4.5 buffer solutions were added to two test tubes containing herbal drink samples. The measurements were taken using a UV-Vis spectrophotometer with a 510 nm wavelength and analyzed by comparing the absorbance values at both pHs. Fat, protein, mineral, and water concentrations in the herbal drink were tested using Soxhlet, Kjeldahl, dry ash, and thermogravimetric methods. Carbohydrate concentration was assessed by subtracting the total percentage (100%) from water, ash, fat, and protein percentages. Calculating the energy content of an herbal drink using the proximate method involves the composition of macronutrients (4 kcal/g carbohydrate, 4 kcal/g protein, and 9 kcal/g fat. Total energy is the sum of energy contributions from each macronutrient. Finally, vitamin C concentration was determined using a spectrophotometric method.

Analysis of delphinidin and hesperidin levels in an herbal drink

Delphinidin and hesperidin levels were measured using the High-Performance Liquid Chromatography (HPLC Rigol L-3000, Germany. The standard delphinidin and hesperidin compounds were obtained from Sigma Aldrich with CAT 43725 and H5254, respectively. The mobile phase used in HPLC analysis to determine delphinidin and hesperidin levels was formic acid and acetonitrile. The protocol of delphinidin followed the method by Khan et al. (2022) with slight modifications. 1 mg delphinidin was dissolved in 5 mL of methanol solution. Serial dilution ranged from 1.25, 2.5, 5, 10, 25, and 50 ppm. 1.5 mL of herbal drink was centrifuged at 13.200 rpm for 15 minutes, from which the pellets were dissolved with 1.5 mL of methanol. After that, the herbal drink solution was injected into a 5µm C18, 150×4.6 mm Diamonsil column (Cat#99901 Ser#3673520, Dikma Technologies Inc., USA), and the flow rate was set at 1 mL per minute. The protocol of hesperidin analysis followed the method by Büyüktuncel (2017) with slight modifications. 25 mg hesperidin was dissolved with 25 mL Dimethyl sulfoxide (DMSO. Serial dilution ranged from 50, 100, 200, 400, and 800 ppm. 1.5 mL of herbal drink was centrifuged at 13,200 rpm for 15 minutes, and the pellets were dissolved with 1.5 mL of DMSO. After that, the herbal drink solution was injected into a 5µm C18, 150×4.6 mm Diamonsil column, and the flow rate was set at 1 mL per minute.

Statistical analysis

The collected data were analyzed using the SPSS (Statistical Package for the Social Sciences) program version 25. Numerical data were presented as mean ± SD. Because data distribution was not normal, we used the Kruskal-Wallis statistical test to compare four formulations of herbal drinks in terms of color, flavor, texture, taste, and overall (Ruiz-Capillas and Herrero, 2021), followed by the Mann-Whitney post hoc test the Mann-Whitney post hoc test, and a significant value was $p<0.05$.

RESULTS AND DISCUSSION

Organoleptic test results of four herbal drink formulas

Four formulations of herbal drink (F1-F4) were evaluated for their acceptability (color, flavor, viscosity, and taste, Table 1). The four formulations were similar in color, flavor, and viscosity, but the F3 formulation (score=4.50±0.527) had the best taste compared to F4 (score=4.30±0.675), F1 (score=2.50±0.527), and F2 (score=2.60±0.516) formulations.

Referring to Table 1, we find that the taste of F3 was not significantly different from F4 but significantly different from F1 and F2 ($p<0.001$). Panelists liked the taste of formulas F3 and F4, but somewhat like formulas F1 and F2. Overall, the F3 formula was the highest panelists' acceptance compared to the other three formulas, resulting in F3 as the best formula for the herbal drink.

Analyses of microbiological, anthocyanin, macro, and micronutrient levels in the herbal drink

The herbal drink is free from pathogenic bacteria, contains 258.48 ppm anthocyanin, has low levels of fat (0.82%), carbohydrates (0.099%), calories (7.38 kcal), and high levels of vitamin C (3.5%). In addition, it contains 98.90% water and 0.34% protein (Table 2).

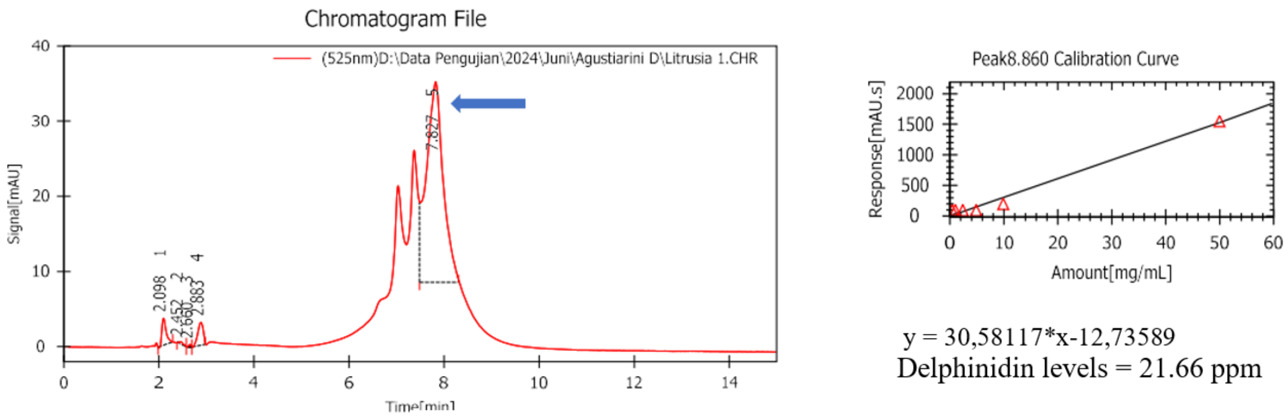
The delphinidin and hesperidin contents of the herbal drink

Figures 2 and 3 present the HPLC assay results of delphinidin and hesperidin in the herbal drink. We detected delphinidin at an 8-minute retention time and hesperidin at a 10-minute retention time. The HPLC results also indicated that the herbal drink contains 21.66 ppm delphinidin and 525.68 ppm hesperidin.

Table 1. Organoleptic test results of the herbal drink

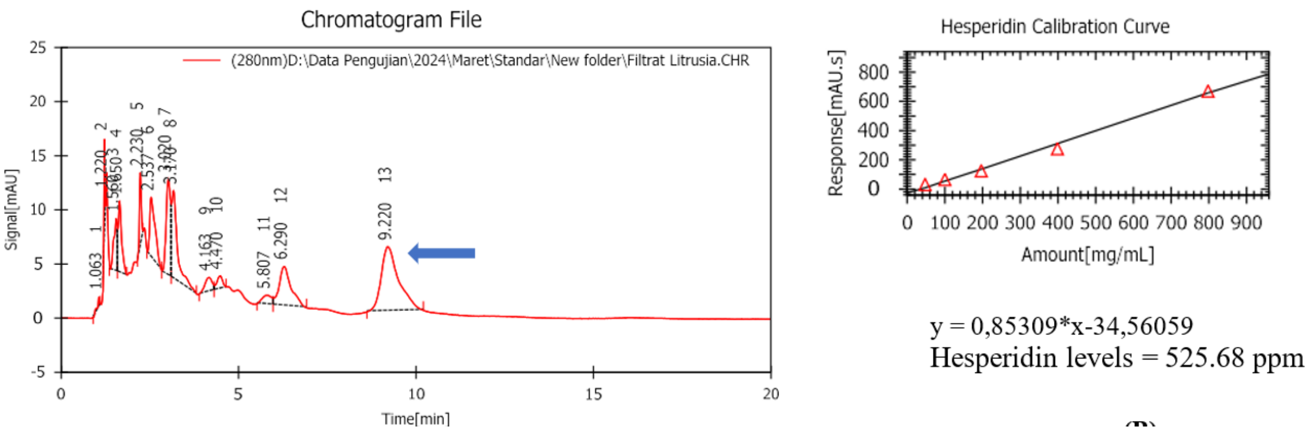
Formulas	Organoleptic test results of herbal drink (mean ± SD)				
	Color	Flavor	Viscosity	Taste	Overall
F1	4.30±0.483	4.00±0.000	4.30±0.483	2.50±0.527 [a), b)]	3.80±0.365 [a), b)]
F2	4.30±0.483	4.00±0.000	4.30±0.483	2.60±0.516 [a), b)]	3.83±0.236 [a), b)]
F3	4.40±0.516	4.00±0.483	4.30±0.483	4.50±0.527 [c), d)]	4.26±0.365 [c), d)]
F4	4.10±0.738	3.70±0.483	4.30±0.483	4.30±0.675 [c), d)]	4.14±0.425 [c), d)]
p-value	0.780	0.076	1.000	<0.001*	0.016*

Note: 1: extremely dislike, 2: dislike, 3: somewhat like, 4: like, 5: extremely like. *Significantly different based on Kruskall Wallis test ($p<0.05$. [a)] significantly different from F3, [b)] significantly different from F4, [c)] significantly different from F1, [d)] significantly different from F2 based on Mann Whitney post hoc test



A B

Figure 2. HPLC chromatogram profile: A. Delphinidin content in herbal drink; and B. A standard curve of delphinidin



A B

Figure 3. HPLC chromatogram profile: A. Hesperidin content in herbal drink; and B. A standard curve of hesperidin

Table 2. Microbiological, acidity, anthocyanin, macronutrient, and vitamin C contents in the (F3) herbal drink

Types of analysis	Analysis method	Analysis results (wb)
Microbiological	Total Plate Count (TPC)	ND[a)]
Acidity	pH meter	2.83
Anthocyanin	Differential pH	258.48 ppm
Water	Thermogravimetry	98.70 %
Mineral	Dry ash	0.036 %
Fat	Soxhlet	0.82 %
Proteins	Kjeldahl	0.34 %
Carbohydrate	By different	0.099 %
Energy	Total energy calculation [b)]	7.38 kcal
Vitamin C	Spectrophotometric	3.5 % [c)]

Note: [a)] ND: not detected, *ppm: mg/1,000 mL. [b)] Total energy is calculated from energy values of carbohydrate (4 kcal), protein (4 kcal), and fat (9 kcal). [c)] A 100-gram sample of herbal drink contains 3.5 grams of vitamin C

Discussion

In this study, we have demonstrated that the herbal drink with an 85:15% combination of butterfly pea flower extract and lemon juice, added with 3.25% liquid stevia sweetener, is the best formulation compared to the other three formulas based on food acceptability assessment. Our herbal drink formulation is safe from pathogenic bacteria, low energy (7.38 kcal/100 mL), low carbohydrates (0.099%), low fat (0.82%), and high vitamin C (3.5%). Based on Perka BPOM RI No. 1, 2022 on the Supervision of Claims on Labels and Advertisements of Processed Food, the herbal drink can be claimed as low-energy (not more than 20 kcal/100 mL), low-fat (not more than 1.5 g/100 mL), 'No Added Sugar' (sucrose, lactose, and fructose not more than 0.5 g/100 mL. A claim of high vitamin C in the beverage product is more than 2× the Recommended Dietary Allowance (RDA of vitamin C = 90 mg. 100 mL of herbal drink contains 3.5 g (3,525 mg) of vitamin C; hence, it can be claimed as a high vitamin C (Perka BPOM RI 2022). Administering high doses of vitamin C intravenous may result in health risks, but consumption of high vitamin C in foods remains safe, as vitamin C is a water-soluble vitamin that the body will excrete through urine if taken in excess (Doseděl et al. 2021). This herbal drink also contains anthocyanin (258.48 ppm), delphinidin (21.66 ppm), and hesperidin (525.68 ppm). Overall, this formulation of herbal drink may be suitable for an alternative diet treatment for obesity.

Our previous research on three formulations of butterfly pea flower and lemon combination (herbal drink): E1 (75:25%), E2 (80:20%), and E3 (85:15%) contained flavonoids and anthocyanins. Our *in vivo* study indicated that administering an herbal drink with 80:20% and 85:15% for 21 days improved lipid profile and reduced body weight in obese rats (Indarto and Utami 2023). Based on the results of the previous study, re-formulation of the herbal drink with a proportion of 80:20% and 85:15% was carried out by adding liquid stevia sweetener to increase its acceptability. The liquid stevia sweetener is a natural sweetener derived from the leaf extract of the *Stevia rebaudiana* plant, containing zero calories and 50-350 times sweeter than sugar (Samuel et al. 2018). Stevia does not increase postprandial glucose levels and reduces appetite. Therefore, it is recommended for weight loss management, such as for obese patients (Farhat et al. 2019). The FDA conferred 'Generally Recognized as Safe' (GRAS) status for stevia leaf extract in 2018. The European Food Safety Authority (EFSA) sets an acceptable daily intake (ADI) of 4 mg/kg/day for Steviol glycosides. There is no clinical evidence on the side effects of Steviol glycoside sweeteners due to the very small amount required for consumption (Orellana-Paucar 2023). In comparison to other natural sweeteners (high fructose derived from corn extract), high consumption of corn sweeteners (fructose intake >50 mg/day) is involved in several human disorders such as increased insulin resistance, hypertriglyceridemia, hypertension, weight gain, non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease (Khorshidian et al. 2021; Taskinen et al. 2019). In contrast to artificial sweeteners such as sucralose, acesulfame K, aspartame, and saccharin, a large cohort study showed that high artificial sweetener intake was associated with higher mortality, the risks of cardiovascular disease, and cancer (Lizuka 2022).

Government Regulation of the Republic of Indonesia No. 86/2019 on Food Safety and SNI 01-3719-1995 on the Indonesian National Standard for fruit juice drinks recommend that laboratory tests be conducted before food or beverage products are circulated to ensure that they do not contain contaminants and food additives that can be detrimental to health (BSN 2014; Peraturan Presiden Republik Indonesia 2019). Based on the SNI 01-3719-1995, the quality requirements for fruit juice drinks, among other things, are free from microbial contamination (total plate count maximum 2×102 colonies/mL). The microbial test on herbal drinks using the TPC method showed an ND result, which means that the herbal drink is free from bacterial contamination. Ewansiha (2020) states that drinks with a concentration of 12.5-25% lemon can inhibit the growth of pathogenic bacteria. Pathogenic bacteria cannot grow in an acidic pH environment (pH<4) because acidic pH damages their cell structure and biochemical functions (Lund et al. 2020). Thus, the herbal drink is safe from pathogenic bacterial contamination due to its lemon juice concentration (15%) and acidic pH (pH=2.83).

The anti-obesity activity of butterfly pea flowers has been studied by Hardinsyah et al. (2023) using a fermented beverage of butterfly pea flower kombucha, and then tested by HPLC-ESI-HRMS/MS. They identified 13 essential compounds, including kaempferol, quercetin, dibenzylamine, and pyrrolidinopropiophenone, showing anti-obesity properties in molecular docking studies against pancreatic lipase and amylase. Widowati et al. (2022) examined a *TeMon* drink made from butterfly pea flowers and lemon tea, which contained 2.97 mg flavonoids and 0.28 mg phenols per 100% sample, with 28.66% antioxidant value, higher than butterfly pea flower tea (17.07%) and lemon tea (25.34%).

The herbal drink has an anti-obesity effect due to its anthocyanins, delphinidin, and hesperidin content. The mechanism of anthocyanins for losing weight is through inhibiting the pancreatic lipase to inhibit lipogenesis, increasing body fat burning through the adenosine monophosphate-activated protein kinase (AMPK) pathway, increasing lipolysis through the Peroxisome Proliferator Activator Receptor (PPAR) pathway, and suppressing the appetite of obese people through the Neuropeptide Y (NPY), Protein Kinase A-α (PKA-α), phosphorylated-Cyclic AMP Response Element Binding protein (p-CREB) pathways (Franco-San Sebastián et al. 2023). Delphinidin can reduce lipid absorption from food through the mechanism of inhibiting pancreatic lipase, and inhibits lipogenesis and adipogenesis through the PPAR-γ and sterol regulatory element-binding transcription factor 1 (SREBP1) (Daveri et al. 2018; Park et al. 2019). Hesperidin is a flavanone glycoside that is anti-obesity by stimulating the growth of intestinal microbes to increase the production of short-chain fatty acid (SCFA), thereby increasing insulin sensitivity and improving glucose homeostasis, suppressing the appetite of obese patients by stimulating the release of cholecystokinin (CCK), an appetite-regulating hormone in enteroendocrine STC-1 cells (Xiong 2019).

The herbal drink contains 258.48 ppm anthocyanins (51.70 mg/200 mL). Administration of 81.16 mg anthocyanins/day from Rosella extract for six weeks in obese men can reduce body weight by -0.77 kg and waist circumference by -3.08 cm (Sari et al. 2018) and administration of *Juçara* pulp powder with 130.7 mg anthocyanins/day for six weeks in obese adults can reduce body weight by -0.58 kg and increase HDL_C levels by 107 mg/dl (Jamar et al. 2020). Delphinidin levels in the herbal drink were 21.66 ppm or 4.34 mg per serving (200 mL). *In vivo* studies have shown that administration of 100 μg delphinidine to rat adipose tissues can reduce lipid accumulation, inhibit lipogenesis and adipogenesis through PPAR-γ, SREBP1, and AMPK without toxicity (Park et al. 2019). In vitro studies have shown that administering 25 μg delphinidine to human adipose tissues has an adipogenesis inhibitory effect similar to the anti-obesity liraglutide drug (Saulite et al. 2019). Herbal drink also contains 525.68 ppm hesperidin. A previous study on 40 amateur cyclists (aged 18-55 years) in Spain, given the 2S-hesperidin intervention (500 mg/day, n=20) and another group taking placebo (microellulose 500 mg/day, n=20) for 8 weeks showed that the hesperidin intervention group had a decrease in body fat percentage (-10.4%, p = 0.035) and a decrease in body weight percentages (-3.7%, p = 0.006) of DXA analysis (Martinez Noguera et al. 2021). The anthocyanin and hesperidin content per serving (200 mL) of the herbal drink was lower than that of the Rosela drink and hesperidin capsules. However, the delphinidin content was higher than that of the delphinidin extract tested in the study by Saulite et al. (2019). The advantage of an herbal drink is that it is made from a brew of butterfly pea flowers and lemon juice, not pure extract, so it contains numerous phytochemical compounds.

The nutritional content of herbal drinks also supports obesity diet therapy because it contains high vitamin C. In obese patients, oxidative stress occurs due to pro-oxidant levels (which come from producing pro-inflammatory cytokines) greater than antioxidant levels in adipose tissues. Therefore, a therapeutic approach using antioxidant foods such as vitamin C to reduce the adverse effects of obesity. Vitamin C administration in obese mice can modulate lipolysis in adipose tissues by activating lipoprotein lipase (Imessaoudene et al. 2022).

The American Heart Association and Diabetes Association recommend low-calorie sweetened beverages to control weight gain and blood sugar because excessive and long-term consumption of sugary drinks leads to obesity and cardiometabolic diseases. The herbal drink is low in carbohydrates (0.099%) and low in calories (7.38 kcal. WHO recommends consuming <10% sugar per day, or equivalent to 50 grams per day for adults with an average calorie requirement of 2,000 kcal, and 25 grams per day is ideal (Chatelan et al. 2024). Stevia contains steviol glycosides, which have a sweet taste 300 times sweeter than granulated sugar and have a non-glycemic effect (Peteliuk et al. 2021). Raghavan et al. (2023) showed that overweight subjects who were treated to replace the commonly consumed granulated sugar with steviol glycosides for 90 days proved to significantly reduce body weight by 2.12 kg and reduce waist circumference by 4.78 cm. The sweetness of 2 g of steviol glycoside contains zero calories; in comparison, 10 g (1 tbsp) of table sugar contains 38 calories. Adopting steviol glycosides as a substitute for table sugar can lower calorie intake by 90 kcal per day, or 2,700 kcal per month, potentially leading to weight loss (Raghavan et al. 2023). Hence, this herbal drink contains 7.38 kcal of energy per 100 mL, 0.82% of fat, 0.34% of protein, 0.099% of carbohydrate, and 3.5% of vitamin C. This herbal drink contains bioactive compounds that have anti-obesity properties, including 258.48 ppm anthocyanins, 21.66 ppm delphinidin, and 525.68 ppm hesperidin. However, herbal drink product has several limitations, as it does not last long at room temperature and change quickly when exposed to sunlight. Secondly, the herbal drink has an acidic pH, so it cannot be given to obese patients who have gastrointestinal disorders.

In conclusion, herbal drinks have low calories, low fats, and high vitamin C. They also contain anthocyanins, delphinidin, and hesperidin, and have the potential as an alternative anti-obesity treatment. The best formula for a herbal drink, determined by an acceptance score of 4.26±0.365, is a combination of 85:15% with 3.25% liquid stevia addition. Liquid stevia is used to enhance the sweetness of the herbal drink without adding extra calories. Further research is needed to improve the durability of herbal drinks by storing them at 4°C and using dark bottles. Moreover, the herbal drink should be consumed after meals and given to obese people without gastrointestinal disorders.

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Mycochemical screening, antioxidant, and cytotoxic properties of *Panaeolus cyanescens*

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Abstract. *Galay KV, Plimaco SMY. 2025. Mycochemical screening, antioxidant, and cytotoxic properties of* Panaeolus cyanescens. *Nusantara Bioscience 17: 137-154.* This study focuses on exploring the subtleties of the hallucinogenic mushroom *Panaeolus cyanescens* Sacc., known for its psychoactive component psilocybin. The study focuses on exploring the phytochemical, antioxidant, and cytotoxic properties of ethanolic extracts through rigorous procedures while considering factors such as chemical composition, physiological characteristics, possible therapeutic applications, and the broader implications of its psychoactive nature. The methods used are mycochemical screening, Gas Chromatography-Mass Spectrometry (GC-MS) analysis, DPPH free radical scavenging assay, and brine shrimp lethality test. The goal of the study is to determine the bioactive components in the mushroom and investigate their potential therapeutic applications, with an emphasis on their anticancer capabilities. The process entails gathering, confirming, and producing the extracts. A wide spectrum of secondary metabolites, including flavonoids, steroids, alkaloids, and fatty acids, are shown in the results, suggesting several potential therapeutic advantages. 25 unique compounds with antibacterial, antioxidant, anticancer, and anti-inflammatory properties have been identified by GC-MS analysis, offering a comprehensive understanding of their bioactivity. With an IC50 value of 91.19 ppm, the DPPH assay exhibits strong antioxidant qualities and notable concentration-dependent antioxidant activity. With an LC50 value of 26.63 ppm, cytotoxicity studies show substantial toxicity and strong anticancer effects. The study highlights *P. cyanescens's* varied bioactive properties, antioxidant capacity, and intriguing anticancer potential while offering crucial insights into the proper dosage and responsible usage of the plant. The findings of the present assessment provide initial scientific validation for the mushroom's historic use in long-standing cultural customs. This study catalyzes additional research and confirmation of its therapeutic value.

Keywords: Antioxidant, brine shrimp lethality test, DPPH free radical scavenging assay, Gas Chromatography-Mass Spectrometry (GC-MS) analysis, mycochemical screening, *Panaeolus cyanescens*, psilocybin

INTRODUCTION

Mushrooms, with their diverse mycochemical compounds, offer a wide range of physiologically active substances that might be exploited as functional components in pharmaceutical formulations to treat various ailments (Kostić et al. 2020). They include different mycochemical compounds such as alkaloids, steroids, flavonoids, terpenoids, tannins, phenols, glycosides, and more (Ogidi and Oyetayo 2016). Mushrooms stand out for their immune-modulating and antitumor properties, which make them particularly promising in the treatment of serious diseases. They also exhibit other therapeutic attributes such as antioxidant, antihypertensive, cholesterol-lowering, liver protection, anti-obesity, anti-inflammatory, antidiabetic, and antimicrobial effects (Shamtsyan 2016). Mushrooms also produce secondary metabolites and antibiotics with various chemical groups, which are well-suited for natural products (Shao et al. 2020; Chugh et al. 2022).

The prospective use of this crucial knowledge in the field of medicine has been largely overlooked. There have been limited ethnomycological research projects conducted in the Philippines. There is a need for scientific validation of such empirical studies (Tantengco and Ragragio 2018; Brown 2019; Zeleke et al. 2020; Živković et al. 2021; Ríos-García et al. 2023). Half of the drugs on the market are thought to be derived from natural sources such as medicinal plants, herbal drugs, animals, fungi, and marine organisms. Only a small fraction of mushrooms with medicinal properties have been studied and evaluated for their therapeutic benefits (Ahmed et al. 2021). As a result, it is believed that many compounds found in plants and mushrooms have antioxidant characteristics.

Psychedelic mushrooms, sometimes referred to as magic mushrooms, contain chemicals that can induce hallucinations; psilocybin may have potential benefits for both physical and mental well-being in addition to producing euphoric effects (Wieczorek 2015; Barrett 2020). *Panaeolus cyanescens* Sacc., from the Galeropsidaceae family, is known as the blue meanie due to its blue staining reaction when bruised. It is a coprophilous fungus found growing on dung in tropical and subtropical regions worldwide. *Panaeolus cyanescens* is well-known for its psychedelic effects due to the presence of the hallucinogenic chemical psilocybin (Awan et al. 2018).

Research has focused on the anti-inflammatory properties, safety profile, and potential therapeutic uses of *P. cyanescens* (Nkadimeng et al. 2020; Nkadimeng et al. 2021). Caution should be exercised when considering the consumption of *P. cyanescens*, as it bears a resemblance to other poisonous species, emphasizing the need for accurate identification and expert supervision (Kaur et al. 2014).

Bustillos et al. (2014) found that *P. cyanescens* have several pharmacological characteristics. It has hallucinogenic effects due to the psychoactive compounds, antioxidant activity to combat free radicals, anti-inflammatory potential, and can suppress the formation of cancer cells. Significant bioactive substances such as psilocybin, psilocin, and baeocystin have been extracted from *P. cyanescens*. Psilocybin and psilocin are the main psychoactive compounds that produce hallucinogenic effects by interacting with serotonin receptors. They hold the potential to assist individuals in overcoming challenging-to-treat or treatment-resistant conditions such as psychogenic diseases and mental health disorders (Dawood Hristova and Pérez-Jover 2023). Psychedelic mushrooms are known for their ability to alter consciousness and induce mystical experiences, which has resulted in their incorporation into many spiritual and medicinal practices (Guzmán 2008; Johnson et al. 2014).

The study extensively examines the ethanolic extracts of *P. cyanescens*, investigating secondary metabolites and bioactive substances using mycochemical screening and GC-MS analysis (Brands et al. 2021). The evaluation involves determining the antioxidant capabilities of the mushroom by the DPPH free radical scavenging assay, which demonstrates its ability to counteract oxidative stress (Gulcin and Alwasel 2023). Brine shrimp lethality assay is used in cytotoxicity studies to evaluate the effectiveness of mushrooms in treating disorders associated with uncontrolled cell development, providing valuable information on their health benefits (Wahab and Hussain 2021). Although *P. cyanescens* has been traditionally used for treating several health disorders, little scientific data is confirming its safety and effectiveness. Hence, further investigation is required to study the pharmacological and toxicological characteristics of these fungi, along with their medication interactions and adverse consequences. The study examined the biological characteristics and antioxidant and cytotoxic activities of *P. cyanescens*. Further research is required to explore their potential in other health-related activities, including anticancer qualities and treatment of mental health issues.

MATERIALS AND METHODS

Study area

Mushrooms of *P. cyanescens* were collected at Dicoloc, Jimenez, Misamis Occidental (Figure 1). To ensure authenticity, the mushroom samples were meticulously documented and subsequently sent to Jennifer M. Niem, the highly experienced Curator of the Mycological Herbarium at the Museum of Natural History, University of the Philippines-Los Banos, for further verification. To prevent deterioration, 230 fresh-weight grams of mushroom samples were submerged in 1000 mL of ethanol subsequent to the harvesting process. The sample underwent filtration using Whatman Number 1. It was subsequently concentrated in a rotary evaporator at a temperature range of 40-50°C while maintaining reduced pressure, resulting in the formation of a semisolid material. The extracted samples were then carefully stored in vials specifically designated for subsequent analysis, ensuring their integrity and reliability.

Mycochemical screening

A qualitative assessment of different components in ethanolic extracts, such as flavonoids, steroids, saponins, alkaloids, tannins, cyanogenic, anthraquinones, and glycosides, was carried out at the Chemistry Laboratory of Mindanao State University's - Iligan Institute of Technology (MSU-IIT). The Handbook of the Philippine Medicinal Plants was used as the foundation for a three-point rating system (+turbid, ++moderate, and +++heavy) (De Padua et al. 2012; Dela Peña et al. 2019).

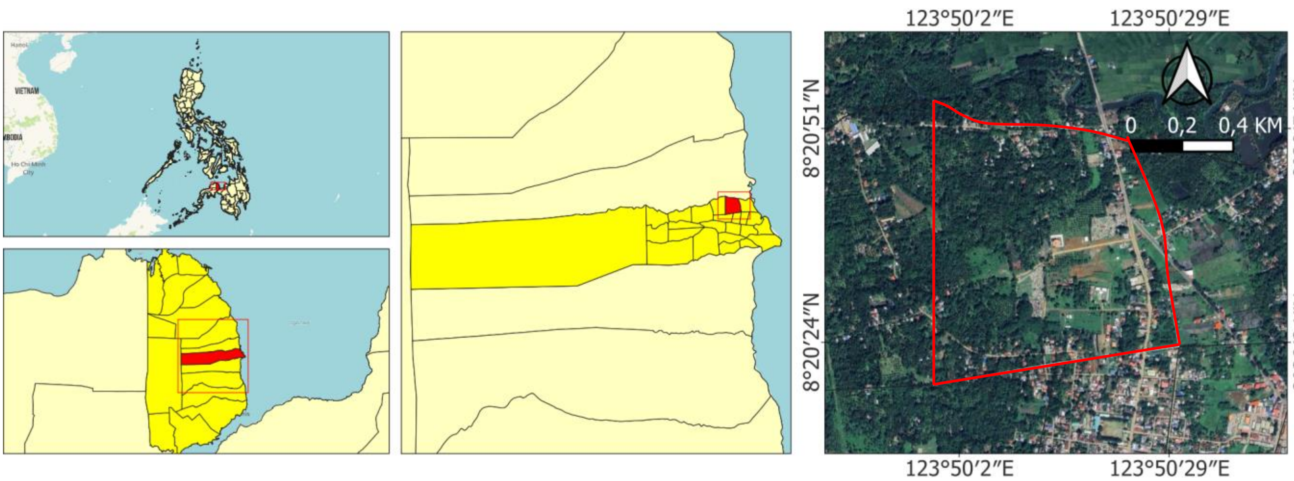

Figure 1. Geographical location of the sampling site. The map of the Philippines is raised in the upper left portion. The map of Dicoloc, Jimenez, and Misamis Occidental, Philippines is inset in the lower left section

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The analysis of the *P. cyanescens* mushroom sample was conducted using Gas Chromatography-Mass Spectrometry (GC-MS) in accordance with the methodology outlined by Chipiti et al. (2015). This approach has previously been employed to detect chemical compounds in mushroom fruiting bodies. The identification of substances was accomplished by the comparison of the mass spectrum of the analyte at a specific retention time with a reference standard in the collection of the National Institute of Standards and Technology (NIST). The qualitative investigation of the ethanolic extracts from *P. cyanescens* mushrooms was undertaken by the Chemistry Analytical and Research Laboratory at Ateneo de Davao University in Davao City.

DPPH – Free radical scavenging assay

The antioxidant properties of *P. cyanescens* mushroom extracts were assessed by measuring their ability to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The evaluation technique was adapted from Jacinto et al. (2011) with a modification of the protocol. Multiple samples with varying concentrations (1,000, 500, 300, 200, 100, 50, 30, 20, and 10 μg/mL) were combined with 3 mL of an ethanolic DPPH solution (0.1 mM). The mixture was vigorously shaken for 10 seconds using a vortex mixer and then left to stand in a dark area at room temperature for one hour. Subsequently, the absorbance was quantified at a wavelength of 517 nm. Control samples were tested using DPPH solution in ethanol, with each sample analyzed three times. The radical scavenging activity was directly related to the DPPH quenching intensity, and the percentage of DPPH discoloration in the sample was determined. The results were quantified as the percentage of inhibition using the formula: % Inhibition = $[(A_{control} - A_{sample}) / A_{control}] \times 100$. Here, $A_{control}$ refers to the absorbance values of the control, while A_{sample} indicates the absorbance values of the sample. The extract concentration or IC_{50} needed to inhibit DPPH radicals by 50% was used to indicate the antioxidant activity of the extracts.

Cytotoxicity test using brine shrimp lethality test

The cytotoxic effects of *P. cyanescens* mushroom extracts were evaluated by the brine shrimp lethality test, following the techniques outlined by Lumogdang et al. (2021) with certain modifications. The Chemistry Department at MSU-IIT evaluated the fatal effects on brine prawns (*Artemia salina* (Linnaeus, 1758)) to determine the possible bioactivity of ethanolic extracts. The brine shrimp larvae were used for the cytotoxicity test two days after hatching. Using ethanol as the extraction solvent, an ethanolic extract was prepared from *P. cyanescens* at three concentrations: 10, 100, and 1000 μg/mL. A stock solution was prepared by dissolving 36.5, 25.18, and 35.4 mg of the extract in ethanol to make a 10,000-ppm stock solution for each concentration. The ethanol extraction solvent was allowed to stand for two days to facilitate evaporation. Additionally, specific quantities of Dimethylsulfoxide (DMSO) were added; next, diluting the prepared stock solution created concentrations of 1,000, 100, and 10 ppm. Three duplicates of the ethanolic extract were made, and 5 mL of sterile filtered seawater was used as the control setup for comparison. Each prepared extract received ten nauplii, and the control setup (sterile seawater) had an additional ten nauplii added. Afterward, the test tubes were examined, and the quantity of died (non-motile) nauplii in each tube was recorded at 6 and 24 hours.

Data analysis

The network analysis conducted in this study utilized Gephi software (0.10.1 202301172018) under the dual license CDDL and GNU General Public License v3, (©2008-2023). The data was imported in the form of an edges table, with commas as the separator and the graph type set to directed. Various statistical analyses were performed using Gephi, including network overview, node overview, edge overview, and community detection. Each dataset was analyzed separately by Gephi due to the unique communities and network graphs associated with each dataset. In this context, bioactive compounds and their respective biological functions were represented as nodes, while the connections between them were depicted as edges. The layout of the resulting graph was adjusted using the Fruchterman-Reingold algorithm. Furthermore, Gephi facilitated the analysis of network centrality and modularity. The final network was examined for its bioactive compounds and functions based on the visual outcomes generated from the network analysis.

RESULTS AND DISCUSSION

Mycochemical screening

Mycochemical screening of the ethanolic extracts of *P. cyanescens* using ethanol as the solvent confirms the potential advantages associated with exposure to secondary metabolites. *Panaeolus cyanescens* extracts were found to contain a range of bioactive compounds, including significant concentrations of antioxidants such as steroids, fatty acids, and alkaloids, believed to contribute to its therapeutic properties. Minimal quantities of flavonoids and saponins were detected, although tannins and anthraquinone were entirely absent in the extracts (Table 1). Previous research has identified comparable chemicals in several species of the Galeropsidaceae family but with minor variations (Bustillos et al. 2014; Nkadimeng et al. 2020a). The chemicals are thought to be accountable for the fungi's remarkable therapeutic characteristics.

Table 1. Mycochemical profile of the ethanolic extracts of *Panaeolus cyanescens*

Phytoconstituents	Interference
Alkaloids	+++
Anthraquinone	-
Cyanogenic-Glycosides	-
Flavonoids	+
Fatty Acids	+++
Saponins	+
Steroids	+++
Tannins	-

Note: + mean signifies present: + = turbid, ++ = moderate, +++ = heavy; - mean signifies absent

Alkaloids, emphasized by Willems et al. (2020), are potent bioactive compounds that are essential for therapeutic purposes and are commonly present in mushrooms. Alkaloids, whether in pure form or synthesized, are used as medicinal agents due to their pain-relieving, muscle-relaxing, and antimicrobial properties (Ogidi et al. 2021). This finding is consistent with previous research by Dulay et al. (2015) that examined *Panaeolus antillarium* (Fr.) Dennis. Alkaloids are effective antioxidants that fight free radicals and show promise for the future development of cancer therapeutic drugs (Nkadimeng et al. 2021). These substances have various pharmacological effects such as pain relief, antibacterial properties, cell division inhibition, malaria treatment, anti-inflammatory effects, antiviral activity, psychotropic effects, and potential anticancer properties (Wink 2016; Ding et al. 2017). Particular alkaloids, such as quetiapine, are essential in psychiatric medications used to treat diseases, including schizophrenia and bipolar disorder (He et al. 2020).

Similarly, flavonoids, another class of bioactive compounds, contribute to the medical properties of *P. cyanescens*. These phenolic compounds are present in a turbid state. Phenolics are recognized for their antioxidant and antibacterial characteristics. Research has shown that flavonoids from ten Malaysian wild mushrooms have significant beneficial effects on human health, demonstrating anti-inflammatory, antimutagenic, and anticarcinogenic properties, free radical scavenging, and acting as reducing agents (Azieana et al. 2017; Ang et al. 2019; Chávez-González et al. 2020; Ekalu and Habila 2020). Flavonoids have been found to offer various medicinal benefits, such as anticancer effects, antidepressant, anticonvulsant, anti-proliferative, antihypertensive, antiallergenic, antiviral, vasodilating actions, antifungal, hepatoprotective, and cytotoxic activity (Dias et al. 2021). The major characteristic of flavonoids is that they exert antidiabetic effects (Panche et al. 2016). Alternatively, phytochemicals like flavonoids have a broad variety of biological roles and can be used as medicines directly (Badshah et al. 2021).

Beyond flavonoids, mushrooms are also rich in natural sources of fatty acids that are essential components in human nutrition (Çayan et al. 2016). Unsaturated fatty acids can counteract diet-induced inflammation and reduce body fat by directly affecting the hypothalamus (Cintra et al. 2012). Analysis of fifteen types of edible mushrooms in Korea shows that fat quality indicators, including the ratio of hypocholesterolemic/hypercholesterolemic fatty acids, the atherogenic index, and the thrombogenic index, demonstrate the health benefits of fats derived from mushrooms (Saini et al. 2021). Unsaturated fats, essential fatty acids obtained from the diet, are necessary for basic bodily functions such as cell growth, organ function, and nerve function. They may also have protective effects against heart disease and diabetes. Arachidonic acid plays a role in blood vessel constriction and blood clotting, while oleic acid has a relatively lower negative impact on carcinogenicity (Kayode et al. 2015). According to Rathore et al. (2017) reported that linoleic acid exhibits anticarcinogenic characteristics in animal models of breast, prostate, and colon cancers, inhibiting tumor cell proliferation at different stages of carcinogenesis.

Saponins are also compounds present in a turbid state in the ethanolic extracts of *P. cyanescens*, which are considered potential anticarcinogens. Saponins are believed to have anticarcinogenic qualities through mechanisms such as direct cytotoxicity, immune-modulatory actions, bile acid binding, and normalization of carcinogen-induced cell proliferation (Podolak et al. 2010). Saponins have various pharmacological effects such as antimicrobial, anti tumor, hemolytic, anti-inflammatory, antidiabetic, antifungal, expectorant, vasoprotective, hypocholesterolemic, hypoglycaemic, molluscicidal, and antispasmodic properties. The substance has been discovered to possess antioxidant, anticancer, and antiviral characteristics (Sharma et al. 2021). It is also utilized as an adjuvant in addition to being employed in the food and cosmetic sectors as an emulsifier or sweetener (Majinda 2012). Saponins have anticancer capabilities by disrupting cellular DNA replication and inhibiting the growth of cancer cells (Yıldırım and Kutlu 2015). The research on saponins in various forms as a treatment for cancer has generated a lot of potential (Thakur et al. 2011). Several saponins have demonstrated significant anticancer properties and have also shown promise in combating neurological illnesses, cardiovascular disease, and renal disease (Zhong et al. 2022).

Furthermore, steroids are a vital phytochemical detected in high levels in the ethanolic extracts being studied. In vitro and in vivo studies show that certain triterpene and steroid compounds, particularly those with unsaturated side chain bonds, have enhanced antitumor activity. Triterpene and steroid compounds derived from mushrooms have a wide range of therapeutic benefits, such as hepatoprotective, hypoglycemic, antifungal, antioxidant, anti-inflammatory, and cholesterol biosynthesis regulation (Nikitina et al. 2016). Additionally, Ogidi et al. (2021) confirmed that steroids present in *Chromolaena* DC. plant species have antibacterial and antiviral properties against *Streptococcus mutans* Clarke, 1924 and *Streptococcus sobrinus* strains. A novel steroid derived from *Ganoderma sinense* J.D.Zhao, L.W.Hsu & X.Q.Zhang has a strong binding affinity to HK2 and significantly binding free energy. It has been identified as an HK2 inhibitor and may be utilized to target HK2 in cancer treatment (Badalyan et al. 2019). Phytochemical analysis of *P. cyanescens* extracts using various solvents resulted in the following findings.

Analysis of aqueous extracts from both the mycelia and fruiting body of *P. cyanescens* detected several mycochemicals such as saponins, alkaloids, and tannins. Flavonoids were solely present in the fruiting body. No phlobatannins or cardiac glycosides were detected in the mycelium or fruiting bodies of *P. cyanescens* (Bustillos et al. 2014). The results are consistent with the study carried out by Nkadimeng et al. (2023) on the cardiovascular safety of extracts from *P. cyanescens*, *Psilocybe natalensis* Gartz, D.A.Reid, M.T.Sm. & Eicker, *Psilocybe cubensis* (Earle) Singer, and *P. cubensis* leucistic A+ strain mushrooms. The ethanol and water extracts from the four psilocybin-containing mushrooms exhibited cardio-protective effects against angiotensin II-induced oxidative stress. The antioxidant and anti-inflammatory qualities of the water and ethanol extracts from the four psilocybin-containing mushrooms were validated by the phytochemical analysis of the extracts. The selection of solvents and extraction methods had a notable influence on the extraction of bioactive chemicals, producing different outcomes in each medicinal species examined (Lezoul et al. 2020). Various solvents extracted varied amounts and types of phytochemicals from various parts of the species, indicating that a single solvent may not be able to extract all the known beneficial components (Madike et al. 2017). The results showed variations in the different extracts, highlighting the importance of choosing the precise solvent to extract the optimal amounts of bioactive compounds (Toiu et al. 2018). Analysis of mycochemical screening in *P. cyanescens* has unveiled a diverse array of mycochemical compounds inherent to this mushroom species. A wide range of mycochemical groups, including flavonoids, saponins, alkaloids, steroids, and tannins, have been found in this investigation. It is important to understand that these secondary metabolites are bioactive substances that exhibit a wide range of physiological effects that significantly increase the overall biological activity of this mushroom. They are more than just their chemical makeup.

These mycochemicals specific and focused impacts on the human body make them fascinating subjects for further scientific study. Research on these chemical compounds' potential for creating innovative treatments is fascinating. These drugs are expected to have minimal adverse effects on human health and provide therapeutic advantages for a broad range of medical disorders. This research aligns with the broader goal of advancing knowledge about mycochemicals, which will result in the development of novel pharmacological treatments. The potential use of these mycochemicals as therapeutic agents heralds a new era in natural product-based medicine and their possible integration into pharmaceutical research, opening a world of possibilities for developing more effective and healthier cures for various illnesses.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS analysis was crucial for pinpointing the precise bioactive ingredients that give the ethanolic extract from *P. cyanescens* its medicinal qualities. Table 2 provides an intricate detail of the complex characteristics of these bioactive compounds and the documented biological effects associated with each. The analysis revealed 25 compounds in the mushroom extract, enhancing our knowledge of its biological makeup.

Figure 2 depicts the interconnectedness of the scientific properties of the twenty-five distinct bioactive compounds identified in the ethanolic extracts of *P. cyanescens*. Fifty distinct nodes signify unique biological attributes or individual bioactive compounds within a complex network uncovered by the Gephi analysis. Besides that, there were 112 edges, and this represents an illustration of how complex the interdependencies are among every bioactive component and its biological feature. It shows interdependence among many components in the biological landscape. To further understand the structure of this network, we estimated the mean degree, which is determined to be 2.24. This metric indicates that, on average, each node is connected to approximately two other nodes, suggesting a relatively sparse yet interconnected network. Such a degree of connectivity can provide insights into the potential interactions and collaborative functions of the bioactive compounds and their biological roles.

As depicted in Figure 2, antimicrobials emerge as a predominantly significant therapeutic category within this network, highlighting their key role in biological connections and potential applications in health and disease management. Furthermore, the analysis reveals that the core node demonstrates a remarkable level of centrality, signifying its vast interconnections with multiple bioactive substances. This central node may represent a key player in the network, potentially influencing various biological processes and interactions, thereby warranting further investigation into its functional implications and therapeutic potential. Overall, the findings from the Gephi analysis provide a valuable framework for understanding the complex interplay between bioactive compounds and their biological attributes, paving the way for future research and applications in the field of biomedicine. The mass peak of the bioactive compounds against its retention time is depicted in Figure 3.

Fatty Acid Methyl Esters (FAMEs) offer a more environmentally friendly substitute for regular fatty acids. They have improved qualities that make them appropriate for use as lubricants, biofuel, and sustainable applications by substituting a methyl ester for carboxylic acid (Röttig et al. 2009). A notable example that highlights the adaptability and importance of FAMEs is the molecule 9,12-Octadecadienoic acid (Z, Z)-, methyl ester. This chemical has strong binding affinity and potential for modulating target proteins, indicating its relevance in drug development. The potential effectiveness of targeting proteins for therapeutic purposes is shown by the docking score of -1.284 kcal/mol seen against the EGFR kinase domain, an essential part of the Epidermal Growth Factor Receptor (EGFR) protein, functioning as a molecular switch that helps transmit signals for cell growth and division, making it a target in drug development as documented by (Reza et al. 2021). Another significant compound known as octadecanoic acid, methyl ester, has been acknowledged for its capacity to inhibit tumor growth, demonstrate cytotoxic properties in the context of cancer treatment, and have anti-inflammatory properties (Shahin et al. 2022).

Table 2. Bioactive substances were identified qualitatively by analyzing the ethanolic extracts of *Panaeolus cyanescens* using GC-MS

Compound name	Retention time	Peak area (%)	Molecular formula	Reported biological properties with reference
Dodecane, 4,6-dimethyl-	6.165	93	$C_{14}H_{30}$	Antibacterial activity (Geethalakshmi and Sarada 2013; Zou et al. 2021); antioxidant (Zou et al. 2021); antimicrobial (Zou et al. 2021); inhibitory potential (Abdel-Ghaffar and Youssef 2013).
Dodecane, 2,6,10-trimethyl-	6.464	90	$C_{15}H_{32}$	Cytotoxic and antimicrobial activity (Jaradat et al. 2021); antifungal (Padma et al. 2019); antibacterial (Faridha Begum et al. 2016; Padma et al. 2019).
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	8.953	92	$C_{10}H_{20}O_4$	No known activity.
Hexadecane, 1-iodo-	10.207	92	C16H331	Antioxidant (Kim et al. 2022; Manzano et al. 2023); antiallergic, inhibitory, antimicrobial, and anticancer (Kim et al. 2022); antibacterial (Yogeswari et al. 2012).
Benzaldehyde, 2,5-bis[(trimethylsilyl)oxy]-	12.851	73	C16H26O2Si2	Inhibitory (Alijani et al. 2020).
Dotriacontane	13.158	82	C32H66	Antioxidant activity (Soosairaj and Dons 2016; Ekambaram et al. 2020; Qanash et al. 2022; Gazwi et al. 2023); antiviral (Qanash et al. 2022; Gazwi et al. 2023); antimicrobial and antibacterial (Asong et al. 2019; Kawuri and Darmayasa 2019; Değırmencı and Erkurt 2020; Ekambaram et al. 2020; Qanash et al. 2022; Gazwi et al. 2023); antispasmodic (Jeyaraman et al. 2018; Ekambaram et al. 2020; Teleb et al. 2022; Gazwi et al. 2023); cytotoxicity (Soosairaj and Dons 2016; El-Fayoumy et al. 2021; Qanash et al. 2022); anticancer (Dhanraj et al. 2021; El-Fayoumy et al. 2021); antifungal (El-Fayoumy et al. 2021); anti-inflammatory (El-Fayoumy et al. 2021); hypercholesterolemic (Ekambaram et al. 2020).
Pentadecanoic acid, methyl ester	13.550	94	C16H32O2	Antioxidant (Gheda and Ismail 2020; Reza et al. 2021); antimicrobial (Nisar et al. 2013; Gheda and Ismail 2020; Reza et al. 2021); antibacterial (Salem et al. 2018); antifungal (Belakhdar et al. 2015).
Triacontane	13.762	88	C30H62	Cytotoxic (Mellado et al. 2019; Paudel et al. 2019; Saud 2023); antioxidant (Paudel et al. 2019; Sadiq et al. 2020; Sharma and Panwar 2022; Mohammed et al. 2021); antihyperlipidemic (Mohammed et al. 2021); antibacterial, antidiabetic, and antitumor (Paudel et al. 2019); antimicrobial (Cunha et al. 2018); anticancer (Nidugala et al. 2015).
Heneicosane	15.159	90	C21H44	Antimicrobial (Añides et al. 2019; Jihene et al. 2020; Kaplan 2021; Riaz et al. 2022); antioxidant (Vanitha et al. 2020); antineoplastic (Subramanian et al. 2020); antibacterial (Ekambaram et al. 2020); inhibitory potential (Okechukwu 2020); anticancer (Yusuf et al. 2020; Kaplan 2021); anti-proliferative (Yusuf et al. 2020).
9,12-Octadecadienoic acid (Z, Z)-, methyl ester	16.042	94	C19H34O2	Anti-inflammatory (Hadi et al. 2016; Iroaganachi et al. 2023); analgesic and ulcerogenic properties (El-Sayed et al. 2023); antioxidant (Chakraborty et al. 2021; Reza et al. 2021; Shahin et al. 2022); antiarthritic (Muflihunna et al. 2021); anticancer, antihistaminic, and hepato-protective (Gheda and Ismail 2020); hypocholesterolemic, antiacne, and antieczemic (Ahmad et al. 2020); antimicrobial (Rahman et al. 2014).
Hexadecane, 2,6,10,14-tetramethyl- (CAS) Phytane	16.276	91	C20H42	No known activity.
Octadecanoic acid, methyl ester	16.450	90	C19H38O2	Antioxidant, antitumor, cytotoxic, antimicrobial, and anti-inflammatory (Shahin et al. 2022); antimycobacterial (Ahmad et al. 2020).
Tetrapentacontane, 1,54-dibromo-	16.754	86	C54H110Br2	Antioxidant (Enema et al. 2019; Tanod et al. 2019; Bensaad et al. 2022); antimicrobial (Bensaad et al. 2022; Oviya et al. 2022); antimutagenic (Bensaad et al. 2022); antifungal (Oviya et al. 2022); antibacterial (Selim et al. 2013; Oviya et al. 2022).
1,3,12-Nonadecatriene	16.990	85	C19H34	Antioxidant (Moustafa et al. 2015; Mangawang et al. 2020; Ogundajo et al. 2022; Truong et al. 2023); hepatoprotective (Moustafa et al. 2015); anti-inflammatory (Truong et al. 2023); antimicrobial (Khan et al. 2019; Lykholat et al. 2021); antifungal (Parveen et al. 2017; Khan et al. 2019; Lykholat et al. 2021).
1-Heptanol, 2,4-diethyl-	17.110	79	C11H240	Antimicrobial (Ruixia et al. 2017; Ferdosi et al. 2023).

Tetrapentacontane	18.846	80	C54H110	Anti-inflammatory (Fairuz et al. 2019; Ali et al. 2021); antioxidant (Xuanji et al. 2016; Fairuz et al. 2019; Ali et al. 2021; Radhika et al. 2022); antimicrobial (Alia and Yusufc 2016; Canli et al. 2020; Lykholat et al. 2021); anticancer (Sezer and Uysal 2018; Sri et al. 2021; Parimalachelvam et al. 2023); antibacterial (Ali et al. 2021); antifungal (Ali et al. 2021).
Tetracosane	18.975	72	C24H50	Cytotoxicity (Uddin et al. 2012; Casuga et al. 2016); antimicrobial (Naragani et al. 2016); antibacterial (Holla et al. 2001); anticancer (Sri et al. 2021).
Eicosane	19.240	86	C20H42	Antiviral (Ramasamy and Gopalakrishnan 2013); antimicrobial (Chuah et al. 2018; Oviya et al. 2022); anti-inflammatory (Chuah et al. 2018; Okechukwu 2020); antifungal (Kokila et al. 1928; Ibrahim et al. 2017; Khattab et al. 2022; Oviya et al. 2022); anti- proliferative (Chuah et al. 2018); antitumor (Ibrahim et al. 2017); analgesic effects (Okechukwu 2020); antipyretic (Okechukwu 2020); antihyperlipidemic (Kokila et al. 1928).
9,10[1',2']-Benzeno-anthracene, 9,10-dihydro-	19.619	61	C18H14	No known activity.
4,5-Dihydrobenzo[e]pyrene	20.291	61	C20H16	Antimutagenic (MacLeod et al. 1979; Wood et al. 1979); anticarcinogenic (MacLeod et al. 1979); cytotoxic (MacLeod et al. 1979).
Decane, 1-iodo-	21.985	62	C10H211	Antioxidant (Olubunmi 2009); antimicrobial (Olubunmi 2009).
Tetratriacontyl pentafluoropropionate	22.992	81	C37H72F5O2	Anticarcinogenic (Orem et al. 2017); antimicrobial (Kumar and Sharma 2021; Mohammed and Al-Katib 2023); antibacterial (Mohammed and Al-Katib 2023); anti-inflammatory (Mohammed and Al-Katib 2023).
Isotridecanol-	23.280	70	C13H280	Antimicrobial (Meenakshi et al. 2012; Senthilkumar et al. 2012; Selkäinaho et al. 2018); antioxidant (Senthilkumar et al. 2012); anticancer (Senthilkumar et al. 2012).
Octatriacontyl pentafluoroproprionate	23.424	84	C41H82F5O2	Antimicrobial (El Shafay et al. 2016; Upadhye 2021; Zulhendri et al. 2022; Ahmad et al. 2023); antibacterial (El Shafay et al. 2016; Albratty et al. 2021; Murniasih et al. 2022); antiviral (H Elwakil et al. 2021); antioxidant (Zulhendri et al. 2022); anti-inflammatory (Zulhendri et al. 2022).
Dodecane, 1-iodo-	24.495	69	C12H251	Antimicrobial (Viraj and Sakshi 2020); antibacterial (Al-rubaye et al. 2020); anti-inflammatory (Chabake and Chaubal 2020); antiviral (Viraj and Sakshi 2020).

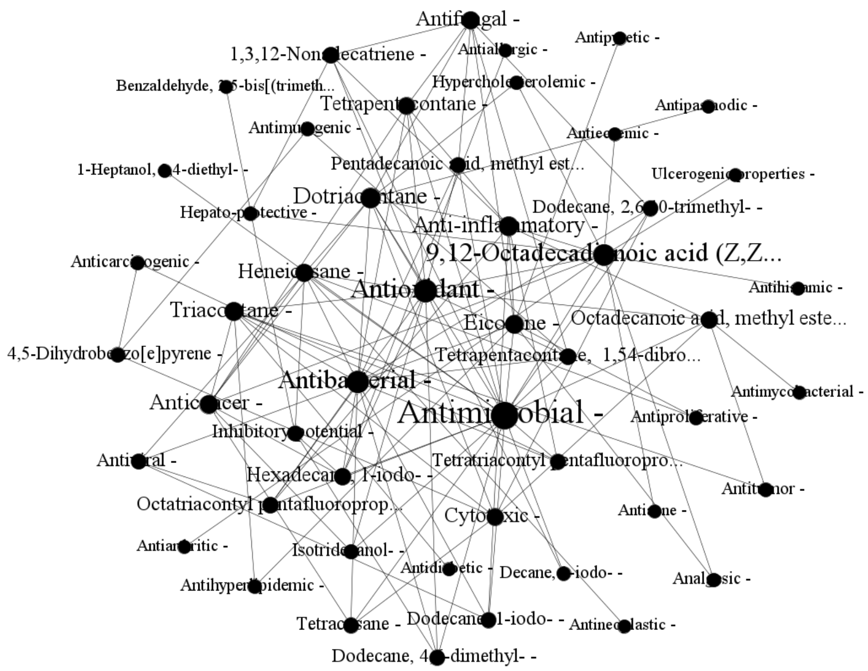

Figure 2. Network analysis of the relationship of the ethnopharmacological properties of *Panaeolus cyanescens* and the bioactive compounds detected

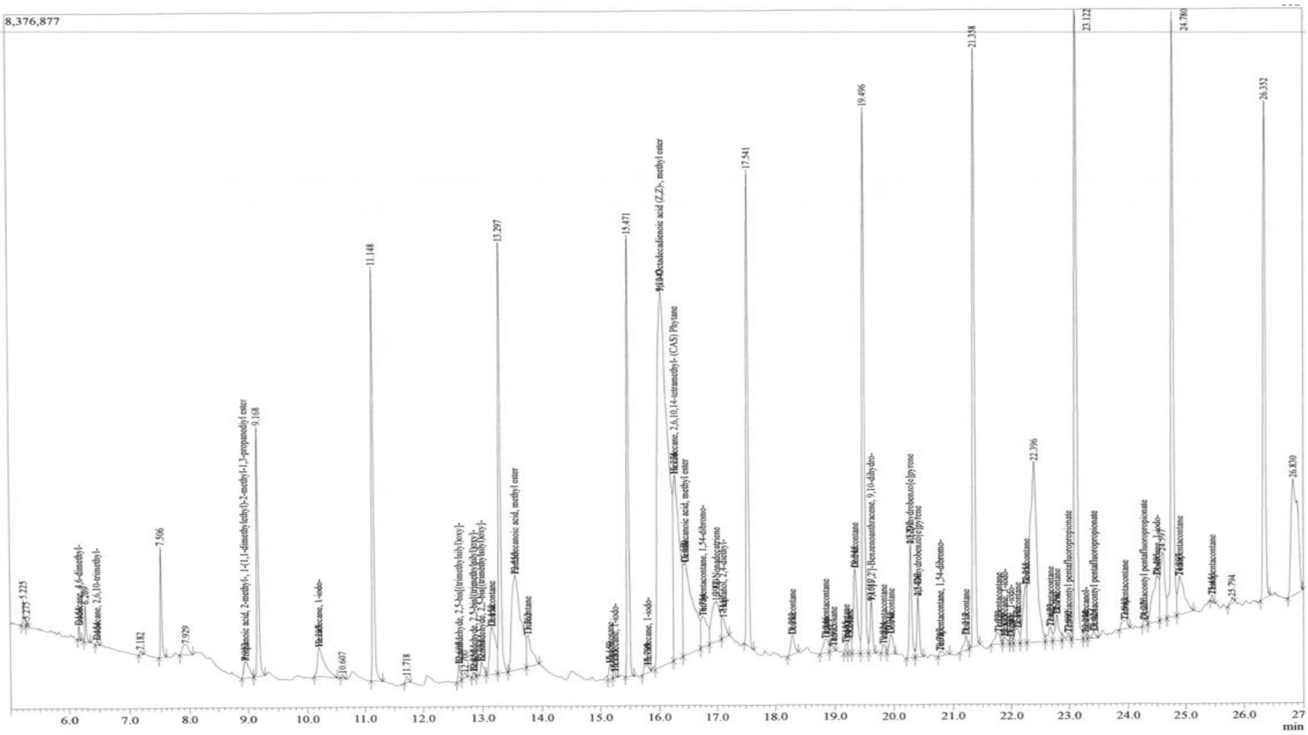

Figure 3. The mass peak of the bioactive compounds against its retention time

Another significant compound, by Abdel-Ghaffar and Youssef (2023), revealed that dodecane, 4,6-dimethyl- exhibits potent effects on glycogen synthase kinase 3-β (GSK-3β), an essential enzyme involved in metabolic and signaling pathways. GSK-3β plays an important role in regulating kinds of cellular processes, including cell survival, gene expression, and metabolism, making it a key target in therapeutic research for various health conditions like diabetes and also brain diseases such as Alzheimer's and Parkinson's. The study highlights a strong interaction between dodecane, 4,6-dimethyl- and the enzyme's active site, as indicated by a free binding energy of -28.49 kcal/mol. The compound forms significant alkyl and µ-alkyl bonds with specific amino acid residues (Tyr134, Ala83, Cys199, Leu188, and Leu132), suggesting a suppression mechanism through targeted binding interactions. These findings highlight the potential of dodecane, 4,6-dimethyl- for the development of GSK-3β-related therapies. Comparably, hexadecane, 1-iodo-, is an aliphatic alkane chemical that shares its classification with dodecane, 2,6,10-trimethyl- (Padma et al. 2019). A study conducted by Kim et al. (2022) revealed that hexadecane, 1-iodo-, a naturally occurring molecule present in the essential oil of *Chrysanthemum boreale*, has potential as a therapeutic intervention for atopic dermatitis, also known as eczema. Their study showed that this chemical can alleviate skin symptoms like those of AD in mice primarily through its anti-inflammatory and skin barrier-enhancing properties. By preventing mast cells from releasing histamine and other important inflammatory proteins and encouraging keratinocytes to produce essential skin barrier proteins, the chemical hexadecane, 1-iodo-, had two distinct effects. According to the results, hexadecane, 1-iodo may prove useful in creating new strategies for the management of AD. Similarly, Heneicosane showed strong antibacterial activity against *Aspergillus fumigatus* Fresen. and *Streptococcus pneumoniae* A, highlighting its potential application in antimicrobial or therapeutic settings (Vanitha et al. 2020). Additionally, in a rat model, Heneicosane, along with other n-alkanes, exhibited analgesic effects via potentially suppressing inflammation. These chemicals have the potential to create analgesic or anti-inflammatory medications (Okechukwu 2020). Similarly, the stem extract of *Dendrobium crepidatum* Lindl. & Paxton contains triacontane, a compound associated with cytotoxic activity. The cytotoxic effects on HeLa and U251 cancer cell lines were assessed in the study. The study revealed that the chloroform extract had the highest level of efficacy in decreasing the proliferation of HeLa cells. In comparison, the hexane extract demonstrated the greatest effectiveness in suppressing the growth of U251 cells. The cytotoxic activity found against cancer cell lines is attributed to the presence of tetracosane, tetracosane, and various phenol derivatives (Paudel et al. 2019).

Moreover, the methanolic extract of the kei apple (*Dovyalis cafra* (Hook.fil. & Harv.) Warb.) revealed that Dotriacontane plays a crucial role in its numerous therapeutic advantages. Its detrimental effects on HepG2 cells demonstrated the anticancer capabilities of Dotriacontane. Additionally, it demonstrated promising antibacterial properties when utilized in conjunction with other constituents of the extract, particularly against *Escherichia coli* E and *Proteus vulgaris* B. The extract's modest antiviral action against human coronavirus 229E was also attributed to the presence of Dotriacontane. The therapeutic significance of Dotriacontane in the kei apple extract was further substantiated by molecular docking research, as evidenced by the findings of Qanash et al. (2022). In another study, Mishra et al. (2019) highlight the presence of Dotriacontane, a high constituent found in the acetone extract of *Curcuma raktakanta*, a relatively obscure species of curcuma originating from Kerala, India. The objective of this study was to evaluate the efficacy of several fractions (acetone, hexane, and ethyl acetate) derived from *C. raktakanta* on cell lines associated with glioma, cervical, and breast cancer. Dotriacontane in the acetone extract reduced the vitality of cancer cells in a concentration-dependent manner, with a particularly strong effect on C-6 glioma cells. Dotriacontane was identified as a prominent constituent in the acetone extract of *C. raktakanta* using GC-MS analysis, suggesting its potential involvement in the plant's anticancer activities. Further research by Kawuri and Darmayasa (2019) emphasizes that *Streptomyces* sp. 9, specifically *Streptomyces capoamus* Goncalves de Lima et al., 1964, had noteworthy biocontrol capabilities in combating banana bacterial wilt disease. In antagonistic tests, Dotriacontane showed a notable inhibition of *Ralstonia solanacearum* (Smith, 1896) Yabuuchi et al., 1996, and in greenhouse-scale trials, it was able to suppress the disease completely.

The study conducted by Alijani et al. (2020) highlights the importance of Benzaldehyde, 2,5-bis[trimethylsilyl)oxy]- as a key component in the volatile chemicals emitted by *Stenotrophomonas maltophilia* B strain UN1512. The GC-MS study of this molecule suggests that it is likely involved in the antifungal actions that have been documented against the pathogen that causes strawberry anthracnose, *Colletotrichum nymphaeae* (Pass.) Aa. Furthermore, it has been shown that volatile compounds, such as Benzaldehyde and 2,5-bis[trimethylsilyl)oxy]-, play a crucial role in promoting the growth of tomato plants by influencing the development of both roots and shoots. This observation underscores the notable contribution of Benzaldehyde, 2,5-bis[trimethylsilyl)oxy]- in augmenting the antifungal properties of the bacteria against strawberry anthracnose and facilitating the growth and maturation of tomato plants.

Alkanes are hydrocarbon compounds consisting of carbon and hydrogen atoms connected by single bonds (Clark et al. 2009). These compounds are used as solvents and lubricants in medicines, cosmetics, and industrial applications because of their non-reactive nature and stability (Van and Funhoff 2007). The ethanolic extracts of *P. cyanescens* included alkane chemicals such as tetrapentacontane, 1,54-dibromotetrapentacontane, tetracosane, and eicosane. The synthesized compounds were tested for their antibacterial, antifungal, and antioxidant activities that conferred resistance to free radicals in yeast (Holla et al. 2001). Alkanes found in *P. cyanescens* contribute to its antioxidant properties, including dodecane, tetradecane, and hexadecane (Bustillos et al. 2014). Prior studies have shown that tetrapentacontane, namely 1,54-dibromo-, an alkane compound, had antioxidant and antibacterial properties when administered to the bacterial species under investigation.

In addition to their role in *P. cyanescens,* tetrapentacontane and its derivatives have been shown to possess potent antimicrobial, antifungal, and bactericidal properties (Oviya et al. 2022). According to Sri et al. (2021), the tetrapentacontane molecule has shown significant efficacy in promoting hair growth, reducing uric acid production, and inhibiting arachidonic acid. The anticancer effects of tetracosane have been shown against many cell lines, including MDA-MB-231, HT-2918, AGS, and NIH 3T3. The chemicals demonstrate antioxidant, anticancer, anti-inflammatory, and antibacterial properties due to their structural attributes, such as the presence of phenolic compounds in antioxidants or specific alkaloids in antimicrobial medications (Özçelik et al. 2011). According to Oviya et al. (2022) and Okechukwu (2020), eicosane, an alkane molecule present in the fungus *P. cyanescens*, has significant anti-inflammatory, antibacterial, analgesic, and antipyretic characteristics.

1,3,12-Nonadecatriene is classified as an alkene, a kind of compound that contains carbon-carbon double bonds (Smith 2016). Alkenes are very desirable initial substances for producing epoxides, which serve as crucial and adaptable intermediate components for the pharmaceutical, flavoring, and polymer sectors. Previous studies have concentrated on using various oxidizing agents and enzymes, such as cytochrome P450 monooxygenases, for alkene epoxidation. Additionally, microbial whole-cell catalysts have been explored despite their limitations (Özçelik et al. 2011; Truong et al. 2013; Babot et al. 2022). This improved the antioxidant capacity of cells by blocking the generation of reactive oxygen species and decreased the inflammatory response by lowering the release of nitric oxide.

Esters are another group of compounds found in mushrooms that exhibit distinct physicochemical features and functionalities (Xu et al. 2022). Ester compounds showed potent antibacterial effects against several oral infections (Huang and Ebersole 2010). Tetratriacontyl pentafluoropropionate and Octatriacontyl pentafluoroproprionate are Esters known for their strong antimicrobial, antibacterial, anti-inflammatory, antioxidant, and anticarcinogenic properties that are also found in the ethanol and water extracts of four psilocybin mushrooms (Zulhendri et al. 2022; Mohammed and Al-Katib 2023; Nkadimeng et al. 2023). The ethanolic extracts of *P. cyanescens* also contain halogenated alkanes such as decane, 1-iodo, and dodecane, 1-iodo, which are known for their antibacterial, antifouling, and repellant activities (Gribble 1994). Saltwater contains certain halogenated alkanes that are not directly affected by human activities (Dembitsky and Tolstikov 2003). According to Dutta and Hyder (2019), the inclusion of halogen substituents has the potential to augment the toxic, mutagenic, and other deleterious characteristics of compounds.

The concentration of alcohol in *P. cyanescens* was evaluated by the utilization of gas chromatography, identifying 1-Heptanol, 2,4-diethyl. The antibacterial activity of the compounds has been well-documented (Ferdosi et al. 2023). According to Mohamed and Farghaly (2014), alcohols have a variety of functions, including flavoring, antifreeze, antiseptic, fuel, preservative, solvent, antioxidant, and antibacterial capabilities. Isotridecanol is a fatty alcohol derived from the isotridecanol hydrocarbon and is present in *P. cyanescens*. According to Lakhera et al. (2022), fatty alcohol molecules are composed of nitro groups that possess the ability to firmly remove electrons and hydroxyl groups that have the ability to donate electrons. Fatty alcohol molecules are widely utilized in medicinal science, insecticides, dyes, cosmetics, and other fields (Federle and Itrich 2006). Isotridecanol, a kind of fatty alcohol, has been categorized as possessing anticancer, antibacterial, antimicrobial, antifungal, phototoxic, and other chemical and therapeutic activities (Kuklev et al. 2003).

Polycyclic Aromatic Hydrocarbons (PAHs) are a type of persistent organic chemical present in *P. cyanescens* mushrooms, known for their significant cancer-causing properties and widespread presence in the environment (Zhou et al. 2020). According to MacLeod et al. (1979), PAH compounds have the potential to create cancers in many species and tissues. Additionally, they may induce mutagenesis and convert cells into malignant cells when grown in a laboratory setting. 9-dihydroanthracene (D-A-D) Polycyclic aromatic hydrocarbons (PAHs) encompass 9,10-dihydrobenzo[e]pyrene and 4,5-dihydrobenzo[e] pyrene. The chemicals possess potential antimutagenic, anticarcinogenic, and cytotoxic properties (MacLeod et al. 1979). Numerous studies have confirmed that Polycyclic Aromatic Hydrocarbons (PAHs) having one, two, or three rings exhibit acute toxicity, whereas those encompassing more than three rings exhibit genotoxicity (Li et al. 2010).

DPPH – Free radical scavenging assay

The DPPH radical scavenging technique is a crucial experiment used to assess the antioxidant ability of natural substances by inhibiting lipid oxidation. This methodology assesses the efficacy of the analyzed substances in counteracting free radicals, providing crucial information on their antioxidant properties. The results of the DPPH radical scavenging activity of ethanolic extracts of *P. cyanescens* are presented in Table 4. The findings of the study indicate that the scavenging activity of the extracts exhibited a concentration-dependent pattern, whereby higher concentrations of the extract resulted in a more pronounced scavenging activity.

Free radicals are chemical entities that possess one or more unpaired electrons in their atomic or molecular orbitals (Qazi and Molvi 2018). Cellular damage caused by unstable atoms can result in illness and the process of aging (De Leon et al. 2020; Adetuyi et al. 2022). Oxygen-derived radicals are the predominant type of radical species generated within biological entities. Oxygen molecule acts as a free radical, causing the production of reactive oxygen species that can harm cells (Sánchez 2017). According to Husain and Kumar (2012), an excessive presence of Reactive Oxygen Species (ROS) can disrupt the equilibrium between antioxidants and oxidants, hence causing oxidative harm to many cellular components such as cell structures, DNA nucleic acid bases, lipids, and proteins. These cells serve as the body's primary defense against damage caused by free radicals (Abd 2012; Khatua et al. 2013).

Bustillos et al. (2014) assessed the antioxidant properties of *P. cyanescens* by varying culture medium and pH levels. The antioxidant activity of the coconut water with a pH of 5 was found to be the highest, as evidenced by its total phenolic content of 25.19 mg AAE/g sample and a radical scavenging activity of 14.40%. The findings underscore the significance of culture medium and pH profiles in influencing the antioxidant characteristics of *P. cyanescens*. The DPPH radical scavenging activity of *P. antillarium* was assessed by Dulay et al. (2015) across several culture media and pH conditions. The antioxidant potential and DPPH radical scavenging activity of potato broth were found to be the greatest, whereas coconut water exhibited the lowest levels. The study determined that the ideal pH level for effective DPPH radical scavenging in *P. antillarium* was 7.0. The findings suggest that the culture media and pH strongly influence the mushroom's capacity to scavenge DPPH radicals. The antioxidant capacity of potato broth was shown to be enhanced by a pH of 7.0. *Marasmius oreades* (Bolton) Fr., in the same taxonomic order as *P. cyanescens* in Agaricales, has notable DPPH free radical scavenging activities, suggesting it might be a valuable natural antioxidant. The findings suggest that the DPPH free radical scavenging activity of *M. oreades* varies depending on the concentration of the extract. The antioxidant capabilities of the ethanol extract derived from *M. oreades* were found to be substantial, as evidenced by scavenging activities ranging from 38.98 to 72.58% across several dosages (0.25, 0.5, 1, and 2). The maximal concentration (2) yielded the highest reported scavenging activity, leading to the neutralization of about 72.58% of the DPPH free radicals. The findings suggest that *M. oreades* might serve as a beneficial natural source of antioxidants with prospective uses (Karalti et al. 2022).

Table 4. DPPH radical scavenging activity of the ethanolic extracts of *Panaeolus cyanescens*

Concentration (μg/mL)	%Inhibition
20	3.00
50	48.21
100	63.82
200	70.98
300	77.23
500	83.58
1000	87.85
IC50	91.19

Table 5. Brine Shrimp Lethality Test of the ethanolic extract of *Panaeolus cyanescens*

Concentration (μg/mL)	Mortality rate (%) 24 hours exposure	LC50 (ppm)
10	1.33%	26.63
100	33%	
1000	33%	

Several investigations have demonstrated that mushrooms contain a diverse range of secondary metabolites, including phenolic chemicals. Phenolic chemicals play a major role in the antioxidant effect of some types of mushrooms (Edeoga et al. 2005). The mycochemical analysis of *P. cyanescens* in this study showed significant levels of antioxidants, secondary metabolites such as fatty acids, alkaloids, and steroids, as well as flavonoids and saponins. The bioactive compounds were thought to be responsible for the mushroom's antioxidant effects. The phenolic content and antioxidant activity of this specific mushroom, which are commonly used to assess antioxidant properties, may offer new insights into the creation of treatment strategies for oxidative diseases (Cheung et al. 2003). Previous studies on *P. cyanescens* have largely examined its medicinal qualities, but information regarding its antioxidant qualities is limited (Bustillos et al. 2014). According to Rajan et al. (2020), the solvent extraction processes have the potential to induce alterations in polarity, phytoconstituents, antioxidant activity, and other biological activities, hence impacting the effectiveness of free radical scavenging. The utilization of ethanol as the solvent in the study of *P. cyanescens* underscores the potential advantages associated with exposure to secondary metabolites. The evaluation of antioxidant activity is commonly conducted by the utilization of 2,2-diphenyl-1-picrylhydrazyl (DPPH), a stable free radical, as described by Dulay et al. (2016). The DPPH radical is an artificial nitrogen radical that possesses extraordinary characteristics. The experimental procedure is predicated upon the phenomenon of electron transfer, wherein an antioxidant compound reduces the oxidant by transferring an electron, resulting in a change in color and subsequent modification in absorbance. The generation of DPPH results in the removal of violet hue, exhibiting a peak absorption at the wavelength range of 517-520 nm (Dwivedi and Paul 2020). The concentration of extracts necessary to inhibit 50% of radicals is determined using the IC50 value. The IC50 value is a widely used metric for quantifying antioxidant activity since it measures the concentration of antioxidants required to lower the initial DPPH concentration by 50% (Molole et al. 2022).

The ethanolic extract exhibited substantial inhibition of DPPH, displaying a highly potent antioxidant activity of 83.58% at a concentration of 500 μg/mL. Peña et al. (2019) classify substances as potent antioxidants if their inhibition rate exceeds 80%, moderate antioxidants if their inhibition rate ranges from 50 to 80%, and weak antioxidants if their inhibition rate is below 50%. The IC50, which stands for half-maximal inhibitory concentration, is a vital metric used in pharmacological research to assess the strength of antagonist drugs. The term refers to the concentration of a pharmaceutical compound required to impede a biological process by 50%. This parameter is frequently assessed using whole-cell assays (Aykul and Martinez-Hackert 2016). The determination of an antioxidant's effectiveness heavily relies on the IC50 value. If a chemical's IC50 value is less than 50 ppm, it is considered highly potent; if it is between 50 and 100 ppm, it is considered strong; and if it is between 101 and 150 ppm, it is considered moderately potent. Sukweenadhi et al. (2020) define weak antioxidants as compounds with an IC50 value greater than 150 ppm. A lower IC50 value indicates higher antioxidant activity of the extracts, suggesting more efficiency in scavenging DPPH (Wright et al. 2017). The findings of this study provide clear evidence that the extract possesses significant antioxidant properties, as seen by its IC50 value of 91.19 ppm. According to the study's findings, *P. cyanescens* may have a biochemical basis that supports its traditional medical use, such as its ability to treat infections and its potential as an anticancer and antioxidant.

Cytotoxicity test using brine shrimp lethality test

The Brine Shrimp Lethality Test (BSLT) is a crucial instrument utilized for evaluating the lethal effects of various chemical substances. The purpose of this test is to assess the potential toxicity of chemicals by quantifying the mortality rates of brine shrimp larvae within a designated timeframe. The results of the BSLT provide crucial insights into the difficult characteristics of the chemicals under examination, where higher fatality rates suggest higher toxicity levels. Table 5 demonstrates that the mortality rates of brine shrimp larvae exposed to ethanolic extracts of *P. cyanescens* exhibited a concentration-dependent manner within 24 hours. The results demonstrated that increased concentrations of the extract had a more pronounced impact on prawn mortality, hence underscoring the hazardous characteristics of the extracts.

The Brine Shrimp Lethality Test (BSLT) is a concise, economical, and simple technique for evaluating the lethality of mushroom extracts. It often has a strong correlation with cytotoxic and antitumor properties (Baravalia et al. 2012). This test is used to predict drugs or extracts that may have anticancer properties, as demonstrated by Moshi et al. (2004). Furthermore, Krishnaraju et al. (2005) have established the efficacy of this method in the identification of fungal toxins and the assessment of mushroom extract toxicity. BSLT's quickness is a significant advantage, along with the resemblances between *Artemia* larvae and mammalian cells, the quick, uncomplicated, and cost-effective testing procedure, the minimal amount of material needed for testing, and the outcomes that closely mimic real-world conditions due to the large number of larvae utilized (Mirzaei et al. 2013).

The toxicity of the ethanolic extract of *P. cyanescens* was assessed using the BSLT. Throughout 24 hours, there was an apparent increase in fatality rates among brine shrimp larvae as the concentration of the ethanolic extract increased, hence underscoring its significant toxicity. The findings exhibited a progressive rise in lethality, with rates of 1.33, 33, and 33% seen at doses of 10, 100, and 1000 μg/mL, correspondingly. The LC_{50} value is the concentration at which 50% of the brine shrimp population dies after 24 hours of exposure, serving as a crucial measure of the extract's toxicity (Tawaha 2006). The concentration of the *P. cyanescens* extract was determined to be 26.63 parts per million (ppm).

Extracts are classified according to their LC_{50} values: categorizing them as extremely hazardous if they fall within the range of 0 to 100 μg/mL, medium toxic if they fall within the range of 100 to 500 μg/mL, and low toxic if they fall within the range of 500 to 1000 μg/mL. Substances having LC_{50} values greater than 1000 μg/mL are classified as non-toxic (Yurasbe et al. 2023). Lestari et al. (2015) and Wanyoike et al. (2004) emphasized that a smaller LC_{50} value indicates more toxicity, whereas a bigger LC_{50} value indicates lesser toxicity. Based on its LC_{50} value of 26.63 ppm, *P. cyanescens* extract is categorized as very hazardous within 24 hours. This result highlights the importance of doing more investigations to evaluate its potential impact. The possible hazards and ethical implications concerning the toxic effects of the ethanolic extracts from *P. cyanescens* are noteworthy and mostly due to their high-hazard classification. There are considerations on its potential impact on human health, the need to obtain consent in a study, and ethical responsibility regarding safety while investigating the curative potential of these substances.

Previous studies have demonstrated that *P. cyanescens* have teratogenic and toxic properties at higher doses when employed as a model organism in zebrafish embryos. The present study proposes that mushrooms of *P. cyanescens* possess teratogens that have promising potential for the development of anticancer pharmaceuticals. The *P. cyanescens* species includes psilocin (0.48%), psilocybin (0.11%), serotonin (0.072%), urea (1.8%), and baeocystin (0.02%), which might potentially have harmful and teratogenic effects on embryos (Bustillos et al. 2016). This finding aligns with the research conducted by Meyer (2017), which suggests that extracts derived from psychedelic mushrooms, specifically *P. cubensis* (which belongs to the same order as *P. cyanescens* within Agaricales), exhibit notable cytotoxic properties on brine shrimp. These effects are observed at high levels of toxicity, regardless of the concentration used. The observed mortality rate of brine prawns upon exposure to extracts derived from *P. cubensis* serves as evidence for the extract's toxic properties in comparison to the control groups. According to the research, all brine prawns in the treatment vials died within 24 hours. The toxicity of *P. cubensis* towards brine prawns and soil bacteria is noteworthy, suggesting that the presence of psilocybin and related compounds may have evolved as a defensive mechanism.

Psychoactive compounds found in *P. cyanescens* are mainly psilocybin and psilocin. These cause adverse effects like hallucinations, anxiety, and possibly neurotoxicity. The process of ethanolic extraction may increase the concentration of such compounds and the risk of toxicity. Acute exposure may result in intense psychological distress, but chronic use might cause severe impairment of cognitive functioning or a severe psychological disorder. Special consideration is required for the vulnerable population, who may already be diagnosed with some mental health condition, since they may be at higher risk of adverse reactions. Therefore, understanding the full spectrum of these effects is crucial for assessing the safety of these extracts.

According to Lumogdang et al. (2021), the cytotoxic effects of ethanolic extracts can be attributed to their elevated levels of mycochemicals, antioxidants, and antibacterial properties. The first evaluation of the bioactive substances found in *P. cyanescens* benefits from the application of the brine shrimp lethality assay. The remarkable efficacy of *P. cyanescens* extract at dangerous concentrations suggests that further investigation is necessary to see whether it could be a good substitute for anticancer treatments. To create new anticancer treatments, more research is required to utilize the extract's cytotoxic properties fully. This emphasizes the necessity of investigating the dual properties of some dangerous substances for possible therapeutic uses. Hence, this underscores the significance of ongoing research in elucidating the cytotoxic characteristics of *P. cyanescens*, thus facilitating the development of sophisticated and efficacious therapeutic interventions for cancer.

The current study has shown promise for developing anticancer drugs from ethanolic extracts of *P. cyanescens* due to its observed cytotoxicity. However, such findings must be translated into clinical applications through a holistic approach involving phytochemical characterization, mechanistic studies, in vivo assessments, formulation development, and rigorous clinical testing. Through this, future researchers will be able to utilize the therapeutic benefits of this bioactive compound, hence furthering efforts toward successful treatments for cancer. Traditional knowledge, in combination with the application of scientific techniques, will bring forth groundbreaking therapeutic interventions, bettering patients' conditions with regard to cancer diseases.

In conclusion, a thorough analysis of ethanolic extracts has revealed important information about the mycochemical, antioxidant, and cytotoxic properties of the hallucinogenic mushroom *P. cyanescens*. The ethanolic extract showed strong antioxidant properties at 1,000 μg/mL, which led to an 87.85% suppression of DPPH. This inhibition's IC_{50} value was found to be 91.19 ppm. The GC-MS analysis conducted in this study revealed the presence of 25 bioactive compounds, including fatty acid methyl esters. These compounds have been well acknowledged for their numerous health benefits, such as their anticancer, anti-inflammatory, and antibacterial properties. As demonstrated by the high toxicity of *P. cyanescens* with an LC_{50} value of 26.63 ppm, indicating significant anticancer properties, the brine shrimp lethality test validated the extract's potential as an anticancer agent. This illustrates only the initial phases of exploring the wide range of possibilities offered by *P. cyanescens*. More research is required to understand the complex mechanisms underlying these impacts. The need for further research is not just a necessity but an exciting opportunity to delve deeper into the potential of *P. cyanescens*. Furthermore, it is imperative to conduct comprehensive clinical trials in order to validate the efficacy and safety of the intervention within a medical framework. To fully realize the potential of this intriguing opportunity, ethical and accurate scientific investigation is required.

Considering the ethical implications of promoting the medicinal use of hallucinogenic mushrooms is vital. Public health and safety must always be a priority. It's important to advocate for responsible use, including proper dosage, setting, and patient education. Clear communication about potential risks and benefits is crucial in fostering an informed public discourse. Collaborating with healthcare professionals, researchers, and policymakers will ensure that any advancement in using these substances is done ethically, prioritizing safety and informed consent. When discussing the variability of natural compounds in clinical applications, it's crucial to advocate for standardized extraction methods and dosing. It's true that individual responses to compounds can differ significantly due to genetic, environmental, and psychological factors. However, this variability also points to the need for personalized medicine approaches, which consider individual differences in treatment plans. Emphasizing ongoing research and the importance of controlled studies can help address concerns regarding inconsistency.

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Exploring the ethnobotany of natural dye plants in Kalibawang Sub-district, Wonosobo District, Indonesia through traditional knowledge, conservation, and community empowerment

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[2]Walai Rukhavej Botanical Research Institute, Mahasarakham University. Kantarawichai District, Maha Sarakham, 44150, Thailand
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Abstract. *Sulton MN, Guzherra RMR, Fahimah RA, Putri RA, Permatasari RI, Saensouk S, Iskandar J, Supriyatna J, Setyawan AD. 2025. Exploring the ethnobotany of natural dye plants in Kalibawang Sub-district, Wonosobo District, Indonesia through traditional knowledge, conservation, and community empowerment. Nusantara Bioscience 17: 155-168.* The use of synthetic dyes can cause environmental and health problems. Natural dyes can be a novelty to replace synthetic dyes because they contain materials that are more environmentally friendly, non-toxic, and sustainable. This ethnobotanical research was carried out to reveal the traditional knowledge of the people in Kalibawang Sub-district, Wonosobo District, Indonesia, regarding the use of various species of plants as a source of natural dyes. The purpose of this study is to analyze the species of plants that can produce natural colors used by the people of Kalibawang Sub-district, as well as to assess their conservation and sustainability and empower the community. The method used is an ethnographic study through in-depth interviews with informants, field observations, and documentation of plant species. The quantitative method used is recording how many people use the coloring plants and calculating the RFC. This study identifies various species of plants used as natural dyes and their utilization. A total of 53 plant species from 37 families have been identified and utilized as color producers in Kalibawang Sub-district. The most widely used plant parts are fruit (36%) and leaves (28%), the plant forms are herbs (49%) and trees (26%), the colors are red (28%) and green (15%), used to color food (49%), and drinks (42%). Most of these plants are intentionally planted (53%), but most of them are rarely used (43%), only a few are frequently used (26%), among the most important are turmeric (*Curcuma longa* L.) (RFC 0.891) for yellow color, and pandan (*Pandanus amaryllifolius* Roxb. ex Lindl.) (RFC 0.633), and green grass jelly (*Cyclea barbata* Miers) (RFC 0.613) for green color. The sustainability of several species needs attention because of their conservation status, namely *Tectona grandis* L.f. (EN), *Coffea arabica* L. (EN), and *Cinnamomum verum* J.Presl (VU). The dye extraction method usually uses water or other natural ingredients. This study shows that natural dyes have the potential to be further innovated. However, this traditional knowledge is in danger of being lost due to lifestyle changes and the lack of interest of the younger generation. Hence, it needs to increase public awareness of the potential of natural dyes as an alternative to synthetic dyes and to engage the community in this important issue.

Keywords: Conservation, *Curcuma longa*, ethnobotany, natural dyes, sustainability, traditional knowledge

INTRODUCTION

Dyes are defined as chromophoric substances that are capable of interacting chemically or physically with a substrate, thereby selectively absorbing certain wavelengths of light and producing a visible color (Alegbe and Uthman 2024). As a country with abundant biodiversity, Indonesia is a country with great potential to provide natural materials that can be used as a source of natural dyes (Maskun et al. 2021). These natural materials are usually called coloring plants. Coloring plants themselves refer to various types of plants that have the ability to produce natural coloring compounds, which can be used in various applications, especially in the food, textile, and cosmetic industries (Mohamad et al. 2019). Generally, natural colorants that are easy to find are derived from plant pigments (Lakshmi 2014). The use of natural dyes provides significant environmental benefits, such as reducing pollution and chemical waste and supporting plant conservation as natural colorants. There are four main groups of pigments from plants, namely chlorophyll (green), carotenoids (yellow, red, orange), flavonoids (anthocyanins, red, blue, purple), and betalains (red, yellow, purple) (Malabadi et al. 2022).

The use of coloring plants has been known for thousands of years and is an important part of the culture and traditions of various societies around the world. More than 2,000 color variations have been produced from various plant components, but only 150 colors are used economically. Colors can be taken from various parts of the plant, such as seeds, fruits, roots, stems, and bark, and produced through several processes, namely boiling, burning, grinding, and direct use (Berlin et al. 2017). Different plant components can produce one or more colors, depending on which part is taken from the plant (Ebrahim et al. 2022). These coloring plants are environmentally friendly and safer compared to synthetic dyes, which often contain harmful chemicals. The use of natural dyes can also support sustainability as the raw materials come from renewable sources (Raturi et al. 2023).

Knowledge of dye plants is often passed down through generations in communities, especially in rural areas that still utilize natural resources traditionally. In Kalibawang, an area known for its rich biodiversity and traditional agricultural practices, dye plants have long been utilized by the community. They use local plants, such as teak leaves, turmeric, and mahogany bark, to produce natural colors that are used for various needs. These natural dye colors are influenced by various factors such as light, the presence of oxygen, and water activity which are a testament to the local knowledge that must be preserved (Novais et al. 2022). Natural dyes are usually categorized by color, solubility, chemical constitution, application, and origin (Sk et al. 2021). The Kalibawang community frequently uses coloring plants to promote appetite, as an element of tradition, and as a means of play for children. The Kalibawang community's primary source of income is farming and animal breeding (Herdananta et al. 2024), which determines how plants are utilized to make dyes, particularly natural food dyes.

In the food sector, natural colorants are used to enhance the visual appeal of food, provide vibrant colors, and sometimes add flavor. From a technological point of view, the addition of colorants is considered natural because some foods undergo discoloration during processing and storage, so colorants are added to restore or adjust the lost color (Ribeiro and Veloso 2021). Coloring plants can also add value to products, increasing marketability in a market that is increasingly concerned with health and sustainability. While natural colorants have many benefits, there are some drawbacks and challenges in their use. Natural dyes do not always provide the desired color variation, and their availability is limited (Pranta and Rahaman 2024). Natural dyes also tend to fade quickly, and their quality may not be as consistent as synthetic dyes (Affat 2021). Because of the consequent color inconsistencies, natural dyes are being phased out. As a result, this local knowledge may be lost if not preserved.

Therefore, the aims of this study were to (i) analyze the species of plants that can produce natural colors used by the people of Kalibawang Sub-district, (ii) assess their conservation and sustainability, and empower the community. The findings of this research aim to preserve dye plants as part of the culture and promote environmentally friendly natural dyes compared to the numerous synthetic dyes available.

MATERIALS AND METHODS

Study area

This research was conducted in the Kalibawang Sub-district, Wonosobo District, Central Java Province, Indonesia (Figure 1). This sub-district has an area of 5,217.6 ha and an altitude of 25-350 meters above sea level (m asl.). This research was conducted in four villages of Kalibawang Sub-district, namely Dempel, Kalialang, Karangsambung, and Mergolangu. The four villages are close together. Dempel Village has an area of 773.2 ha, Kalialang Village has an area of 656.9 ha, Karangsambung Village has an area of 643.4 ha, and Mergolangu Village has an area of 502.8 ha. The selection of these four villages is due to the similarity of the landscape area, which is still natural with hilly areas, dominated by agroforestry and production forests. Kalibawang Sub-district is one of the farthest sub-districts from the nearest city center, and it has a strong influence on Javanese culture.

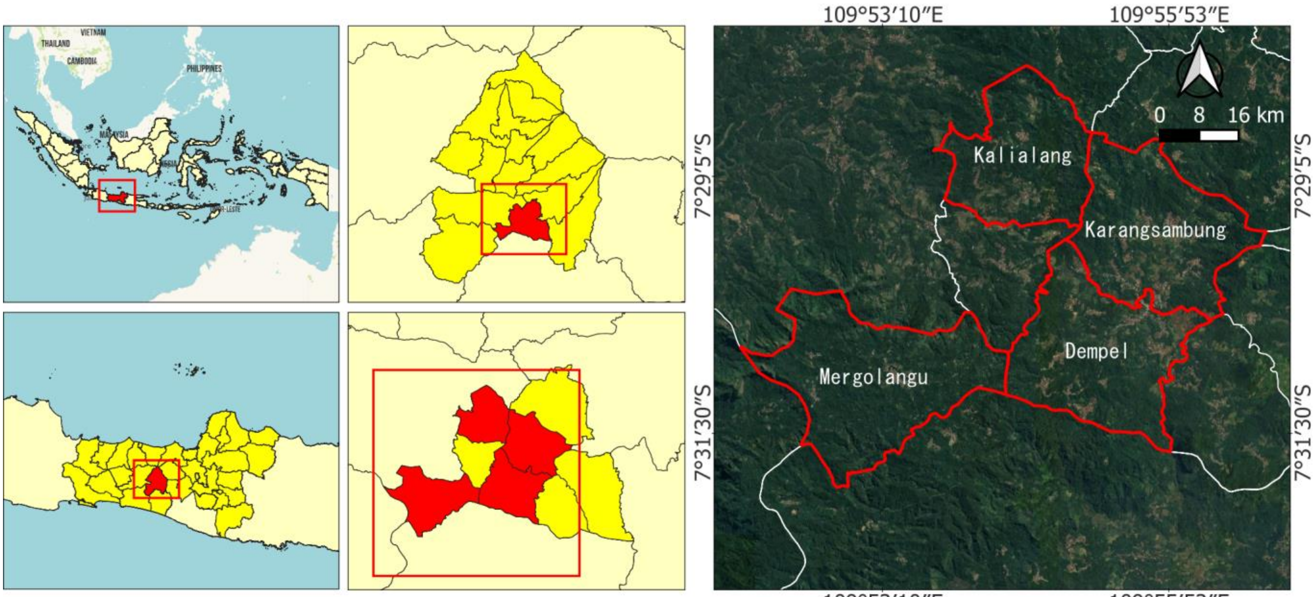

Figure 1. Administrative map of Dempel, Kalialang, Karangsambung, Mergolangu Villages in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

Data collection

The method used is an ethnographic study through in-depth interviews with informants, field observations, and documentation of plant species. We picked respondents from local communities through purposive sampling who use and are familiar with plants as natural dyes and are at least 18 years of age. We used Slovin's formula to calculate the total number of respondents (Yamane 1967). There are 9829 households in Kalibawang Sub-district. We obtained 101 respondents and 5 key informants in this study. In addition, we used village heads and stakeholders as key informants to investigate dye plant management using Forum Group Discussion (FGD). Interviews were conducted by asking several questions, such as what plants are used as dyes, which parts are used, what colors are produced, what is the plant habitus, where it comes from, how to extract it, how often it is used, and what it is used for (food, drinks, medicine, etc.). The frequency of use is determined by the degree of community utilization in one year. If it is used only 1-2 times per year, it is rarely used; if it is used 3-6 times per year, it is used sometimes; and if it is used more than 6 times per year, the community frequently uses it (Luong et al. 2023). We target our respondents with a group of 5 levels of education, namely not in school, elementary school, junior high school, high school, and university.

In addition to interviews, field observations and plant recording were conducted to directly identify the types of plants that grow and are used by the local community for their use of natural dye plants. We utilized the observational data and plant species documentation to supplement and reinforce the information provided by the respondents. The names of local plants obtained were then identified by their scientific names using Plants of the World Online (POWO) (https://powo.science.kew.org/) and Global Biodiversity Information Facility (https://www.gbif.org/).

To determine the conservation status of the species found, we used data from the IUCN Red List 2024 (https://www.iucnredlist.org/) and Indonesian protected animals and plants regulations (P.106/2018). We also conducted semi-structured interviews with key informants to examine indigenous knowledge on natural dye plants and their implications for community empowerment.

Data analysis

The quantitative method used is recording how many people use the coloring plants and calculating the RFC (Relative Frequency of Citation). The RFC (Relative Frequency of Citation) is a calculation used to measure how important a plant species is in the research area (Ahmad et al. 2014). This calculation is done by dividing the number of respondents who know or use plant species by the total number of respondents interviewed (Jan et al. 2022).

The calculation formula is as follows:

$$RFC = \frac{FC}{N}$$

Where: FC: Number of respondents who know or use the plant species; N: Total of all respondents

The results of the RFC calculation become a measure of the frequency of community use of a species. The closer the value of RFC is to 1, the more frequently the species is used by the community, while the closer the value is to 0, the less frequent to never.

RESULTS AND DISCUSSION

Sociodemographic characters

Based on the data collected, the distribution of respondents in this study shows that the majority of respondents are women, which is 71 people (71%) of the total respondents. In comparison, male respondents only amounted to 30 people (30%) (Table 1). This shows that women's participation in this study is still dominant, which is influenced by the characteristics or needs of the study that are more relevant to women, or social and cultural conditions that cause more women to be involved.

Furthermore, the data on the respondents' village of origin also showed considerable variation. Most respondents came from Dempel village, at 33%, followed by Mergolangu Village, which accounted for 31% of the total respondents. Kalialang and Karangsambung Villages contributed 20% and 17%, respectively, to the overall sample. Dempel and Mergolangu appear to be the two villages that participated the most, which could be due to geographical factors, higher levels of awareness or socialization in these villages, or the characteristics of a larger population compared to other villages. The respondents' level of education shows an alarming pattern. A significant 47% of the respondents have only an elementary school education, and 13% did not attend school at all. This data underscores the urgent need to address the issue of low formal education in the community.

The prevalence of low educational attainment in Kalibawang Sub-district is strongly associated with the predominance of traditional agriculture as the primary livelihood among its population (59%). Statistical data from the Central Bureau of Statistics (BPS 2023) indicate that over 54% of Kalibawang's residents rely on the agricultural sector for subsistence. Further supporting this observation, Nuraini et al. (2024) argue that limited formal education constrains the local community's capacity to sustainably harness natural resources, including the utilization of flora for daily necessities.

As many as 30% of respondents are junior high school graduates, which is the first level of secondary education. Only 6% have completed high school, and even fewer, at 5%, have attended university. In addition, 13% of respondents have not had the opportunity to attend school at all. This data underscores the significant challenges in accessing education in the study area and the socio-economic factors that restrict opportunities for individuals to pursue higher education. This data provides a clear picture that the majority of respondents have relatively low levels of education, which may have an impact on their perceptions, knowledge, and ability to engage with the issues we're exploring in this study.

Table 1. Respondent information was obtained from four villages in Kalibawang, Wonosobo, Central Java, Indonesia (n = 101)

Variable	Freq	Percentage
Gender		
Male	30	30%
Female	71	71%
Village		
Kalialang	20	20%
Karangsambung	17	17 %
Dempel	33	33%
Mergolangu	31	31%
Last education		
No school	13	13%
Elementary School	47	47%
Junior High School	30	30%
High School	6	6%
University	5	5%
Occupation		
Farmers	59	58.4%
Housewives	15	14.9%
Laborers	12	11.9%
Traders	8	7.9%
PNS/Polri/TNI	6	6.0%
Others	1	1.0%

Plant diversity

Table 2 shows that the food coloring plants found in Kalibawang Sub-district, Wonosobo District, especially in Kalialang, Mergolangu, Karangsambung, and Dempel Villages, are 53 species from 37 families. More than 30 different plant families reflect the diversity of the flora in this region. Species-rich families include Fabaceae, Arecaceae, and Solanaceae (3 species, 5.66%). Amaranthaceae, Brassicaceae, Cucurbitaceae, Lauraceae, Myrtaceae, Piperaceae, Poaceae, Rosaceae, Rubiaceae, and Rutaceae represent 2 species (3.77%), and the other 24 families were represented by one species each.

Our study of plant families uncovers a wealth of practical uses from well-known families such as Zingiberaceae, Fabaceae, Solanaceae and Myrtaceae. Each family is home to a variety of plant species, each with its own unique applications. For instance, the Zingiberaceae family is represented by *Curcuma longa* L. (turmeric or *kunyit*), which is widely known as a food coloring ingredient and traditional medicine. The Rubiaceae family is represented by the coffee plant (*Coffea arabica* L.), which is an important commodity in mountainous areas such as Wonosobo. Genu\era such as *Curcuma*, *Amaranthus*, *Capsium* and *Persea* each contribute a species that is often used as a natural food coloring. In addition, some species from the Fabaceae family provide us with species like *Clitoria ternatea* L. (butterfly pea or *telang*) and *Biancaea sappan* (L.) Tod. (sappanwood or *secang*). Local communities often use these renowned, well-known sources of natural dyes, connecting them to their cultural heritage.

Part used

Based on Figure 2 reveals the plant parts used for coloring. From the available data, fruit is the most dominant part used for coloring, with 19 species (36%). Leaves came in second with 15 species (28%), showing the importance of leaves in the preparation of natural dyes. Flowers and bulbs were used by 7 species each, representing 13% of the total. Seed parts were utilized by 3 species (6%), while bark was used by only 2 species (4%). Rhizome was the least used part, with only 1 species (2%). This shows that fruits and leaves are the most utilized parts in the preparation of natural dyes.

Fruits and leaves are the plant parts most commonly used in the manufacture of natural dyes that are not just functional but also stunningly beautiful. Leaves, with their high pigment content like chlorophyll and carotenoids, offer a stunning range of green and yellow dyes. Fruits are also widely utilized for their natural pigment content, such as anthocyanins, which provide red to purple colors. Some fruits, such as dragon fruit or *buah naga* (*Hylocereus undatus* (Haw.) Britton & Rose) and pangi or *kluwek* (*Pangium edule* Reinw.) seeds, are used to produce dyes with brown and red color variations that are truly intriguing (Tamilarasi and Banuchitra 2021).

Life form

Based on Figure 3, the species composition of dye plants consists of four main categories based on their form, namely herbs (49%), trees (26%), shrubs (17%), and vines or lianas (8%). Herbaceous plants dominate as the most commonly used form of dye plants, like pandan (*Pandanus amaryllifolius* Roxb. ex Lindl.) produces green dye from its leaves, and turmeric (*C. longa*), which is well-known as a source of yellow natural dye. Herbaceous plants, like pandan and turmeric, are the most commonly used form of dye plants. They are abundant, with their soft, non-woody stems making them easy to find and fast to grow. This abundance should inspire you to explore the world of natural dyeing. Tree-shaped plants, which account for 26% of the composition, for example, are avocado (*Persea americana* Mill.), which produces green dye from its fruit, and teak tree or *jati* (*T. grandis*), whose leaves can produce a red color. Shrub-shaped plants, meanwhile, occupy 17%, which includes species such as Paper flower or *bunga kertas* (*Bougainvillea glabra* Choisy) which produces red color, and also rubus (*Rubus* sp.), which produces red color from its fruit. In addition, vines or lianas, which account for the smallest proportion (7%) but are nonetheless important in the ecosystem and natural dye industry, such as green grass jelly or *cao* (*Cyclea barbata* Miers) species used in green dyeing. These plants are found in various geographical regions; some are more prevalent in tropical climates, while others thrive in temperate zones. Altitude and climate factors can affect plant growth (Negari et al. 2023; Sulton et al. 2024).

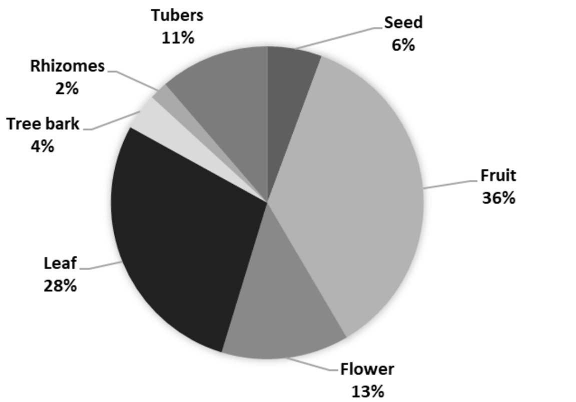

Figure 2. Part used of dye plants in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

Table 2. Composition of coloring plants found in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia, namely Dempel Village, Kalialang Village, Karangsambung Village, and Mergolangu Village

Family	Species name	Common name	Local name	Parts used	Life form	Color	Origin	Extraction	Frequency	Benefits	RFC
Achariaceae	*Pangium edule* Reinw.	Pangi	*Kluwek-pocung*	Fruit	Tree	Black	Wild	Boiled	Rarely used	Food	0.049
Amaranthaceae	*Amaranthus tricolor* L.	Chinese spinach	*Bayam*	Leaf	Herb	Green	Wild	Cooked	Frequently used	Food	0.227
Amaranthaceae	*Beta vulgaris* L.	Beet	*Bit*	Tubers	Herb	Red	Planted	Boiled, blended	Rarely used	Food	0.019
Anacardiaceae	*Mangifera indica* L.	Mango	*Mangga*	Fruit	Tree	Orange	Planted	Blended	Sometimes	Food	0.069
Apiaceae	*Daucus carota* L.	Carrot	*Wortel*	Tubers	Herb	Orange	Planted	Boiled, cooked	Sometimes	Food	0.039
Araceae	*Colocasia esculenta* (L.) Schott	Taro	*Talas*	Tubers	Herb	Chocolate	Planted	Boiled	Rarely used	Food	0.009
Arecaceae	*Cocos nucifera* L.	Coconut	*Kelapa*	Fruit	Herb	Chocolate	Wild, planted	Boiled	Frequently used	Food	0.198
Arecaceae	*Arenga pinnata* (Wurmb) Merr.	Sugar palm	*Aren*	Flower	Tree	Chocolate	Planted	Boiled	Frequently used	Drinks	0.049
Arecaceae	*Areca catechu* L.	Betel-nut palm	*Pinang*	Fruit	Tree	Red	Wild, planted	Chewed	Rarely used	Food	0.029
Asparagaceae	*Dracaena angustifolia* (Medik.) Roxb.	Narrow-leaf dracaena	*Daun suji*	Leaf	Shrub	Green	Wild	Pounded	Rarely used	Food	0.009
Asteraceae	*Lactuca sativa* L.	Red lettuce	*Selada merah*	Leaf	Herb	Red	Planted	Cooked	Rarely used	Food	0.009
Balsaminaceae	*Impatiens balsamina* L.	Garden balsam	*Pacar air*	Flower	Herb	Red	Wild	Pounded	Rarely used	Nail colorant	0.198
Basellaceae	*Anredera cordifolia* (Ten.) Steenis	Madeira vine	*Binahong*	Leaf	Herb	Green	Wild, planted	Boiled, tea	Rarely used	Drinks	0.009
Brassicaceae	*Brassica rapa* L.	Mustard green	*Sawi*	Leaf	Herb	Green	Planted	Cooked	Frequently used	Food	0.019
Brassicaceae	*Brassica oleracea* L.	Red cabbage	*Kol ungu*	Leaf	Herb	Purple	Planted	Cooked	Rarely used	Food	0.009
Cactaceae	*Hylocereus undatus* (Haw.) Britton & Rose	Dragon fruit	*Buah naga*	Fruit	Herb	Purple	Planted	Blended	Frequently used	Drinks	0.207
Caricaceae	*Carica papaya* L.	Papaya	*Pepaya*	Fruit	Herb	Green	Planted	Cooked	Frequently used	Food	0.178
Clusiaceae	*Garcinia mangostana* L.	Mangosteen	*Manggis*	Fruit	Tree	Purple	Planted	Dried	Sometimes	Drinks	0.118
Convolvulaceae	*Ipomoea batatas* (L.) Lam.	Sweet potato, purple yam	*Ubi ungu, ubi kuning*	Tubers	Herb	Purple, Yellow	Planted	Boiled	Sometimes	Food	0.475
Cucurbitaceae	*Cucurbita moschata* (Duchesne) Duchesne ex Poir.	Pumpkin	*Waluh-labu*	Tubers	Vines	Orange	Planted	Boiled	Sometimes	Food	0.356
Cucurbitaceae	*Citrullus lanatus* (Thunb.) Matsum. & Nakai	Watermelon	*Semangka*	Fruit	Herb	Red	Planted	Blended	Rarely used	Drinks	0.009
Euphorbiaceae	*Manihot esculenta* Crantz	Cassava	*Singkong*	Tubers	Herb	White	Wild, planted	Dried	Frequently used	Food	0.019
Fabaceae	*Clitoria ternatea* L.	Butterfly pea	*Telang*	Flower	Herb	Blue	Wild	Boiled, tea	Rarely used	Food	0.069
Fabaceae	*Glycine max* (L.) Merr.	Soybean	*Kedelai*	Seed	Herb	White	Planted	Boiled	Sometimes	Food	0.009
Fabaceae	*Biancaea sappan* (L.) Tod.	Sappanwood	*Secang*	Tree bark	Tree	Red	Wild, planted	Boiled, tea	Rarely used	Drinks	0.009
Lamiaceae	*Tectona grandis* L.f.	Teak	*Daun jati*	Leaf	Tree	Red	Wild, planted	Boiled	Rarely used	Food	0.148
Lauraceae	*Persea americana* Mill.	Avocado	*Alpukat*	Fruit	Tree	Green	Planted	Blended	Rarely used	Drinks	0.178
Lauraceae	*Cinnamomum verum* J.Presl	Cinnamon	*Kayu manis*	Tree Bark	Tree	Chocolate	Planted	Boiled, tea	Rarely used	Drinks	0.009
Malvaceae	*Theobroma cacao* L.	Cocoa	*Coklat*	Fruit	Tree	Chocolate	Wild	Dried	Sometimes	Drinks	0.089
Menispermaceae	*Cyclea barbata* Miers	Green grass jelly	*Cao*	Leaf	Vines	Green	Wild, planted	Boiled	Sometimes	Drinks	0.613
Moraceae	*Abelmoschus moschatus* (L.) Medik.	Musk mallow	*Waron*	Flower	Herb	Red	Wild	Pounded	Rarely used	Nail colorant	0.029

Myrtaceae	*Psidium guajava* L.	Guava	*Jambu*	Leaf	Tree	Red	Planted	Blended	Rarely used	Drinks	0.059
Myrtaceae	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	Clove	*Cengkeh*	Flower	Tree	Chocolate	Planted	Boiled	Sometimes	Drinks	0.108
Nyctaginaceae	*Bougainvillea glabra* Choisy	Paper flower	*Bunga kertas*	Flower	Shrub	Red	Wild, planted	Pounded	Rarely used	Nail colorant	0.019
Pandanaceae	*Pandanus amaryllifolius* Roxb. ex Lindl.	Pandan	*Pandan*	Leaf	Herb	Green	Wild, planted	Pounded, blender	Frequently used	Food	0.633
Passifloraceae	*Passiflora edulis* Sims	Passion fruit	*Markissa*	Fruit	Vines	Yellow	Wild, planted	Raw	Sometimes	Drinks	0.009
Piperaceae	*Piper betle* L.	Betel	*Sirih*	Leaf	Vines	Red	Wild	Boiled, chewed	Sometimes	Drinks	0.227
Piperaceae	*Piper sarmentosum* Roxb.	Wild pepper	*Senggani*	Flower	Shrub	Purple	Wild, planted	Pounded	Rarely used	Nail colorant	0.009
Poaceae	*Cymbopogon citratus* (DC.) Stapf	Lemongrass	*Sereh*	Leaf	Herb	Chocolate	Wild, planted	Boiled	Sometimes	Drinks	0.009
Poaceae	*Oryza sativa* L.	Rice	*Pari*	Seed	Herb	Black	Planted	Burned	Sometimes	Food	0.019
Rosaceae	*Rubus* sp.	Rubus	*Rubus*	Fruit	Shrub	Red	Wild	Pounded	Rarely used	Food	0.009
Rosaceae	*Fragaria ×ananassa* (Weston) Rozier	Strawberry	*Strawberry*	Fruit	Herb	Red	Planted	Blended	Sometimes	Food	0.019
Rubiaceae	*Coffea arabica* L.	Arabica coffee	*Kopi*	Seed	Shrub	Black	Wild, planted	Dried	Frequently used	Drinks	0.287
Rubiaceae	*Uncaria gambir* (W.Hunter) Roxb.	Gambir	*Gambir*	Leaf	Shrub	Chocolate	Wild, planted	Boiled	Sometimes	Drinks	0.019
Rutaceae	*Citrus* sp/	Citrus	*Jeruk*	Fruit	Tree	Orange	Planted	Blended	Frequently used	Drinks	0.089
Rutaceae	*Citrus ×limon* (L.) Osbeck	Lemon	*Lemon*	Fruit	Shrub	Yellow	Planted	Blended	Rarely used	Drinks	0.009
Sapotaceae	*Phyllanthus reticulatus* Poir.	Potato-bush	*Mangsi*	Fruit	Tree	Black	Wild	Pounded	Rarely used	Ink	0.009
Solanaceae	*Capsicum frutescens* L.	Chili pepper	*Cabai*	Fruit	Herb	Red	Planted	Blended	Frequently used	Food	0.356
Solanaceae	*Solanum lycopersicum* L.	Tomato	*Tomat*	Fruit	Herb	Red	Planted	Blended	Frequently used	Food	0.277
Solanaceae	*Nicotiana tabacum* L.	Tobacco	*Mbako*	Leaf	Herb	Chocolate	Wild, planted	Dried	Rarely used	Food	0.019
Theaceae	*Camellia sinensis* (L.) Kuntze	Tea	*Teh*	Leaf	Shrub	Chocolate	Planted	Boiled, tea	Frequently used	Drinks	0.267
Vitaceae	*Vitis vinifera* L.	Grape	*Anggur*	Fruit	Shrub	Purple	Planted	Blended	Sometimes	Drinks	0.009
Zingiberaceae	*Curcuma longa* L.	Turmeric	*Kunyit*	Rhizomes	Herb	Yellow	Planted	Boiled, cooked	Frequently used	Drinks	0.891

Color

This investigation discovered 9 different colors from dye plants used by the community (Figure 4). Red is the most frequently utilized color, which includes 15 species (28%), followed by brown (10 species, 19%) and green (8 species, 15%). Blue is the least common color acquired from plants, accounting for only one species (2%). Sweet potato/purple yam or *ubi ungu* (*Ipomoea batatas* (L.) Lam.) produces two color pigments used by the community, i.e.: yellow and purple. The community commonly uses sweet potato as a culinary coloring. Butterfly pea produces the only blue color in its flowers, which is commonly used as a refreshing drink in the community.

Origin

Figure 5 shows the distribution of the origin of plant species, which is divided into three main groups: Planted (53%), wild (28%), and planted and wild (19%). From these results, it can be seen that more than half of the species are maintained through cultivation (planted), indicating a significant impact of human actions on the distribution of plant species.

These include species such as turmeric, chili or *cabai* (*Capsicum frutescens* L.), sweet potato, etc. A total of 28% of the species are found naturally in the wild, indicating that almost a third of the species still grow without human intervention. Wild plants include species such as betel or *sirih* (*Piper betle* L.), and garden balsam or *pacar air* (*Impatiens balsamina* L.). Meanwhile, 19% of species can be found both in cultivated form and the wild (planted and wild), reflecting a balance between exploitation and conservation efforts for some species. Species in this category, such as cloves or *cengkeh* (*Syzygium aromaticum* (L.) Merr. & L.M.Perry) and green grass jelly, can grow in the wild as well as be specially planted.

Frequency

Figure 6 demonstrates that 23 species (43%) are rarely utilized, 16 species (30%) are sometimes utilized, and 14 species (26%) are frequently used by community. The frequency of use of dye plants varies from frequent use, such as turmeric and pandan, to infrequent use, such as sappanwood for red color. Most of these plants are utilized for food and beverage coloring, while some are explored for broader uses in textile or craft dyes. The predominance of herbs in the composition of dye plants indicates their superiority in availability and ease of utilization. However, the presence of trees, shrubs, and vines also offers further development potential for a more diverse source of natural dyes. This study showed that plants of various morphological types (herbaceous, tree, shrub, and vine) play an important and diverse role in the provision of natural dyes, with the greatest dominance coming from herbs. This diversity in nature's provision of natural dyes is something to be appreciated. The morphological diversity of these plants supports conservation efforts and more sustainable use in the natural color industry (Yadav et al. 2023). The results of research on plant species that can be used as food coloring in the villages of Kalialang, Mergolangu, and Dempel, show that this region has a diversity of plants that are not only useful for food but also produce natural dyes that are safe and environmentally friendly.

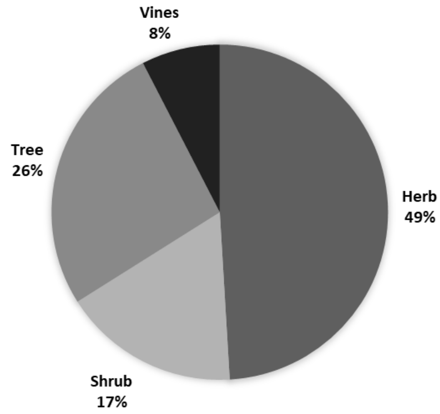

Figure 3. Life form of dye plants in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

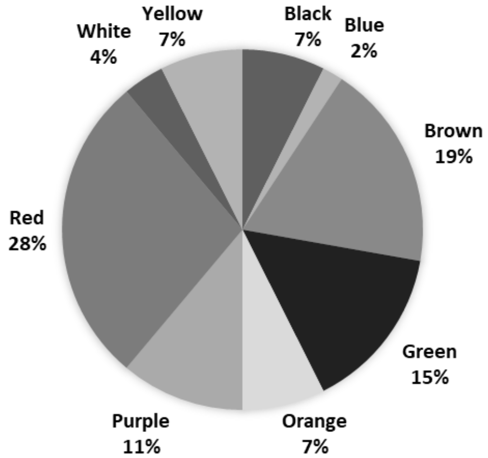

Figure 4. Color diversity of dye plants in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

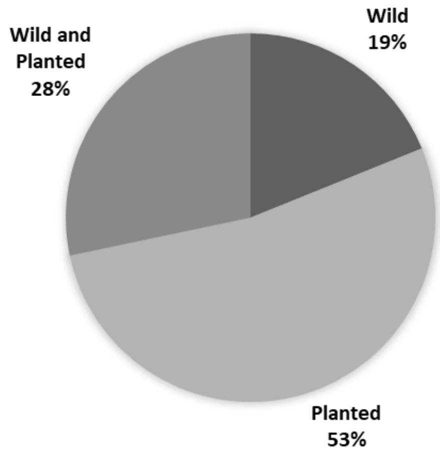

Figure 5. Origin distribution of dye plants in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

Dye plants utilization

The majority of the dye plants in Kalibawang Sub-district are utilized for food and beverage coloring. Food colorings rank highest with 26 species (49%), followed by drink colorings (22 species, 42%), nail polish (4 species, 8%), and ink colorings (1 species, 2%) (Figure 7). Natural plant dyes for food coloring are widely used by the community for traditional and cultural ceremonies, including religious ritual offerings such *merdi dusun* and *sadranan*, as well as traditional offerings at weddings, birth celebrations, and funerals. Turmeric has been widely known as a natural food colorant in traditional culinary practices. Empirical evidence obtained from structured interviews revealed that turmeric is mostly used in the preparation of *nasi kuning* (yellow rice), *opor ayam* (Javanese chicken curry in coconut milk), and *tempe goreng* (fried tempeh), due to its stable pigmentation and cultural prevalence in Indonesian cuisine. We also found the use of the plant as a medium for children's play, especially as a temporary nail polish from the paper flower plant. Our study also identified the use of potato bush as a source of natural ink. This plant species grows wild and is increasingly underutilized in local communities, despite its potential applications.

Extraction

Figure 8 shows that boiling was the most common method (23%, 12 species), followed by blending (21%, 11 species) and pounding (13%, 7 species). Additionally, five species (10%) were prepared as tea, while five species (10%) were dried to obtain color. The dye extraction method usually uses water or other natural ingredients. The dye extraction techniques in Kalibawang remain relatively simple, with water-based methods such as boiling and blending being predominant. Coconut and green grass jelly are traditionally processed through water extraction (boiling). Coconut sap, when thermally reduced, produces coconut sugar—a culturally important sweetener and an important source of income for local communities. Similarly, green grass jelly forms a gelatinous matrix after boiling, which is innovatively used in ethnobotanical practices as a digestive agent and a base for traditional drinks, underlining its dual role in local gastronomy and herbal medicine. The small-scale, household-level use of dye plants in this region explains the limited adoption of modern extraction methods. According to Ballabh and Pullaiah (2017), the preference for simple techniques is influenced by the primary application of these dyes in food and beverages, where minimal processing is favored. Methods like boiling and blending are not only cost-effective but also perceived as healthier due to the absence of synthetic chemicals. Jamaludin et al. (2023) further support this practice, noting that plant-based dyes are preferred for their environmental sustainability and ability to preserve food quality.

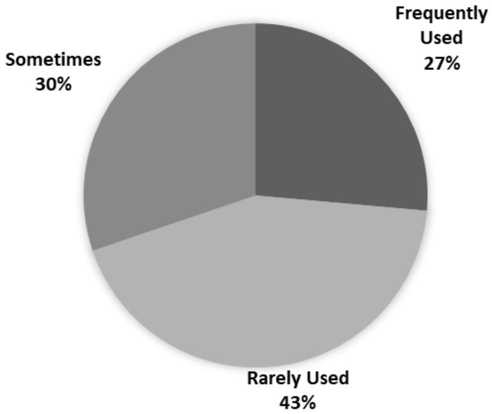

Figure 6. The frequency of use of dye plants in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

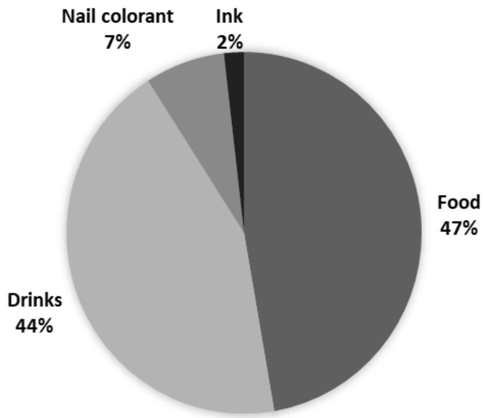

Figure 7. Dye plants utilization in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

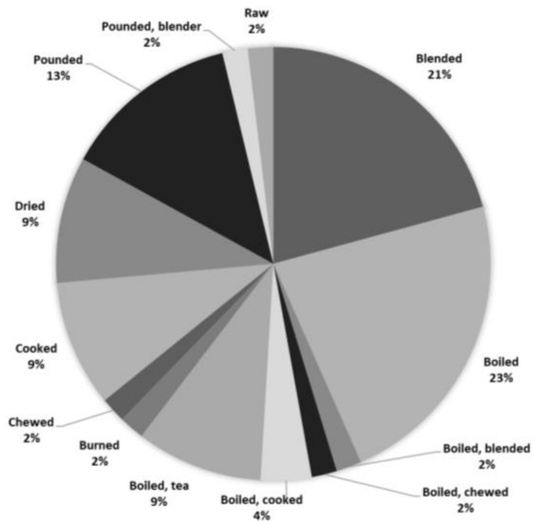

Figure 8. Extraction of dye plants in Kalibawang Sub-district, Wonosobo, Central Java, Indonesia

RFC

The RFC (Relative Frequency of Citation) value in Table 3 is an indicator that describes the level of importance of a plant species based on the frequency of its use by the local community. The higher the RFC value, the more often the plant is used as a natural dye in the life of the Kalibawang community, highlighting their reliance on these plant species. The plant with the highest RFC value is turmeric, with a value of 0.891, followed by pandan with a value of 0.633.

Turmeric has long been the main choice of the Kalibawang community as a natural coloring in food. The curcumin content in turmeric produces a distinctive bright yellow color, giving an attractive appearance to various traditional foods such as yellow rice, *opor* (a traditional cuisine is made from chicken cooked in coconut milk and numerous spices), and wet cakes. Besides beautifying dishes, turmeric also offers health benefits thanks to its safe nature and antioxidant effects. Its abundant availability in Kalibawang is another reason why turmeric is widely used. The plant is easily cultivated in yards or fields without special care, making it a cheap and accessible ingredient for household needs, especially in cooking food.

Meanwhile, pandan is also a plant often used as a natural dye in Kalibawang. Pandan leaves are rich in chlorophyll pigments that produce a soft green color, perfect for enhancing traditional foods such as wet cakes, *jenang* (sticky Indonesian toffee), or drinks. Not only does pandan provide color, but it also produces a distinctive fragrant aroma, enhancing the taste of food. In addition, pandan plants thrive in the Kalibawang environment and can be grown in home yards without intensive care, making it a practical and economical ingredient.

Turmeric and pandan are considered healthier natural dyes than artificial dyes, which often contain harmful chemicals. By utilizing these two plants, people not only maintain the authenticity of the taste and appearance of food but also ensure the health of the family. The combination of benefits, availability, and safety makes turmeric and pandan an important part of the daily life of the Kalibawang community.

Rarity

Table 3 outlines the conservation status of various plant species used as natural dyes based on data from the International Union for Conservation of Nature (IUCN). Only 28 species were recorded on the IUCN Red List. There are a number of species that have Data Deficient (DD) status, such as *Mangifera indica* L., *Brassica rapa* L., *Brassica oleracea* L., *Hylocereus undatus* (Haw.) Britton & Rose, *Camellia sinensis* (L.) Kuntze, *Carica papaya* L., *Garcinia mangostana* L., *Ipomoea batatas* (L.) Lam., *Manihot esculenta* Crantz, *Pandanus amaryllifolius* Roxb. ex Lindl., and *Curcuma longa* L. indicating a lack of sufficient data to assess the level of threat they face. Therefore, further research is needed to more accurately determine their conservation status (Li et al. 2022).

Table 3. Conservation species based on IUCN

Family	Species name	Local name	IUCN	P.106/2018
Achariaceae	*Pangium edule* Reinw.	*Kluwek-pocung*	LC	Not protected
Amaranthaceae	*Beta vulgaris* L.	*Bit*	LC	Not protected
Anacardiaceae	*Mangifera indica* L.	*Mangga*	DD	Not protected
Apiaceae	*Daucus carota* L.	*Wortel*	LC	Not protected
Araceae	*Colocasia esculenta* (L.) Schott	*Talas*	LC	Not protected
Arecaceae	*Arenga pinnata* (Wurmb) Merr.	*Aren*	LC	Not protected
Arecaceae	*Areca catechu* L.	*Pinang*	LC	Not protected
Brassicaceae	*Brassica rapa* L.	*Sawi*	DD	Not protected
Brassicaceae	*Brassica oleracea* L.	*Kol ungu*	DD	Not protected
Cactaceae	*Hylocereus undatus* (Haw.) Britton & Rose	*Buah naga*	DD	Not protected
Theaceae	*Camellia sinensis* (L.) Kuntze	*Teh*	DD	Not protected
Caricaceae	*Carica papaya* L.	*Pepaya*	DD	Not protected
Clusiaceae	*Garcinia mangostana* L.	*Manggis*	DD	Not protected
Convolvulaceae	*Ipomoea batatas* (L.) Lam.	*Ubi ungu, Ubi kuning*	DD	Not protected
Euphorbiaceae	*Manihot esculenta* Crantz	*Singkong*	DD	Not protected
Fabaceae	*Biancaea sappan* (L.) Tod.	*Secang*	LC	Not protected
Lamiaceae	*Tectona grandis* L.f.	*Jati*	EN	Not protected
Lauraceae	*Persea americana* Mill.	*Alpukat*	LC	Not protected
Lauraceae	*Cinnamomum verum* J.Presl	*Kayu manis*	VU	Not protected
Moraceae	*Abelmoschus moschatus* (L.) Medik.	*Waron*	LC	Not protected
Myrtaceae	*Psidium guajava* L.	*Jambu*	LC	Not protected
Pandanaceae	*Pandanus amaryllifolius* Roxb. ex Lindl.	*Pandan*	DD	Not protected
Rubiaceae	*Coffea arabica* L.	*Kopi*	EN	Not protected
Rutaceae	*Citrus ×limon* (L.) Osbeck	*Lemon*	LC	Not protected
Sapotaceae	*Phyllanthus reticulatus* Poir.	*Mangsi*	LC	Not protected
Solanaceae	*Capsicum frutescens* L.	*Cabai*	LC	Not protected
Vitaceae	*Vitis vinifera* L.	*Anggur*	LC	Not protected
Zingiberaceae	*Curcuma longa* L.	*Kunyit*	DD	Not protected

Note: LC: Least Concern. DD: Data Deficient. VU: Vulnerable. EN: Endangered

Some species fall under the Least Concern (LC) category, such as *Pangium edule* Reinw., *Areca catechu* L., *Beta vulgaris* L., *Daucus carota* L., *Colocasia esculenta* (L.) Schott, *Arenga pinnata* (Wurmb) Merr., *Biancaea sappan* (L.) Tod., *Persea americana* Mill., *Abelmoschus moschatus* (L.) Medik., *Psidium guajava* L., *Citrus ×limon* (L.) Osbeck, *Phyllanthus reticulatus* Poir., *Capsicum frutescens* L., and *Vitis vinifera* L. indicating that these species are still relatively safe and do not face major threats. In addition, *Cinnamomum verum* J.Presl (*kayu manis*) has a Vulnerable (VU) status, indicating that although they are not yet critically endangered, they are vulnerable to extinction if threats to their habitat or population are not properly addressed. Other species, such as *Coffea arabica* L., and *Tectona grandis* L.f. are categorized as Endangered (EN), indicating that they are under high threat to survival. None of the species documented in the study area are included in Indonesia's protected species list (P.106/2018). Although IUCN classifies *Cinnamomum verum* (Vulnerable/VU), *Tectona grandis* (Endangered/ EN), and *Coffea arabica* (Endangered/EN), these species are not listed under Indonesian protected species regulations (PP 106/2018).

Discussion

Most widely used plant

The findings of this study indicate that several plant species are employed as dye plants in Kalibawang Sub-district, Wonosobo. There are 53 species, with 37 families. Sapariani et al. (2023) discovered 26 species from 22 families in Raut Muara Village, Sanggau District, which are commonly used as food coloring. The Baiko Yao people of China use 23 species of natural dye plants from 19 families (Hu et al. 2022). The Zingiberaceae family is widely utilized, both in this research and in the study by Sapariani et al. Zingiberaceae is a family of plants that thrive in tropical regions and is extensively cultivated by local communities (Syafira et al. 2024). The most commonly used plants are turmeric, pandan leaves, and green grass jelly leaves. Turmeric, pandan leaves, and *cao* are plants that are most often used as natural coloring sources, especially as food coloring in various regions, especially in the Asian Region.

Turmeric (*C. longa*) is a shallow-rooted Herbaceous plant with thick and fleshy rhizomes (Nwaekpe et al. 2015). Turmeric is popular for its curcumin content, which provides a long-lasting yellow color that is often used as a natural food coloring. In addition to the curcumin content, turmeric also contains 3-5% essential oil and 2.5-6% yellow pigment (Bora et al. 2019). The part that is used as a colorant is the rhizome. In the four villages visited, turmeric is often used as a natural food coloring for their dishes. In Kalibawang Sub-district, Wonosobo, a very famous turmeric-based dish is *tempe kemul*, which is processed fried tempeh with flour, usually mixed with turmeric to give it a yellow color (Figure 9.A). Turmeric has anticancer, antidiabetic, antioxidant, and anti-inflammatory properties in addition to being utilized as a food coloring (Iweala et al. 2023; Nuraini et al. 2024).

Another plant that is widely used by the community is pandan leaves (*P. amaryllifolius*). Pandan plants in Indonesia are known as *pandan wangi* or *pandan rampe* (Dalimunthe 2022). Pandan leaves produce a distinctive green color so they are widely used to make foods such as *jenang* (Figure 9.B). Pandan is processed by boiling to extract the juice and then mixing it with the dough. Pandan is widely used as a food coloring in Southeast Asia. Pandan can also be utilized in medicine, cosmetics, cockroach repellent, and traditional ceremonies (Wang et al. 2024).

In addition to turmeric and pandan leaves, *cao* leaves are also popular in the community for their use as natural dyes. Green grass jelly (*C. barbata*) is very much found in various regions, especially in Indonesia (Sofyan et al. 2020). Three types of *C. barbata* are known to the Indonesian people, namely green grass jelly, black grass jelly, and shrub grass jelly. Because the leaves are thin and limp and easy to squeeze to produce a gel, Indonesian people love *C. barbata*. In Kalibawang Sub-district, Wonosobo, green grass jelly leaves are widely used as a natural colorant for green or black fresh drinks, such as ice jelly drinks (Figure 9.C). The use of green grass jelly leaves as a natural coloring agent is increasingly rare because traditional culture is slowly fading in the community, and the population of green grass jelly is decreasing in the area due to lack of cultivation, and its existence is starting to become difficult to find.

Utilization of plants

They use natural ingredients such as pumpkin or *waluh* (*Cucurbita moschata* (Duchesne) Duchesne ex Poir.) and sweet potato/purple yam for *bolu kukus* or sponge cakes (Figure 9.D), and teak leaves and guava or *jambu* leaves (*Psidium guajava* L.) for *gudeg*, a Javanese food made from young jackfruit (*Artocarpus heterophyllus* Lamk.) and cooked with coconut milk (*Cocos nucifera* L.) and spices, which are usually served in traditional ceremonies (Figure 9.E). Foods that use plants as natural colors are usually served at celebrations and weddings (Luu-Dam et al. 2016). This is an important component of the culinary tradition that has rich cultural significance. Pumpkin and purple yam are used in making sponge cakes to provide orange and purple colors. These ingredients not only add color but also contribute to the nutritional value of the dish.

Meanwhile, teak leaves and guava leaves are also used as natural red color producers to make *gudeg* by steaming young jackfruit along with the leaves until the color turns brownish red. The leaves, in addition to their color-producing properties, are rich in antioxidants, adding a healthful element to the dish. Traditional communities have unique ways of utilizing their natural resources. This tradition is still inherent in the Kalibawang Sub-district, Wonosobo community, which also shows that there is a balance between taste, cultural meaning, and, of course, aesthetics. The use of natural dyes is also a way to respect nature and the heritage of the ancestors. Natural dyes incorporated into food are useful as food protectors because these natural ingredients have many benefits (Echegaray et al. 2023). This also shows that Kalibawang people prefer to use natural ingredients and do not contain synthetic and harmful chemicals, which makes food safer and certainly sustainable for daily life.

Figure 9. Some utilization of natural dye plants in Kalibawang, Wonosobo, Indonesia. A. Turmeric for food, B. pandan leaves for food, C. *Cao* leaves for beverages, D. Pumpkin and purple yam for *bolu kukus* cake, E. *Pacar air* for nail colorant

In addition to being used as natural food coloring, the people of Kalibawang Sub-district, Wonosobo utilize dye plants for beverages. Beyond quenching thirst, these natural dyes enhance the visual appeal of drinks, improve palatability, and provide health benefits. The tradition of using dye plants for beverages has been passed down through generations and remains preserved in Wonosobo, particularly in the Kalibawang Sub-district. The local community believes that certain plants can be brewed into beverages that promote vitality and improve well-being. One notable example is *wedang uwuh*, a traditional herbal drink blended from various plant parts, such as ginger, sappanwood, clove, cinnamon, etc. valued for its therapeutic properties. This practice aligns with the findings of Tsurayya et al. (2025), who observed that *wedang uwuh* is still commonly traded in Wonosobo's traditional markets and widely consumed by the community for its direct health benefits.

Benefits

Wonosobo is an area rich in natural resources, including agriculture (Larasati et al. 2024). Wonosobo's climate tends to be cool because of its location in the mountains and the fertility of the soil, which can support plant growth. Kalibawang Sub-district is located in a hilly area with several mixed gardens and producing forests (Ndofah and Santosa 2023; Herdananta et al. 2024). Mixed-garden areas outperform other types of land uses. Pulungan et al. (2017) found that mixed gardens have a beneficial relationship to enhanced soil nutrients, which has an impact on agricultural sustainability. Rough slopes further increase the risk of landslides. Hence mixed gardens are the best option (Negari et al. 2023). The Kalibawang community uses mixed gardens to grow valuable plants, including dye plants.

The use of turmeric, pandan leaves, and green grass jelly leaves as natural dyes is influenced by various factors, ranging from the availability of raw materials to the traditions of local communities that have been passed down from generation to generation. The majority of Wonosobo's population are farmers, so natural ingredients from plants are very easy to find there and will continue to be cultivated. In addition, their easy availability makes them the right choice that is practical and economical to use as a natural food coloring. The use of the three types of plants as food coloring in Kalibawang Sub-district, Wonosobo is related to the culinary tradition in the area. Many traditional foods from Wonosobo use turmeric, pandan, and green grass jelly as natural dyes. The uses of plants vary for each community, depending on each community's traditional knowledge of the matter. Traditional knowledge is the knowledge that is passed down from one generation to the next (Jamaludin 2023). Likewise, in the community of Kalibawang, the purpose of use and the reasons for use are based on their hereditary traditions, which are not modified due to a lack of environmental education and limited knowledge.

Some plants, in addition to serving as food coloring, can be used as textile dyes. For example, turmeric can be utilized as a fabric dye (Inyom et al. 2024) and an alternative to eosin (Suryawanshi et al. 2017), whilst pandan has the potential as a natural sensitizer for solar cells (Al-Alwani et al. 2017) and a green dye for fabrics

(Berghuis et al. 2024). Because natural dyes yield delicate hues, mordants are required to keep the color pigments in fabrics intact. Color changes will occur when using different types of mordants. Natural mordants, such as alum or plant extracts, are chosen since they are safer for the environment (Singh and Singh 2018). In addition to the type of mordant, light exposure, washing and rubbing methods, and fabric type all have an impact on color fastness (Saeed et al. 2023).

In the four villages, Dempel, Karangsambung, Kalialang, and Mergolangu, plants were use as small dyes for cosmetics and textiles. The use of garden balsam or *pacar air* as a natural nail colorant is still frequently used by children as a medium for playing (Figure 13). According to research (Sapariani et al. 2023), color-producing plants are not only used as food dyes but also as dyes for cosmetics and textiles such as water henna, noni, and henna inai. This research is certainly contrary to this, where color-producing plants are only limited to being used as food coloring and have not penetrated the textile and cosmetic fields. The field of cosmetics and textiles is a strong sector in the industry, given the increasing attention to body image (Guerra et al. 2018). This could be an opportunity to create jobs for local people and improve the region's economy. Better environmental education and the development of environmentally friendly and accessible technologies are needed to address this gap.

The rarity of natural dyes

This research found that several plants are conserved to maintain their existence. Based on the IUCN Red List, some plants are already categorized as Endangered (EN). The International Union for Conservation of Nature (IUCN) Red List is a global authority on the risk of global species extinction (Betts et al. 2019). Endangered (EN) is a category that has the highest risk and requires action to prevent the extinction of the species (Geyle et al. 2018). Plants that fall into this category are *C. arabica* and *T. grandis*. Previous research conducted by Seran et al. (2024) on dye plants in Belu District, Indonesia also found the use of T. grandis (EN) as a traditional fabric dye.

Tectona grandis or teak, a native plant of South and Southeast Asia, has long been introduced to Indonesia since the 14th century and now grows abundantly in central and eastern Java (Verhaegen et al. 2010). Javanese people, including in Kalibawang, consider teak as a valuable asset and often plant it in their yards (Roshetko et al. 2013). Teak is also considered as a family savings, which helps avoid excessive logging considering its long growing time. In addition to its wood for furniture, teak leaves are also used as food wrappers and coloring. This non-timber utilization is a way to preserve the existence of teak in Kalibawang. The agroforestry system practiced in Kalibawang, which integrates teak and pine (*Pinus merkusii* Jungh. & de Vriese) with seasonal crops such as rice and cassava, has been recognized as a sustainable approach to preserving teak populations in Java. Furthermore, teak contributes to soil fertility enhancement and provides significant economic benefits (Pachas et al. 2019; Herdananta et al. 2024). To further optimize conservation efforts, we recommend expanding this agroforestry model by incorporating *C. arabica* and *C. verum*. Both species are currently under conservation concern, and agroforestry presents a viable strategy to ensure their sustainability while maintaining ecological and economic productivity.

Natural dyes challenges

The use of plants as natural dyes does have many advantages because it has health benefits beyond the ability to color (Martins et al. 2016). However, despite the health benefits of natural colorants, the use of plants as colorants has challenges in its application (Li et al. 2022). Inconsistent color in plants can be a challenge because colors in plants have poor stability against heat, light, and pH conditions. In addition, the storage period of natural dyes tends to be short before the dyeing process (Eskak and Salma 2020). Based on interviews the key informants and FGDs, the availability of raw materials is also a challenge, as many coloring plants are seasonal, and the rarity of different plants varies. The last challenge is higher production costs than the use of synthetic dyes because it requires higher costs for the process of cultivating plants and processing them to produce intense colors. The solution to overcome these challenges is to cultivate dye plants with efficient and sustainable cultivation techniques, implement color quality standardization so that colors are better maintained, and establish collaboration between producers, researchers, the government, and stakeholders in order to maintain the availability of dye plants.

Kalibawang Sub-district boasts distinctive community-managed specialty products, notably *leye* (a cassava-based rice analogue) and palm sugar (derived from *Arenga pinnata*), both cultivated and preserved by local communities due to their high economic value. Recognizing the need to maintain product quality and quantity, farmers have implemented conservation measures, primarily through village-level farmer groups that serve as platforms for knowledge exchange, agricultural improvement, and value enhancement while preserving product integrity. Previous research by Herdananta et al. (2024) demonstrates these farmer groups' significant role in developing human capital and supporting local plant conservation initiatives in Kalibawang.

In conclusion, we documented 53 species from 37 families of plants that are used as natural dyes by the people of Kalibawang Sub-district. Turmeric is the most valuable species of dye plants in this area. Especially at traditional ceremonies such as celebrations and weddings, they need to present food colored with natural dyes from plants. This traditional knowledge about natural dyes and how to process them is passed down from generation to generation. However, this traditional knowledge is in danger of being lost due to lifestyle changes and the younger generation's lack of preservation of local culture. Therefore, it is necessary to create awareness to preserve and systematize this traditional knowledge for the younger generation.

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Optimizing bitter gourd (*Momordica charantia*) production through carrageenan-fertilizer synergy

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Abstract. *Anayatin AB, Pabiona MG, Cristobal JU, Tan R, Labajo JRN. 2025. Optimizing bitter gourd (Momordica charantia) production through carrageenan-fertilizer synergy. Nusantara Bioscience 17: 169-177.* This study evaluates the synergistic effects of combining radiation-modified Carrageenan Plant Growth Promoter (PGP) with the Recommended Rate of Inorganic Fertilizer (RRIF) on the growth, nutrient dynamics, and yield of *Momordica charantia* L. (bitter gourd or *ampalaya*). Through a Randomized Complete Block Design (RCBD) replicated three times, treatments integrating RRIF and Carrageenan PGP were tested for their impact on plant height, soil properties, nutrient content, and fruit morphology. Results revealed that *Full RRIF + 4.5L ha^{-1} Carrageenan PGP* significantly enhanced fruit yield (64.50 tons ha^{-1}) and achieved the highest return on investment (ROI: 435.30%), underscoring its economic viability. Meanwhile, ½ RRIF + 9L ha^{-1} Carrageenan PGP produced the largest fruit diameter (49.44 mm) and highest total nitrogen (2.60%) and crude protein (16.24%) content, highlighting its role in improving nutritional quality. Mid-growth plant height (29 DAT) exhibited strong correlations with fruit weight (r = 0.475), length (r = 0.482), and diameter (r = 0.647), emphasizing the critical role of vegetative vigor in later stages for optimizing fruit development. Notably, earlier flowering (26 DAT under Full RRIF) correlated negatively with fruit weight (r = -0.558) and length (r = -0.532), suggesting accelerated reproductive phases enhance resource allocation to fruit traits. Carrageenan PGP applications stabilized soil moisture (92.30-93.82%) and maintained adequate phosphorus/potassium levels while significantly boosting soil organic matter (1.48%) and nitrogen content (0.074%) in Full RRIF treatments. The stability of these soil parameters, coupled with enhanced nutrient partitioning efficiency, positions Carrageenan PGP as a sustainable alternative to conventional fertilization. This study advocates for integrating Carrageenan PGP with reduced inorganic fertilizers to balance productivity, soil health, and economic returns. Future research should explore long-term impacts on soil microbial diversity, carbon sequestration, and scalability across diverse agroecological zones to refine protocols for sustainable *M. charantia* cultivation.

Keywords: Carrageenan, kappa-Carrageenan, *Momordica charantia*, nutrient content, plant growth promoter, soil properties

Abbreviations: OM: Organic Matter, PGP: Plant Growth Promoter, RMKC: Radiation-Modified Kappa-Carrageenan, ROI: Return on Investment, RRIF: Recommended Rate of Inorganic Fertilizer

INTRODUCTION

Through the years, farmers have optimized yield using inorganic fertilizers alone. Typically, the essential nutrients from fertilizer inputs form part of the optimum production of crops. Still, it also contributes to a significant portion of total crop input costs, leading to many farmers having difficulty. Still, they also contribute to a significant portion of total crop input costs, leading to many farmers having difficulty financing their agricultural inputs. Managing fertilizer inputs is essential as it can constitute a considerable portion of total crop input costs. Research also showed that the dependent use of inorganic fertilizers as a source of nutrients and nourishing agricultural lands resulted in soil degradation. The soil's pH level has changed significantly due to the excessive application of inorganic fertilizers. Most agricultural lands have become unproductive and infertile.

The soil degradation problem calls for mandatory fertilizer application in growing crops. Today, half of the world's population depends on synthetic fertilizers, especially nitrogen (Erisman and Sutton 2008). However, it was mentioned by Dikitanan et al. (2017) that in addition to being very costly, continuous use of synthetic fertilizer could also damage the environment by building up toxic and hazardous chemicals in soil, air, and groundwater, thereby threatening biodiversity. Thus, organic farming is suggested by (Mavuri et al. 2020) to help attain the Sustainable Development Goal (SDG-15) (life on land).

Recent studies have emphasized the advantages of combining organic and inorganic fertilizers. Combining these fertilizers has enhanced soil health by improving microbial biomass, leading to more sustainable soil productivity (Tao et al. 2015; Ozlu et al. 2019). This integrated approach is essential in vegetable production, where high nutrient demands necessitate a balance between maintaining soil fertility and achieving high yields. Experiments conducted across various countries have demonstrated that relying solely on inorganic fertilizers is insufficient for sustaining soil productivity under intensive cropping systems. The need for a continued increase in production and per-hectare yield of vegetables requires increased nutrients (Fernández et al. 2022; Miñoza et al. 2023). The results of several studies on inorganic and organic fertilizers showed that very intensive farming systems could not be sustained by inorganic fertilizers alone (Ilahi et al. 2020; Qaswar et al. 2020). Therefore, combined organic and inorganic fertilizers fix that issue by providing proper nutrients as necessary; essential macro elements like NPK can help the plants grow quickly and strongly (Shaji et al. 2021; Francisco et al. 2022).

An emerging area of interest is plant-derived extracts, such as seaweed-based fertilizers, to reduce dependence on synthetic fertilizers. In the Philippines, the Department of Science and Technology's Philippine Nuclear Research Institute (DOST-PNRI) developed a seaweed emulsion called Carrageenan, derived from red edible seaweed. When this undergoes radiation, natural bioactive agents develop. The process produces oligo-carrageenan polymers with shorter chains, which have been shown to have antibiotic, antioxidant, and Plant Growth-Promoting (PGP) properties and are high in Potassium (K), Magnesium (Mg), and Calcium (Ca), which help to improve the growth, development, and immune system of rice. According to (Gatan et al. 2020), the red seaweed *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva is a significant source of κ-carrageenan. It contains Nitrogen, Potassium (K2O), Calcium, Manganese, Copper, Zinc, Magnesium (MgO2), Iron, and gibberellic acid. Research conducted by various Philippine agricultural institutions has demonstrated the effectiveness of Radiation-Modified Kappa-Carrageenan (RMKC) in enhancing crop yields. Studies have shown that applying RMKC significantly increases the yield of rice, peanuts, and mung beans (Abad et al. 2018a; Gatan et al. 2020). These findings suggest that Carrageenan Plant Growth Promoter (PGP) can be a valuable addition to the range of inputs used in farming, offering a cost-effective and environmentally friendly alternative to traditional inorganic fertilizers.

This study aims to evaluate the impact of Carrageenan PGP on the growth and yield performance of *M. charantia*. Given the rising costs of inorganic fertilizers, there is a pressing need for alternative solutions that optimize crop yields while being economically viable and environmentally sustainable. Specifically, this research seeks to (i) assess the growth and yield of *M. charantia* Mestiza F1 under varying combinations of inorganic fertilizer and Carrageenan PGP; (ii) determine which combination rate offers the highest return on investment; (iii) evaluate the post-harvest chemical properties of the soil; and (iv) analyze the nutrient content of *M. charantia* fruits in response to different treatment combinations. This study will provide essential scientific data to support *M. charantia* farming with innovative and sustainable agricultural practices.

MATERIALS AND METHODS

Study area

The study was conducted at Purok Mabuhay, Barangay San Isidro, Koronadal City, South Cotabato, Philippines, from April 14 to August 9, 2021, as shown in Figure 1. The area is approximately 6°26'34.4"N 124°51'21.6" E on the Island of Mindanao, with an elevation of about 227.4 meters above sea level (masl). Koronadal City has a tropical climate and significant yearly rainfall, even during the driest months.

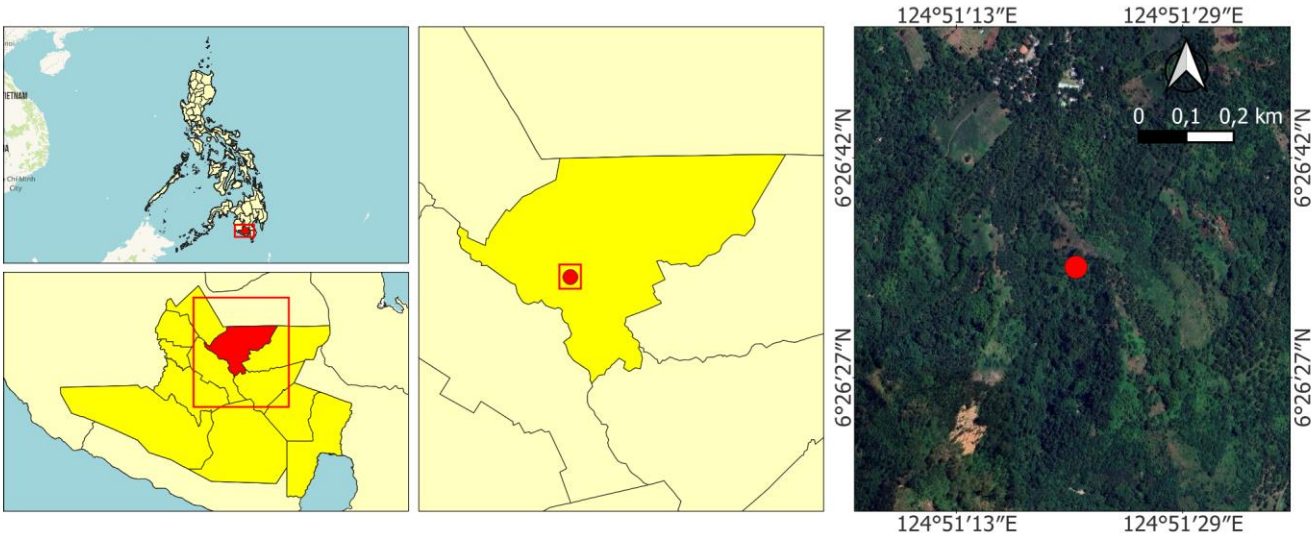

Figure 1. Location of the study site at Koronadal City, South Cotabato, Philippines

Experimental design and treatments

The study was conducted in an area of 870 m^2. Soil samples were taken from the experimental area for initial soil characterization one 1 month before transplanting. Composite soil samples were analyzed at the Soil and Plant Analysis Laboratory (SPAL) at the Department of Soil Science, College of Agriculture, Central Mindanao University, Musuan, Maramag, Bukidnon, to obtain the Recommendation Rate of Inorganic Fertilizer (RRIF). The study was laid out in a Randomized Complete Block Design (RCBD), with seven (7) treatments replicated three (3) times. Mestiza F1 hybrid *ampalaya*/bitter gourd seeds (*Momordica charantia* L.) were used. The experimental area was divided into three (3) blocks, which measured three (3) meters in width by six (6) meters long, with a buffer zone of two (2) meters distance observed between treatments and each block. Varying amounts of Carrageenan PGP per hectare served as a variable. VitalGro® Carrageenan PGP is a radiation-modified plant growth promoter developed by the Philippine Nuclear Research Institute (PNRI) under the Department of Science and Technology (DOST). It is derived from *K. alvarezii*, a red edible seaweed ("guso" or "gulamang dagat") native to the Philippines (Mariano Marcos State University 2018; Philippine Nuclear Research Institute 2022). The treatments were as follows: T_1- Absolute Control; T_2- Full RRIF (60-30-50); T_3- ½ RRIF; T_4- 9 L ha^{-1} Carrageenan PGP; T_5- ½ RRIF + 9L ha^{-1} Carrageenan PGP; T_6- ½ RRIF + 4.5L ha^{-1} Carrageenan PGP; and, T_7- Full RRIF + 4.5L ha^{-1} Carrageenan PGP.

Cultural and management practices

The cultivation practices detailed for *M. charantia* (bitter melon) encompass a comprehensive approach to optimize growth and yield. The preparation of the planting area involved thorough plowing and harrowing, creating well-prepared beds covered with plastic mulch to maintain soil moisture and suppress weeds. Seedling preparation was meticulous, with sowing in trays containing sterilized media and precautionary fungicide treatment to prevent damping off. Transplanting followed rigorous protocols, including vermicompost application for nutrient enrichment, precise spacing, and immediate watering for establishment. Fertilization strategies were tailored to soil analysis, employing deep placement techniques to minimize nutrient loss, supplemented by Carrageenan Plant Growth Promoter (PGP) application was done as foliar in the 3rd, 5th, 7th, 9th, and 11th week after transplanting; it was applied separately in the application of inorganic fertilizer, for enhanced growth and stress tolerance. Trellising and pruning techniques were used to promote vine growth and fruit development (Khan et al. 2022). Integrated pest management techniques were also used to control pests and diseases. Regular water management, weeding, and harvesting protocols ensured optimal plant health and fruit quality throughout the cultivation cycle, demonstrating a holistic approach to sustainable *M. charantia* production.

Collection and preparation of soil and fruit samples for analysis

After harvest, a soil sample was collected between hills in each experimental plot. Soil sampling was done from the surface to 10-15 cm depth. The collected soil sample was placed in a plastic bag and air-dried at room temperature for at least one week. The soil sample was sieved using a 2 mm mesh sieve and brought to the Soil and Plant Analysis Laboratory (SPAL) for soil analysis. Fruit samples were taken from each experimental plot in the eighth harvest; they were cleaned and dried immediately using dry clothes or tissue paper. Drying was done using a forced draft oven with a temperature of 65-70°C. The sample was pulverized and dried again overnight at 70°C before analysis.

The following soil properties were determined from the soil samples: soil pH using the Potentiometric Method (1:5 soil-water ratio) as described by Biddle (1997); OM content using the Walkley-Black Method (PCARRD 1991); extractable phosphorus (P) using the Bray P2 method (PCARRD 1991); exchangeable potassium (K) using 1 N NH4OAc at pH 7; and initial soil texture analysis. For the plant samples, the analyses included total Nitrogen (N) using the Micro Kjeldahl method, total phosphorus (P) using the Dry Ashing Vanadomolybdate method with a UV-VIS Spectrometer, and total potassium (K) using Dry Ashing and Atomic Absorption Spectroscopy (AAS) (PCARRD 1991).

Cost and return analysis

The economics of the production process was determined by computing the Return on Investment (ROI). The net income was derived from the difference between the gross income and the total cost of production used in the study. The ROI was computed using the formula. ROI Net income divided by the cost of production × 100. Where: Net income = Gross income – Total cost of production.

Statistical analysis

Data was analyzed using ANOVA in a Randomized Complete Block Design (RCBD). The HSD Test was used to compare treatment means that differed significantly. All statistical analyses were performed using the IRRI STAR Nebula software.

RESULTS AND DISCUSSION

Horticultural and characteristics of *Momordica charantia* as affected by the treatment

Plant height of Momordica charantia

The plant height of *M. charantia* at 15 and 29 days after transplanting, as influenced by the different treatment combinations, is presented in Table 1. The result showed no variation among the treatments on the plant height at 15 days after transplanting, with mean values ranging from 45.03 to 51.75 cm. However, the T_1 absolute control obtained a highly significant difference among the treatments, while in T_2, T_3, T_4, T_5, T_6, and T_7, no significant differences were observed among the treatment means on the height of the plants at 29 days after transplanting. In contrast, the plants applied with ½ RRIF + 9L ha^{-1} Carrageenan PGP (T_5), ½ RRIF + 4.5L ha^{-1} Carrageenan PGP (T_6), Full RRIF + 4.5L ha^{-1} Carrageenan PGP (T_7), 9 L ha^{-1} Carrageenan PGP (T_4), Full RRIF (T_2), and ½ RRIF (T_3) produced the tallest plants having mean values ranging from 227.19 to 231.14 cm, respectively. The shortest plants were found on the control plants (T_1) with a mean value of 183.69 cm.

Despite insignificant variances among treatments, the application of Carrageenan PGP caused greater height than that without Carrageenan PGP. The result indicates that Carrageenan PGP is promising because its effect is similar to that of inorganic fertilizer regarding *M. charantia* plant height. The results are consistent with the research of Khan et al. (2009), which states that seaweed formulations are widely used as biostimulants in crop production and that seaweed products have growth-stimulating properties. These materials promote plant growth when applied in small quantities. The cellular metabolism of treated plants is influenced by seaweed components, including macro- and microelements, amino acids, vitamins, cytokinins, auxins, and compounds that resemble abscisic acid (ABA). This increases plant growth and yield (Kocira et al. 2019; Chaturvedi et al. 2022).

Days of the first flowers appearance

The days of the first flower appearance after transplanting, as influenced by the different treatment combinations, are presented in Table 1. There is no significant difference among treatments. The first flower appearance was observed 26 days after transplanting (T2) with the application of the full Recommended Rate of Inorganic Fertilizer (RRIF). Among the seven treatments, the last flower appearance was observed in Treatment 1 (absolute control), 31.67 days after transplanting. The same flower appearance was observed 27.33 days after transplanting in the treatment T4, the application of 9 L ha^{-1} Carrageenan PGP, and T6, the application of ½ RRIF + 4.5L ha^{-1} Carrageenan PGP.

Average weight, length, diameter, and fruit yield of *Momordica charantia*

Table 2 presents *M. charantia* fruits' average weight, length, and diameter, as influenced by the different treatment combinations. The results showed no significant difference between treatments regarding *M. charantia* fruits' average weight and length. The average weight of *M. charantia* fruits ranges from 210.33 grams to 240.48 grams. In contrast, the average size of *M. charantia* fruits ranges from 255.43 to 277.50 mm.

The combination of treatments influenced the fruit diameter. The plants applied with ½ RRIF + 9L ha^{-1} Carrageenan PGP (T_5) and full RRIF (T2) applied the biggest, most significant mean values of 49.44 and 48.91 mm. It was followed by ½ RRIF + 4.5L ha^{-1} Carrageenan PGP (T6), Full RRIF + 4.5L ha^{-1} Carrageenan PGP (T_7), ½ RRIF (T_3), and 9 L ha^{-1} Carrageenan PGP (T_4) with mean values of 48.63, 48.46, 47.24, and 46.36 mm. The smallest diameter was observed at absolute control (T_1) with 44.68 mm. As a result of its composition of macro and microelements, vitamins, cytokinins, auxins, and abscisic acid (ABA)-like growth substances, Carrageenan PGP was found to have a significant impact on the fruit development of *M. charantia*. This enhanced growth and crop yield in treated plants (Butay 2017; Mondal et al. 2020; Chaturvedi et al. 2022).

It was generally observed that Treatment 5, the application of ½ Recommended Rate of Inorganic Fertilizer (RRIF) plus 9 liters of Carrageenan PGP per hectare, obtained the highest average length of 277.50 mm and diameter of 49.44 mm. In contrast, T_2- Full application of the Recommended Rate of Inorganic Fertilizer (RRIF) alone recorded the highest average weight of 246.90 g. In comparison, absolute control (T_1) acquired the lowest average weight, length, and diameter of 210.33 g, 255.43 mm, and 44.68 mm, respectively.

Table 1. Plant height (cm) of *Momordica charantia* plant 15 and 29 DAT, and days of first flower appearance as affected by the different treatment combination

Treatment	Plant height 15 (DAT)	Plant height 29 (DAT)	Days of first flowers appearance (DAT)
T_1- Absolute Control	47.83±1.83	183.69±3.94 b	31.67±2.52
T_2- Full RRIF	45.03±2.35	228.33±19.74 a	26.00±2.65
T_3- ½ RRIF	48.86±5.39	227.19±14.11 a	29.00±3.61
T_4- 9 L ha^{-1} Carrageenan PGP	47.03±2.71	229.97±5.34 a	27.33±1.53
T_5- ½ RRIF + 9L ha^{-1} Carrageenan PGP	48.61±3.17	231.14±10.40 a	28.67±3.79
T_6- ½ RRIF + 4.5L ha^{-1} Carrageenan PGP	49.11±2.36	230.78±9.76 a	27.33±5.13
T_7- Full RRIF + 4.5L ha^{-1} Carrageenan PGP	51.75±5.81	230.56±32.97 a	28.00±5.57
Mean	48.32±2.06	223.09±17.43	28.29±1.79
F-test	ns	*	ns
CV, %	7.14	4.87	12.46

Note: Data are presented as mean ± standard deviation (n = 3 replications). Means within a column followed by the same letter are not significantly different at *p* < 0.05 (Tukey's HSD). RRIF: Recommended Rate of Inorganic Fertilizer; PGP: Plant Growth Promoter; ns: not significant; *: significant; CV: Coefficient of Variation

Table 2. Average weight, length, diameter, fruit yield, and % ROI of *Momordica charantia* fruits as affected by the different treatment combinations

Treatment	Average fruit weight (g)	Average fruit length (mm)	Average fruit diameter (mm)	Fruit Yield (tons ha^{-1})	% ROI
T_1- Absolute Control	210.33±2.89	255.43±1.74	44.68±0.51 b	28.49±5.21 d	154.25±46.50d
T_2- Full RRIF	246.90±15.03	276.20±6.41	48.91±1.55 a	53.48±10.98 ab	371.19±128.42ab
T_3- ½ RRIF	228.47±7.16	269.15±4.32	47.24±1.38 ab	41.62±2.88 bc	252.14±25.64bcd
T_4- 9 L ha^{-1} Carrageenan PGP	224.01±32.05	263.51±20.61	46.36±1.99 ab	34.01±6.34 cd	191.91±53.89cd
T_5- ½ RRIF + 9L ha^{-1} Carrageenan PGP	240.10±7.05	277.50±2.00	49.44±1.69 a	44.87±7.79 bc	279.11±64.85bcd
T_6- ½ RRIF + 4.5L ha^{-1} Carrageenan PGP	239.42±7.84	276.98±5.24	48.63±1.02 ab	49.62±7.65 b	317.29±65.73abc
T_7- Full RRIF + 4.5L ha^{-1} Carrageenan PGP	240.48±7.09	275.82±7.89	48.46±2.16 ab	64.50±6.17 a	435.30±51.62a
Mean	232.81±12.62	270.66±8.46	47.67±1.69	45.23±12.10	287.53±98.77
F-test	ns	ns	*	**	**
CV, %	5.59	3.21	3.06	9.71	17.17

Note: Data are presented as mean ± standard deviation (n = 3 replications). Means within a column followed by the same letter are not significantly different at *p* < 0.05 (Tukey's HSD). RRIF: Recommended Rate of Inorganic Fertilizer; PGP: Plant Growth Promoter; ns: not significant; *: significant; **: highly significant; CV: Coefficient of Variation

Table 3. Soil pH, OM, and total nitrogen content of the soil after harvest as affected by the different treatment combinations

Treatment	pH	Organic matter %	Total Nitrogen %	Extractable P (mg kg $^{-1}$)	Exchangeable K (cmol kg $^{-1}$)
T_1- Absolute Control	6.61±0.11	0.59±0.02 c	0.030±0.00 c	98.05±4.30	0.71±0.13
T_2- Full RRIF	6.57±0.10	1.48±0.14 a	0.074±0.01 a	108.87±9.34	0.64±0.14
T_3- ½ RRIF	6.54±0.10	0.96±0.08 b	0.048±0.00 b	107.50±8.87	0.77±0.01
T_4- 9 L ha^{-1} Carrageenan PGP	6.82±0.08	0.90±0.02 b	0.045±0.01 b	111.27±7.45	0.67±0.03
T_5- ½ RRIF + 9L ha^{-1} Carrageenan PGP	6.71±0.25	0.69±0.03 c	0.034±0.01c	113.30±4.74	0.79±0.08
T_6- ½ RRIF + 4.5L ha^{-1} Carrageenan PGP	6.81±0.05	0.58±0.01 c	0.029±0.00 c	110.20±6.08	0.85±0.17
T_7- Full RRIF + 4.5L ha^{-1} Carrageenan PGP	6.85±0.03	0.98±0.05 b	0.049±0.00 b	109.32±7.50	0.82±0.10
Mean	6.07±0.13	0.88±0.31	0.044±0.01	108.36±4.91	0.75±0.08
F-test	ns	*	*	ns	ns
CV, %	1.66	7.67	7.66	5.67	14.20

Note: Data are presented as mean ± standard deviation (n = 3 replications). Means within a column followed by the same letter are not significantly different at *p* < 0.05 (Tukey's HSD). RRIF: Recommended Rate of Inorganic Fertilizer; PGP: Plant Growth Promoter; ns: not significant; *: significant; CV: Coefficient of Variation

Return on investment

The return on investment of *M. charantia*, as influenced by the different treatment combinations, is presented in Table 2. The result showed that there is a highly significant difference among treatments. Treatment 7, applying full RRIF plus 4.5 liters per hectare of Carrageenan PGP, obtained a fruit yield of 64.50 tons ha^{-1} with a corresponding return on investment of 435.30%. The lowest return on investment of 154.25% was recorded in treatment 1 (absolute control), with a corresponding yield of 28.49 tons ha^{-1}. Treatment 7 obtained a highly significant difference among the treatments. According to Abad (2018b), carrageenan PGP increased rice yields by as much as 30% compared to average farmer practices. In general findings, carrageenan PGP is also effective in *M. charantia* production as a supplement.

Chemical properties of the soil after harvest

Soil properties, including pH, OM percentage, total nitrogen percentage, extractable phosphorus (P), and exchangeable potassium (K) affected by different treatments, are shown in Table 3. The treatments involve varying Reduced-Rate Inorganic Fertilizer (RRIF) and Carrageenan Plant Growth Promoter (PGP) combinations. The results show significant differences in OM and total nitrogen content among the treatments. At the same time, pH, extractable phosphorus, and exchangeable potassium remain unaffected. Specifically, the application of Full RRIF (T2) significantly increased OM (1.48%) and total Nitrogen (0.074%) compared to the control and other treatments. Treatments involving half RRIF with or without PGP showed moderate improvements in these soil properties. However, extractable phosphorus and exchangeable potassium did not show significant differences across treatments, suggesting that these elements are stable irrespective of the applied treatments.

The variations in soil OM and total Nitrogen, as shown in Table 3, are crucial for the growth and development of *M. charantia* (bitter melon). This research underscores the importance of soil OM and nitrogen content, mainly observed in the Full RRIF treatment (T2), which can enhance soil fertility and improve nutrient availability, essential for *M. charantia* plants' strong growth. Higher OM enhances soil structure, water retention, and microbial activity, creating a more conducive environment for root development and nutrient uptake. The significant increase in total Nitrogen, a critical component of chlorophyll and amino acids, can promote vigorous vegetative growth, leading to healthier plants and potentially higher yields.

Table 4. Moisture content and dry matter (%) of *Momordica charantia* fruits as affected by the different treatment combinations

Treatment	Moisture content (%)	Dry matter (%)
T_1- Absolute Control	93.63±0.81	6.37±0.81
T_2- Full RRIF	93.31±0.75	6.69±0.75
T_3- ½ RRIF	92.30±0.95	7.70±0.95
T_4- 9 L ha^{-1} Carrageenan PGP	93.82±0.54	6.18±0.54
T_5- ½ RRIF + 9L ha^{-1} Carrageenan PGP	93.74±0.44	6.26±0.44
T_6- ½ RRIF + 4.5L ha^{-1} Carrageenan PGP	93.52±0.26	6.48±0.26
T_7- Full RRIF + 4.5L ha^{-1} Carrageenan PGP	93.58±0.76	6.42±0.76
Mean	93.41±0.52	6.59±0.52
F-test	ns	ns
CV, %	0.7075	10.03

Data are presented as mean ± standard deviation (n = 3 replications). Means within a column followed by the same letter are not significantly different at *p* < 0.05 (Tukey's HSD). RRIF: Recommended Rate of Inorganic Fertilizer; PGP: Plant Growth Promoter; ns: not significant; CV: Coefficient of Variation

While the pH, extractable phosphorus, and exchangeable potassium levels show no significant differences among treatments, the consistent levels across treatments indicate that these soil properties are inherently stable in the given conditions. A farmer's understanding of soil pH is crucial since many plants and soil organisms prefer either alkaline or acidic conditions (Tale and Ingole 2015). The lack of significant change in phosphorus and potassium suggests that the existing soil conditions already provide adequate nutrients for *M. charantia* growth. This stability can be beneficial as it prevents potential nutrient imbalances affecting plant health. However, the enhanced OM and nitrogen content from the Full RRIF treatment provides a more nutrient-rich soil environment, which could lead to improved growth and productivity of *M. charantia*. Therefore, for optimal growth and yield. Focusing on treatments that boost OM and Nitrogen without disrupting other soil properties could benefit *M. charantia* cultivation.

Moisture content and dry matter of *Momordica charantia* fruit as affected by the treatments

The table presents an analysis of moisture content (%) and dry matter (%) in *M. charantia* subjected to various agricultural treatments in Table 4. These treatments include combinations of Reduced-Rate Inorganic Fertilizers (RRIF) and Carrageenan Plant Growth promoters (PGP). The results indicate minimal variation in moisture content across treatments, with values ranging from 92.30 to 93.82%. The F-test showed no significant differences among treatments, suggesting that RRIF and Carrageenan PGP applications did not markedly affect the moisture levels of the fruit. Conversely, significant differences were observed in dry matter content, ranging from 6.18 to 7.70%. Treatments with half the recommended RRIF dose (T3) exhibited the highest dry matter content, indicating increased nutrient concentration and potential improvements in fruit quality. These results emphasize the significance of nutrient management strategies in influencing the physiological aspects of *M. charantia*, such as nutrient uptake and assimilation, which directly impact on its market value and consumer acceptance.

Moisture content is crucial in determining *M. charantia* post-harvest quality and storage stability. The consistent moisture levels observed across treatments suggest that the variations in RRIF and Carrageenan PGP applications did not disrupt water uptake or translocation within the fruit. This stability is beneficial as it ensures uniformity in texture and juiciness, contributing to consumer satisfaction and marketability. In contrast, the significant differences in dry matter content highlight the influence of fertilization practices on the nutritional composition of *M. charantia*. Higher dry matter content, particularly in treatments with reduced RRIF applications, indicates enhanced nutrient utilization efficiency and potentially improved yield and economic returns for farmers.

Plant tissue chemical properties

Table 5 presents the effects of various treatments on nutrient content and crude protein levels in *M. charantia* (bitter melon). The treatments included Reduced-Rate Inorganic Fertilizer (RRIF) and Carrageenan Plant Growth Promoter (PGP) combinations. Significant differences were observed in total nitrogen (N) and crude protein content among the treatments. Full RRIF (T2) showed the highest values for both parameters. Total Nitrogen ranged from 1.53% in control (T1) to 2.60% in T2, while crude protein content ranged from 9.58% in T1 to 16.24% in T2. Conversely, no significant differences were noted in total phosphorus (P) and total potassium (K) content among the treatments, indicating stable levels of these nutrients across different treatment applications.

The significant increase in total Nitrogen and crude protein content in the Full RRIF treatment (T2) has important implications for the growth and productivity of *M. charantia*. Higher nitrogen levels enhance vegetative growth, leaf chlorophyll content, and overall plant vigor, leading to more robust and productive plants. The increased crude protein content also suggests improved nutritional quality of *M. charantia*, which can benefit both human consumption and market value. These improvements are crucial as they directly impact yield and quality, making Full RRIF a valuable treatment for maximizing the agricultural potential of *M. charantia*. Nitrogen application is more critical than other essential nutrients for successful crop production (Javed et al. 2022). Slow development of plant and early leaf senescence due to deficient N can cause decreased both crop production and quality (Dong et al. 2012), but excess N prolongs the vegetative growth period, delays maturity, decreases sugar content, and attracts insect pests and causes disease epidemics (Anas et al. 2020). Phosphorus is classified as a significant nutrient. Despite crops needing it in relatively high amounts, it is frequently insufficient for crop output. It is an excellent source of magnesium, folic acid, zinc, phosphorus, manganese, and vitamins B1, B2, and B3. It has high dietary fiber (Asna et al. 2020). It was reported that bitter melon is an excellent source of phenolic chemicals, which have strong antioxidant properties (Aminah and Anna 2011). Fruits are relatively high in proteins, minerals, vitamins, and many other nutrients required in the human diet (Krishnendu and Nandini 2016).

Despite the variations in nitrogen and protein content, the stability of phosphorus and potassium levels across treatments ensures that these essential nutrients remain available to support various physiological processes in the plant. Phosphorus is vital for energy transfer and photosynthesis, while potassium is crucial in enzyme activation and osmoregulation. The consistent levels of these nutrients indicate that the different treatment combinations do not disrupt their availability, allowing for balanced growth and development of *M. charantia*. Therefore, while increasing nitrogen availability is important for raising yield and quality, keeping potassium and phosphorus levels steady is just as important for the crop's general health and productivity.

The correlation matrix revealed significant relationships between key growth, phenological, and yield parameters of *M. charantia* shown in Table 6. Plant height at 29 DAT exhibited moderate to strong positive correlations with fruit weight 0.475, length 0.482, and diameter 0.647, suggesting that vegetative growth during later stages strongly influences fruit development. Studies on bitter gourd cultivars under Karaj conditions demonstrated that taller plants (e.g., Hybrid No. 486 at 355.33 cm) produced higher yields and larger fruits, aligning with the role of mid-growth vigor in resource allocation for fruit development (Valyaie et al. 2021). Notably, days to first flowering showed a highly significant negative correlation with fruit weight -0.558 and length -0.532, indicating that earlier flowering promotes larger and heavier fruits. Among yield components, fruit weight, length, and diameter were strongly intercorrelated (0.857-0.901), highlighting their synergistic contribution to overall fruit morphology. Fruit yield demonstrated highly significant correlations with weight 0.674, length 0.676, and diameter 0.554, underscoring the cumulative impact of these traits on productivity.

The weak correlation between early vegetative growth (15 DAT) and yield parameters (0.165-0.304) implies that early-stage plant height is less predictive of final yield compared to mid-growth (29 DAT) and phenological traits. The negative association between days to flowering and yield-related traits suggests that treatments accelerating flowering could enhance fruit development. However, the non-significant correlation between early growth (15 DAT) and yield (0.184) may reflect variability in treatment responses during initial growth phases.

Table 5. Total N, P, K, and crude protein content of *Momordica charantia* fruits as affected by the different treatment combinations, (%)

Treatment	Total N	Total P	Total K	Crude protein
T_1- Absolute Control	1.53±0.14d	0.35±0.04	2.58±0.09	9.58±0.90d
T_2- Full RRIF	2.60±0.29a	0.32±0.04	2.77±0.41	16.24±1.81a
T_3- ½ RRIF	2.27±0.01abc	0.36±0.05	2.94±0.47	14.21±0.04abc
T_4- 9 L ha^{-1} Carrageenan PGP	2.17±0.14abc	0.30±0.03	2.07±0.35	13.57±0.88abc
T_5- ½ RRIF + 9L ha^{-1} Carrageenan PGP	1.83±0.04cd	0.32±0.05	2.95±0.33	11.46±0.25cd
T_6- ½ RRIF + 4.5L ha^{-1} Carrageenan PGP	2.13±0.16bc	0.27±0.12	2.06±0.74	13.34±0.96bc
T_7- Full RRIF + 4.5L ha^{-1} Carrageenan PGP	2.49±0.16ab	0.24±0.03	2.38±0.87	15.57±1.00ab
Mean	2.15±0.37	0.31±0.04	2.54±0.38	13.42±2.30
F-test	**	ns	ns	**
CV, %	7.34	18.03	19.20	7.34

Data are presented as mean ± standard deviation (n = 3 replications). Means within a column followed by the same letter are not significantly different at *p* < 0.05 (Tukey's HSD). RRIF: Recommended Rate of Inorganic Fertilizer; PGP: Plant Growth Promoter; ns: not significant; *: significant; **= highly significant; CV: Coefficient of Variation

Table 6. Correlation analysis of growth, phenological, and yield parameters in *Momordica charantia* as affected by the different treatment combinations

Variable	Plant height 15 (DAT)	Plant height 29 (DAT)	Days of first flowers appearance	Average fruit weight	Average fruit length	Average fruit diameter	Fruit yield
Plant height 15 (DAT)	1.000						
Plant height 29 (DAT)	0.402	1.000					
Days of first flowers appearance (DAT)	-0.365	-0.384	1.000				
Average fruit weight	0.165*	0.475*	-0.558**	1.000			
Average fruit length	0.304	0.482*	-0.532*	0.901**	1.000		
Average fruit diameter	0.234	0.695	-0.526*	0.857**	0.798**	1.000	
Fruit yield	0.184	0.289	-0.412	0.674**	0.676**	0.554**	1.000

Correlation coefficients (Pearson's *r*) are shown; *: significant at *p* < 0.05, **: highly significant at *p* < 0.01. DAT: Days After Transplanting. Data derived from 3 replications per treatment

In conclusion, the study results indicate that the application of Recommended Rate Inorganic Fertilizer (RRIF) and Carrageenan Plant Growth Promoter (PGP) significantly influences various aspects of *M. charantia* cultivation. The findings highlight that specific combinations of RRIF and PGP can effectively enhance plant growth parameters, promote early flowering, improve soil pH and OM content, increase nutrient availability, and enhance protein levels in *M. charantia*. These results highlight the importance of tailored fertilization strategies and PGP applications in optimizing crop yield, quality, and nutritional value. This study demonstrates that integrating Carrageenan-based Plant Growth Promoters (PGP) with inorganic fertilizers significantly enhances the growth, yield, and nutritional quality of *M. charantia* while improving soil health. Key findings reveal that treatments combining reduced inorganic fertilizer (½ RRIF) with Carrageenan PGP (T5: ½ RRIF + 9L ha^{-1} PGP) optimized fruit diameter (49.44 mm) and nutrient content, whereas full RRIF with PGP (T7) achieved the tallest plants (260.42 cm) and highest yield (64.50 tons/ha). Notably, carrageenan PGP mitigated the reliance on synthetic fertilizers, improving soil organic matter (OM) and nitrogen content without destabilizing pH or phosphorus/potassium levels. The strong correlation between mid-growth vigor (29 DAT) and fruit traits (0.475-0.857**) underscores the importance of late-stage vegetative development in yield outcomes. However, early growth (15 DAT) showed minimal predictive value for productivity, emphasizing the need for targeted mid-growth interventions. These results validate carrageenan PGP as a sustainable alternative to conventional practices, balancing economic viability (435% ROI for T7) with environmental stewardship. Moreover, the study reveals that while the moisture content of the fruits remained relatively stable across different treatments, the dry matter content varied significantly, with the highest dry matter observed in treatments with reduced RRIF.

To optimize *M. charantia* production sustainably may explore integrating carrageenan PGP with inorganic fertilizers, such as prioritizing combinations like ½ RRIF + 9L ha^{-1} PGP (T5) for enhanced fruit quality or Full RRIF + 4.5L ha^{-1} PGP (T7) to maximize yield and vegetative growth, depending on market needs. Prioritizing mid-growth monitoring (29 DAT) over early-stage assessments could improve yield predictions, as plant height at this stage strongly correlates with fruit traits. Exploring long-term soil health impacts, including microbial diversity and carbon sequestration under carrageenan PGP use, alongside trials across diverse crops, may validate broader applicability. Conducting cost-benefit analyses tailored to smallholder farmers can refine adoption strategies, balancing economic and environmental benefits. Integrating Carrageenan PGP into existing practices offers a pathway to harmonize productivity, profitability, and ecological resilience in *M. charantia* cultivation. Future research might also examine long-term effects on soil fertility, environmental sustainability, and dietary benefits to enhance agricultural practices for similar crop systems.

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Melatonin partly reverses differentiation and apoptosis resistance caused by chemical hypoxia

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Abstract. *Liu X, Liu H, Qi Y, Zhang S, Ma Y. 2025. Melatonin partly reverses differentiation and apoptosis resistance caused by chemical hypoxia. Nusantara Bioscience 17: 178-184.* Hypoxia, a condition characterized by a deficiency of oxygen reaching tissues, is a key feature of malignant solid tumors. Tumor cells under a hypoxic microenvironment exhibit more malignant phenotypes to adapt to the hypoxic environment for survival by regulating gene expression involved in cell proliferation, differentiation, invasion, migration, angiogenesis, and also chemoresistance. Melatonin is a hormone that has been proven to induce apoptosis and differentiation in various types of malignant cells. The present study aims to investigate the role and potential molecular mechanism of melatonin in mediating chemical hypoxia-induced differentiation and apoptosis resistance. Cobalt chloride ($CoCl_2$) was used to generate an in vitro hypoxia culture model, as verified by the increased expression of hypoxia-inducible factor-1α. The cells treated with melatonin and $CoCl_2$ were collected to determine differentiation and apoptosis levels through LDH (Lactate Dehydrogenase) activity measurement and Hoechst staining. GRP78, p53, and endocan levels were detected using Western blot and ELISA (Enzyme Linked Immunosorbent Assay) to elucidate the potential molecular mechanisms by which melatonin induces differentiation and resistance to apoptosis in response to chemical hypoxia. The significant elevation of a hypoxia marker protein successfully demonstrated ($CoCl_2$-induced hypoxia). The chemical hypoxia environment resulted in a less differentiated phenotype and a lower apoptotic ratio in gastric cancer cells. Melatonin converted the differentiation and apoptosis resistance phenotypes in $CoCl_2$-treated gastric cancer cells. Changes in the expression of endocan, LDH, p53, and GRP78 were observed. Melatonin-regulated differentiation and apoptosis-related genes partly reverse differentiation and apoptosis resistance caused by chemical hypoxia induced by $CoCl_2$. The study provides a new perspective on the anti-tumor effect of melatonin.

Keywords: $CoCl_2$, gastric cancer, GRP78, Lactate Dehydrogenase, p53

Abbreviations: LDH: Lactate Dehydrogenase, MLT: Melatonin

INTRODUCTION

Malignant tumor cells are more proliferative than normal cells. The surrounding blood vessels are compressed due to the abnormal proliferation and rapid growth of the tumor, resulting in lower oxygen pressure in the tumor compared to normal tissues (< 7.5 mmHg vs. > 40 mmHg). This phenomenon is known as hypoxia (Lugano et al. 2020). Thus, hypoxia is one of the most important features of malignant solid tumors (Jing et al. 2019). To adapt to a hypoxia environment, genes to regulate differentiation, proliferation, mobility, and angiogenesis were differentially expressed in cancer cells under the hypoxia microenvironment, which made the cells more malignant and more likely to resist chemotherapy (Paredes et al. 2021). Lots of research has indicated that the poor prognosis of solid tumors is considered to be related to the adaptive resistance of tumor cells caused by local hypoxia in tumor tissues (Zheng and Gao 2019).

Gastric cancer is one of the most frequent malignancies worldwide, with high morbidity as well as high mortality (Machlowska et al. 2020) with 968350 newly diagnosed cases and a total of 659,853 deaths per year according to Global Cancer Statistics (GLOBOCAN) 2022 (Bray et al. 2024). Although a variety of treatments such as surgery, chemotherapy, and radiotherapy, have been applied to the treatment of gastric cancer, the prognosis for patients remains poor (Shi and Gao 2016). Especially in patients with metastatic, disseminated, or advanced gastric cancer, there is still a low 5-year survival rate, and the median survival is less than 2 years even after palliative chemotherapy (Guan et al. 2023). Hypoxic condition in gastric cancer tissues results in higher malignant phenotypes and constitute one of the main causes leading to drug resistance and the failure of clinical therapy (Rankin and Giaccia 2016). Thus, more effective therapeutic strategies against gastric cancer by targeting or reversing hypoxia with lower toxicity are still urgently needed.

Melatonin (N-acetyl-5-methoxytryptamine, MLT) is a kind of indole neurohormone mainly synthesized by the pineal gland and has been proven to have a wide range of biological effects (Minich et al. 2022). It has been shown to possess differentiation inducer and chemotherapeutic potential in human cancers, including gastric cancer, by modulating multiple signal transduction pathways associated with cell apoptosis, cell growth, and migration and invasion capabilities (Woo et al. 2015; Cheng et al. 2023). However, few studies have focused on whether melatonin could reverse the increased malignant phenotypes caused by hypoxia microenvironment. The present study was designed to explore whether melatonin can attenuate the higher malignancy caused by chemical hypoxia in gastric cancer cells and to investigate the possible molecular mechanisms by which melatonin exerts this effect.

Cell dedifferentiation or blocked differentiation is a key biological characteristic in the process of malignant tumor development and progression in addition to malignant proliferation (Marsafy and Larghero 2023). LDH (Lactate Dehydrogenase) is an important enzyme known to play a crucial role in regulating gastric cell differentiation, with upregulated activities in gastric cancer cells and decreased activity during the process of normal gastric cell differentiation. LDH was shown to have high activity in low-differentiated cells. These indicated that LDH functions as a dedifferentiation factor in the stomach and is recognized as a marker enzyme for gastric cancer cell differentiation level (Jin et al. 2020). The secreted proteoglycan, endocan, was reported to be positively correlated with gastric cancer differentiation level (Zhang et al. 2012). Exogenous endocan promoted the differentiation of gastric cancer cells (Zhang et al. 2012). Under a chemical hypoxic condition mimicked by $CoCl_2$, gastric cancer cells exhibited a poorer differentiation state, as indicated by increased Lactate Dehydrogenase (LDH) activity and a lower level of secreted endocan in the culture medium's supernatants. This study confirmed that endocan and LDH are involved in the reversal effect of melatonin on dedifferentiation caused by chemical hypoxia in gastric cancer cells. Cell apoptosis is co-mediated by various apoptotic pathways. For example, P53 is a proapoptotic protein that activates the intrinsic apoptosis pathway (Ozaki and Nakagawara 2011), and GRP78 is an endoplasmic reticulum stress-associated apoptotic protein (Zhang et al. 2020).

Cobalt chloride ($CoCl_2$), a recognized chemical hypoxia inducer (Chen et al. 2018), was used in the present study to treat human gastric cancer cells to generate an in vitro hypoxia model verified by elevated hypoxia marker protein, hypoxia-inducible factor-1α (HIF-1α) (Gao et al. 2023). The effects of melatonin on differentiation and apoptosis of $CoCl_2$ treated gastric cancer cells, and also the potential molecular mechanisms were investigated here. We found that melatonin treatment attenuated the more malignant behavior caused by $CoCl_2$ in gastric cancer cells at least partly by altering the expression of differentiation and apoptosis-related proteins.

MATERIALS AND METHODS

Materials

The present research was conducted at the Laboratory of the Department of Biochemistry and Molecular Biology, Anhui Medical University (AHMU), Hefei, China. The human gastric cancer cell line SGC7901 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). High glucose DMEM (Dulbecco's modified Eagle's medium) was obtained from Gibco (Carlsbad, CA, USA). FBS (Fetal bovine serum) was from Clark Bioscience. Melatonin (MLT), DMSO (Dimethyl Sulfoxide), and MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). Cobalt chloride ($CoCl_2$) was obtained from Tianjin Guangfu Technology (Tianjin, China). Primary antibodies against HIF-1α, GRP78, p53, and β-actin were from Santa Cruz (Dallas, TX). All corresponding secondary antibodies were from Thermo Fisher Scientific.

Cell culture

SGC7901 cells were cultured in high-glucose DMEM supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (40 U/mL penicillin and 100 U/mL streptomycin) in an incubator with 5% CO2 and 100% humidity at 37°C under corresponding conditions.

Cell proliferation assessment

SGC7901 cells grown in 96-well plates (5×10^3 cells/well) were treated with $CoCl_2$ at different final dilutions (0, 100, 200, 300, 400, 500, and 600 μM) for 48 h to select a suitable concentration to induce cytochemical hypoxia without affecting cell viability. MTT was added to each well, followed by a 4 h incubation at 37°C. Formazan crystals formed in wells were dissolved in 100 µL DMSO for 10 min at 37°C, and the absorbance was measured at 570 nm using an absorbance microplate reader (Thermo Scientific). The growth inhibition ratio of each group was calculated as: Inhibition ratio (%) = $[1 - (\text{mean } OD_{\text{treated groups}} - \text{mean } OD_{\text{blank controls}}) / (OD_{\text{control groups}} - OD_{\text{blank controls}})]$.

After the appropriate concentration of $CoCl_2$ was determined, cells were treated as follows: (i) Normoxia group: Cells cultured routinely; (ii) hypoxia group: Cells treated with $CoCl_2$; (iii) Melatonin group: Cells cultured in 10^{-4} M melatonin without being exposed to $CoCl_2$; and (iv) Hypoxia induction group: Melatonin in the concentration of 10^{-4} M was given simultaneously to treat $CoCl_2$-exposed gastric cancer cells.

Quantification of endocan

Endocan concentration in the culture supernatants was measured using a commercial ELISA Kit (CUSABIO) according to the manufacturer's instructions. Briefly, a 100 mL standard or supernatant sample from each group was incubated in the ELISA plate at 37°C for 2 h, followed by Biotin-antibody binding and HRP-avidin incubation. TMB substrate and stop solution were added in sequence, and the absorbance of each well was read using a microplate reader at 450 nm (Thermo Scientific).

Lactate Dehydrogenase (LDH) assay

After the various treatments mentioned above, a culture medium was collected for the determination of LDH activity using the microplate method. According to the instructions of the LDH assay kit, LDH activity was calculated as ($OD_{\text{of the unknown sample}} - OD_{\text{of the blank control}}$) / ($OD_{\text{of the standard sample}} - OD_{\text{of the blank control}}$) × the concentration of the standard (0.2 μmol/mL) × 1000 based on the absorbance values read at 450 nm.

Hoechst 33258 staining to detect cell apoptosis

SGC7901 cells grown on sterilized coverslips were fixed and stained with Hoechst 33258 for 5 minutes at 37°C after 48 hours of different treatments. The stained cells were visualized and pictured under a fluorescence microscope.

Western blot analysis

SGC7901 cells treated for 48 h, as mentioned above, were lysed in RIPA (Radio Immunoprecipitation Assay) buffer and centrifuged to collect the supernatant. The total protein concentration was determined by BCA assay. An equal amount of total protein of the cell lysate from each group was separated by SDS-PAGE and transferred onto the PVDF membrane. Expression levels of target proteins were detected by incubating specific antibodies against HIF-1α (1:500), GRP78 (1:500), p53 (1:500), or β-actin (1:1000), followed by HRP-conjugated secondary antibodies and visualization using enhanced chemiluminescence. Western blot data were quantified using Quantity One software. β-actin was accepted as a reference gene.

Data analysis

Each experiment was repeated three times, and all data were expressed as mean ± standard deviation. Data analysis was performed with SPSS 17.0 Software (IBM SPSS, USA). The differences among the groups were analyzed using one-way Analysis of Variance (ANOVA) and Least Significant Difference (LSD). $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

$CoCl_2$ simulated an anoxic microenvironment successfully

To select a suitable concentration of $CoCl_2$ that effectively induces hypoxia without significant cytotoxicity, an MTT assay and Western blot were performed to assess cell viability and HIF-1α levels, respectively. HIF-1α is used here as a marker of hypoxia to confirm if $CoCl_2$ treatment could result in a chemically hypoxic microenvironment in cells. $CoCl_2$ at 100 μM could successfully induce HIF-1α expression in SGC7901 cells, but did not cause decreased cell viability (Figures 1 and 2). Therefore, $CoCl_2$ at 100 μM was used as an optimal dose to generate a hypoxic state in SGC7901 cells in further experiments.

Melatonin could promote SGC7901 cell apoptosis and differentiation under a chemically hypoxic environment

LDH and endocan are known molecular differentiation-related markers of gastric cancer cells. Secreted endocan concentration and LDH activity in culture supernatant were detected to evaluate the differentiation level of cells in each group (Figures 3 and 4). The concentration of secreted endocan was obviously reduced, and LDH activities were increased in the cells exposed to $CoCl_2$ when compared to normoxic cells, which mean that gastric cancer cells under chemical hypoxic environment show lower differentiation degree. Melatonin significantly attenuates $CoCl_2$-induced lower differentiation, as evidenced by a higher secreted endocan level and lower LDH activity in the cell culture medium, compared to chemically hypoxic cells treated with $CoCl_2$ alone.

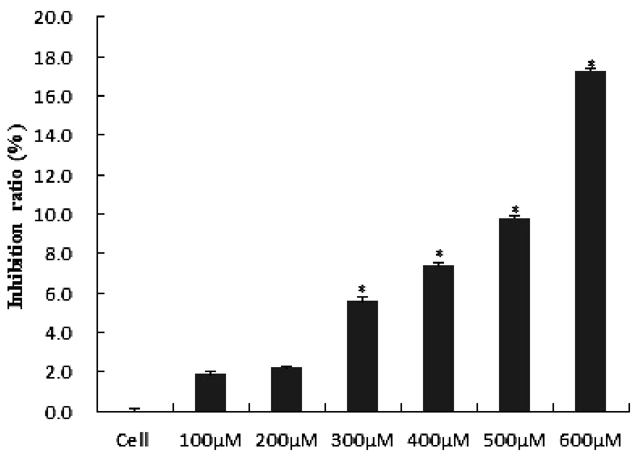


Figure 1. The effect of $CoCl_2$ on the viability of SGC7901 cells was assessed using the MTT assay. SGC7901 cells were treated with $CoCl_2$ at various concentrations (0, 100, 200, 300, 400, 500, and 600 μM). n = 3. Data were analyzed using ANOVA with LSD test for multiple groups comparisons. *p < 0.01 compared to control cells

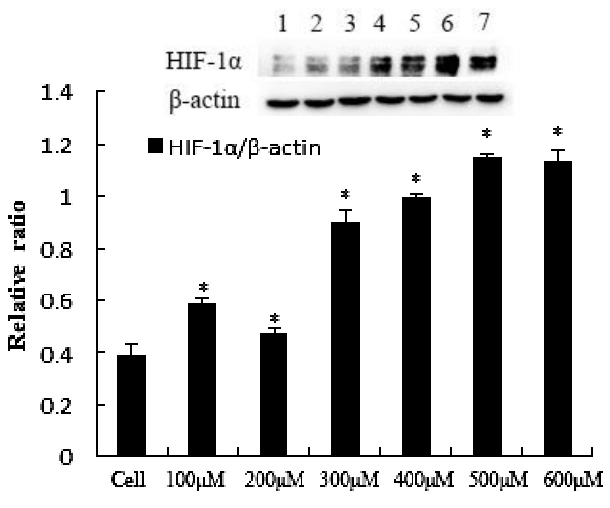


Figure 2. The expression of HIF-1α after $CoCl_2$ treatment at different concentrations was detected by western blot. 1: Cell control; 2: 100 μM $CoCl_2$; 3: 200 μM $CoCl_2$; 4: 300 μM $CoCl_2$; 5: 400 μM $CoCl_2$; 6: 500 μM $CoCl_2$; 7: 600 μM $CoCl_2$. n = 3. Data were analyzed using ANOVA with LSD test for multiple groups comparisons. *p < 0.01 compared to control cells

Hoechst staining was used to stain the cell nucleus, allowing for the observation of apoptotic cells, which could be distinguished from uniformly stained non-apoptotic cells. Apoptosis resistance was observed in $CoCl_2$-induced hypoxic cells, characterized by the presence of few apoptotic cells, as indicated by nuclear shrinkage and apoptotic corpuscles detected by Hoechst staining throughout the entire field of view. Melatonin could promote SGC7901 cell apoptosis under both normoxic and hypoxic states, as shown in Figure 5, by which the reversal role of melatonin on apoptosis resistance caused by $CoCl_2$-induced hypoxia was ascertained.

GRP78 and p53 are involved in apoptosis resistance in hypoxic gastric cancer cells

GRP78 and p53 are famous genes that mediate the endoplasmic reticulum apoptotic pathway and p53-dependent mitochondrial apoptosis, respectively. The Western blot assay revealed an obvious upregulation of GRP78 and a downregulation of p53 in $CoCl_2$-treated cells (Figure 6). Treatment with melatonin attenuated the upregulation of GRP78 and downregulated p53 in cells under a chemically hypoxic environment induced by $CoCl_2$.

Discussion

Gastric cancer has become a public health problem worldwide as a major gastrointestinal malignancy with high mortality and incidence (Siegel et al. 2023). In China, gastric cancer is the third leading cause of cancer mortality, with a mortality rate the second highest among all the malignancies (Yang et al. 2023). The causes and pathogenic factors of gastric cancer occurrence are very complex, including dietary habits, the surrounding environment, *Helicobacter pylori* infection, and so on (Whitmire and Merrell 2019). Many gastric cancers are diagnosed in the middle or late stages due to the complex pathogenesis and the difficulty in early diagnosis. At the same time, its poor response or resistance to chemotherapy drugs in advanced and disseminated gastric cancer patients is a tough problem for effective clinical treatment of gastric carcinoma (Shi and Gao 2016).

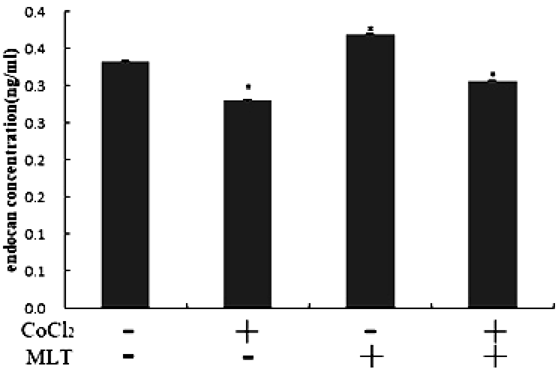


Figure 3. The concentration of endocan in the cultural supernatant of the SGC7901 cells was detected by ELISA assay. $^{*}p<0.05$ compared to cell control, $^{\#}p<0.05$ compared to $CoCl_2$ treated cells

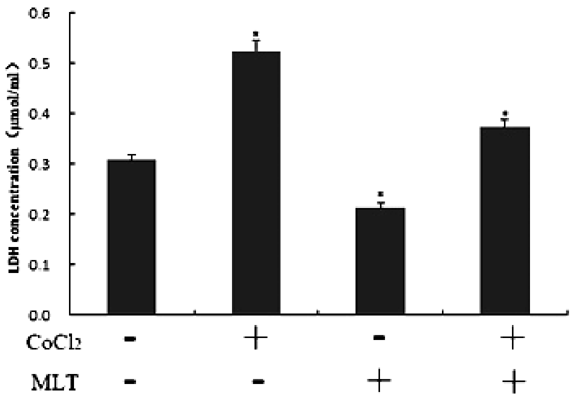
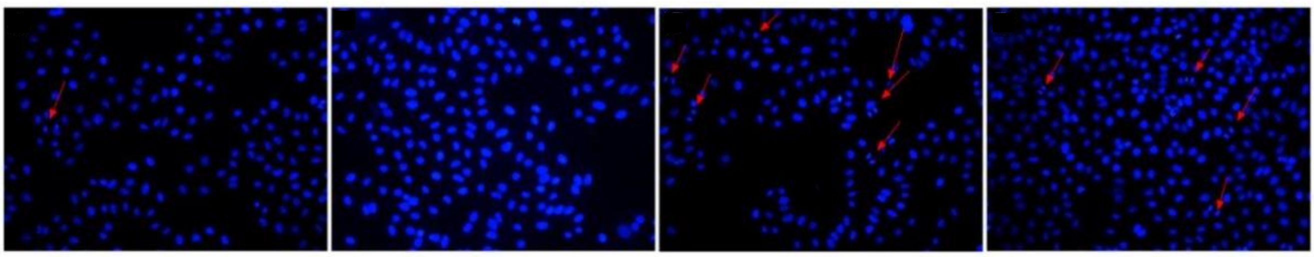


Figure 4. The activities of LDH in SGC7901 cells. $^{*}p<0.05$ compared to cell control, $^{\#}p<0.05$ compared to $CoCl_2$ treated cells



A B C D

Figure 5. The morphology of apoptosis was detected by Hoechst staining. The arrows point to typical apoptotic cells, characterized by nuclear shrinkage, pyknosis, or karyorrhexis. A: Cell control; B: $CoCl_2$; C: MLT; D: $CoCl_2$+MLT. (×200)

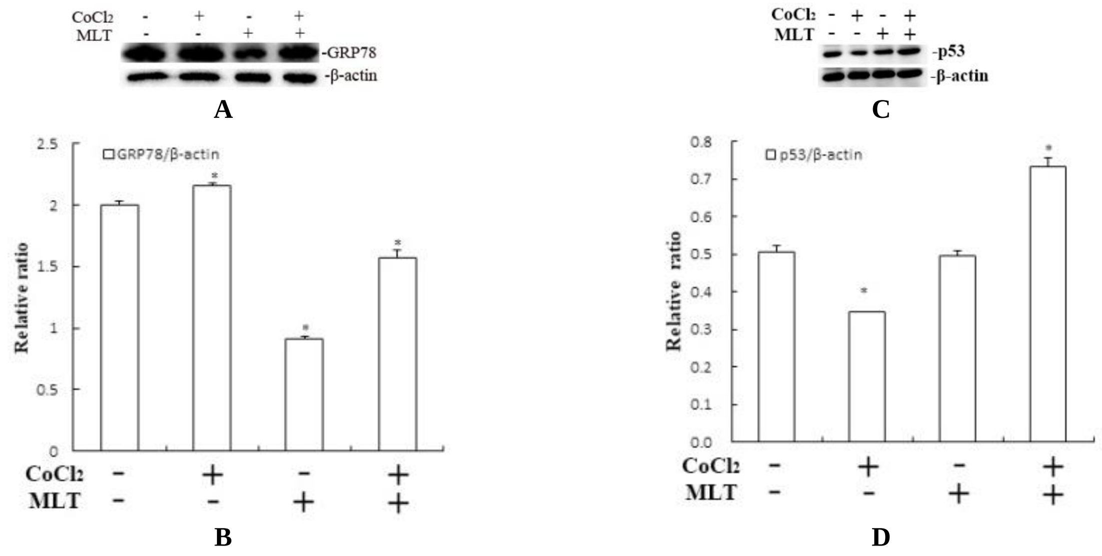


Figure 6. Western blot assay was performed to detect the expression level of GRP78 and p53. A. Western blot bands of GRP78; B. The relative gray values of GRP78; C. Western blot bands of p53; D. The relative gray values of p53. *p<0.05 compared to cell control, #p<0.05 compared to $CoCl_2$ treated cells

Studies have found that malignant cells in a hypoxic environment, caused by excessively rapid growth and insufficient blood supply, are prone to develop resistance to chemotherapy drugs due to the adaptive changes of tumor cells to hypoxia (Kopecka et al. 2021; Paredes et al. 2021; Wei et al. 2021). Under a hypoxic microenvironment, malignant cells adapt to the hypoxic environment by regulating the expression levels of genes that contribute to cell proliferation, apoptosis, differentiation, and resistance to chemotherapy (Borsi et al. 2015). Thus, cancer cells under hypoxia exhibit higher malignant phenotypes, including resistance to apoptosis, more vigorous proliferation, increased neovascularization, lower differentiation levels, and a greater likelihood of developing local invasion and distant metastasis. This adaptation was related to hypoxia-induced expression of HIF-1α in solid tumors. HIF-1α is known as a transcription factor that affects the expression of more than hundreds of downstream target genes related to hypoxia adaptation and tumor growth and thereby increasing tumor proliferation, invasion, and metastasis ability (Feng et al. 2019). Thus, finding safe and effective agents that can alleviate or overcome the more malignant phenotypes and chemotherapy resistance caused by hypoxia is especially important.

The application of melatonin provides a new direction in drug therapy for malignant tumors (Cheng et al. 2023). Melatonin is an indole heterocyclic compound acting as a kind of natural hormone secreted mainly by the human pineal gland (Kubatka et al. 2018). The therapeutic properties of melatonin on malignant tumors have been confirmed both in clinical and basic research (Li et al. 2015). Melatonin has significant value in preventing the occurrence of tumors and delaying their growth and progression. It delayed the malignant transformation of cells to modulate cancer initiation and promoted tumor cell differentiation and apoptosis to restrain cancer promotion through receptor-dependent or receptor-independent mechanisms (Hsieh et al. 2020). Other scientists and our group have shown that MLT inhibited malignant behaviors by different signaling pathways to regulate numerous gene expressions involved in apoptosis, proliferation, differentiation, angiogenesis, and so on (Cheng et al. 2019). It was reported that melatonin can decrease HIF-1α, thereby preventing the formation of vasculogenic mimicry and the Epithelial-Mesenchymal Transition (EMT) in cancer cells under hypoxic conditions, and is considered beneficial in cancer treatment (Maroufi et al. 2020). In the present study, we attempted to demonstrate that melatonin exerts therapeutic sensitization by alleviating the apoptosis and differentiation inhibition of tumor cells caused by hypoxia.

$CoCl_2$ has almost the same biochemical response as physiological hypoxia in gastric cancer cells (Rath et al. 2016), which is suitable for inducing cell hypoxia models. Hence, we chose $CoCl_2$ to simulate the hypoxic environment of gastric cancer cells *in vitro*, and increased HIF-1α level was used as an indicator of successfully induced hypoxia. The HIF-1α level detected by Western blot assay revealed that the hypoxia culture model in vitro was successfully established by $CoCl_2$ treatment at all concentrations used (100, 200, 300, 400, 500, and 600 μM). MTT assay showed that $CoCl_2$ did not conspicuously inhibit the viability of SGC7901 cells at 100 μM. These results could suggest that $CoCl_2$ at 100 μM induced a successful chemical hypoxia model with no obvious cytotoxicity. In the subsequent experiments of the present study, $CoCl_2$ at 100 μM was used as the optimal dose to induce chemical hypoxia in cells.

Melatonin at 10^{-4} M (Zhang et al. 2013) was used to treat gastric cancer cells with or without exposure to $CoCl_2$. Hypoxic cells exhibited a decreased differentiation level (Figure 4) and lower levels of apoptosis (Figure 5) compared to the control cells. MLT promoted the differentiation of hypoxic SGC7901 cells, as indicated by decreased LDH activity and increased endocan levels in the culture medium. Malignant cells are more likely to obtain energy through anaerobic glycolysis instead of aerobic oxidation (Shafaee et al. 2015). As an important enzyme catalyzing glycolysis, the serum level of LDH directly or indirectly reflects the strength of glycolysis, thereby predicting the ability of oncocyte proliferation and tumor development. This provides a theoretical basis for ascertaining the degree of malignancy and evaluating tumor prognosis. It has been reported that the serum level of LDH is significantly increased in tumor patients, and those with higher LDH have more poorly prognosis (Liu et al. 2017). Thus, LDH is a well-known enzyme that negatively correlates with the differentiation level of gastric cancer cells.

Endocan, initially named endothelial cell-specific molecule-1 (ESM-1), is a secreted proteoglycan (Lassalle et al. 1996) involved in molecular interactions. Endocan has a wide range of biological activities that are crucial for biological processes of tumorigenesis or metastasis, such as cell adhesion, migration, proliferation, and angiogenesis (Lin et al. 2017). Previously, our team reported that endocan levels were positively correlated with differentiation levels of gastric cancer and colorectal carcinoma (Zhang et al. 2012). Over-expression of functional endocan promoted the differentiation-induced cell apoptosis, but reduced the migration of gastric cancer cells, with the opposite effect when endocan was knocked down (Sumei et al. 2016). The apoptosis-associated protein p53 and caspase 3, together with migration-associated protein MMP-9, were proved to participate in an endocan-mediated anti-carcinogenic role, and melatonin promoted differentiation of gastric cancer cells at least partly through upregulating endocan expression (Zhang et al. 2012). We found that MLT increased endocan secretion (Figure 3) in hypoxic SGC7901 cells, suggesting that endocan may also be involved in MLT-induced cell differentiation and apoptosis in hypoxic gastric cancer cells.

Furthermore, apoptosis-associated proteins crucial to different apoptotic pathways, such as p53 and GRP78, were detected using Western blot. P53 is a famous tumor suppressor gene involved in various signaling pathways to regulate cell proliferation and cell death (Tanikawa et al. 2017). As reported in numerous studies, increased expression of p53 protein is vital for apoptosis induction (Nakanishi et al. 2014; Tanikawa et al. 2017; Vaddavalli and Schumacher 2022). Western blot analysis revealed that p53 expression was decreased in $CoCl_2$-treated cells, indicating that hypoxia exposure downregulated p53 expression, which is involved in the apoptosis resistance induced by hypoxia. Also, abnormal expression of GRP78 is involved in tumor occurrence and progression (La et al. 2018). Increased GRP78 in tumor tissues promoted cell migration and proliferation and was correlated with poor survival in many cancer types (Niu et al. 2015). Moreover, GRP78 promoted therapeutic resistance in cancer (Clarke et al. 2019). Mechanically, GRP78 prevented cell apoptosis by suppressing caspase-7 activation (Zhang et al. 2020). Reducing GRP78 expression in tumor cells or suppressing its anti-apoptotic properties has profound significance for apoptosis induction and migration inhibition, and is considered an effective approach to reversing resistance to anti-cancer treatments. The lacking of oxygen in malignant cells induces GPR78 expression through the endoplasmic reticulum stress pathway (Dauer et al. 2019). We found an increased expression of GRP78 in gastric cancer cells under hypoxia. This indicates that both the GRP78-mediated endoplasmic reticulum stress apoptotic pathway and the p53-dependent mitochondrial apoptosis pathway are involved in hypoxic apoptosis resistance. Melatonin worked to upregulate p53 and down-regulate GRP78 when combined with $CoCl_2$. Thus, it can be seen that melatonin can mitigate the abnormal expression of p53 and GRP78 in hypoxic gastric cancer cells exposed to $CoCl_2$, thereby attenuating the apoptosis resistance caused by hypoxia.

In conclusion, melatonin significantly alleviates the reduced apoptosis and differentiation levels caused by chemical hypoxia by regulating p53-dependent mitochondrial apoptosis and GRP78-mediated endoplasmic reticulum stress apoptotic pathway. We revealed a possibility that melatonin serves as an effective adjuvant therapy for treating gastric cancer by overcoming higher malignancy and chemotherapy resistance caused by a hypoxic microenvironment in tumor tissues. The results obtained from the present study provide clues for further exploration on the intimate molecular mechanisms in subsequent studies.

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