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Introduction is about 600 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for a manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgements** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

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The usage of "et al." in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al. 1999. Future of health insurance. *N Engl J Med* 325: 325-329. DOI: 10.1007/s002149800025.

Book:

Rai MK, Carpinella C. 2006. *Naturally Occurring Bioactive Compounds*. Elsevier, Amsterdam.

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:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.). *Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island*. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

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.

Secondary metabolites of rhizospheric fungal isolate <i>Aspergillus carneus</i> ABRF4 regulate the antibacterial and anti-proliferative activity against cancer cells MAHENDRA KUMAR SAHU, RAGINI YEERAVALLI, AMITAVA DAS, HARIT JHA	137-142
Effectiveness test of <i>Diploknema oligomera</i> extracts to the decrease in glucose levels in alloxan-induced BALB/c male mice SITI FAIZAH, KARTINI ERIANI, ROSNIZAR	143-148
Antibacterial activities of <i>Polyalthia longifolia</i> leaf extracts on multiple antibiotic-resistant bacteria isolated from hospital fomites in Akure, Nigeria OLAMIDE JOSHUA BABATUNDE, AYODELE OLUYEMISI OGUNDARE, TINUOLA TOKUNBO ADEBOLU	149-160
Classification of forest vegetation in Yaralıgöz Education and Observation Forest, Kastamonu, Türkiye MUSTAFA KARAKÖSE, SALIH TERZIOĞLU	161-171
Chemical composition of <i>Corymbia citriodora</i> ABDULRAHMAN MAHMOUD DOGARA	172-178
Habitat characteristics, population structure, distribution, and regeneration status of <i>Taxus wallichiana</i> Zucc. in South Vietnam NGUYEN VAN HOP, CHEN CHANG XIONG, NGUYEN VAN QUY, NGUYEN THI LUONG	179-188
The link of socioeconomic importance to the conservation status of the Mediterranean endemic plants in Egypt KAMAL SHALTOUT, HEBA BEDAIR, MOHAMED MAHMOUD EL-KHALAFY, AMR KESHTA, MARWA W.A. HALMY	189-211
Avifaunal diversity of Northwestern Himalayas (Jammu & Kashmir) and Trans Himalayas (Ladakh) of India PARVAIZ YOUSUF, SEMRAN PARVAIZ, NISHEET ZEHBI, SABIA ALTAF, BILAL NASIR ZARGAR, ROUF RATHER, JAVEED TANTRAY, MOHAMMAD LATEEF	212-231
The initial complete chloroplast genome of <i>Ludisia discolor</i> (Orchidaceae) in Vietnam VIET THE HO, MINH PHUONG NGUYEN, THI HUONG TRINH	232-237
Screening for endophytic bacteria from Ambon Banana (<i>Musa paradisiaca</i>) as biocontrol agent of anthracnose (<i>Colletotrichum gloeosporioides</i>) on bananas fruit ARTINI PANGASTUTI, HESTI PRATIWI, RATNA SETYANINGSIH	238-244
Effect of phosphate solubilizing bacteria and nitrogen fixing bacteria on pontianak siam citrus (<i>Citrus nobilis</i> var. <i>microcarpa</i>) seed germination RETNO FIKRIA IDIL ADHYANINGTYAS, RAHMAWATI, MUKARLINA	245-250
The potency of Berastagi sweet orange (<i>Citrus sinensis</i>) peel extract for obesity treatment: An experimental study in male rats WINDA RIZKI PEBRINA BATUBARA, TRI NUGRAHA SUSILAWATI, DONO INDARTO	251-257
Population genetic structure of Kawakawa <i>Euthynnus affinis</i> (Cantor, 1849) in Malaysian waters based on COI gene KHALED BINASHIKHBUBKR, AHMAD DWI SETYAWAN, DARLINA MD NAIM	258-268

Morphometric variations of <i>Kawakawa</i> (<i>Euthynnus affinis</i> Cantor, 1849) in Malaysian and Yemeni waters	269-277
KHALED BINASHIKHBUBKR, , AHMAD DWI SETYAWAN, DARLINA MD NAIM	
Optimization of L-asparaginase production from endophytic bacteria isolated from the mangrove <i>Rizophora mucronata</i>	279-287
ARIN NAFISATURRAHMAH, ARI SUSILOWATI, ARTINI PANGASTUTI	
Potential effects of fermented moringa (<i>Moringa oleifera</i>) leaf extract to increase the color brightness of the Oranda goldfish (<i>Carassius auratus</i>)	288-296
SITI UMAIYAH, ACHMAD NOERKHAERIN PUTRA, MUH. HERJAYANTO, MAS BAYU SYAMSUNARNO	

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Secondary metabolites of rhizospheric fungal isolate *Aspergillus carneus* ABRF4 regulate the antibacterial and anti-proliferative activity against cancer cells

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Abstract. Sahu MK, Yeeravalli R, Das A, Jha H. 2023. Secondary metabolites of rhizospheric fungal isolate *Aspergillus carneus* ABRF4 regulate the antibacterial and anti-proliferative activity against cancer cells. *Nusantara Bioscience* 15: 137-142. The medicinal capabilities of plants are influenced by soil chemistry, genotype, and climate. Many biotic and abiotic factors affect soil composition. Microorganisms constituting the soil microflora indicate a mutualistic relationship with plant rhizospheric region, and they play an important role in plant secondary metabolite production, yield, and efficacy. They are the major resources for structurally unique bioactive natural metabolites. The aim of the present study was to evaluate the bioactivity of the secondary metabolite extracted from the rhizospheric fungal isolate *Aspergillus carneus* ABRF4 isolated from the Achanakmar Biosphere Reserve, Chhattisgarh, India. The fungal secondary metabolites were extracted using several solvents by soxhlet extraction techniques. The crude and partially purified column fractions of *A. carneus* ABRF4 were characterized by Gas Chromatography-Mass Spectroscopy (GC-MS), Fourier Transform Infra-Red (FTIR) spectrum, and Thin Layer Chromatography (TLC). Results showed that the acetonitrile fraction had an antimicrobial activity with the variable zone of inhibition against human pathogens such as *Bacillus circulans* (MTCC-7906), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC-96), and *Ralstonia eutropha* (MTCC-2487). The crude extracts and the identified secondary metabolite, trans-1,3-dimethyl-Cyclohexane, possess different anti-proliferative activity against human tissue-specific cancer cell lines, including breast cancer (MDA-MB-231, MDA-MB-468, and MCF-7), liver cancer (HepG2), lung cancer (A-549), and prostate cancer (DU-145) suggesting a potential therapeutic application of the isolated rhizospheric fungi.

Keywords: Antimicrobial activity, anti-proliferative activity, *Aspergillus* sp., fungus

INTRODUCTION

The rhizosphere is the region around the plant roots with higher nutrients, so it has a higher microbial diversity than other soil areas. The plant roots benefit from several microorganisms residing in the rhizospheric region. These microorganisms create a microenvironment that inhibits the growth of pathogenic microorganisms. Rhizospheric microbes also impact the host plant's physiology and metabolism by changing the metabolic flux. They improve nitrogen fixation and subsequent nutrient uptake, including micronutrients and humus. It was previously reported that rhizospheric microbes might induce and modify the aromatic secondary metabolite pathways (Retnowati et al. 2018; Kong and Liu 2022). The rhizospheric microorganisms use the nutrients from the roots. The soil microenvironment, composition, temperature, and moisture affect its diversity. Fungi, such as mycorrhiza or free-living rhizospheric fungi, dominated the rhizospheric microbiome. The seasonal variation and anthropogenic activities may also affect the growth and rhizospheric fungi diversity. It has an impact on the growth of crops and medicinal plants. The Rhizospheric fungi reside in healthy

living root tissue of medicinal plants with mutual beneficial effects. They produce biologically active secondary metabolites. The secondary metabolites of the Rhizospheric fungi have been reported to have a broad spectrum of applications in biotechnology, pharmaceuticals, food, and agriculture (Radhika and Rodrigues 2010; Ferris and Tuomisto 2015). They provide a natural and efficient alternative to conventional and xenobiotic chemicals and products. Rhizospheric fungi must synthesize metabolites to compete with co-emerging organisms, hosts, and pathogens to colonize the host and for nutrition. Fungus produces metabolites that may kill bacteria and regulate the metabolism of other fungi and plant cells (Zeilinger et al. 2016). These metabolites might be used to defeat diseases and cancer cells. They may control the growth of cancer cells or kill the cancer cells.

Exploring the resources of various functional chemicals is crucial as the primary sources of structurally distinct bioactive natural metabolites with broad therapeutic potential, including alkaloids, enopyranones, benzoquinones, flavonoids, phenols, steroids, terpenoids, and tetralones (Pandit et al. 2018). They are a good source of biological substances with therapeutic potential, such as

antibacterial (Takahashi and Nyvad 2008), antifungal (Nicoletti et al. 2007), antiviral (Parshikov et al. 2015), and anticancer, cholesterol-lowering agents (Noman et al. 2021). Using morphological, microscopic, and the ITS 18S rDNA sequences, the *Aspergillus* strain (Isolate No. ABRF4) that was isolated from the Achanakmar Biosphere Reserve, Chhattisgarh, India, was identified as *Aspergillus carneus*. Previous research showed that compounds from *A. carneus* are effective against Gram-positive bacteria (Özkaya et al. 2018). The current investigation was conducted to determine the antimicrobial activity of *A. carneus* against four pathogenic bacteria, i.e., *Bacillus circulans* (MTCC-7906), *Bacillus subtilis* (MTCC 441), *Ralstonia eutropha* (MTCC-2487) and *Staphylococcus aureus* (MTCC-96), and proliferative activity against several cancer cell lines.

MATERIALS AND METHODS

Ethics statement

Ethics approval and consent to participate are not applicable as this study did not involve human participants or animals.

Chemicals and reagents

Chemicals and reagents used in this study were: Potato Dextrose Agar, Potato Dextrose Broth, and Czapekdox Agar (Himedia, India). 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethyl benzothiazole-6-sulphonic acid) (ABTS⁺), acarbose, potassium persulfate, were purchased from Sigma-Aldrich, USA. All reagents and chemicals were analytical grades. In addition, all the solvents (toluene, chloroform, ethyl acetate, methanol, and acetonitrile) used for extraction were HPLC grade.

Fungal isolation and identification

As previously described, a pure fungal strain ABRF4 was isolated from the rhizospheric soil of Achanakmar Biosphere, Bilaspur, CG, India (Radhika and Rodrigues 2010). It was identified as *A. carneus*.

Source of microorganisms for therapeutic studies

The antibacterial activity of fungal metabolites was tested against four pathogenic bacterial strains and one yeast isolate. Bacterial strains and yeast were obtained from microbial-type culture collection (MTCC, CSIR-IMTECH, Chandigarh, India). Bacteria isolates and yeast used in this study were *B. circulans* (MTCC-7906), *B. subtilis* (MTCC 441), *R. eutropha* (MTCC-2487), *S. aureus* (MTCC-96). All bacterial cultures were cultivated overnight on Nutrient Agar Medium (NA) slants and kept at 4°C as described previously (Ferris and Tuomisto 2015; Sahu et al. 2020; Sahu et al. 2022).

Fermentation

The pure fungal strain was inoculated into 500 mL of Yeast Extract Sucrose Broth (YESB) medium in a 1,000 mL Erlenmeyer and cultured for 14 days at 28°C. The culture was filtered using laboratory filter paper after

incubation. The antibacterial and anti-proliferative activity of the column-purified fractions of the fungal extract was investigated.

Selection of optimum culture medium

To evaluate the best media for secondary metabolite growth, development, and production, fungal isolate ABRF4 was cultivated for 15 days in five different fungal growth media. Five media used in this study were CzapekDox Broth (CDB), CzapekDox Yeast Broth (CDYB), Malt Extract Broth (MEB), Potato Dextrose Broth (PDB), and Yeast Extract Sucrose Broth (YESB) (Sahu et al. 2022). Fungal culture achieved maximum growth and secondary metabolites production in YESB medium incubated at 28°C for 15 days.

Screening

At post-incubation, culture media was added with 1.5% tween-80 and shaken for 30 min at ambient temperature for homogeneous cell wall disruption of the fungal biomass and subsequent release of intracellular secondary metabolites into media. As described previously, the fungal biomass was screened for intracellular and extracellular secondary metabolites (Alkhulaifi et al. 2019). The filtrates were collected and concentrated up to 20% of their original volume (v/v) using a vacuum evaporator at 50 ± 5°C. The residue was concentrated and stored before diluting it as a working solution in sterile distilled water for further studies.

Extraction

Fungal biomass was extracted using ethanol on a soxhlet extractor, followed by filter sterilization before concentration and drying off the ethanol extract.

The ethanol extract was purified by further procedures to obtain active metabolites. Briefly, 1g of dry extract was solubilized in ethanol (1:1 w/v) and subjected to adsorption chromatography on a glass column packed with silica gel (60-120 mesh size) in toluene. Next, elution was carried out by standard method with increasing polarity of toluene, chloroform, ethyl acetate, methanol, and acetonitrile. Fractions obtained from each solvent were collected and subjected to spectrophotometric evaluation. Finally, a rotatory evaporator further concentrates selected fractions. Since the preliminary study results showed that the maximum activity was obtained in the ethanolic extracts, it was further analyzed. The crude ethanolic extract was mixed with toluene (1:1 w/v) and subjected to adsorption chromatography on a glass column packed with silica gel (60-120 mesh size) with different eluents according to the increasing polarity of toluene, chloroform, ethyl acetate, methanol, and acetonitrile. In addition, TLC and Gas Chromatography-Mass Spectroscopy (GC-MS) methods were used to identify active secondary metabolites in the extracts.

Purification of ethanolic extract

Thin layer chromatography (TLC)

The ethanol extract was transferred on a TLC plate and developed using an 18:80:2 (v/v) solvent system of

acetonitrile, water, and acetic acid. The chromatogram profile was examined under UV at 365nm to determine the R_f value. The fractions with similar TLC profiles were grouped, and a single spot on the TLCs indicated that the compounds were relatively pure (Azerang et al. 2019).

Identification of compound using spectroscopic methods

Fourier Transform Infrared (FTIR) spectroscopy

A Fourier Transform Infra-Red Spectrometer (105 Nicolet Avatar 370, ThermoScientific, USA) was used to determine the infrared spectra of the pure compound at room temperature. Pure chemical (5 mg) was combined with spectroscopic grade KBr (95 mg) for pellet manufacturing. The IR spectra were captured in transmission mode at a 4000-400 cm⁻¹ frequency range. A KBr pellet with no sample was employed as the control (Sahu et al. 2020).

Gas Chromatography-Mass Spectroscopy (GC-MS)

The samples were analyzed using GC-MS (Shimadzu GC-MS-QP2020; Kyoto, Japan) for qualitative and quantitative analysis utilizing the electron impact ionization (70 eV) method and mass spectra (Shimadzu GC-MS-QP2020; Kyoto, Japan). First, the compounds were identified by comparing their relative index to the mass spectra of standards in the National Institute of Standards and Technology's (NIST; Gaithersburg, Maryland, United States) GCMS library. Then, the proportion of the compound was calculated based on the peak area.

Bioactive properties of the isolated compound

In all the tests, the antimicrobial and anti-proliferative properties of extract and pure compounds from the strain ABRF4 were assessed in triplicates with appropriate blanks and controls.

Assessment of antibacterial activity of the fungal extract

The antibacterial activity of the fungal extract and fractions was determined using the agar well diffusion method (Balouiri et al. 2016) against human pathogenic bacteria, i.e., *Bacillus circulans* (MTCC-7906), *B. subtilis* (MTCC 441), *S. aureus* (MTCC-96), and *R. eutropha* (MTCC-2487). The vehicle control was ethanol, while the positive control was streptomycin (1 mg/mL) (Sahu and Jha 2020a,b).

Anti-proliferative activity

The cytotoxicity of the fractions was assessed using the Sulforhodamine B assay in various human tissue-specific cancer cell lines, including breast cancer (MDA-MB-231, MDA-MB-468, and MCF-7), liver cancer (HepG2), lung cancer (A-549), prostate cancer (DU-145) and primary control cell line (HEK-293). Briefly, 5×10³ cells/ well were seeded onto a 96-well plate. After a 24 h incubation period, cells were treated for 48 h with increasing concentrations (1, 10, 100, and 300 mg/mL) of methanol, and ethanol fractions, followed by fixation and SRB staining of treated cells and vehicle controls. Doxorubicin was used as a positive control. A multimode reader (Perkin

Elmer, Germany) was used to detect the optical density at 510 nm, and IC₅₀ was calculated using GraphPad Prism 9.0 (Manupati et al. 2017).

Statistical analysis

All experiments were carried out in triplicate. Therefore, all the statistical data are the average of triplicate determination ± SD.

RESULTS AND DISCUSSION

Determination of secondary metabolites using TLC, GC-MS analysis, and FTIR

A fungus isolated from the Achanakmar Biosphere of Chhattisgarh was identified as *A. carneus* (isolate no. ABRF4) based on macroscopic and microscopic properties and the ITS 18S rDNA sequences (Sahu and Jha 2020a,b). Its secondary metabolites were screened for their bioactivities. The ethanolic extract of fungal biomass was fractionated using various solvents ranging from polar to non-polar solvents, i.e., acetonitrile, ethyl acetate, methanol, chloroform, and toluene. Fractions were tested for biological activity.

ABRF4 ethanolic extract (Spot A) and fractions of ethyl acetate and acetonitrile produced fluorescent spots under UV light (Spots B and C). The R_f values for Spots A, B, and C were 0.451, 0.351, and 0.283, respectively.

Ethyl acetate extract of *A. carneus* ABRF4 had maximum activity. Therefore, it was further spotted on TLC to isolate and purify the compounds (Figure 1.A). The results showed the presence of four peaks in purified fractions (Figure 1.B). A unique peak in the ethyl acetate fraction was found at 1190 cm⁻¹, which represented C–OH stretching and C–C stretching due to esters. COO-symmetric stretching was suggested by absorption at 1449.19 cm⁻¹, whereas alkane and amine group stretching was indicated by absorption at 2979.32 cm⁻¹. The IR spectrum revealed the presence of an aromatic ring replacing the ester bond, clearly indicating the predicted functional group of cycloalkanes structure (Figure 1.C-F). The GC-MS of the ethyl acetate fraction revealed the presence of cyclohexane, 1,3-dimethyl-, trans (Molecular weight 112 Da) (Table 1, Figure 1.G). This secondary metabolite has been hypothesized as the bioactive compound, while the remaining compounds are either primary metabolites or chemical compounds not involved in biological activity.

Antibacterial activity

The acetonitrile, ethyl acetate, methanol, chloroform, and toluene fractions of ABRF4 isolate were tested for antibacterial activity at 100 µg/mL. It inhibited the growth of *B. circulans* (MTCC-7906), *B. subtilis* (MTCC-441), *R. eutropha* (MTCC-2487) and *S. aureus* (MTCC-96) (Figure 2). A clearance zone indicated antibacterial activity. The diameter of inhibition against *S. aureus* was 17.18 ± 1.3 mm, and the diameter against *R. eutropha* was 15.95 ± 1.03 mm. The ethyl acetate fraction had the highest antibacterial activity (Table 2).

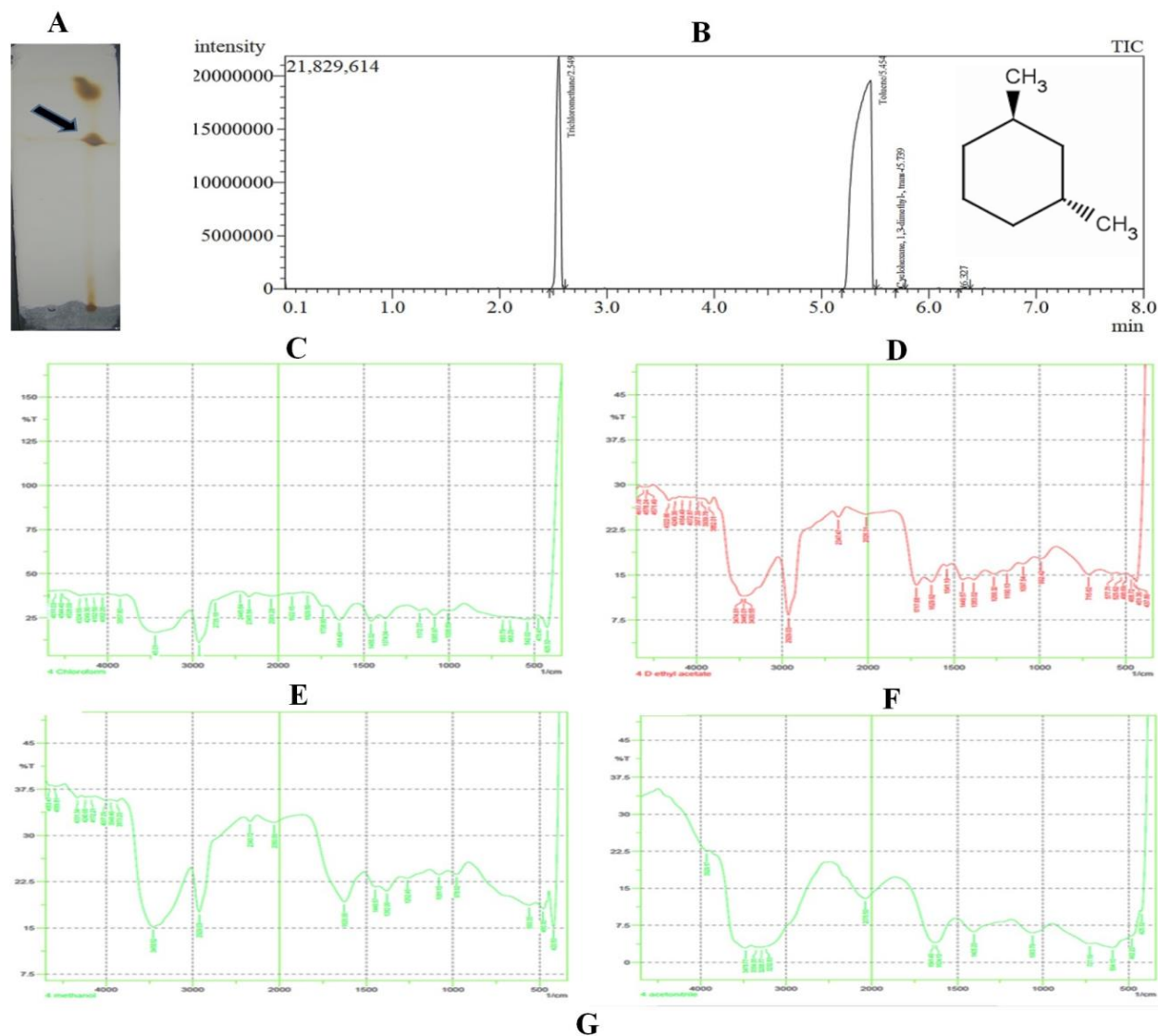


Figure 1. A-G. Chromatographic and spectroscopic analysis of the purified compound from *Aspergillus carneus* ABRF4

Table 1. Retention time of *Aspergillus carneus* (ABRF4) secondary metabolites and their respective retention time obtained by GCMS

Peak	Retention time	Area	Area percent	Name
1	2.549	59467953	21.73	Trichloromethane
2	5.454	213778647	78.11	Toluene
3	5.739	198449	0.07	Cyclohexane, 1,3-dimethyl-, trans-
4	6.327	256562	0.09	
		273701611	100	

Table 2. Antibacterial activity of *Aspergillus carneus* (ABRF4) activity

Bacterial pathogen	Zone of inhibition ^e					Positive ^g control	Negative ^g control
	Column fraction of ethanolic extract of <i>Aspergillus carneus</i> (ABRF4)						
	Toluene	Cholororm E.	Acetate	Methanol	Acetonitrile		
<i>B.circulans</i> (gram +ve)	Nd	11 ± 1.3	15.4 ± 1.25	Nd	16.16 ± 1.1	24.28±1.25	Nd
<i>B.subtilis</i> (gram +ve)	Nd	Nd	14.7 ± 1.15	Nd	15.81± 1.8	24.21±0.93	Nd
<i>S. aureus</i> (gram +ve)	Nd	Nd	17.18 ± 1.3	Nd	17.16 ± 0.2	24.62±1.05	Nd
<i>R. eutrophae</i> (gram –ve)	Nd	Nd	Nd	Nd	15.95 ± 1.0	25.33 ± 1.7	Nd

Note: Nd: not detected. The superscript letters are significantly different (p < 0.05). ²Inhibition zone excluding disc. ⁶6 mm disc as standard

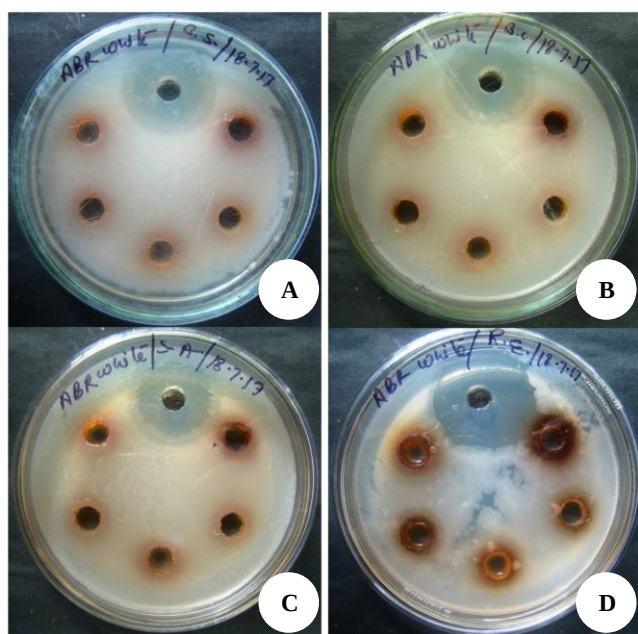


Figure 2. Antimicrobial activity of *Aspergillus carneus* ABRF4 against A. *Bacillus subtilis*, B. *Bacillus circulans*, C. *Staphylococcus aureus*, D. *Ralstonia eutropha*

Determination of anti-proliferative activity

SRB assay was used to test all three fractions of the fungal isolate ABRF4 against tissue-specific cancer cell lines. Fraction A (ethyl acetate fraction) exhibited relatively moderate anti-proliferative activity against lung cancer cell line, A-549, breast cancer cell lines, MCF-7, and MDA-MB-468, with an IC_{50} of 14.69, 15.01, and 18.93 $\mu\text{g/mL}$, respectively. Fraction B (methanol fraction) had an IC_{50} of 14.3, 15.66 $\mu\text{g/mL}$ against the breast cancer cell line, MDA-MB-231, and prostate cancer cell line, DU-145, respectively. Finally, fraction C (ABRF4, ethanol fraction) exhibited an IC_{50} of 11.83, 12.75, and 13.82 $\mu\text{g/mL}$ against DU-145, MDA-MB-231, and HepG2, respectively. These three fractions showed an IC_{50} value approximately five times higher than the control primary cell line, HEK-293,

suggesting these fractions had anti-proliferative activity (Table 3).

Discussion

The fungus *A. carneus* isolate-ABRF4 was identified based on morphological and sequencing similarity. Extracellular and intracellular extracts were tested for their bioactivities. Intracellular extracts had higher anti-proliferative activity than extracellular extracts (data not shown). The fractions were investigated against Gram-positive and Gram-negative bacteria. The results showed that the fractions of ethyl acetate, methanol, and acetonitrile inhibit the growth of both Gram-positive and Gram-negative bacteria. The TLC profile of the ethyl acetate fraction revealed a single spot with an R_f value of 0.5, like alternariol-like compounds isolated from fungi (Wei et al. 2017; Tulsawani et al. 2020). Trans-1,3-dimethyl-cyclohexane was one of the metabolites identified in the fraction that might have biological activity. Therefore, it needs further analysis. FTIR was used to validate the identity of the compound and its structural group. Subsequently, the GC-MS of ethyl acetate fraction confirmed the presence of trans-1,3-dimethyl-cyclohexane. Esters exhibit antibacterial activities (Tomar et al. 2019). Cyclohexane and its derivatives have various biological activities such as antioxidant, anticancer, cytotoxic, analgesic, anti-inflammatory, and anti-thrombin activities (Modak et al. 2011; Shoaib et al. 2021). The previous study by Lallo et al. (2014) showed 3-acetyl-4-benzoyl-1-benzoyloxymethyl-1,6-diepoxy-cyclohexan-2,3,4,5-tetrol has cytotoxic activity against pancreatic and breast cancer but not against normal cell lines. The 1,1-disubstituted cyclohexane-1-carboxamides also exhibited anticancer activity against various cancer cell lines, including MCF-7, HepG2, A549, and HTC-116. The compounds were considered potent apoptotic inducers interfering with extrinsic and intrinsic apoptotic pathways (Abd-Allah and Elshafie 2018; Abd-Allah et al. 2019). The results of the present study showed that trans-1,3-dimethyl-cyclohexane isolated from ethyl acetate fraction of *A. carneus* ABRF4 has anti-proliferative properties by inducing an apoptotic regulation in the cancer cells.

Table 3. IC_{50} value of *Aspergillus carneus* (ABRF4) fractions against various cancer and non-cancerous cell lines

<i>Aspergillus carneus</i> (ABRF4) HPLC fractions	IC_{50a} ($\mu\text{g/mL}$)						
	MCF-7 ^b	MDA-MB-468 ^c	MDA-MB-231 ^d	DU-145 ^e	HEPG-2 ^f	A-549 ^g	HEK-293 ^h
Fraction A	15.01 $\pm$ 0.35	18.93 $\pm$ 2.12	41.87 $\pm$ 1.98	53.22 $\pm$ 2.73	25.67 $\pm$ 3.73	14.69 $\pm$ 2.76	90.82 $\pm$ 1.93
Fraction B	44.28 $\pm$ 1.10	29.74 $\pm$ 2.42	14.31 $\pm$ 1.53	15.66 $\pm$ 2.47	57.61 $\pm$ 2.82	79.58 $\pm$ 2.92	82.09 $\pm$ 5.45
Fraction C	19.26 $\pm$ 1.48	22.48 $\pm$ 3.26	12.75 $\pm$ 0.02	11.83 $\pm$ 0.01	13.82 $\pm$ 1.89	18.67 $\pm$ 0.55	77.3 $\pm$ 1.28
Doxorubicin (μM)	5.78 $\pm$ 0.44	0.77 $\pm$ 0.06	1.33 $\pm$ 0.02	0.74 $\pm$ 0.15	0.90 $\pm$ 0.07	2.98 $\pm$ 0.25	37.95 $\pm$ 4.50
Positive control							

Note: Fraction A: n-valeric acid (methanol), B: ethyl oleate (methanol), C: Cyclohexane, 1,3-dimethyl-, trans- (Ethanol). ^a50% inhibitory concentrations and mean $\pm$ SEM of IC_{50} ($\mu\text{g/mL}$) values of different fractions represent the mean of three replications. ^bLuminal-A (ER⁺/PR⁺/Her2⁻) breast cancer. ^cBasal (low claudin) Triple-negative (ER⁻/PR⁻/Her2⁻) breast cancer. ^dBasal Triple negative (ER⁻/PR⁻/Her2⁻) breast cancer. ^eModerate metastatic potential (PSA⁺) Androgen-independent prostate cancer. ^fLiver hepatocellular carcinoma. ^gAdenocarcinoma human alveolar basal epithelial cells lung cancer. ^hNon-cancerous primary human embryonic kidney cells.

In conclusion, the *A. carneus* ABRF4 contains several potent secondary metabolites, including trans-1,3-dimethyl-cyclohexane (Molecular weight 112 Da), which has anti-proliferative activity suggesting its potential therapeutic application.

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Effectiveness test of *Diploknema oligomera* extracts to the decrease in glucose levels in alloxan-induced BALB/c male mice

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Abstract. Faizah S, Eriani K, Rosnizar. 2023. Effectiveness test of *Diploknema oligomera* extracts to the decrease in glucose levels in alloxan-induced BALB/c male mice. Nusantara Bioscience 15: 143-148. People with diabetes mellitus usually consume chemical drugs to lower their blood glucose levels which cause side effects and require a reasonably high cost. Therefore, alternative natural medicines (phytopharmaceuticals) that are safer and more affordable are needed. Punti fruit (*Diploknema oligomera* H.J Lam) has the potential as an antihyperglycemic agent that can lower glucose levels in the blood because the punti fruit contains flavonoid compounds, tannins, terpenoids, and saponins. These secondary metabolites can reduce blood glucose levels. This research used an experimental method with a completely randomized design, six treatments, and four replications. This study used 24 BALB/C (*Mus musculus* Linnaeus, 1758) male mice grouped into six treatments, namely normal control treatment (P1), negative control by giving alloxan 150 mg/kg bw (P2), positive control by giving glibenclamide dose of 0.65 mg/kg body weight (bw) (P3), ethanol extract of punti fruit dose of 250 mg/kg bw (P4), ethanol extract of punti fruit dose of 500 mg/kg body weight (P5) and ethanol extract of punti fruit dose of 1,000 mg/kg bw (P6). The results proved that administering punti extract (P3, P4, P5) for 14 could reduce blood glucose levels in male diabetic mice with a percentage reduction of 33.6%, 37.4%, and 52.7%, and treatment-positive control decreased blood glucose by 32.6%. The conclusion showed that the ethanol extract of punti fruit at a dose of 1,000 mg/kg bw could effectively reduce blood glucose levels in diabetic mice compared to a dose of 250 mg/kg bw, 500 mg/kg bw and 0.65 mg/kg glibenclamide kg bw. Therefore, punti fruit can be used as a drug candidate to be developed as phytopharmaceuticals in treating diabetes mellitus.

Keywords: Antioxidant, blood glucose, diabetes mellitus, hyperglycemia, hypoglycemia

INTRODUCTION

Hyperglycemia is one of the causes of Diabetes Mellitus (DM). It is a degenerative disease that causes the most deaths in the world. Based on the International Diabetes Federation (IDF), there were 425 million people with diabetes worldwide in 2007, and Indonesia was ranked seventh with 10.7 million DM sufferers in 2019. The number increased by 19.5 million in 2021 and was ranked fifth worldwide (IDF 2021; Sun et al. 2022); hyperglycemia of DM sufferers is caused by diet. Widiyanto and Rahayu's (2019) research result on 17 respondents at the Sidomulyo Health Center in Pekanbaru indicated that 51.5% of respondents had irregular eating patterns. Rahati et al. (2014) stated that consuming foods containing excess carbohydrates has a high risk of diabetes mellitus. The high consumption of carbohydrates results in a buildup of glucose in the blood (Hawash et al. 2020), which causes the pancreas to work harder to produce insulin (Rahman et al. 2021). If this happens continuously, the pancreas will be damaged, and insulin cannot be produced optimally. These conditions lead to diabetes mellitus (Feinman et al. 2015).

Moreover, people with diabetes mellitus must lower their blood glucose levels by consuming chemical drugs, such as glibenclamide, acarbose, and metformin. However, metformin has side effects: impaired liver and kidney

function and digestive disorders, such as flatulence, vomiting, abdominal pain, diarrhea, and constipation (Ghosal and Ghosal 2019). Likewise, glibenclamide's side effects include diarrhea, nausea, vomiting, and constipation (Rani et al. 2014). In addition, chemical drugs take longer and are quite expensive to treat hyperglycemia. Therefore, alternative therapeutic solutions from plants that are safer, more affordable, and effective in dealing with hyperglycemia in diabetes mellitus patients are needed.

One of the plants with the potential to an antihyperglycemic is punti (*Diploknema oligomera* H.J. Lam). Punti contains secondary metabolites, such as phenolic compounds, flavonoids, saponins, tannins, and terpenoids (Furqan and Putri 2020). These secondary metabolites can lower blood glucose (Lisdiani et al. 2022) by increasing insulin secretion (Martin and Ramos 2021) and/or regenerating pancreatic β cells (Ghorbani et al. 2019). In addition, these secondary metabolites also lower blood glucose by increasing insulin sensitivity through the stimulation of insulin receptors (Xu et al. 2018) on insulin target cells, such as adipose cells (Kang and Chiang 2022), liver cells, and muscle cells (Hu et al. 2014).

Several studies have investigated various plants' activity with secondary metabolites in reducing blood glucose levels. For example, Njogu et al. (2018) reported that *Kigelia africana* (Lam.) Benth. leaves have hypoglycemic activity due to phytochemicals detected in leaf powder.

These phytochemical compounds include phenolics, saponins, tannins, flavonoids, and alkaloids. Likewise, Morada et al. (2016) found that tannins extracted from *Sonneratia alba* Sm. leaves (16 mg/g body weight) were given to hyperglycemic mice for 6 hours and 12 hours; it showed a significant decrease in blood glucose levels (39,6% and 56,4%). Meanwhile, Nardia et al. (2020) reported the ethanol extract of *Persea americana* Mill. seeds contain polyphenols, tannins, flavonoids, triterpenoids, quinones, monoterpenoids, and sesquiterpenoids. Furthermore, these phytochemicals proved in these secondary metabolites could lower blood glucose in diabetic mice.

Another study conducted by Song et al. (2022) revealed that terpenoid compounds extracted from the bark of *Dillenia indica* L could stimulate insulin receptors so that they have the potential as antihyperglycemic agents. Meanwhile, research by Latief et al. (2021) showed that the ethanol extract of Sungkai leaves (*Peronema canescens* Jack) contains alkaloids, steroids, terpenoids, flavonoids, saponins, tannins, and phenolics. These phytochemicals could reduce glucose levels by inhibiting the α -glucokinase enzyme, increasing antioxidant enzyme activities, and regenerating pancreatic β cells. It is in line with research by Yuliana et al. (2021) that the ethanol extract of *karamunting* (*Rhodomyrtus tomentosa* (Aiton) Hassk.) leaves could reduce blood glucose levels in diabetic rats. The *karamunting* leaves contain phenolic compounds, such as flavonoids, saponins, tannins, steroids, and triterpenoids (Dona et al. 2020). Matusin et al. (2021) examined the methanol extract of *Dillenia excelsa* leaves. These leaves have antihyperglycemic properties because of the triterpenoid compound effects: flavonoids, tannins, and phenolics (Lisdiani et al. 2022). Meanwhile, Furqan and Putri's (2020) found that the ethanol extract of punti contains flavonoids, terpenoids, phenolics, tannins, and saponins. However, punti as an antihyperglycemic agent has not been studied scientifically. Therefore, research on punti (*D. oligomera*) as an antihyperglycemic is paramount so that the findings can later provide information regarding the treatment of diabetes mellitus.

MATERIALS AND METHODS

Sample extractions

Punti powder of 150 g was soaked in one liter of 96% ethanol and then allowed to stand for three days in a glass container protected from light. Then it was filtered with filter paper to get the filtrate. The filtrate was then concentrated using a rotary evaporator at 60°C for $\pm$ 3 hours.

Management of experimental animals

The experimental animals used in this study were 24 BALB/c male mice weighing 20-30 g. All experimental animals were given water and all feed-4 based on body weight (ad libitum) and placed in cages 20 cm x 13 cm x 10 cm covered with sawdust. The cage was enclosed with iron wire measuring 35 cm x 35 cm and cleaned every two

days.

Diabetes induction in experimental animals

Before intraperitoneal induction of alloxan monohydrate (Sigma-Aldrich, USA), BALB/c male mice fasted for 24 hours. Alloxan with a 150 mg/kg BW dose was dissolved in 1 mL of 0.9% NaCl (Widastuti et al. 2017). After 72 hours of induction, the blood glucose levels of BALB/c male mice were checked with the EasyTouch GCU. The positive control and treatment groups included blood glucose values above > 200 mg/dL.

Experimental animal treatments

Experimental animals were divided into six groups consisting of four BALB/c male mice. The normal control group was given water and all feed-4 (P1). In comparison, alloxan monohydrate (P2) induced the negative control group. The positive control group was induced by alloxan monohydrate and glibenclamide administration of 0.65 mg/kg bw (P3). Groups P4, P5, and P6 were induced by alloxan and given punti extracts orally at a dose of 250 mg/kg bw for P4, 500 mg/kg bw for P5, and 1,000 mg/kg bw for P6. All treatments were carried out at 10.00 WIT for 14 days.

Measurement of glucose levels

Blood samples were taken by cutting a small part of BALB/c male mice's tails. The tails were cleaned first with cotton given 70% alcohol. Then, the blood from the tail vein was dripped onto the strips attached to the Easy Touch GCU glucometer. Blood measurements were carried out on days 0, 4, 11, and 18. Blood sugar levels on day zero were glucose levels after fasting for 16 hours before alloxan was induced. Meanwhile, on the fourth day, it was diabetic blood sugar levels after alloxan induction and recovery blood sugar levels after treatment of punti extracts and glibenclamide administration on the 11th and 18th days.

Data analysis

Blood glucose levels obtained were analyzed using the SPSS 25 software with a one-way Analysis of Variance (ANOVA) test. The ANOVA test aims to determine differences in blood glucose levels between treatments. If there is a significant difference, proceed with Duncan's multiple range test at a 5% confidence level (Eriani et al. 2021).

RESULTS AND DISCUSSION

The results of the 6 treatments showed blood glucose levels in mice for 18 days of treatment, as shown in Figure 1. After 72 hours of alloxan induction, the blood glucose examination with a dose of 150 mg/kg body weight to the treatment (P2, P3, P4, P5, and P6) showed that blood glucose levels increased by > 200 mg/dL on the 4th day. The highest increase in blood glucose levels was found in the negative control treatment on day 11 and day 18, with an average of 442.5 ± 66.28 mg/dL and 455.50 ± 38.46 mg/dL. Duncan's 5% confidence level test showed that the

average glucose levels on day 11 and day 18 in the negative control treatment were significantly different from the other treatments (Table 1).

The positive control treatment with glibenclamide of 0.65 mg/kg in diabetic mice showed a decrease in blood glucose levels of 401.75 ± 58.60 mg/dL on day 11 and continued to decrease to 269.75 ± 38.87 mg/dL on day 18, reduced by 32.9%. In addition, the results of Duncan's multiple range test with a 5% confidence level showed that the average blood glucose levels on day 11 and day 18 in the positive control treatment significantly differed from the negative control treatment (Table 1).

The examination of blood glucose levels in diabetic mice after adding punti fruit ethanol extract in the treatments (P4, P5, and P6) showed a decrease in blood glucose levels on the 11th and 18th days by 33.6%, 37.4%, and 52.7%, respectively. The percentage value of decreasing blood glucose was measured by subtracting the average blood glucose levels from day 11 to day 18. The reduction results were then divided by 100% to obtain the percentage value of decreasing blood glucose levels (Chandrika et al. 2006). The Duncan test with a 5% confidence level proved that the punti fruit ethanol extract treatment (P4, P5, P6) significantly differed from the negative control treatment (Table 1).

The admission of punti ethanol extract to diabetic mice in treatment (P6) for 14 days showed a higher decrease by 52.7% compared to the positive control treatment (32.9%). For other treatments (P4 and P5), it was 33.6% and 37.4%. These percentages indicated treatment 6 had the highest blood glucose reduction compared to the positive control treatment (P4 and P5). The Duncan test with a 5% confidence level also revealed that, on average, blood glucose levels at P6 significantly differed from the positive control treatments (P4 and P5) (Table 1).

Discussion

This study proved that mice induced with alloxan starting from 150 mg/kg showed an increase in blood glucose levels >200 mg/dL on the 4th day. That aligns with Widastuti et al. (2017) reported that alloxan at 150 mg/kg increased blood glucose levels in mice >200 mg/dL on day 4. The increase in blood glucose levels is due to the alloxan mechanism. Alloxan's structure is similar to glucose; it can enter pancreatic β cells via glucose transporter 2 (GLUT2)

(Berraouan et al. 2015). In addition, alloxan also perforates the pancreatic β cells through the cell membrane because the structure of alloxan consists of substituted N atoms bound to lipophilic alkyl groups, easily penetrating all parts of the cell membrane. Furthermore, the lipophilic nature of alloxan leads to free radical toxicity (Ighodaro et al. 2017), which occurs through Reactive Oxygen Species (ROS). ROS undergo dismutation into hydrogen peroxide (H_2O_2) and hydroxyl radicals ($*OH$). Hydroxyl radicals are more reactive, so these radicals destroy pancreatic β cells (Eguchi et al. 2021), leading to reduced insulin production (Udia et al. 2016) and hyperglycemia (Abdulgani et al. 2014).

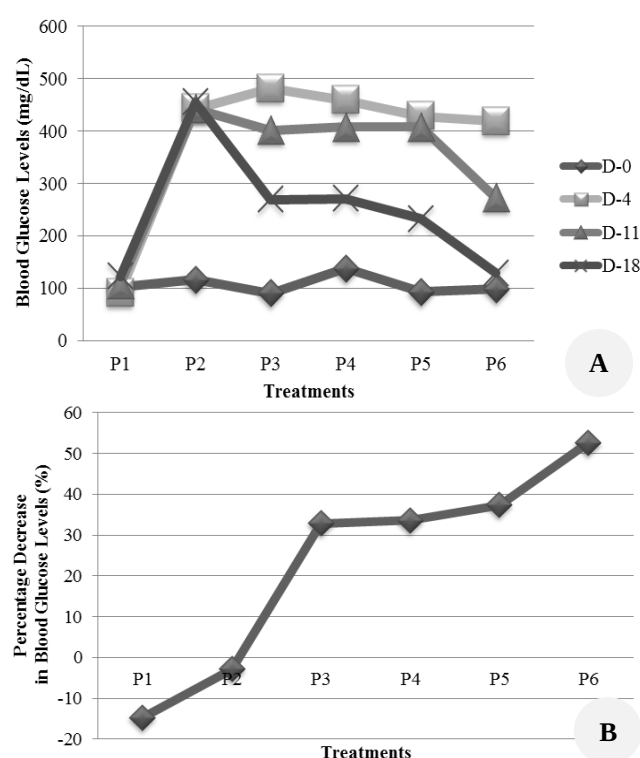


Figure 1. Blood glucose levels of 1 to 6 treatments A. Average blood glucose levels during the 18 days of treatment, B. Percentage reduction in average blood glucose levels from day 11 to day 18

Table 1. Analysis of average blood glucose levels in BALB/c male mice during the treatments

Treatments	Blood glucose levels (mg/dL) ($\bar{x} \pm SD$)				Decrease %
	0 day	4 th day	11 th day	18 th day	
P1	102.75 ± 16.68	91.750 ± 22.90	107.25 ± 27.60^a	123.00 ± 27.30^a	-14.68
P2	116.25 ± 30.33	443.00 ± 77.59	442.50 ± 66.28^c	455.50 ± 38.46^c	-2.9
P3	90.00 ± 7.300	481.25 ± 34.1	401.75 ± 58.60^b	269.75 ± 38.87^b	32.9
P4	137.75 ± 42.5	458.75 ± 96.2	408.00 ± 90.80^b	270.75 ± 81.70^b	33.6
P5	93.50 ± 28.55	428.50 ± 26.51	408.00 ± 90.80^b	233.00 ± 44.02^b	37.4
P6	98.50 ± 11.26	418.75 ± 9.630	272.75 ± 78.40^a	129.00 ± 59.50^a	52.7

Note: Numbers followed by different letters in the same column state significant difference at α of 0,05 (Duncan). P1 = Normal control, P2 = Negative control, P3 = Positive control, P4 = Mice given punti fruit extract 250 mg/kg BW, P5 = Mice given punti fruit extract 500 mg/kg BW, P6 = Mice given punti fruit extract 1,000 mg/kg BW

Adding glibenclamide to diabetic mice in the positive control treatment decreased the average blood glucose level due to its mechanism. Glibenclamide could lower blood glucose through sulfonylurea 1 (SUR1) receptor inhibition, which closes the ATP-sensitive potassium channels (KATP) in the pancreatic β cell membrane. That causes depolarization of the pancreatic β -cell membrane and Ca^{2+} channels opening. As a result, Ca^{2+} ions enter the cell and increase the intracellular Ca^{2+} ions. The increasing Ca^{2+} in pancreatic β cells will stimulate insulin-carrying granules; therefore, insulin is released, and blood glucose decreases (Yau et al. 2021).

In this study, punti fruit was extracted using ethanol solvent. According to Yusnawan (2013), using polar solvents (ethanol) for maceration can produce a diverse group of secondary metabolites. Furthermore, polar solvents include alcohol groups generally used to extract secondary metabolites in plants. Polar solvents can increase cell permeability and penetrate the cell, resulting in extraction of more endocellular secondary metabolites, both polar and less polar compounds, than non-polar solvents (Seidel 2012). Research by Azmir et al. (2013) reported that ethanol, as a polar solvent, can attract polyphenolic compounds, tannins, and terpenoids. Meanwhile, research conducted by Hikmawanti et al. (2021) proved that ethanol solvent could attract phenolic and flavonoid compounds in the *katuk* leaf plant (*Sauropus androgynus* (L.) Merr.). Likewise, Furqan and Putri's research (2020) reported that ethanol solvents produced saponin, tannin, phenolic, and flavonoid compounds in punti fruit plants.

The administration of punti ethanol extract to diabetic mice in the treatments (P4, P5, and P6) reduced the average blood glucose level. That is presumably due to the effects of the flavonoids, terpenoids, saponins, and phenolic compounds in the ethanol extract of punti. Xu et al. (2018) reported that saponin compounds could increase insulin sensitivity through the stimulation of insulin receptors (insulin receptor substrate-1 (IRS-1). IRS-1 stimulation activates phosphoinositide 3-kinase (PI3K) phosphorylation and protein kinase B (PKB)/AKT activation. PKB/AKT activation can also be done by phenolic flavonoids (Deng et al. 2020) and terpenoids (Song et al. 2022). Hu et al. (2014) argued that IRS-1 stimulation with PI3K and AKT activation could cause GLUT4 translocation to cell membranes, increasing glucose uptake in liver and skeletal muscle cells and decreasing blood glucose.

Saponins and phenolic compounds also lower blood glucose by stimulating adenosine 5' monophosphate-Activated Protein Kinase (AMPK), causing glucose absorption in adipose tissue and affecting blood glucose reduction (Xu et al. 2018; Kang and Chiang 2022). Meanwhile, tannin lowers blood glucose with its ability to inhibit glucose-6-phosphatase, increases glucose absorption in hepatocyte cells and myoblast cells, and acts as an inhibitor of the α -glucosidase enzyme that can delay glucose absorption in the small intestine wall (Sheikh et al. 2019). Saponin and flavonoids also can inhibit the α -glucosidase enzyme (Sohretoglu et al. 2018; Nabil et al. 2019).

Flavonoid also increases insulin secretion (Khedher et al. 2018) through the effect of regenerating pancreatic β cells (Ghorbani et al. 2019), which in turn lowers blood glucose (Kasmawati et al. 2019). Flavonoids carry out the regeneration mechanism of pancreatic β cells from the flavone group. Flavones consist of rings C (C2, C3) and B (C4), which are thought to stimulate glucagon-like peptide-1 (GLP-1) (Wang et al. 2018). The activation of GLP-1 leads to PI3K and AKT phosphorylation (Campbell and Drucker 2013). In addition, Phosphorylated AKT will increase the expression of the pancreatic duodenum homeobox-1 (PDX-1) gene. This gene can regulate the regeneration of pancreatic β cells through the processes of proliferation, differentiation, and transdifferentiation (Seeberger et al. 2014; Guney and Lorberbaum 2020). In addition, the pancreatic β cell regeneration activity can increase the amount of insulin secretion, decreasing blood glucose (Campbell and Drucker 2013).

Furthermore, phenolic can act as an antioxidant by protecting pancreatic β cells from damage caused by alloxan (Adiba et al. 2021). Phenolic has hydroxyl groups of one or more aromatic ring structures (Singh et al. 2015). Based on the number of hydroxyl groups, it is grouped into several compounds, such as tannins and flavonoids (Singh et al. 2016). Phenolic compounds, such as flavonoids with hydroxyl groups (OH^-), can donate hydrogen atoms (H) to stabilize free radicals (Vo et al. 2019). In addition, free radicals are unstable and highly reactive compounds (Kia et al. 2020) that can cause cell damage (Yu et al. 2012). Phenolic can deactivate free radicals, stopping damage to pancreatic β cells (Hasanah 2016) and decreasing blood glucose levels (Golovinskaia and Wang 2023).

The Duncan test results with a 5% confidence level in the punti ethanol extract treatment (P6) showed a significant difference from other treatments (P4 and P5). The content of secondary metabolites in P4 (dose 250 mg/kg bw) and P5 (500 mg/kg bw) was at a low concentration to improve the performance of pancreatic β cells so that the reduction in blood glucose levels had not yet reached the maximum effect. Nasution (2015) argued that administering the dose required an optimal condition to achieve the desired effect. Thus, the dose of punti ethanol extract to best reduce blood glucose levels is 1,000 mg/kg body weight.

The Duncan test with a 5% confidence level on the average blood glucose level in the punti ethanol extract treatment (P6) and the positive control treatment (P3) also indicated a significant difference. Glibenclamide only works to lower blood glucose (Liem et al. 2015) through the release of insulin by pancreatic β cells (Yau et al. 2021) without increasing the regeneration of pancreatic β cells (Ibrahim et al. 2021).

In conclusion, the ethanol extract of punti of 1,000 mg/kg body weight improved blood glucose levels in diabetic mice by 52.7% compared to Glibenclamide administration (32.9%). Therefore, the ethanol extract of punti at a dose of 1,000 mg/kg BW works more optimally in lowering blood glucose than glibenclamide. This study's findings prove that punti can be used as a drug candidate in treating diabetes mellitus.

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Antibacterial activities of *Polyalthia longifolia* leaf extracts on multiple antibiotic-resistant bacteria isolated from hospital fomites in Akure, Nigeria

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Abstract. Babatunde OJ, Ogundare AO, Adebolu TT. 2023. Antibacterial activities of *Polyalthia longifolia* leaf extracts on multiple antibiotic-resistant bacteria isolated from hospital fomites in Akure, Nigeria. *Nusantara Bioscience* 15: 149-160. *Polyalthia longifolia* (Sonn.), an ornamental plant, is said to be therapeutic while searching for new medications to treat infections caused by multiple Antibiotic-Resistant Bacteria (MAR). This plant's leaves were investigated for prospective antibacterial activity against MAR isolated from fomites in selected hospitals in Akure and their pharmacological properties. Standard microbiological methods were used to isolate and identify bacteria from fomites. Disc diffusion was performed to test their sensitivity to conventional antibiotics and *P. longifolia* leaf extracts made with ethanol and water. Ciprotab® was used as the control during the antibacterial assay. Therefore, GC-MS analysis was carried out using standard methods to identify the chemicals in the plant leaf extracts. *Staphylococcus aureus* (29.17%), *Streptococcus pyogenes* (20.83%), *Pseudomonas aeruginosa* (14.28%), *Escherichia coli* (14.28%), *Salmonella typhi* (12.5%) and *Klebsiella pneumoniae* (8.33%) were the bacterial species isolated from the fomites sampled in this study. Crude ethanol leaf extract of *P. longifolia* (100mg/mL) inhibited the growth of these organisms with the greatest effect on *P. aeruginosa* with a value of 23.83 ± 0.44 mm, which is superior to that medicated by the control antibiotic (ciprofloxacin). The GC-MS analysis of the purified leaf extracts of *P. longifolia* revealed the presence of bioactive compounds such as n-hexadecanoic acid and phytol, among others. The study revealed that the leaf extracts of *P. longifolia* can inhibit the growth of the isolated MAR from fomites with an added cidal effect, and the inhibition increase with the increase in concentration and exposure time.

Keywords: Fomites, hospital, MAR bacteria, *Polyalthia longifolia*

INTRODUCTION

Over the past ten years, there has been a significant increase in the focus placed on the role that contaminated hospital surfaces and environments play in transmitting Hospital-Acquired Infections (HAIs) (Sen and Sen 2020). According to Walji et al. (2021), a surface that has been polluted could become a reservoir for various pathogens; as a result, it has the potential to be a significant source of HAI spreading. Furthermore, these pathogens can remain infectious on fomites for weeks, even in extreme circumstances. They can survive for months due to the generation of spores, which could increase the risk to the hospital patients exposed to them (Dancer and Kramer 2019).

The development of antibiotic resistance among the bacterial pathogens responsible for community- and hospital-acquired illnesses is a major concern for public health (CDCP 2013). Furthermore, nosocomial infections are more frequently associated with the presence of Multiple Antibiotic-Resistant (MAR) organisms, and these infections contribute to an increase in patients' mortality and morbidity (Peters et al. 2019; Dunachie et al. 2020). There have also been reports of organisms responsible for nosocomial infections exhibiting resistance

to several medicines, making it harder to treat bacterial infections. In addition, new patients who move into a room previously occupied by a patient with MAR infections are at an increased risk of contracting these pathogens because they are more likely to come into contact with them (Penrice et al. 2021).

Creating new antimicrobial agents is one of the actions planned to slow the ongoing spread of antibiotic resistance (Persaud et al. 2019; Babatunde et al. 2022). According to the Global Health Organization (WHO) estimate, more than 75 percent of the globe's population relies entirely or partially on herbal medicines for their health care (WHO 2013). As a result, plants have been utilized in developing pharmaceuticals and have served as the basis for various sophisticated conventional medical systems (Akinjogunla et al. 2011). However, according to Odugbemi (2006), some of the recognized medicinal plants have been utilized in an antique manner, which means that the scientific community has not evaluated them. These plants have traditionally been used to treat fever symptoms, cough, and stomach issues. However, only several medicinal plants like *Allium sativum* (Adebolu et al. 2011), *Vernonia amygdalina* (Ogundare 2011), *Ocimum gratissimum* (Bhavani et al. 2019), *Andrographis paniculata* (Isunu et al. 2023), among others, have been evaluated scientifically.

Moreover, *P. longifolia*, which has been selected in this study, has been reported by some researchers to have medicinal values (Thenmozhi and Sivaraj 2010; Nagore et al. 2021; Firdous et al. 2022). On the other hand, there is a paucity of data about their antibacterial efficacy against microorganisms resistant to MAR. Therefore, the purpose of this study was to investigate the level of antibacterial activity exhibited by the ethanol and aqueous leaf extracts of *P. longifolia* on MAR bacteria isolated from hospital fomites in Akure, Nigeria. In addition, an investigation of the chemical ingredients of the plant's components responsible for its antibacterial properties was also carried out.

MATERIAL AND METHODS

Isolation and identification of bacteria

Therefore, a total of 88 Swab samples were obtained from door knobs, beddings, laboratory gowns, hospital stretchers, bedside lockers, toilet seats, and railings from different hospitals in Akure, Ondo State. The hospitals include the University of Medical Sciences Teaching Hospital (UNIMEDTH), ChristBay Consultant (CBC) Hospital, Unity Clinic (UC), Comprehensive Health Centre (CHC), Arakale, and FUTA Health Centre (FHC). In addition, the isolation of bacteria was carried out as described by Aderanti et al. (2019), while the presumptive identification of the isolated bacteria was carried out using the biochemical characterization described by Olutiola et al. (2000).

Standardization of inoculum

Standardization of the culture to 0.5 McFarland's standard (10^6 CFU/mL) was done as described by Isunu et al. (2022).

Antibiotic sensitivity testing

The antibiotic sensitivity test was performed using the method described by Bauer (1966). First, the zone of inhibition's diameter was measured with a calibrated ruler and then compared with the Clinical and Laboratory Standards Institute's for their sensitivity or resistance standards (CLSI 2017).

Collection of plant materials

The leaves of *P. longifolia* were collected at the Obanla campus of the Federal University of Technology, Akure (FUTA), Nigeria, where they were planted for ornamental purposes. The leaves were after that authenticated at the Department of Crop, Soil, and Pest Management, FUTA.

Preparation of plant extracts

The air-dried leaf samples of *P. longifolia* were powdered using a Marlex blender (Electroline model IS 4780, CM/L 7902804). First, the powdered plant materials were extracted using ethanol, hot water, and cold water. Next, 100 g of the ground samples were weighed into three containers and labeled according to their solvents. Afterward, 1,000 mL of each solvent was added to respective containers, covered, and shaken intermittently

for 72 hours. Next, the Solvents were drained using a muslin cloth and filtered with Whatman No. 1 filter paper. Finally, the semi-solid extracts were obtained using a rotary evaporator (RE-52A Union Laboratories, England).

Phytochemical analysis

The leaf extracts of *P. longifolia* were analyzed for the presence or absence of different phytochemicals such as alkaloids, saponins, tannins, anthraquinone, flavonoids, steroids, terpenoids, and cardiac glycosides using standard methods described by AOAC (2011).

Antimicrobial activity of *Polyalthia longifolia*

The assay for the antibacterial activity of *P. longifolia* extracts was carried out as described by Isunu et al. (2022) with modifications. First, the extracts' reconstitution was done according to the various concentration intended for use in this study. For instance, to get 100 mg/mL of each extract, 1 g was dissolved in 10 ml of 30% Dimethyl Sulfoxide (DMSO). Sterile perforated filter papers were then impregnated with the reconstituted extracts and placed accordingly on Mueller-Hinton agar plates streaked with the test organisms. Positive control was maintained with 2mg/mL of Ciprotab®. The plates were then incubated for 18 hours at 37 °C, and the diameter of inhibition zones was measured in mm.

Determination of Minimum Inhibitory Concentration (MIC)

This assay used the broth dilution method with peptone water broth. A series of test tubes containing different concentrations of the extracts of *P. longifolia*, ranging from 6.25 mg/mL to 100 mg/mL, were inoculated with the standardized bacteria and incubated for 18-24 hours. The lowest concentration of *P. longifolia* extracts dissolved in the broth medium and inoculated with no visual turbidity was recorded as the MIC.

Determination of Minimum Bactericidal Concentration (MBC)

The MBC of *P. longifolia* extracts was determined using the method developed by Bosso and Innalegwu (2018). The test tubes from the MIC test that did not show visible growth were aseptically inoculated on different sterile Muller-Hinton agar plates and incubated at 37 °C for 24 hours. The MBC was chosen as the lowest extract concentration, resulting in no visible growth of the bacterial isolates on the plate.

Determination of the killing rate of extracts

Using the pour plate technique described by Ogundare (2011), the killing rate of ethanol extract on *P. longifolia* leaves on the bacterial isolates was determined at 0, 10, 20, 30, 60, 120, 240, 360, and 480 mins, after which observation was made for microbial growth and colony count was carried out.

Identification of chemical compounds using GC/MS

The method of Olusola-Makinde et al. (2021) was used to evaluate the chemical components and their percentage

abundance using a Varian GC – MS equipment (Varian 4000 mass spectrometer, USA) alongside a mass spectrometer (MS) 3000 equipped with Agilent MS capillary column (30 m × 0.25 mm, i.e., film thickness).

Statistical analysis

Data obtained in this study were subjected to a one-way Analysis of Variance (ANOVA), and differences between means were compared by Duncan's New Multiple Range Test at a 95% confidence interval using Statistical Package for Social Sciences (SPSS) version 26.0.

RESULTS AND DISCUSSION

Identification of bacterial isolates

The biochemical characterization tests revealed the identity of the bacteria isolated from fomites in this study. The identified bacteria include *Staphylococcus aureus* (n=14), *Streptococcus pyogenes* (n=10), *Escherichia coli* (n=7), *Pseudomonas aeruginosa* (n=7), *Salmonella typhi* (n=6) and *Klebsiella pneumoniae* (n=4) as shown in Table 1.

Rate of occurrence of bacteria isolated from hospital fomites

The percentage occurrence of the bacteria isolated from the hospital fomites. The *S. aureus* was observed as the most frequently isolated bacteria (29.17%), followed by *S. pyogenes* (20.83%), while *K. pneumoniae* (8.33%) was the least frequently occurring among the isolates obtained in this study (Figure 1).

The percentage occurrence of the bacterial isolates from each hospital sampled is shown in Figure 2. Each bacterial isolate was found to be more frequently occurring at UNIMEDTH. On the contrary, at Christ Bay Clinic and Unity Clinic, *S. pyogenes* had a 0% rate of occurrence. However, the same bacteria had 70%, 20%, and 10% at UNIMEDTH, Comprehensive Health Centre, and FUTA Health Centre, respectively.

The occurrence rate of bacteria on each fomite sampled is displayed in Figure 3. The *E. coli* (71.43%) was found to occur more frequently on toilet seats. *S. aureus* (50%), *K. pneumoniae* (50%), and *P. aeruginosa* (28.57%) were found to be more frequently occurring on beds, door knobs, and railings, respectively.

In healthcare settings, particularly hospitals, antimicrobial-resistant bacteria are becoming a growing concern. These microorganisms can survive on fomites,

surfaces, or objects that transmit infections. For example, there are frequent fomites in hospitals, doorknobs, bed rails, and medical equipment. Patients can contract antimicrobial-resistant bacteria in contact with these contaminated fomites (Dalton et al. 2020). Antimicrobial-resistant bacteria can cause hospital-acquired infections that are severe and difficult to treat. In addition, patients already weakened due to illness or injury may be more susceptible to these infections, resulting in longer hospital stays, higher medical costs, and even death (Brown et al. 2020).

This study analyzed the distribution and antimicrobial resistance pattern of bacteria isolated from hospital fomites in Akure, Nigeria. In addition, the frequency of occurrence of these bacterial pathogens was estimated. The bacteria isolated in this study from hospital fomites were *S. aureus*, *S. pyogenes*, *K. pneumoniae*, *P. aeruginosa*, *S. typhi*, and *E. coli*. In addition, the frequency of occurrence of these bacterial pathogens was estimated. These findings are similar to the report by Moges et al. (2014) in which *Klebsiella* sp., *Pseudomonas* sp., *E. coli*, *Citrobacter* sp., *S. aureus*, and two species of *Shigella* were isolated from hospital environments. However, although most of the bacteria isolated in this study were similar to what they identified, the percentage occurrence of each differed. Similarly, a study by Prasad et al. (2018) presented the presence of multiple antibiotic-resistant *P. aeruginosa*, *S. pyogenes*, *E. coli*, *S. aureus*, *S. typhi*, and *K. pneumonia* in a hospital environment.

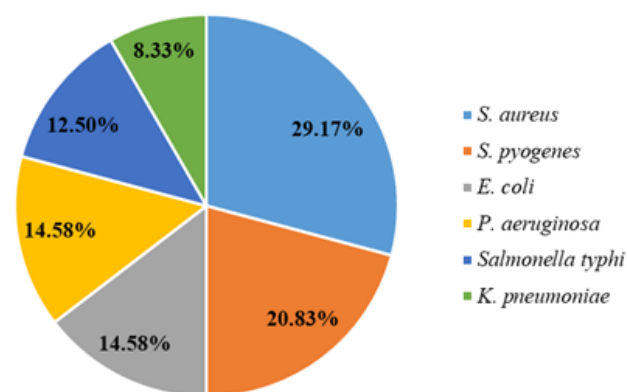


Figure 1. Percentage occurrence of bacteria isolated from Hospital fomites

Table 1. Biochemical characterization of bacterial isolates

Shape	GR	CA	CI	CO	MO	H ₂ S	UR	IN	OX	FU	GU	GA	SU	LA	MA	MN	GAS	Probable organisms
Cocci	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	-	<i>Staphylococcus aureus</i>
Rod	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	+	+	<i>Escherichia coli</i>
Cocci	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	<i>Streptococcus pyogenes</i>
Rod	-	+	+	-	-	-	+	-	-	-	+	+	+	+	+	+	+	<i>Klebsiella pneumoniae</i>
Rod	-	+	+	-	+	-	-	-	+	-	-	-	-	-	-	+	-	<i>Pseudomonas aeruginosa</i>
Rod	-	+	-	-	+	+	-	-	-	-	+	-	-	-	+	+	-	<i>Salmonella typhi</i>

Note: GR: Gram's reaction, CA: Catalase, CI: Citrase, CO: Coagulase, MO: Motility, H₂S: Hydrogen Sulphide, UR: Urease, IN: Indole, OX: Oxidase, FU: Fructose, GA: Galactose, GU: Glucose, SU: Sucrose, LA: Lactose, MA: Maltose, MN: Mannitol

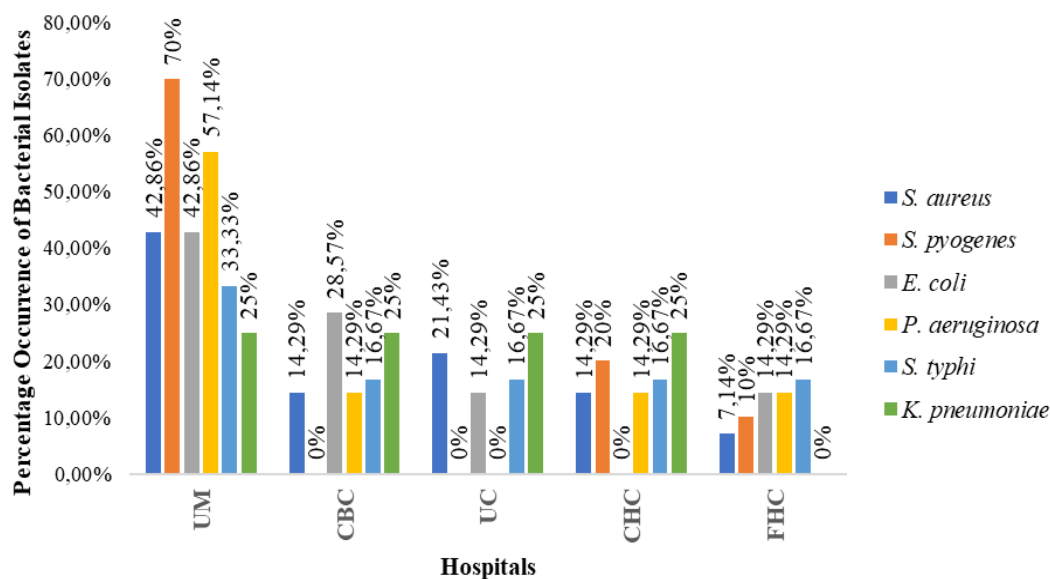


Figure 2. Distribution of bacteria isolated across different hospitals. Note: CBC: Christ Bay Clinic, CHC: Comprehensive Health Centre, FHC: FUTA Health Centre, UC: Unity Clinic, UNIMEDTH: University of Medical Sciences Teaching Hospital

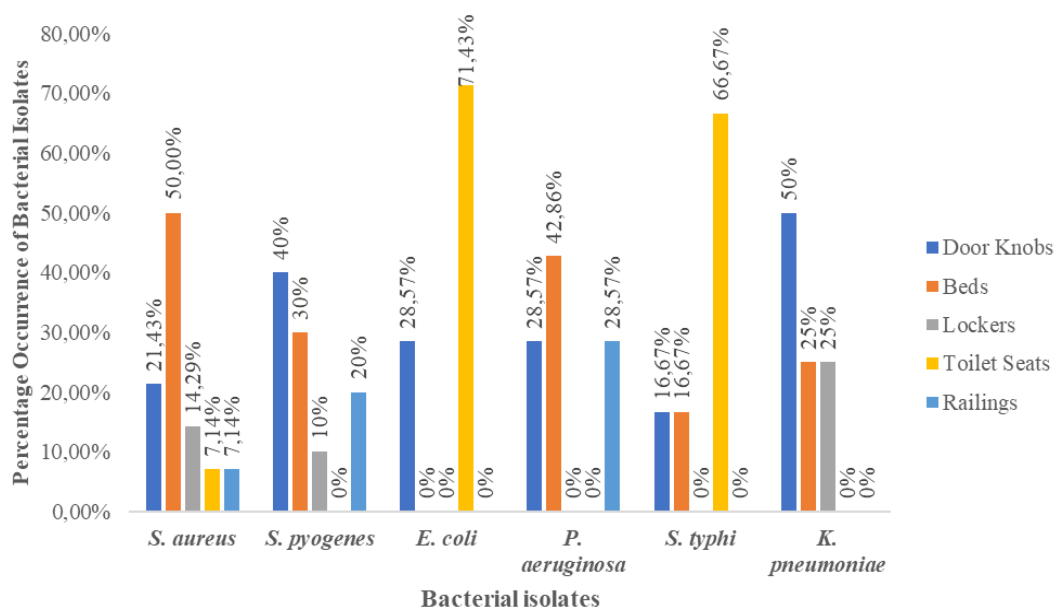


Figure 3. Distribution of bacteria isolated across different fomites

Antibiotic sensitivity pattern of bacterial isolates

The antibiotic sensitivity test results are shown in Tables 2 and 3 for Gram-negative and Gram-positive bacteria, respectively. All the bacterial isolates were more susceptible to ciprofloxacin, with the inhibition zone diameter ranging from 13.50 ± 0.29 mm against *S. aureus* to 29.33 ± 1.45 mm against *K. pneumoniae*. In addition, the isolates were heavily resistant to augmentin and amoxicillin except for *K. pneumoniae*, with an inhibition zone of 14.17 ± 0.73 mm by amoxicillin.

Most isolated bacteria demonstrated multiple antibiotic resistance to the conventional antibiotics used in this study. This is in line with the report of Oliveira and Damasceno (2010), who identified the antibiotic-resistance pattern of bacteria from hospital environments due to the heavy presence of antimicrobial agents in such environments, leading the bacteria to develop resistance. The high rate of resistance demonstrated by the isolated bacteria to antibiotics further lends credence to the fact that almost all known bacterial species are fast becoming resistant to available antibiotics.

Table 2. Antibiotic sensitivity profile of gram-negative bacterial isolates

Isolates	PEF	OFX	S	SXT	CH	SP	CPX	AM	AU	GN
<i>E. coli</i>	20.67±0.33 ^{cd}	18.83±0.60 ^c	12.50±0.76 ^b	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	21.50±0.50 ^b	0.00±0.00 ^a	0.00±0.00 ^a	21.17±1.09 ^c
<i>K. pneumoniae</i>	29.67±0.88 ^e	27.83±0.73 ^{de}	18.83±1.74 ^c	21.67±0.33 ^c	27.83±0.73 ^{de}	30.00±0.58 ^e	29.33±1.45 ^e	14.17±0.73 ^b	0.00±0.00 ^a	23.33±0.67 ^{cd}
<i>P. aeruginosa</i>	17.83±1.01 ^b	17.83±0.17 ^b	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	21.17±0.60 ^c	0.00±0.00 ^a	0.00±0.00 ^a	19.67±0.88 ^{bc}
<i>S. typhi</i>	21.83±0.17 ^d	19.17±0.93 ^c	13.33±0.17 ^b	0.00±0.00 ^a	0.00±0.00 ^a	14.5±0.50 ^b	26.17±1.09 ^e	0.00±0.00 ^a	0.00±0.00 ^a	22.17±0.17 ^d

Note: PEF: Pefloxacin (30µg), OFX: Ofloxacin (10µg), S: Streptomycin (30µg), SXT: Septrin (30µg), CH: Chloramphenicol (30µg), SP: Sparfloxacin (10µg), CPX: Ciprofloxacin (30µg), AM: Amoxacillin (30µg), AU: Augmentin (10µg), GN: Gentamycin (30µg). Values are presented as mean±SE of triplicates; values in the same row carrying the same superscript are not significantly different ($p < 0.05$) according to Duncan's New Multiple Range Test

Table 3. Antibiotic sensitivity profile of gram-positive bacterial isolates

Isolates	PEF	GN	APX	Z	AM	R	CPX	S	SXT	E
<i>S. aureus</i>	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	13.50±0.29 ^b	21.33±0.33 ^c	31.67±0.33 ^d	0.00±0.00 ^a
<i>S. pyogenes</i>	20.17±0.17 ^d	20.17±0.44 ^d	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	20.33±0.17 ^d	16.00±0.00 ^c	0.00±0.00 ^a	13.33±0.33 ^b

Note: PEF: Pefloxacin (10µg), GN: Gentamycin (10µg), APX: Ampiclox (30µg), Z: Zinnacef (20µg), AM: Amoxacillin (30µg), R: Rocephin (25µg), CPX: Ciprofloxacin (10µg), S: Streptomycin (30µg), SXT: Septrin (30µg), E: Erythomycin (10µg). Values are presented as mean±SE of triplicates; values in the same row carrying the same superscript are not significantly different ($p < 0.05$) according to Duncan's New Multiple Range Test

Phytochemical constituents of the extracts

The qualitative phytochemical analysis revealed that both flavonoids and steroids are absent while confirming the presence of saponins, tannins, anthraquinones, terpenoids, alkaloids, and cardiac glycosides, as shown in Table 4. Phytochemicals such as saponins, tannins, anthraquinones, terpenoids, alkaloids, and cardiac glycosides were found present in the various extracts. Jothy et al. (2013) reported a similar observation about the phytochemical composition of the plant. The synthesis of secondary metabolites with antibacterial properties is an important defense mechanism for plants, and these chemicals have potential medical and agricultural benefits (O'Bryan et al. 2015).

Antimicrobial activity of *Polyalthia longifolia*

The antibacterial activities of *P. longifolia* leave extracts at 100 mg/mL showed that crude ethanol extract produced the widest zone of inhibition against *P. aeruginosa* with a value of 23.83 ± 0.44 mm. In contrast, the control against the same isolate produced a zone of inhibition of 0.00 ± 0.00 mm, indicating that the crude ethanol extract is more effective than the control against the isolate (Table 5). At a concentration of 25 mg/mL, the activities of the extracts were reduced, as shown in Table 6. The control antimicrobial agent was maintained at 2 mg/mL throughout the experiment.

Leaves extracts of *P. longifolia* had antibacterial effects on the isolates at various concentrations. There was a significant difference (at $p < 0.05$) in the activities of the various extracts against the bacterial isolates by comparing the mean values of the inhibition zones they mediated. Additionally, the differences were significant when these values were compared to the mean values of inhibitory zones mediated by the control antibiotic.

This study's ethanol extract of *P. longifolia* leaves is more antibacterial than hot and cold water extracts against the test isolates. At 100 mg/mL concentration, the ethanol extract mediated an inhibition zone of 18.17 ± 0.17 mm against *S. typhi*, while the cold water extract produced the smallest zone of 12.00 ± 0.00 mm. The control was, however, more effective against the isolate with a mean inhibition zone of 24.50 ± 0.76 mm. Similarly, the control antibiotic mediated a wider inhibition zone against *S. aureus*, *K. pneumoniae*, *E. coli*, and *S. pyogenes* with mean inhibitory values of 28.17 ± 0.17 mm, 30.33 ± 0.33 mm, 26.50 ± 0.29 mm, and 23.00 ± 0.58 mm, respectively. These values were observed to be greater than that of the ethanol extract with values of 21.67 ± 0.33 mm, 15.00 ± 0.58 mm, 21.50 ± 0.76 mm, and 18.50 ± 0.29 mm, respectively. In contrast, the leaf extracts mediated wider inhibition zones (23.83 ± 0.44 mm, 17.67 ± 0.44 mm, and 14.33 ± 0.33 for ethanol, hot water, and cold water, respectively) while the control antibiotic mediated a zone of inhibition 0.00 ± 0.00 mm indicating a strong resistance. These findings agree with the study by Thenmozhi and Sivaraj (2010), who revealed that the extracts of *P. longifolia* produce significant inhibition zones against the bacterial isolates tested, indicating that they are good antibacterial agents.

Table 4. Qualitative phytochemical constituents of *Polyalthia longifolia* extracts

Phytochemical	Ethanol	Hot water	Cold water
Saponins	+	+	-
Tannins	+	-	-
Flavonoids	-	-	-
Steroids	-	-	-
Terpenoids	+	+	+
Alkaloids	+	+	+
Cardio glycosides	+	+	-
Anthraquinones	+	+	+

Table 5. Antibacterial activity of the various extracts of *Polyalthia longifolia* at 100 mg/mL concentration

Isolates	Crude ethanol (mm)	Hot water (mm)	Cold water (mm)	Control (mm)
<i>S. typhi</i>	18.17 ± 0.17^b	12.50 ± 0.29^a	12.00 ± 0.00^a	24.50 ± 0.76^c
<i>S. aureus</i>	21.67 ± 0.33^c	18.00 ± 0.58^b	15.83 ± 0.17^a	28.17 ± 0.17^d
<i>P. aeruginosa</i>	23.83 ± 0.44^c	17.67 ± 0.44^{bc}	14.33 ± 0.33^b	0.00 ± 0.00^a
<i>K. pneumoniae</i>	15.00 ± 0.58^b	13.83 ± 0.73^{ab}	12.33 ± 0.17^a	30.33 ± 0.33^c
<i>E. coli</i>	21.50 ± 0.76^c	16.50 ± 0.29^b	10.67 ± 0.33^a	26.50 ± 0.29^d
<i>S. pyogenes</i>	18.50 ± 0.29^b	14.67 ± 0.33^a	14.33 ± 0.17^a	23.00 ± 0.58^c

Note: Values are presented as mean $\pm$ SE of triplicates; values in the same row carrying the same superscript are not significantly different at $p < 0.05$ according to Duncan's new multiple range test. Control: CiproTab (2mg/mL)

Table 6. Antibacterial activity of the various extracts of *Polyalthia longifolia* at 25mg/mL concentration

Isolates	Crude ethanol (mm)	Hot water (mm)	Cold water (mm)	Control (mm)
<i>S. typhi</i>	12.17 ± 0.17^b	8.33 ± 0.17^a	8.33 ± 0.33^a	24.50 ± 0.76^c
<i>S. aureus</i>	15.00 ± 0.29^b	13.67 ± 0.33^{ab}	12.50 ± 0.29^a	28.17 ± 0.17^c
<i>P. aeruginosa</i>	16.00 ± 0.58^c	12.00 ± 0.29^{bc}	10.00 ± 0.00^b	0.00 ± 0.00^a
<i>K. pneumoniae</i>	10.50 ± 0.29^c	7.83 ± 0.17^b	0.00 ± 0.00^a	30.33 ± 0.33^d
<i>E. coli</i>	12.50 ± 0.50^c	8.50 ± 0.29^b	0.00 ± 0.00^a	26.50 ± 0.29^d
<i>S. pyogenes</i>	10.67 ± 0.88^b	9.33 ± 0.33^b	7.67 ± 0.33^a	23.00 ± 0.58^c

Note: Values are presented as mean $\pm$ SE of triplicates; values in the same row carrying the same superscript are not significantly different at $p < 0.05$ according to Duncan's new multiple range test. Control: CiproTab (2mg/mL)

Minimum inhibitory and bactericidal concentration

The MIC values ranged from 6.25 to 12.5 mg/mL for the crude ethanol extract against all the organisms tested. On the other hand, the purified fraction of the ethanol extract, hot water extract, and cold water extract all ranged in MIC values between 6.25 and 25 mg/mL. The lowest MBC value obtained in this study is 6.25 mg/mL, which is that of crude ethanol extract against *P. aeruginosa*. The hot and cold water extracts have an MBC value of 50 mg/mL, recorded against *S. pyogenes* (Tables 7 and 8).

The MIC test conducted for this study showed that ethanol, hot water, and cold water extracts of *P. longifolia* leave possess in vitro antibacterial properties at different concentrations. This validates the findings of Okwulehie et al. (2017) that the antibacterial properties of plant extracts are concentration-dependent. Therefore, the MIC and MBC values of a plant extract are indicators of the extract's antibacterial activity, as Bosso and Innalegwu (2018) stated. In addition, on comparing MIC and MBC values, a plant's ability to fight bacteria is indicated by how low those values are. This investigation showed that the Minimum Effective Concentration (MIC) values were lower than the Minimum Bactericidal Concentration (MBC) values. That indicates the extracts were bacteriostatic at lower doses but bactericidal at higher ones.

The killing rate of bacterial isolates

The killing rate of the organisms by the extracts at 50 mg/mL indicated that the number of viable cells decreased with time (Figures 4-6). The rate at which the extracts were killing the bacteria was investigated, and it was found that the number of viable cells was decreasing throughout the experiment. The number of bacterial cells almost completely disappeared throughout the experiment proves

that the extracts have a cidal impact, provided they are exposed to them long enough. These results are consistent with those obtained by Ogundare (2011). Hence, the longer the bacterial cells are exposed to the extracts of *P. longifolia* leaves, the higher the ability of the extract to inhibit some of the bacteria that were previously resistant to the extract at the beginning of the exposure.

Profile of chemical compounds in the ethanol extract of *Polyalthia longifolia*

The GC-MS analysis of the purified extract of *P. longifolia* revealed the presence of bioactive compounds such as n-hexadecanoic acid, phytol, dibutyl phthalate, palmitoleic acid, 1,2-Benzenedicarboxylic acid, Tetradecanoic acid among others. The compounds with their molecular formula and structures are shown in Table 9, while the GC-MS chromatographic spectra are shown in Figure 7.

The GC-MS analysis of the purified ethanol extract of *P. longifolia* revealed the presence of bioactive compounds such as n-hexadecanoic acid, which Sermakkani and Thangapandian (2012) reported having antimicrobial activity. In addition, oleic acid was also detected in the plant, which has been previously reported by Rahdar et al. (2020) to have some antimicrobial properties, thus justifying why the plant exhibited antibacterial activities against the tested bacterial isolates. Furthermore, other compounds detected have been reported to have antibacterial properties. They include 2-Hydroxy-5-methylbenzaldehyde (Sang and Lin 2019), 1, 2-Benzenedicarboxylic acid (Fadipe et al. 2014), Palmitoleic acid (Watanabe et al. 2021), Phytol (Pejin et al. 2014), Squalene (Nazemi et al. 2022) and Dibutyl phthalate (Khatiworka et al. 2012).

Table 7. Minimum Inhibitory Concentration (MIC) value for each extract and isolate

Isolates	Crude ethanol (mg/mL)	Purified ethanol (mg/mL)	Hot water (mg/mL)	Cold water (mg/mL)
<i>Salmonella typhi</i>	12.5	12.5	25	25
<i>Staphylococcus aureus</i>	6.25	25	6.25	6.25
<i>Pseudomonas aeruginosa</i>	6.25	12.5	12.5	12.5
<i>Klebsiella pneumoniae</i>	12.5	6.25	6.25	6.25
<i>Escherichia coli</i>	12.5	6.25	6.25	6.25
<i>Streptococcus pyogenes</i>	12.5	12.5	12.5	25

Table 8. Minimum Bactericidal Concentration (MBC) value for each extract and isolate

Isolates	Crude ethanol (mg/mL)	Purified ethanol (mg/mL)	Hot water (mg/mL)	Cold water (mg/mL)
<i>Salmonella typhi</i>	25	12.5	25	50
<i>Staphylococcus aureus</i>	12.5	25	25	25
<i>Pseudomonas aeruginosa</i>	6.25	12.5	25	25
<i>Klebsiella pneumoniae</i>	25	12.5	25	25
<i>Escherichia coli</i>	25	12.5	12.5	12.5
<i>Streptococcus pyogenes</i>	25	25	50	50

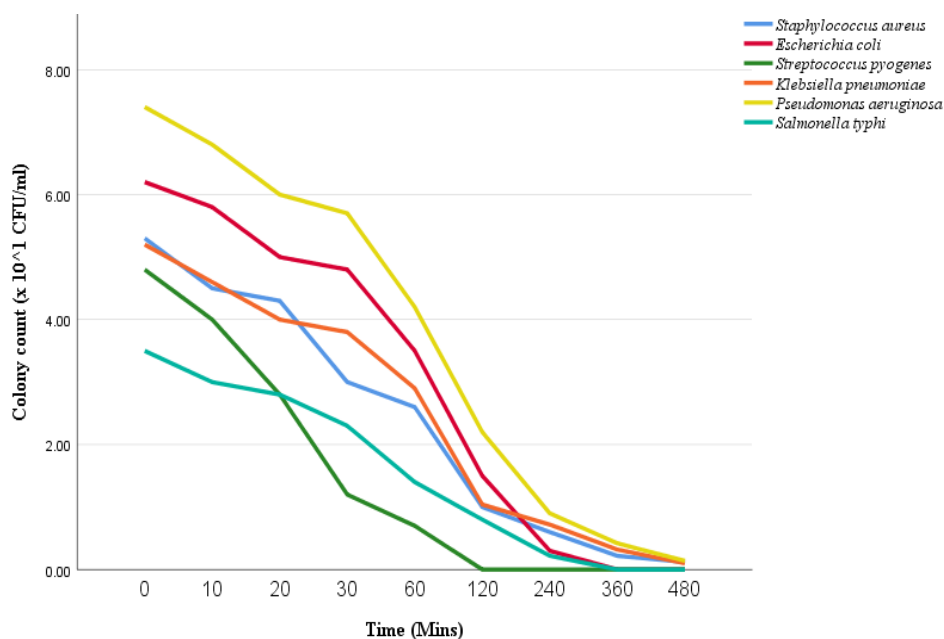


Figure 4. The killing rate of bacterial isolates by 50 mg/mL of crude ethanol extract of *Polyalthia longifolia*

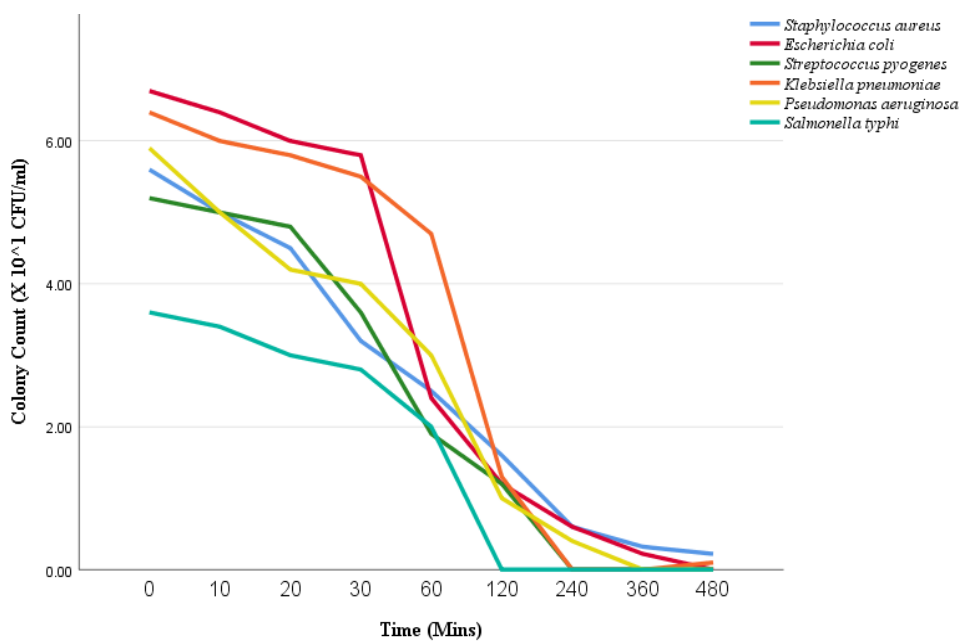


Figure 5. The killing rate of bacterial isolates by 50 mg/mL of hot water extract of *Polyalthia longifolia*

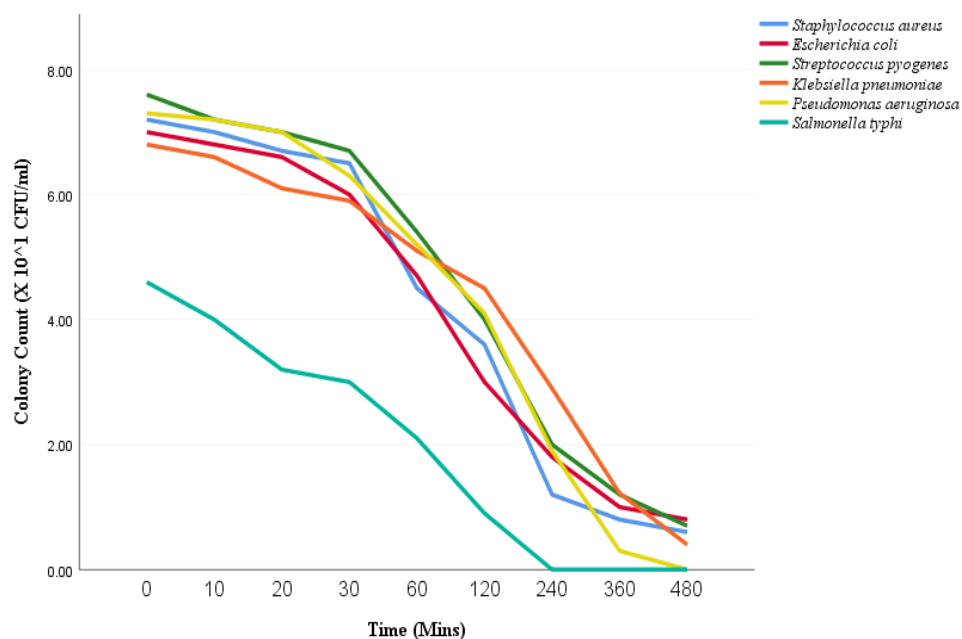


Figure 6. The killing rate of bacterial isolates by 50 mg/mL of cold water extract of *Polyalthia longifolia*

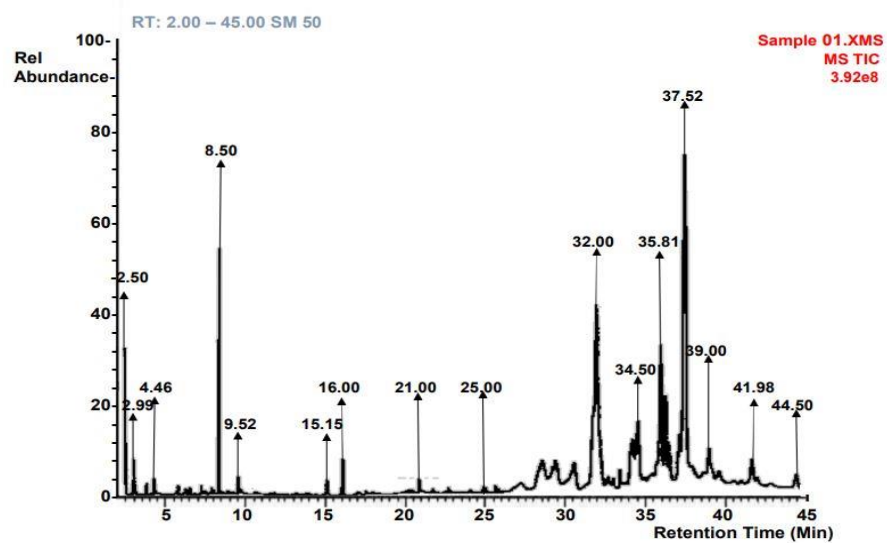
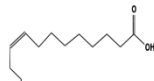
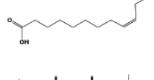
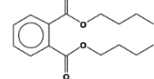
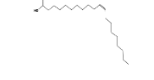
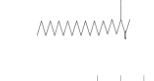


Figure 7. GC-MS chromatographic spectra of ethanol leaf extract of *Polyalthia longifolia*

Table 9. Compounds detected in the Gas Chromatography-Mass Spectrometry of *Polyalthia longifolia* purified ethanol extract

Peak #	RT (Mins)	Compound detected	Mol. formula	MW	Peak area %	Structures
1	2.50	Benzene, 1-ethyl-2-methyl-	C ₉ H ₁₂	120	10.18	
2	2.99	2-Hydroxy-5-methylbenzaldehyde	C ₈ H ₈ O ₂	136	2.73	
3	4.46	1,2-Benzenedicarboxylic acid	C ₈ H ₆ O ₄	166	1.24	
4	8.50	9,12-Octadecadienoic acid (Z,Z)-	C ₁₈ H ₃₂ O ₂	280	17.38	
5	9.52	Palmitoleic acid	C ₁₆ H ₃₀ O ₂	254	1.43	
6	15.15	Tridecanoic acid, methyl ester	C ₁₄ H ₂₈ O ₂	228	1.30	
7	16.00	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	C ₁₈ H ₃₀ O ₂	278	2.61	
8	21.00	Phytol	C ₂₀ H ₄₀ O	296	1.37	
9	25.00	9-Octadecenoic acid, (E)-	C ₁₈ H ₃₄ O ₂	282	1.12	
10	32.00	Oleic Acid	C ₁₈ H ₃₄ O ₂	282	13.66	
11	34.50	Tetradecanoic acid	C ₁₄ H ₂₈ O ₂	228	4.96	
12	35.81	Dibutyl phthalate	C ₁₆ H ₂₂ O ₄	278	10.55	
13	37.52	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256	23.59	
14	39.00	cis-11-Eicosenoic acid	C ₂₀ H ₃₈ O ₂	310	3.10	
15	41.98	Eicosanoic acid, methyl ester	C ₂₁ H ₄₂ O ₂	336	2.48	
16	44.50	Squalene	C ₃₀ H ₅₀	410	2.30	

In conclusion, this study revealed the presence of multiple antibiotic-resistant bacteria on fomites of the selected hospitals sampled. It further revealed that the different extracts of the leaves of *P. longifolia* used in this study possess some phytochemicals and bioactive compounds that make them good antibacterial agents, as verified using in vitro agar well diffusion assay. Therefore, these extracts might be good sources of more effective drugs to checkmate the activities of MAR on fomites.

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Classification of forest vegetation in Yaralıgöz Education and Observation Forest, Kastamonu, Türkiye

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Abstract. Karaköse M, Terzioğlu S. 2023. Classification of forest vegetation in Yaralıgöz Education and Observation Forest, Kastamonu, Türkiye. *Nusantara Bioscience* 15: 161-171. Forest ecosystems, which contain approximately half of the 12,975 plant taxa known to be distributed in Türkiye, are very important in terms of biological diversity. The fact that forests are seen as wood-producing ecosystems and have been planned for these purposes for many years has resulted in forests being kept away from being evaluated in terms of ecosystem and socio-economic aspects. Today, forest management plans have begun to be prepared in which all components of the forest are considered, and biological diversity is reflected. Based on this understanding, this investigation was performed during 2011-2012 to analyze the forest vegetation of Yaralıgöz Education and Observation Forest. The study area is located in the north-western part of Türkiye within the Euro-Siberian phytogeographic region. Phytosociological studies were carried out using the classical Braun-Blanquet approach, and 51 relevés were collected from the research area. The database contains 184 vascular plant taxa, dominated by the Euro-Siberian elements and Hemipterophytes. The relevés were classified using the Modified TWINSpan, and the forest vegetation types' distribution was analyzed using non-metric multi-dimensional scaling. A comprehensive comparison among all described syntaxa within the study area and other closely related forests was provided. Topographic factors and mean Ellenberg indicator values were evaluated to interpret the ecological relationships among forest plant associations. Four associations, two (*Daphniphyllum-Pinetum hamatae* and *Galio rotundifolii-Abietum equi-trojani*) of which are new syntaxa to science, were defined as belonging to the Euxine Province within *Erico-Pinetea*, *Carpino-Fagetea sylvaticae*, *Quercetea pubescentis*, and *Vaccinio-Piceetea*. The distribution of these four forest associations was mostly affected by light intensity, nutrients, moisture content, and altitude. The results of this vegetation study provided precious knowledge for monitoring vegetation change and nature conservation projects in the Yaralıgöz.

Keywords: Euro-Siberian, Euxine, JUICE, syntaxonomy, TURBOVEG

Abbreviations: Education and Observation Forest (EOF), Ellenberg indicator values (EIVs), Forest Planning Unit (FPU), International Code of Phytosociological Nomenclature (ICPN), Non-metric Multi-Dimensional Scaling (NMDS), Paleontological Statistics (PAST)

INTRODUCTION

Forests are not just made up of trees; they are large and complex ecosystems where shrubs and herbaceous species are supported by soil structure and the abiotic environment. The vegetation types that make up the forests exhibit a dynamic structure and can change as spatiotemporal. Vegetation relevés or plots are used extensively by researchers to study vegetation types. Relevés allow us to have a wealth of basic information to help us understand the vegetation change that has occurred over time, the restoration of a piece of forest that has been destroyed as a result of any disturbance (fire, clear cutting, etc.), or classification of habitats (Chytrý et al. 2020).

Biodiversity is under great threat globally. Incorrect land uses, increased demand for natural resources, and excessive pressure on ecosystems can cause fragmentation of habitats. It also decreased the goods and services obtained from ecosystems. The settlement of alien plants in new ecosystems and global warming make this situation even more dangerous (Karaköse 2019). Therefore, by

allowing researchers to access these databases, it will be easier to detect biodiversity on a global scale, and it will provide important contributions to decision-makers and nature conservationists to create necessary conservation projects, monitor vegetation change, and evaluation studies to identify and to reduce existing threats (Sabatini et al. 2021).

Türkiye is located entirely within the Holarctic realm and has around 12,975 taxa of ferns and seed plants and around 4,157 endemic plant taxa (Güner et al. 2012) and thus, is one of the countries with extraordinarily rich floristic diversity in the world. In Türkiye, the presence of three floristic regions (Euro-Siberian, the Mediterranean, and Irano-Turanian) has been one of the main reasons for the formation of this richness. Each floristic region also has various vegetation types depending on their internal dynamics. In Türkiye, the Euro-Siberian phytogeographic region is represented by the Euxine Province (Quézel et al. 1980). It is floristically closely related to the Central European, Balkans (Illyrian), and the Hyrcanian (South Caspian) provinces (Loidi et al. 2021).

Forests are the climatic vegetation type of the Euxine Province and occur throughout the Black Sea, Istranca Mountains of Thrace, and in Marmara Regions. Humid-temperate broad-leaved forests exist in the northern exposures at low altitudes, and humid/sub-humid and winter-tolerating coniferous forests at higher altitudes (Karaköse 2019). The mountainous vegetation structures of Türkiye, with their enormous and unique diversity, have aroused much interest from many vegetation researchers. Foreign researchers conducted the first vegetation studies in the western Black Sea region. Later, many vegetation studies were conducted by Turkish researchers in various regions of Türkiye (Karaköse 2019). Specifically, the research conducted by Akman (1995) and Quézel et al. (1980) is readily accepted as the touchstone of Türkiye's forest vegetation. However, since all of these studies were conducted before (Theurillat et al. 2021), they have some mistakes and deficiencies (Karaköse 2019). But, some of them were typified. The syntaxonomy of the Turkish forest vegetation has not yet been resolved (Akman 1995). However, with new numeric and digital technologies, some of them have been eliminated with the vegetation studies carried out in recent years (Kavgacı et al. 2012; Kenar and Ketenoğlu 2016; Hüseyinova and Yalçın 2018; Çoban and Willner 2019; Karaköse 2019; Yıldırım et al. 2019; Kenar et al. 2020; Tunçkol et al. 2020; Bonari et al. 2021; Çoban et al. 2021; Karaköse and Terzioğlu 2021; Kavgacı et al. 2021; Eminağaoğlu et al. 2022; Yıldırım et al. 2022). However, there are still some scientific gaps in the forest vegetation of Türkiye. For example, although distribution mapping of phytosociological units based on alliance in Europe was made by Preislerová et al. (2022), only the

Thrace (European part) section of Türkiye has been mapped.

The Regional Directorate of Forestry of Kastamonu has created an area (Yaralığöz Education and Observation Forest) for ecotourism purposes in Yaralığöz Mountain and its vicinity (Karaköse and Terzioğlu 2019). With this present study, we aimed to study the forest vegetation of Yaralığöz EOF, and thus the western part of northern Anatolia, and contribute new knowledge to Türkiye's Forest Vegetation Database. Moreover, as a result of this study, the relevés to be obtained about vegetation types will provide valuable information to the forest planners and managers for nature conservation activities.

MATERIALS AND METHODS

Study area

Yaralığöz EOF (in Türkiye), affiliated with the Kastamonu Regional Directorate of Forestry, is located within the boundaries of Devrekani FPU, Şeyhsaban FPU, Tezcan FPU, and Karacakaya FPU. Yaralığöz EOF, found in the eastern of the important plant area of the Küre Mountains as a geographical location, ends at Koru Mountain in the east. Yaralığöz EOF consists of 11,548.2 ha, of which 7,862.8 ha is forested, and 3,685.4 ha is non-forested. Yaralığöz EOF is geomorphologically mountainous and steep terrain; furthermore, the area's elevation varies between 773 and 2,019 meters above sea level/masl (Figure 1).

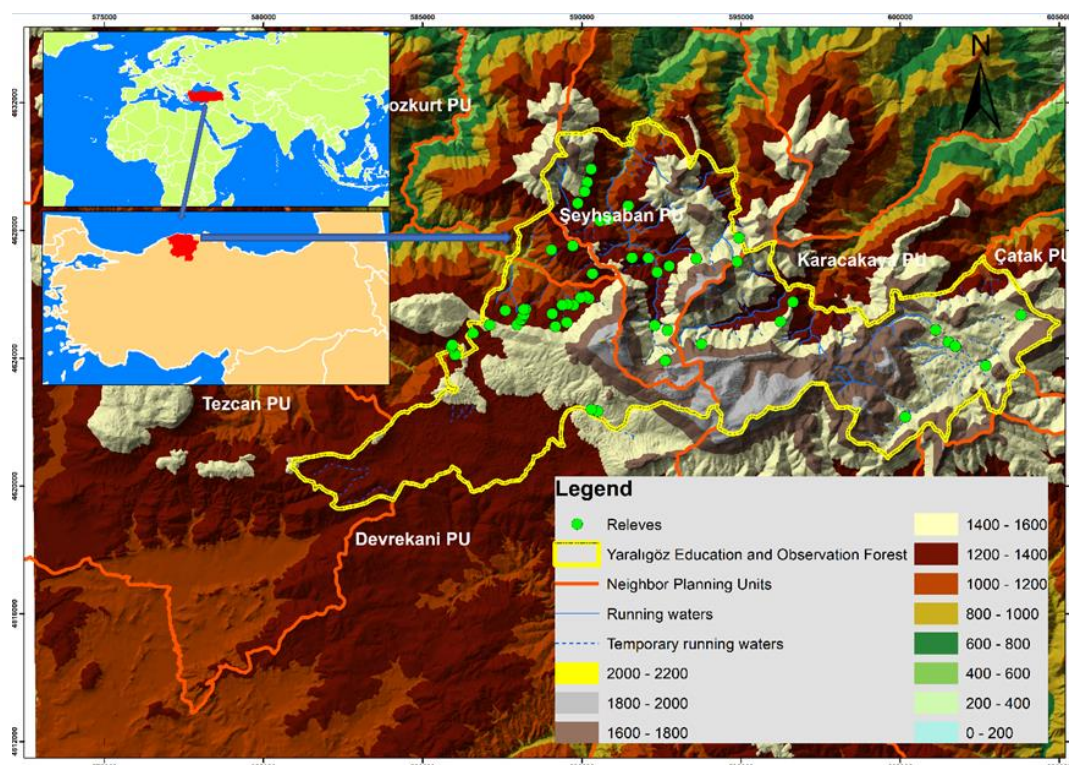


Figure 1. Location of the study area in Yaralığöz EOF, Türkiye

Yaralığöz EOF is located along the A5 square according to the western Black Sea region's grid system (Davis 1965). The research area has no ready weather network to ensure that climate characteristics are examined according to elevation and aspect variations. Closest to the study area are Devrekani (1,050 m), Turkeli (50 m), and Bozkurt meteorological stations (167 m), where long-term observations and measurements were made (Karaköse and Terzioğlu 2019). The average annual temperature for the research area is 7.6°C in Devrekani, 13.5°C in Turkeli, and 12.9°C in Bozkurt. Devrekani, Turkeli, and Bozkurt's mean annual precipitation values are 533.1 mm, 743.4 mm, and 1,259.1 mm, respectively. According to Devrekani, Turkeli, and Bozkurt meteorological data, precipitation regimes in the study area are identified as Spring-Summer-Fall-Winter (semi-continental), Fall-Winter-Summer-Spring (oceanic type 1), and Fall-Winter-Spring-Summer (west Mediterranean). Considering the meteorological data close to the study area, there is a transition climate where Mediterranean, oceanic, and continental climates are felt in Yaralığöz EOF (Karaköse and Terzioğlu 2019).

Field sampling and data assessment

Relevés were taken between 2011 and 2012 to determine the vegetation structures in the study area. The relevés were sampled using the Braun-Blanquet method (Guarino et al. 2018). For vegetation studies, 51 relevés were applied in Yaralığöz EOF from areas homogeneous enough in floristic composition and structure. Minimum area sizes have been determined as 20×20 m (Akman 1995). Flora of Turkey and the East Aegean Islands (Davis 1965) and Turkish Plant List (Güner et al. 2012) were used to identify, nomenclature, and determine the phytogeographic regions of plant taxa. In addition, life forms of plant taxa were determined according to the system of Raunkiaer (Raunkiaer 1934). Field data for the vegetation analyses were first stored to TURBOVEG vegetation database management program (Hennekens and Schaminée 2001). The data set was later exported and uploaded to the JUICE program for hierarchical classification and ordination analysis. For the hierarchical classification and for the analysis of heterogeneity of

vegetation types, the Modified TWINSpan package program integrated into the JUICE program was used. Pseudospecies cut levels were set to five (0, 2, 5, 10, and 20) and with Whittaker's beta diversity. Diagnostic and character species were determined by taking into account the fidelity values of vegetation types based on the cluster dendrogram created using the JUICE program. The diagnostic species for particular vegetation types were calculated with the help of presence/absence data using the phi-coefficient (threshold value of $\phi=0.40$).

The hierarchical classification result was transferred to an ordination plane using NMDS. Ordination was calculated using the R program (version 3.6.1) and vegan package (version 2.5-6) integrated into the JUICE program. Original EIVs were assigned to the species data in the JUICE program. Mean EIVs of vegetation types were compared using the Modified Permutation Test script using the JUICE-R function (Zelený and Schaffers 2012). Ellenberg Indicator Values (EIVs) were used to determine the ecological differences of the identified vegetation types. Similarities between the newly detected vegetation types in terms of floristic composition were compared with the help of Sorensen's similarity index. Upper syntaxonomic groups of the identified vegetation types and their character species were mainly determined according to the following articles (Akman 1995; Mucina et al. 2016; Çoban and Willner 2019; Kavgacı et al. 2021). The nomenclature of vegetation types newly detected in Yaralığöz EOF was made according to the ICPN (Theurillat et al. 2021).

RESULTS AND DISCUSSION

Within the scope of this vegetation study, 51 relevés consisting of 184 vascular plant taxa were sampled from the woodlands in the study area (supplementary material). Using cluster analysis, TWINSpan distinguished four vegetation types into three generally interpretable main groups. Classification analysis results are summarized in the dendrogram (Figure 2).

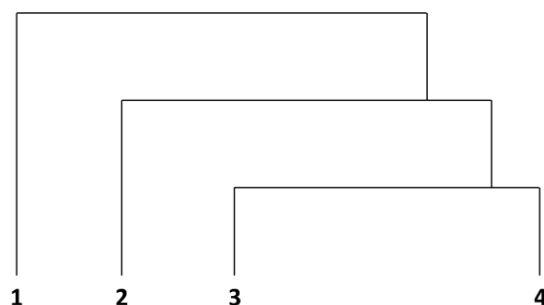


Figure 2. Dendrogram of the clusters in Yaralığöz EOF, Türkiye. Cluster 1. *Juniperus oxycedrus* subsp. *oxycedrus*-*Pinus nigra* subsp. *pallasiana* vegetation type; Cluster 2. *Daphne pontica*-*Pinus sylvestris* var. *hamata* vegetation type; Cluster 3. *Galium odoratum*-*Fagus orientalis* vegetation type; Cluster 4. *Galium rotundifolium*-*Abies nordmanniana* subsp. *equi-trojani* vegetation type

It is possible to read the dendrogram in 2 parts. The first cluster on the left is the *Pinus nigra* subsp. *pallasiana* (Lamb.) Holmboe, and the three clusters on the right represent the *Pinus sylvestris* var. *hamata* Steven (2. cluster), *Fagus orientalis* Lipsky (3. cluster), and *Abies nordmanniana* subsp. *equi-trojani* (Asc. & Sint. ex Boiss.) Coode & Cullen (4. cluster) vegetation types, respectively. The NMDS ordination also reflects this division (Figure 3). The NMDS diagram indicates that the four clusters have a minimum overlap (stress 0.18).

Furthermore, Figure 3 shows that the changes in vegetation types among the related ones are mainly on the second axis, and the effect of ecological and topographic factors corresponds to the second axis. The order of influence of the topographic factors affecting the plant composition and spatial distribution of the spreading forest vegetation types in the Yaralıgöz EOF is altitude, inclination, and aspect. When ecological factors (EIVs) are examined, the impact of temperature and soil reaction is low. However, light intensity, nutrient content, moisture content, and continentality have significant effects (Figure 3).

The *P. nigra* subsp. *pallasiana* vegetation type is markedly separated from the other three vegetation types in the study area regarding floristic composition. This

vegetation type is built of plant taxa with heliophilous, nutrient-poor, xerophilous, and partly continental and thermophilous species such as *Quercus pubescens*, *Clinopodium nepeta* subsp. *glandulosum*, *Teucrium polium*, *Salvia tomentosa*, and *Crataegus orientalis* var. *orientalis*. The *P. sylvestris* var. *hamata* vegetation type is positively associated with light intensity, altitude, and continentality. It consists of more moisture and nutrient-rich taxa such as *Rosa canina*, *Pyrus elaeagnifolia*, *Filipendula vulgare*, *Poa trivialis*, *Dactylis glomerata* subsp. *glomerata*, *Galium verum*, and *Helianthemum nummularium* subsp. *nummularium* than the *P. nigra* subsp. *pallasiana* vegetation type. The *F. orientalis* vegetation type consists of nutrient-poor, heliophilous but thermophilous plant taxa such as *Quercus petraea* subsp. *iberica*, *Galium odoratum*, *Crataegus monogyna* subsp. *monogyna*, *Rhododendron luteum*, *Veronica chamaedrys*, and *Cardamine quinquefolia* at low altitudes. The *A. nordmanniana* subsp. *equi-trojani* vegetation type is positively associated with moisture and nutrients-rich plant taxa such as *Mespilus germanica*, *Lonicera orientalis*, *Helleborus orientalis*, *Ilex colchica*, *Laurocerasus officinalis*, *Lathyrus laxiflorus* and spreads in the northern-facing aspect at the altitude between the *P. sylvestris* var. *hamata* and *F. orientalis* vegetation types (Figure 3).

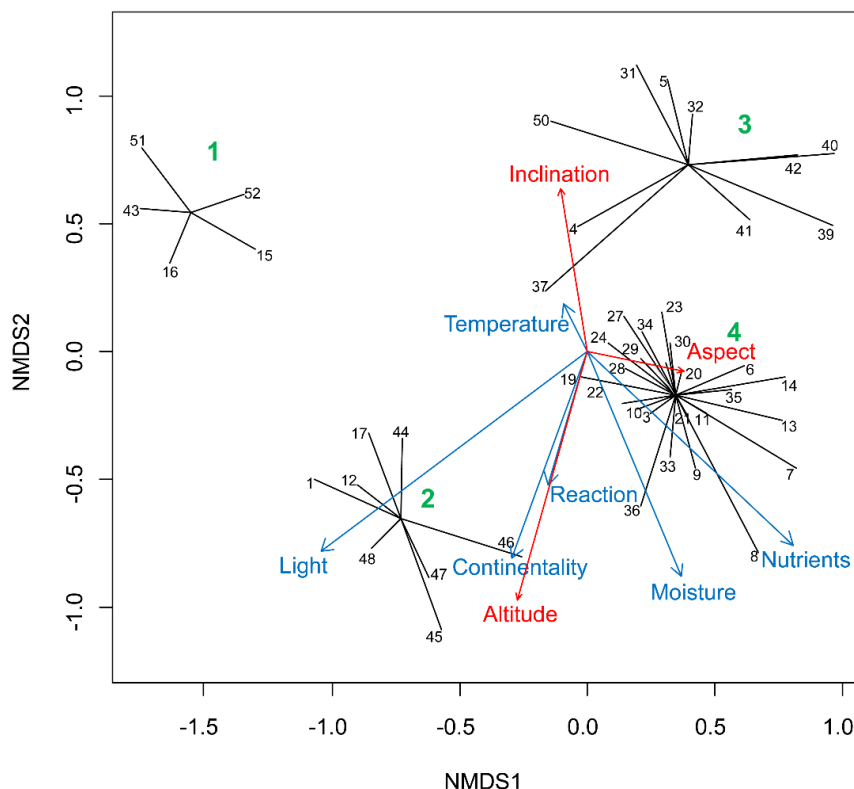


Figure 3. NMDS diagram of clusters. Cluster 1. *Juniperus oxycedrus* subsp. *oxycedrus*-*Pinus nigra* subsp. *pallasiana* vegetation type; Cluster 2. *Daphne pontica*-*Pinus sylvestris* var. *hamata* vegetation type; Cluster 3. *Galium odoratum*-*Fagus orientalis* vegetation type; Cluster 4. *Galium rotundifolium*-*Abies nordmanniana* subsp. *equi-trojani* vegetation type

Cluster 1: *Juniperus oxycedrus* subsp. *oxycedrus*-*Pinus nigra* subsp. *pallasiana* vegetation type

This vegetation type consists of five relevés and 61 vascular plant taxa.

Diagnostic species: *Asyneuma limoniifolium* subsp. *pestalozzae*, *Berberis crataegina*, *C. nepeta* subsp. *glandulosum*, *Campanula rapunculus* subsp. *lambertiana*, *Centaurea urvillei* subsp. *stepposa*, *Clematis vitalba*, *Daphne oleoides* subsp. *oleoides*, *Digitalis lamarkii*, *Globularia trichosantha* subsp. *trichosantha*, *Juniperus oxycedrus* subsp. *oxycedrus*, *Laser trilobum*, *Leontodon hispidus* subsp. *hispidus*, *Medicago lupulina*, *Onosma bornmuelleri*, *Pilosella hoppeana* subsp. *troica*, *P. nigra* subsp. *pallasiana*, *Q. pubescens*, *Salvia sclarea*, *S. tomentosa*, *Sorbus umbellata*, *Tanacetum poteriifolium*, *Taraxacum butleri*, *Teucrium chamaedrys* subsp. *chamaedrys*, *T. polium*, *Thesium arvense*, *Thymus sipyleus*.

Constant species: *Galium paschale*, *L. orientalis*, *Sorbus torminalis* var. *torminalis*.

Dominant species: *P. nigra* subsp. *pallasiana*.

The *P. nigra* vegetation type shows semi-arid semi-continental climate characteristics. This vegetation type has a fragmented distribution with an average inclination of 60-70%, between approximately 1,150 and 1,500 masl altitudes on limestone bedrock in the south-facing slopes in the study area. This vegetation type is partly located in Tezcan FPU, mostly in Devrekani FPU, on the foothills of Yaralığöz EOF, and around Elmacikkalesi Hill. The vegetation type comprises tree, shrub, and herb layers. The tree layer is represented by *P. nigra* subsp. *pallasiana* has a total coverage of between 70 and 90% and 11-18 m in tall. The shrub layer is 150-300 cm tall, and average cover values vary between 20% and 55%. The overall appearance of the shrub layer is made up of *J. oxycedrus* subsp.

oxycedrus, *B. crataegina*, *Q. pubescens*, *L. orientalis*, *Pyracantha coccinea*, and *Rubus hirtus*. The herb layer is 30-100 cm tall and covers 10-65%. The dominant species in the herb layer are *T. poteriifolium*, *T. chamaedrys* subsp. *chamaedrys*, *S. tomentosa*, *Campanula rapunculus* subsp. *lambertiana*, *T. polium*, *M. lupulina*, and *T. sipyleus*. The vegetation type looks highly affected by anthropogenic effects. The abundance of low-frequency, cosmopolitan / widely distributed plant taxa in the vegetation type supports these anthropogenic effects (Table 1). Although the Hemicryptophytes dominate the vegetation type, Phanerophytes and Chamaephytes indicate that the vegetation type is also rich in woody flora (Table 2).

Cluster 2. *Daphne pontica*-*Pinus sylvestris* var. *hamata* vegetation type

This vegetation type contains eight relevés and 83 vascular plant taxa.

Diagnostic species: *Alchemilla mollis*, *Bromus tomentellus*, *D. glomerata* subsp. *glomerata*, *Daphne pontica*, *Filipendula vulgaris*, *G. verum* subsp. *verum*, *Geranium asphodeloides* subsp. *asphodeloides*, *H. nummularium* subsp. *nummularium*, *Juniperus communis* var. *saxatilis*, *Myosotis arvensis* subsp. *arvensis*, *P. sylvestris* var. *hamata*, *P. trivialis*, *Polygala major*, *Rumex tuberosus* subsp. *horizontalis*, *Trifolium pratense* var. *pratense*.

Constant species: *A. nordmanniana* subsp. *equi-trojani*, *Digitalis ferruginea* subsp. *ferruginea*, *Fragaria vesca*, *G. paschale*, *H. orientalis*, *Knautia involucrata*, *Lapsana communis* subsp. *intermedia*, *R. canina*, *T. chamaedrys* subsp. *Chamaedrys*.

Dominant species: *P. sylvestris* var. *hamata*.

Table 1. The dispersion of taxa, according to Raunkiaer's life forms

Vegetation type	Life forms (number / (%))				
	Ph	Ch	H	Cr	Th
<i>Juniperus oxycedrus</i> subsp. <i>oxycedrus</i> - <i>Pinus nigra</i> subsp. <i>pallasiana</i>	15 (24.5)	9 (14.8)	35 (57.4)	0 (0.0)	2 (3.3)
<i>Daphne pontica</i> - <i>Pinus sylvestris</i> var. <i>hamata</i>	14 (17.0)	10 (12.0)	47 (56.6)	3 (3.6)	9 (10.8)
<i>Galium odoratum</i> - <i>Fagus orientalis</i>	14 (22.6)	4 (6.5)	32 (51.6)	9 (14.5)	3 (4.8)
<i>Galium rotundifolium</i> - <i>Abies nordmanniana</i> subsp. <i>equi-trojani</i>	25 (21.7)	3 (2.6)	63 (54.8)	17 (14.8)	7 (6.1)

Table 2. Chorological dispersion of the clusters in the Yaralığöz EOF, Türkiye

Vegetation type	Phytogeographical regions (Number / (%))			
	Circumboreal	Mediterranean	Irano-Turanian	Widely distributed
<i>Juniperus oxycedrus</i> subsp. <i>oxycedrus</i> - <i>Pinus nigra</i> subsp. <i>pallasiana</i>	19 (31.1)	3 (4.9)	7 (11.5)	32 (52.5)
<i>Daphne pontica</i> - <i>Pinus sylvestris</i> var. <i>hamata</i>	40 (48.2)	3 (3.6)	2 (2.4)	38 (45.8)
<i>Galium odoratum</i> - <i>Fagus orientalis</i>	37 (59.7)	1 (1.6)	2 (3.2)	22 (35.5)
<i>Galium rotundifolium</i> - <i>Abies nordmanniana</i> subsp. <i>equi-trojani</i>	67 (58.3)	1 (0.9)	1 (0.9)	46 (40.0)

The *P. sylvestris* var. *hamata* vegetation type shows semi-humid, semi-arid, and cold climate characteristics. In the study area, it spreads well on conglomerate and limestone bedrock, especially in Karacakaya FPU, around Koru Mountain and İmamçuğu Hill and its surroundings on the south-facing slopes with an average inclination of 15-65% between the altitudes of approximately 1,250 and 1,600 m asl. The vegetation type physiologically shows a three-layered structure consisting of tree, shrub, and herb layers. The tree layer of the vegetation type has an average tall of 10-20 m and covering degrees of 75-95%. The dominant species of the tree layer is *P. sylvestris* var. *hamata*, accompanied by the youth of *A. nordmanniana* subsp. *equi-trojani*. The shrub layer has a 50-200 cm tall, and average cover values range from 15 to 50%. The *J. communis* var. *saxatilis* is dominant on the shrub layer and is accompanied by *D. pontica*, *R. hirtus*, *Rubus idaeus*, *L. orientalis*, *P. coccinea*, and *R. canina*. The herb layer has a 30-50 cm tall and a 15-75% cover. The most common species are *H. orientalis*, *K. involucrata*, *Sanicula europaea*, and *Carex sylvatica* in this layer (supplementary material). The abundance of low-frequency taxa belonging to the vegetation type indicates that the vegetation type is open to human intervention. This disturbance can also explain the high cover-abundance value of *J. communis* var. *saxatilis* within the vegetation type. Most of the taxa that spread in the vegetation type are parallel with the study area in the Euro-Siberian region. In addition, most of the taxa in the vegetation type show the Hemicryptophyte character, and the taxa with Phanerophyte and Chamaephyte characters are also noteworthy (Table 2).

Cluster 3. *Galium odoratum*-*Fagus orientalis* vegetation type

This vegetation type occurs with 10 relevés and 62 vascular plant taxa.

Diagnostic species: *Cardamine impatiens* subsp. *pectinata*, *Carpinus betulus*, *F. orientalis*, *G. odoratum*, *Pteridium aquilinum*, *Q. petraea* subsp. *iberica*, *Silene vulgaris* var. *vulgaris*.

Constant species: *A. nordmanniana* subsp. *equi-trojani*, *Argyrolobium biebersteinii*, *Campanula latifolia*, *C. sylvatica* subsp. *sylvatica*, *Cirsium hypoleucum*, *D. ferruginea* subsp. *ferruginea*, *Dorycnium graecum*, *Euphorbia amygdaloides* var. *amygdaloides*, *L. laxiflorus* subsp. *laxiflorus*, *R. idaeus*, *Salvia forskahlei*.

Dominant species: *F. orientalis*, *Q. petraea* subsp. *iberica*.

The *F. orientalis* vegetation type is perhumid to humid and has cold winter climate characteristics. It is well represented in the vegetation type in the study area between approximately 850 and 1,400 masl, on sandstone, limestone, and rarely serpentine bedrocks on the north-facing slopes with an average inclination of 45-75%, within Şeyhşaban and Tezcan FPUs. This vegetation type also has a three-layered structure. The total coverage of the tree layer ranges from 90 to 100%, and the mean tall is 10-30 m (supplementary material). The dominant taxa of the tree layer are *F. orientalis* and *Q. petraea* subsp. *iberica*, accompanied by *A. nordmanniana* subsp. *equi-trojani*, and

C. betulus. The shrub layer has a 0-400 cm tall, and average cover values range between 0 and 30%. The shrub layer generally consists of *R. hirtus*, *R. idaeus*, *D. pontica*, and *R. luteum*. The total coverage and tall of the herb layer are 10-65% and 30-40 cm, respectively. The *G. odoratum*, *C. impatiens* subsp. *pectinata*, *E. amygdaloides* var. *amygdaloides*, *L. laxiflorus* subsp. *laxiflorus*, and *D. ferruginea* subsp. *ferruginea* can be seen commonly in the herb layer. The 60.3% of the taxa determined within the vegetation type belong to the Euro-Siberian phytogeographical region (Table 1). However, within the vegetation type, Hemicryptophytes are predominant, and it is a rich vegetation type in terms of woody flora. Cryptophytes are also significant in this *F. orientalis* vegetation type (Table 2).

Cluster 4: *Galium rotundifolium*-*Abies nordmanniana* subsp. *equi-trojani* vegetation type

It is the most widely distributed forest vegetation type in Yarılgöz EOF. This vegetation type consists of 28 relevés and 115 vascular plant taxa.

Diagnostic species: *A. nordmanniana* subsp. *equi-trojani*, *Astragalus glycyphylloides*, *C. sylvatica* subsp. *sylvatica*, *C. hypoleucum*, *Euonymus latifolius* subsp. *latifolius*, *Galium rotundifolium*, *Lilium martagon*, *S. europaea*, *Valeriana alliariifolia*, *Viola sieheana*.

Constant species: *Clinopodium grandiflorum*, *C. latifolia*, *D. pontica*, *E. amygdaloides* var. *amygdaloides*, *F. vesca*, *G. odoratum*, *H. orientalis*, *L. orientalis*, *P. sylvestris* var. *hamata*, *R. hirtus*, *S. forskahlei*.

Dominant species: *A. nordmanniana* subsp. *equi-trojani*

The *A. nordmanniana* subsp. *equi-trojani* vegetation type shows perhumid to humid and very rainy-cool climate characteristics. It develops optimally on sandstone and conglomerate bedrocks on humid north-facing slopes with an average inclination of 10-75% between 1,050 and 1,750 masl (supplementary material). A good formation of this forest vegetation type is observed around Yarılgöz EOF and its vicinity. However, this vegetation type is well represented in the vicinity of Koru Mountain, Mamatlar Village, and Gümüş Hill. The vegetation type physiologically shows a three vertical-layered structure consisting of tree, shrub, and herb layers. The tree layer of this vegetation type has a coverage of 85-100 m, and the tall of the tree layer varies from 12 to 25 m. The *A. nordmanniana* subsp. *equi-trojani* is the dominant species of the tree layer and, depending on the altitude, forms mixed forests with *P. sylvestris* var. *hamata* and *F. orientalis*. The scrub layer has a 30-400 cm tall, and coverage values range from 5 to 45%. Physiognomically, the shrub layer comprises *L. orientalis*, *R. hirtus*, *D. pontica*, and *R. idaeus*, but some shrub species are also included in the vegetation type with a low frequency. The herb layer is quite rich and has a 30-150 cm tall and a 40-80% coverage. The *G. rotundifolium*, *S. europaea*, *C. sylvatica*, *G. odoratum*, *C. grandiflorum*, *C. hypoleucum*, *V. sieheana*, *H. orientalis*, *E. amygdaloides*, and *F. vesca* are commonly found on the herb layer. The vast majority (57.4%) of the taxa that make up the vegetation type are in the Euro-Siberian floristic region (Table 1). Although

Hemicryptophytes are observed as the majority in this vegetation type, it is rich in woody flora as in the *P. nigra* vegetation type. Cryptophytes also have found the opportunity to spread within the vegetation type (Table 2).

Discussion

According to Quézel et al. (1980), Yaralığöz EOF has located within mountain pre-pontic and Euxinian hilly belts; these are also affected by the pre-pontic supra-Mediterranean belt. In this study, four forest vegetation types were defined in the Yaralığöz EOF with their effect on the climate condition depending on the altitude. This definition is compatible with some studies conducted in Türkiye (Kurt et al. 2015; Sezer et al. 2015; Korkmaz et al. 2016; Kavgaç et al. 2017; Özdeniz et al. 2017). In the Yaralığöz EOF, the sub-Euxine dry *J. oxycedrus*-*P. nigra* subsp. *pallasiana* vegetation type spreads between the elevation of about 1,150 and 1,500 masl. The *J. oxycedrus*-*P. nigra* subsp. *pallasiana* association was first identified from Kunduz forests in Samsun-Vezirköprü (Özen and Kılınç 2002), and the floristic similarity with the association was found to be 23.1% in this study. This low similarity rate is because the research conducted in the Kunduz forests was conducted in a different climate type in the lower elevation zone. In Türkiye, *P. nigra* associations in terms of phytosociology are linked with the orders of *Quercus cerridis*-*Carpinetalia orientalis* Akman, Quézel and Barbéro 1980 and *Quercus pseudocerridis*-*Cedretalia libani* Barbéro, Loisel and Quézel 1974 within the class of *Quercetalia pubescentis* Doing-Kraft ex Scamoni et Passarge 1959 (Akman 1995). Indeed, when phytosociological studies on *P. nigra* in the west and central Black Sea regions were examined, *Quercus-Carpinetalia orientalis* was mostly preferred under the *Q. pubescentis* class. In addition, in a study conducted in Köroğlu Mountain, *Quercetalia pubescentis* and *Q. pubescentis* order and class were found appropriate (Akman 1995). In all studies in the west and central Black Sea regions, *P. nigra* associations were assigned to the *Carpino betuli*-*Acerion hyrcani* Quézel, Barbéro and Akman 1978, *Cisto laurifolii*-*Pinion*

pallasiana Akman et al. ex Quézel et al. 1992, and *Quercion anatolicae* Akman, Barbéro et Quézel 1979. Unfortunately, there are some mistakes and deficiencies in these phytosociological studies conducted in the past (Karaköse 2019). However, with studies conducted in recent years (Kavgaç et al. 2012; Mucina et al. 2016; Çoban and Willner 2019), mistakes in syntaxonomic groups have been corrected. Indeed, it was reported by Çoban and Willner (2019) that the order of *Q. cerridis*-*C. orientalis* and the *C. betuli*-*A. hyrcani* were incorrectly named. Within the scope of the same study, the order of *Q. cerridis*-*C. orientalis* and the alliance of *C. betuli*-*A. hyrcani* were placed as synonyms of *Quercetalia pubescentis-petraeae* Klika 1933 and *Trachystemono orientalis-Carpinion betuli* Çoban and Willner 2019, respectively.

Therefore, due to some taxa (in addition to *P. nigra* subsp. *pallasiana* and *J. oxycedrus* subsp. *oxycedrus*) belonging to *C. laurifolii*-*P. pallasiana* such as *B. crataegina*, *T. chamaedrys*, *Asyneuma limonifolium* subsp. *pestalozzae*, *G. trichosantha* subsp. *trichosantha*, *L. trilobum*, *S. tomentosa*, *T. poteriifolium*, and *S. tomentosa* to coordinate with Çoban and Willner (2019) and Kavgaç et al. (2021), the *P. nigra* association determined in the study was linked to the *C. laurifolii*-*P. pallasiana* and *Erico-Pinetalia* Horvat 1959.

Sub-Euxine *D. pontica*-*P. sylvestris* var. *hamata* association distributes between 1,250 and 1,600 masl at the supra-Mediterranean and mountain pre-pontic belts in Yaralığöz EOF. The association was first mentioned around the Devrez stream and its vicinity (Kılınç 1985). This association was later determined in Bafra-Nebyan Mountain (Kutbay and Kılınç 1995), Samsun-Kunduz forests (Özen and Kılınç 2002), and Bilecik (Türe et al. 2005). Sorensen similarity rates between *D. pontica*-*P. sylvestris* var. *hamata* associations were calculated as 24.1% (Kılınç 1985), 26.3% (Özen and Kılınç 2002), 29.4% (Kutbay and Kılınç 1995), and 22% (Türe et al. 2005) (Table 3).

Table 3. The comparison of the vegetation types with similar associations by Sorensen similarities

Defined associations in the study area	<i>Daphno ponticae</i> - <i>Pinetum hamatae</i>	<i>Galio-Fagetum</i> <i>orientali</i>	<i>Galio rotundifolii</i> - <i>Abietum equi-trojani</i>
Related previous studies	Sorensen similarity rates (%)		
Devrez stream and its vicinity (Kılınç 1985)	24.1%		27.9%
Bafra-Nebyan Mountain (Kutbay and Kilic 1995)	29.4%		
Samsun-Kunduz forests (Özen and Kilic 2002)	26.3%	28.3%	
Bilecik (Türe et al. 2005)	22%		
Samsun-Kastamonu, Ilgaz Mountain, Tokat (Quézel et al. 1980)			35.7%-32.7%-22.4%
Tavşan Mountain (Yıldırım et al. 2019)			22.4%
Ayryaka-Düzce (Çoban and Özalp 2012)			31.9%

Furthermore, the similarity rates show very low similarities with the associations reported from different areas. The study areas are located at different altitudes, and local edaphic and climatic factors vary even at short distances. However, this association was invalidly published (Art. 5) by Kılınç (1985). It was decided to accept the *P. sylvestris* var. *hamata* vegetation type detected in Yaralıgöz EOF as a new association (*Dahpno ponticae-Pinetum hamatae* ass. nova *holotypus hoc loco* description in appendix). In Türkiye, *P. sylvestris* var. *hamata* distributes due to different ecological conditions in wide areas, making its syntaxonomy quite difficult (Karaköse 2019). But it has a limited distribution at semi-continental or Mediterranean-tending transition zones in north-western Anatolia (Akman 1995). Previous *D. pontica*-*P. sylvestris* var. *hamata* vegetation types were evaluated within two different classes, namely *Querco-Fagetea sylvaticae* Br.-Bl. et Vlieger in Vlieger 1937 and *Q. pubescentis*. From this point of view, other plant associations identified except for the Kunduz (Özen and Kılınç 2002) region (the order of *Rhododendro pontici-Fagetalia orientalis* within the class of *Q.-F. sylvaticae*) are included in the order of *Q. cerridis*-*C. orientalis* within the class of *Q. pubescentis*. In all studies except those in Yaralıgöz EOF, *D. pontica*-*P. sylvestris* var. *hamata* vegetation types were associated with the *C. betuli*-*A. hyrcani*. However, as mentioned earlier, *Q. cerridis*-*C. orientalis* and *C. betuli*-*A. hyrcani* have been incorrectly published (Çoban and Willner 2019). In the *P. sylvestris* var. *hamata* vegetation type, the floristic composition of the understory is richer than that in the *P. nigra*, and the number of species with continental characteristics is increasing. As a result of the decrease in the impact of the oceanic climate, the *P. sylvestris* var. *hamata* vegetation type, compared to *A. nordmanniana* subsp. *equi-trojani* vegetation type has acquired a more arid character in which the semi-continental climate is felt. Accordingly, the influence of the mesophilous *Carpino-Fagetea sylvatica* has also decreased significantly. When the association detected in Yaralıgöz EOF is examined from a floristic point of view, the representation of taxa belonging to the *Vaccinio-Piceetea* Br.-Bl. in Br.-Bl. et al. 1939, and *Q. pubescentis* remains low. On the contrary, within the vegetation type, taxa of heliophilous and continental characteristics belonging to *Erico-Pinetea* due to being located near the transition zone to the Irano-Turanian phytogeographical region and also the sub-alpine region are quite well represented together with the *C. laurifolii*-*P. pallasianae*. For that reason, the association of *P. sylvestris* var. *hamata* has been linked to *C. laurifolii*-*P. pallasianae* and *Erico-Pinetalia*.

On the mountain pre-pontic and the pre-pontic supra-Mediterranean belts of the eu-Euxine and sub-Euxine zones in the Yaralıgöz EOF, *F. orientalis* is represented by the forest vegetation type, thermo to mesophilous, formed with *G. odoratum* at an altitude of 850-1,400 masl. The *G. odoratum*-*F. orientalis* association was first identified in Kunduz forests in Samsun-Vezirköprü (Özen and Kılınç 2002), and the floristic similarity with the association was found to be 28.3% in this study. This oriental beech

association was later determined by Kavgacı et al. (2013) from the Aladağlar region, and the floristic similarity with this vegetation type was calculated as 12.5%. In previous studies in Türkiye, *F. orientalis* associations are included in the *Querco-Fagetea* and *Q. pubescentis* classes. The *F. orientalis* associations are linked to the *Pino-Piceetalia* in the eastern Black Sea region (Quézel et al. 1980). It has been evaluated under the order of *Rhododendro-Fagetalia orientalis* and *Q.-C. orientalis* in central and western Black Sea, south Marmara, and Mount Ida (Akman 1995). At the alliance level, there is no representation of the *Querco-Fagetea* class, while the *Carpino-Acerion* and *Quercion frainetto* were used in the *Q. pubescentis*. On the other hand, in a comprehensive study by Kavgacı et al. (2012), oriental beech associations in the Black Sea region were interpreted under four alliances (*Fa. orientalis*, *Staphyleo-Buxion*, *Violo-Fa. orientalis*, and *Carpinio-Fagion orientalis*). However, the two alliances (*V.-Fa. orientalis* and *C.-Fa. orientalis*) described by Kavgacı et al. (2012) were accepted as synonyms of the *Fa. orientalis* Soó 1964 by Mucina et al. (2016). Kavgacı et al. (2012) suggested that the *F. orientalis* associations on the basic bedrock in the montane Euxine zone be linked to the Euxinian *Fa. orientalis*. The *F. orientalis* associations spreading in Bulgaria were first included in the *Fa. orientalis* Quézel et al. 1992, and later the *V.-Fa. orientalis* and *C.-Fa. orientalis* (Kavgacı et al. 2012). However, *F. orientalis* associations were transferred to *Fa. orientalis* Soó 1964 by Willner et al. (2017). When the vegetation type determined in Yaralıgöz EOF is examined in terms of floristic composition, the mesophilous and nutrient-demanding taxa belonging to the *Fa. orientalis* and *R. pontici-Fag. orientalis* Passarge 1981 such as *G. odoratum*, *C. sylvatica* subsp. *sylvatica*, *Dryopteris filix-mas*, *C. impatiens* subsp. *pectinata*, *Cardamine bulbifera*, and *Geranium robertianum* are represented well. Similarly, under the *Carpino-Fagetea sylvaticae*, there are representations of the *D. pontica*, *S. forskahlei*, and *V. sieheana* belonging to the *T. orientalis*-*C. betuli*. Although there are several taxa belonging to the *Q. pubescentis*, their representation has remained low in the association. Therefore, this vegetation type determined here was connected to the *Fa. orientalis* and *R. pontici-Fag. orientalis* under *C.-F. sylvaticae*.

The forest vegetation type formed by *A. nordmanniana* subsp. *equi-trojani* with *G. rotundifolium* spreads between 1,050 and 1,750 masl at the mountain pre-pontic and Euxinian hilly belts of the sub-Euxine zone in Yaralıgöz EOF. Numerous forest associations belonging to *A. nordmanniana* subsp. *equi-trojani* (Syn.: *A. nordmanniana* subsp. *bornmuelleriana* (Mattf.) Coode & Cullen) have been previously identified from the western Black Sea and southern Marmara in Türkiye. Floristic similarities between the *A. nordmanniana* subsp. *equi-trojani* vegetation type is defined within the scope of this study, and the other associations vary between 22.4% and 52.7% (Table 3). Forest associations formed by *A. nordmanniana* subsp. *equi-trojani* are mostly very homogeneous in terms of floristic diversity, and the similarity rates have, therefore, been close. The highest similarity rate was calculated with the association (*A. nordmanniana* subsp. *bornmuelleriana*-

Mercurialis perennis) recorded by (Yurdakulol et al. 2002). However, the nomenclature of this association was made inappropriately (ICPN-Art. 5) (Theurillat et al. 2021), according to the rules. Similarly, an association (*Saniculo-Abietetum bornmuellerianae*), recorded around Gerze-Boyabat (Özen and Kılınç 1995) close to Yaralığöz EOF, is also published invalidly (ICPN arts. 4 and 5). In addition, some studies conducted in north-western Anatolia were also published as invalid according to the ICPN (Art. 5) rules (Akman 1995; Çoban and Willner 2019). Based on this information, it was decided to define the *A. nordmanniana* subsp. *equi-trojani* vegetation type identified in Yaralığöz EOF as a new association (*Galio rotundifolii-Abietum equi-trojani* ass. nova *holotypus hoc loco* description in appendix). There are lots of research in the regions where *A. nordmanniana* subsp. *equi-trojani* taxa spread. The *A. nordmanniana* subsp. *equi-trojani* associations were included in the mostly *Quercus-Fagetum* and *Q. pubescentis* in these previous studies. The *A. nordmanniana* subsp. *equi-trojani* associations were interpreted under *R.-Fag. orientalis*, *Vaccinio-Piceetalia orientalis*, *Fagetalia sylvaticae*, and *Q.-C. orientalis* orders. Akman (1995) drew attention to the difficulty in the phytosociological interpretation of *A. nordmanniana* subsp. *equi-trojani* associations. Akman (1995) stated that, based on local conditions, climate, and bedrock factors in the study areas, *A. nordmanniana* subsp. *equi-trojani* was assigned to the order of *R.-Fag. orientalis* on siliceous bedrock and to the order of *Q.-C. orientalis* on limestone bedrock. Although the connection at the alliance level was low, *Carpino-Acerion*, *Staphyleo-Buxion* Quézel et al. 1992, and *Quercion frainetto* alliances were preferred in some studies (Akman 1995). However, Çoban and Willner (2019) stated that it is not appropriate to classify mesic fir forests within deciduous forests. Mucina et al. (2016) evaluated mesic *Abies* associations on nutrient-rich soils (as in Yaralığöz EOF) in Europe within the *Vaccinio-Piceetum*. For that reason, within the *Vaccinio-Piceetum*, the order of *Abieti nordmannianae-Piceetalia orientalis* and the *Lonicero caucasicae-Piceion orientalis*, which cover all *A. nordmanniana* associations, were formed by Çoban and Willner (2019). In this direction, when we examine the *A. nordmanniana* vegetation type, the representation of the species belonging to the *L. caucasicae-P. orientalis* alliance and the order of *A. nordmannianae-Pi. orientalis* such as *G. rotundifolium*, *L. orientalis*, *V. alliariifolia*, *Phedimus stoloniferus* (S.G.Gmel.) t Hart, *R. luteum*, and *Vaccinium arctostaphylos* is quite well. Similarly, *C. sylvatica* subsp. *sylvatica*, *S. europaea*, *E. amygdaloides* var. *amygdaloides*, *G. odoratum*, and *C. grandiflorum* belonging to the *Vaccinio-Piceetum* class are also distributed in this vegetation type. Although the *Fa. orientalis* and the *R.-Fag. orientalis* were also found in the vegetation type, their representation remained low. Taxa belonging to the *Q. pubescentis*, *Erico-Pinetea*, *Festuco-Brometum* Br.-Bl. et Tx. ex Soó 1947, and *Astragalo microcephali-Brometum tomentelli* Quézel 1973 em Parolly also spread throughout the vegetation type. As a result, the *A. nordmanniana* subsp. *equi-trojani* vegetation type determined in Yaralığöz EOF was linked to the *L.*

caucasicae-P. orientalis and the *A. nordmannianae-Pi. orientalis*.

Based on this knowledge, the syntaxonomic info summarizes given below:

Erico-Pinetea Horvat 1959

Erico-Pinetalia Horvat 1959

C. laurifolii-P. pallasianae Akman et al. ex Quézel et al. 1992

i. *Junipero-Pinetum nigrae* Özen et Kılınç 2002

ii. *Daphno ponticae-Pinetum hamatae* Kılınç ex Karaköse et Terzioğlu ass. nova

Syn. *Daphno-Pinetum sylvestris* Kılınç 1985 *nom. inval.* (ICPN Art. 5)

Type: Karaköse et Terzioğlu 2023 (Description in appendix), *holotypus hoc loco*.

Carpino-Fagetum sylvaticae Jakucs ex Passarge 1968

Rhododendro pontici-Fagetalia orientalis Passarge 1981

Fagion orientalis Soó 1964

iii. *Galio-Fagetum orientalis* Özen & Kılınç 2002

Vaccinio-Piceetum Br.-Bl. in Br.-Bl. et al. 1939

Abieti nordmannianae-Piceetalia orientalis Çoban & Willner 2019

Lonicero caucasicae-Piceion orientalis Çoban & Willner 2019

iv. *Galio rotundifolii-Abietum equi-trojani*

Karaköse et Terzioğlu ass. nova

Syn. *Mercurialo-Abietetum bornmuellerianae* Yurdakulol, Demirörs & Yıldız 2002 *nom. inval.* (ICPN Art. 5)

Type: Karaköse et Terzioğlu 2023 (Description in appendix), *holotypus hoc loco*.

Appendix

The nomenclature type of the association. *Daphno ponticae-Pinetum hamatae* ass. nov. *holotypus*: M.Karaköse (21.07.2012), Relevé no: 48, Plot size: 400 m, Altitude: 1538 m asl, Aspect: NE, Inclination: 21°, bedrock: limestone, N: 34°20'45.62" N, 41°7'44.143" E, Cover tree layer: 90%, Cover shrub layer: 35%, Cover herb layer: 40%.

Tree layer: *P. sylvestris* var. *hamata*: 4.

Shrub layer: *J. communis* var. *saxatilis*: 2, *R. idaeus*: 1, *R. canina*: +, *D. pontica*: +.

Herb layer: *A. limonifolium* subsp. *pestalozzae*: 1, *P. trivialis*: 1, *G. robertianum*: +, *A. biebersteinii*: +, *Vicia cracca* subsp. *tenuifolia*: +, *Trifolium repens* var. *repens*: +, *T. pratense* var. *pratense*: +, *Onobrychis armena*: +, *F. vulgaris*: +, *Microthlaspi perfoliatum*: +, *F. vesca*: +, *Carduus nutans* subsp. *falcato-incurvus*: +, *L. communis* subsp. *intermedia*: +, *H. nummularium* subsp. *nummularium*: +, *Cynoglossum officinale*: +, *T. chamaedrys* subsp. *chamaedrys*: +, *Clinopodium vulgare* subsp. *arundanum*: +, *T. sipyleus*: +, *G. odoratum*: +, *G. verum* subsp. *verum*: +, *G. paschale*: +, *Milium vernale* subsp. *vernale*: +, *V. sieheana*: +, *Dactylis glomerata* subsp. *glomerata*: +.

The nomenclature type of the association. *Galio rotundifolii-Abietum equi-trojani* ass. nov. *holotypus*:

M.Karaköse (08.07.2011), Relevé no: 29, Plot size: 400 m, Altitude: 1,134 m asl, Aspect: W, Inclination: 35°, bedrock: sandstone+limestone, N: 34°07'448" N, 41°77'181" E, Cover tree layer: 100%, Cover shrub layer: 20%, Cover herb layer: 55%.

Tree layer: *A. nordmanniana* subsp. *equi-trojani*: 4, *P. sylvestris* var. *hamata*: 1, *F. orientalis*: +, *Acer heldreichii* subsp. *trautvetteri*: +, *Acer campestre* subsp. *campestre*: +.

Shrub layer: *L. orientalis*: 1, *D. pontica*: 1, *E. latifolius* subsp. *latifolius*: +.

Herb layer: *H. orientalis*: 1, *S. europaea*: 1, *Trachystemon orientalis*: 1, *G. rotundifolium*: 1, *L. martagon*: 1, *C. sylvatica* subsp. *sylvatica*: 1, *C. hypoleucum*: +, *C. latifolia*: +, *Atropa belladonna*: +, *E. amygdaloides* var. *amygdaloides*: +, *A. glycyphylloides*: +, *G. odoratum*: +, *V. sieheana*: +.

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Chemical composition of *Corymbia citriodora*

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Abstract. Dogara AM. 2023. Chemical composition of *Corymbia citriodora*. *Nusantara Bioscience* 15: 172-178. Aromatic plants, particularly those in the Myrtaceae family, are widely used both traditionally and commercially to lengthen food's shelf life and safety. The current investigation was prompted by a lack of information on the composition of the plant's oil, which has traditionally been used to treat and manage cancer, malaria, typhoid fever, and various other ailments. The study thoroughly examines the chemical composition of the essential oil obtained from *Corymbia citriodora* (Hook.) K.D.Hill & L.A.S.Johnson. Hydro distillation was used to extract essential oil from the leaves, which were then analyzed using gas chromatography coupled with mass spectrometry. Fourier Transform Infrared spectroscopy (FTIR) was used to identify the functional group in the essential oil. According to the findings, monoterpene compounds make up (0.8137%), sesquiterpenoids (0.6568%) and other compounds (95.7207% of the total). The most abundant substance was 1-Octadecene (7.83%), followed by Oleic acid, 9-Octadecenoic acid, (E)-, (6.16%), Octadecanal, Disparlure, and 1-Octadecene (all of which were at or below 4%), and all other substances. The *C. citriodora* essential oil yielded 11 spectra. The extracts had sharp peaks at 900 cm⁻¹ (phenyl), 1400 (mono-, oligo-, and carbohydrates), and 2900 (lipid methoxy compounds of CH₃ and CH₂, which have distinctive C-H stretching vibrations). OH groups from water, alcohols, phenols, polysaccharides, and peroxides are fingerprinted at 3400 cm⁻¹. The study concludes that oleic acid-rich oil from *C. citriodora* leaves could be used as an economical source of oleic acid; the study lays the groundwork for future research on the plant in this issue.

Keywords: *Corymbia citriodora*, essential oils, medicinal plants, oleic acid

INTRODUCTION

Due to their potential use as pharmaceuticals in treating various illnesses, medicinal plants are gaining popularity worldwide (Paw et al. 2020). Due to the lack of available common medicines to treat infectious diseases, numerous studies have recently concentrated on evaluating natural products as the source of novel biologically active molecules (Ginovyan and Trchounian 2019). Using medicinal plants to create novel herbal drugs or nutraceuticals has received more attention over the past 20 years (Behbahani et al. 2020). Due to bioactive components in plant parts or the entire plant, plants that have medicinal value have become the primary resource for treating and enhancing human health worldwide (Mahmoud et al. 2019). Secondary metabolites identified in abundance in plants with intriguing biological activity are present in various structural configurations and serve various functions (Mahmoud et al. 2019). Flavonoids, saponins, resins, oleoresins, phenolic compounds, alkaloids, sesquiterpenes, essential oils, and lipids are only a few of the many chemical components found in plants (Paw et al. 2020; Da Silva et al. 2021).

Volatile and possessing a distinct aroma and flavor, essential oils are a byproduct of the secondary metabolites found in plants (Ferreira et al. 2022). Aromatic plants are commonly used because they contain volatile chemicals and essential oils (Mahmoud et al. 2019). Essential oils derived from plants have recently been used extensively in various businesses, including food and beverage, cosmetics,

etc. Essential Oils (EOs) and the chemicals that make them up could be used in food items because they are antifungal, antibacterial, and antioxidant (Salem et al. 2018). There has been a worldwide search for natural products like natural EOs that have several applications due to the negative effects of synthetic antibacterial and antioxidant treatments (Da Silva et al. 2021). EOs are employed as preservatives and antioxidants in food preparation, with effects ranging from moderate to powerful (Elansary et al. 2016; Ferreira et al. 2022). The biological activity of these volatile oils is due to the range of chemical components included in them (Ferreira et al. 2022). Essential oils extracted from medicinal herbs have a wide range of applications in ethnomedicine preservation, cosmetics, food, scent, drinks, and pharmaceuticals.

There are around 55,000 species of trees and shrubs in the Myrtaceae family, divided among 2 subfamilies, 17 tribes, and 124 genera (Abdulrahman et al. 2018a). The southern hemisphere, including Australia, Central America, and South America, is home to the greatest concentration of these plants (Abdulrahman et al. 2018a). Despite the diversity of its species, relatively little is known about this genus (Dewijanti et al. 2020). *Corymbia*, a native plant of Australia, is a distinctive genus of towering trees and shrubs with over 800 species widely grown worldwide; now grown and harvested globally (Goodine and Oelgemöller 2020). The *Citriodora* genus has great form, a well-shaped crown, and thin foliage, especially for the essential oil with a lemon aroma (Nogueira et al. 2021). It is cultivated on a large scale for its citronellal-rich essential oil, which is

used in the perfume and flavoring industries (Batish et al. 2006).

Naturally, citriodora essential oil is used to scent rooms, creating essences, perfumes, disinfectants, soaps, and detergents (Nogueira et al. 2021). However, to the best of our knowledge, and as evidenced by the literature, there aren't many papers that specify the chemical makeup of *Corymbia citriodora* (Hook.) K.D.Hill & L.A.S.Johnson (Luqman et al. 2008; Salem et al. 2018) from other regions of the world, particularly Nigeria. Therefore, the study aimed to provide the essential oil chemical composition of *C. citriodora* from northern Nigeria.

MATERIALS AND METHODS

Study area

The northern Nigerian state of Kaduna (10° 35' N, 7° 19' E) is home to 6,066,562 people across 46,056 km². The area experiences two distinct types of weather: dry (from October to May) and wet (from June to August), even though the weather varies with the season. Most of the people in the country are members of the nomadic Hausa and Fulani tribes (Abdulrahman et al. 2022). Their main sources of income come from the government, agriculture, animal husbandry, fishing, and hunting (Abdulrahman et al. 2022).

Plants material

The *C. citriodora* leaves (Figure 1) were taken from the wild in Kaduna state, Nigeria, at the same time. The medicinal plants obtained in the field were identified by comparison with existing literature and thoroughly confirmed by botanists at Ahmadu Bello University (ABU Zaria), after which the identified herbarium specimen was put in the University herbarium (ABU02886).

Essential oil distillation

Fresh leaves were collected, gently washed to eliminate plant stains, dried at room temperature in the shade, and then processed into a fine powder using a blade mill. Next, 100 g of the leaves were dissolved in 2L of water in a round-bottom flask with Clevenger-type equipment. The essential oil was hydro distilled for 5 hours, then dried with anhydrous sulphate before being put in a dark bottle and kept at -4°C for future analysis (Abdulrahman et al. 2022).

Equation illustrates the yield computation as follows (Kheawfu et al. 2021):

$$\text{The yield of the extract} = \frac{\text{Weight of extract}}{\text{Dry weight of the leaves}} \times 100$$



Figure 1. *Corymbia citriodora*

Gas Chromatography Mass Spectrometry (GC-MS) conditions

Agilent Technologies (Malaysia) 7890A/5975C gas chromatography/coupled mass spectrometer was used for the GC/MS analysis. The chemical mixtures were separated on a column in a temperature programmed: HP-5MS 30 m x 0.25 mm, 0.25 mm film thickness. Start at 60°C for 10 minutes, then increase the temperature by 3°C every minute, holding it at 230°C for 1 minute. The injector temperature was 245°C, and the carrier-helium gas flow rate was 1 mL per minute (Abdulrahman et al. 2022). The MS was performed with an ion source and analyzer temperature of 260°C and 70 E V, respectively.

Fourier Transform Infra-Red spectroscopy (FTIR)

The FTIR spectra were generated from the IRPREATIGE-21 model (Malaysia). The FTIR spectra were collected using a Perkin Elmer Spectrum 400 Infrared spectroscopy (Malaysia) equipped with an air-cooled Deuterated Triglycine Sulphate (DTGS) detector, and the spectra were scanned at mid-IR with a spectral range of 4000-400 cm⁻¹. There were eleven technical replicates. Attenuated Total Reflectance (ATR) scans with 16 scans and 4 cm⁻¹ resolutions were used on all samples (Abdulrahman et al. 2018b). Each FTIR spectrum was baseline adjusted before being converted to an ASCII file, greatly reducing the spectral difference that could have resulted from baseline shifts. The spectra were evaluated considering the existing literature.

RESULTS AND DISCUSSION

Essential oil composition

Phytochemicals are natural plant compounds that can treat and prevent various diseases (Oladunmoye et al. 2018). Plant resources must be genuine and safe to produce herbal items or modern medications. Quality control is vital in ethnopharmacology to check the quality and authenticity of plants used to create modern pharmaceuticals or herbal supplements. Identifying therapeutic plants requires a scientific understanding of their chemical makeup. Traditionally, morphoanatomical features have been used to categorize plants in the plant taxonomy system (Abdulrahman 2022). Therefore, to evaluate the presence of many substances in each sample, scientists use Gas Chromatography-Mass Spectrometry (GC-MS), a hybrid analytical approach that combines the separation capabilities of gas-liquid chromatography with the detection capability of mass spectrometry (Uka et al. 2022). GC can separate a sample's volatile and thermally stable substitutes, and MS can fragment the target analyte for mass-based identification (Uka et al. 2022). New applications for GC-MS have been made possible by its improved sample identification, higher sensitivity, a wider range of analyzable samples, and faster findings (Uka et al. 2022). Chemicals with widespread physiological and bioactive properties may be derived from plant-derived natural materials (Dewijanti et al. 2020). Plants have both primary and secondary metabolism (Dewijanti et al. 2020).

The primary metabolism produces chemicals used in the biosynthetic process, whereas the secondary metabolism produces medicinally active chemicals; therefore, it is necessary to identify each medicinal plant chemically. Based on dry weight, the *C. citriodora* leaves yielded 1.8% of the distilled oil, produced as a yellow oil through hydro distillation. Furthermore, 82 compounds were identified from qualitative results with a 97.1912% recovery rate (Table 1). The oil yield recovery from the leaf demonstrates that the plant is a good source of essential oil. The findings of our study are congruent with those of earlier research (Tolba et al. 2015; et al. 2018). The extraction method, extraction solvent, chemicals present, and polarity of metabolites all play major roles in the variation in extract yield from medicinal plant parts (Abdulrahman et al. 2019). Climate, geographic distribution, plant genetics, plant part used, degree of freshness, drying period, and extraction process are only (some of the many) factors that affect EO yields, making direct comparisons impossible (Sreepian et al. 2022). According to morpho-anatomical research, the abundance of trichomes in the leaves of *C. citriodora* may be related to the abundance of essential oil in the leaves (Mahmoud et al. 2019). As shown in Table 1 and Figure 2, monoterpenes compounds account for (0.8137%), sesquiterpenoids (0.6568%), and other compounds (95.7207%). Furthermore, 1-Octadecene was the dominant compound identified (7.83%), Oleic Acid and 9-Octadecenoic acid, (E)-, (6.16%, respectively), Octadecanal, Disparlure, 1-Octadecene (4%, respectively) and all other compounds below 4% (Figure 3).

According to location, harvesting season, and leaf age, citriodora oils have different chemical compositions (Goodine and Oelgemöller 2020). Differences in the plant's atmosphere and environment, such as sunlight and rainfall, can affect the plant extract content (Dewijanti et al. 2020). Major components of the leaf EO of *C. citriodora* from Chandigarh, India, include the monoterpenoids citronellal, citronellol, and isopulegol (Salem et al. 2018). Due to harvesting seasons and geographical sources, certain species' essential oils have different chemical compositions (Hamad et al. 2017). Moreover, the cosmetics, flavoring, lubricant, fragrance, and suppository industries all use octadecanoic acid due to its hypocholesterolemic properties (Arora and Meena 2018). Oleic acids are used in vitamin E and K1 productions, both of which play crucial roles in the human body and are required as an ingredient in their production. Various biological properties, such as antifungal, antibacterial, antioxidant, anti-inflammatory, and anti-tumor, are supported by the presence of these key phytoconstituents in the leaf extract, proving the plant's traditional therapeutic usage (Uka et al. 2022). Because of their antibacterial qualities, EOs have been widely employed in traditional medicine and to prolong the shelf life of food (Wińska et al. 2019). The high demand for all-natural substances has dramatically increased widespread use in recent years (Singh and Pulikkal 2022). Therefore, to the best of our knowledge, there is no literature on the chemical composition of *C. citriodora* in northern Nigeria. The current inquiry was motivated by a lack of information on the composition of the plant's oil, as the plant is reported

to have traditionally been used to cure and manage cancer, malaria and typhoid fever. The study includes a detailed analysis of the chemical makeup of the essential oil isolated from *C. citriodora* leaves. Therefore, the study provides a foundation for future research on the plant benefits.

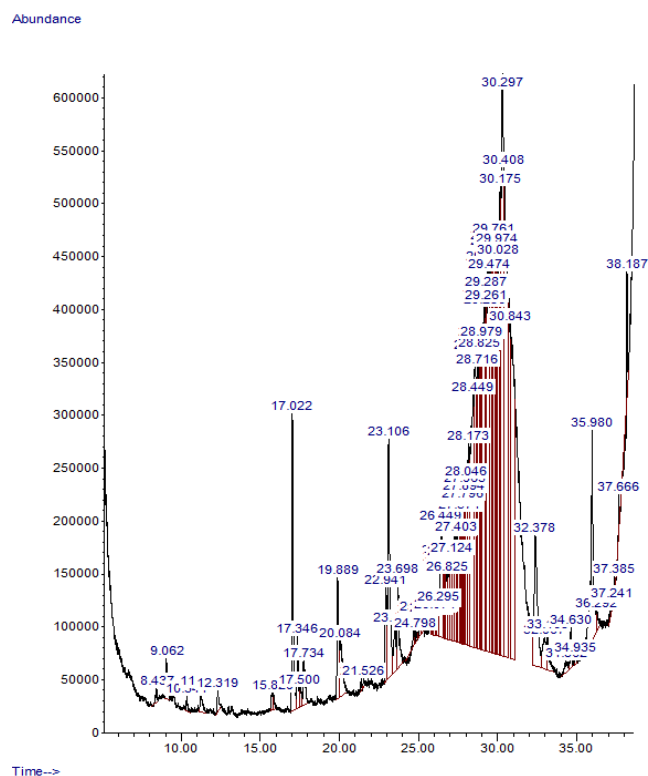


Figure 2. Chromatogram of the essential oil from *Corymbia citriodora* leaves

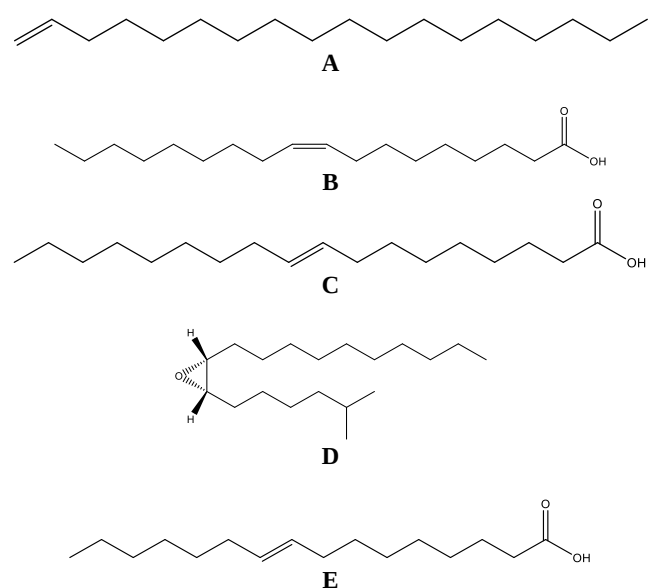


Figure 3. Major compounds in *Corymbia citriodora*: A. 1-Octadecene, B. Oleic acid, C. 9-Octadecenoic acid, (E)- D. Disparlure, E. 9-Hexadecenoic acid

Table 1. The essential oil composition of *Corymbia citriodora*

PK	RT	Compound	Area Pct	Chemical structure
1	8.4371	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-	0.1101	C ₁₅ H ₂₄ O
2	9.062	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	0.3074	C ₁₅ H ₂₄
3	10.3412	(E,Z)-.alpha.-Farnesene	0.098	C ₁₅ H ₂₄
4	11.2315	Dodecanoic acid, methyl ester	0.271	C ₁₃ H ₂₆ O ₂
5	12.3192	Trans-Z-.alpha.-Bisabolene epoxide	0.1673	C ₁₅ H ₂₄ O
6	15.7241	Nonanoic acid, methyl ester	0.1413	C ₁₀ H ₂₀ O
7	15.8295	Undecanoic acid, methyl ester	0.114	C ₁₂ H ₂₄ O ₂
8	17.0223	Alpha.-Phellandrene, dimer	1.0821	C ₂₀ H ₃₂
9	17.3462	Alpha.-Phellandrene, dimer	0.2884	C ₂₀ H ₃₂
10	17.5001	Gamma.-Terpinene	0.1846	C ₁₀ H ₁₆
11	17.734	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	0.4878	C ₁₀ H ₁₆
12	19.8889	Hexadecanoic acid, methyl ester	1.1065	C ₁₈ H ₃₆ O ₂
13	20.0839	1,2-Benzenedicarboxylic acid, butyl octyl ester	0.8025	C ₂₀ H ₃₀ O ₄
14	21.3819	Heptadecyl heptafluorobutyrate	0.0529	C ₂₁ H ₃₅ F ₇ O ₂
15	21.5262	Tert-Hexadecanethiol	0.0504	C ₄₈ H ₉₉
16	22.9413	8,11-Octadecadienoic acid, methyl ester	0.7533	C ₁₉ H ₃₄ O ₂
17	23.1063	11-Octadecenoic acid, methyl ester	2.4952	C ₁₉ H ₃₆ O ₂
18	23.4926	Pentyl triacontyl ether	0.5103	C ₃₅ H ₇₂ O
19	23.6984	Methyl stearate	0.8867	C ₁₉ H ₃₈ O ₂
20	24.7092	E,E-10,12-Hexadecadien-1-ol	0.0795	C ₁₆ H ₃₀ O
21	24.798	Cis-9,10-Epoxyoctadecan-1-ol	0.0779	C ₁₈ H ₃₆ O ₂
22	25.0445	Docosyl propyl ether	0.1339	C ₂₅ H ₅₂ O
23	25.1903	Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy-	0.1049	C ₂₃ H ₃₀ N ₂ O ₅
24	25.8192	2-Methyl-Z,Z-3,13-octadecadienol	0.0649	C ₁₉ H ₃₆ O
25	25.9834	1,15-Hexadecadiene	0.1469	C ₁₆ H ₃₀
26	26.0744	Cyclododecane, ethyl-	0.1074	(CH ₂) ₁₂
27	26.2473	Z-8-Methyl-9-tetradecenoic acid	0.2418	C ₁₅ H ₂₈ O ₂
28	26.2951	Z-8-Methyl-9-tetradecenoic acid	0.0708	C ₁₅ H ₂₈ O ₂
29	26.4485	cis-9-Tetradecenoic acid, propyl ester	1.3343	C ₁₇ H ₃₂ O ₂
30	26.5788	Cyclopentadecanone, 2-hydroxy-	0.3223	C ₁₅ H ₂₈ O ₂
31	26.6499	2-Methyl-Z,Z-3,13-octadecadienol	0.5179	C ₁₉ H ₃₆ O
32	26.7373	8-Hexadecenal, 14-methyl-, (Z)-	0.3795	C ₁₇ H ₃₂ O
33	26.8248	Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy-	0.1457	C ₂₃ H ₃₀ N ₂ O ₅
34	26.9375	Heptadecanoic acid, heptadecyl ester	0.6526	C ₃₄ H ₆₈ O ₂
35	27.1244	Cyclopropaneoctanal, 2-octyl-	0.854	C ₁₉ H ₃₆ O
36	27.2318	Oleic Acid	0.9393	CH ₃ -(CH ₂) ₇ -CH=CH-(CH ₂) ₇ -COOH
37	27.3442	Oleic Acid	0.5391	CH ₃ -(CH ₂) ₇ -CH=CH-(CH ₂) ₇ -COOH
38	27.4025	Oleic Acid	0.5245	CH ₃ -(CH ₂) ₇ -CH=CH-(CH ₂) ₇ -COOH
39	27.571	2-Methyl-Z,Z-3,13-octadecadienol	1.5979	C ₁₉ H ₃₆ O
40	27.6405	Oleic Acid	0.3642	CH ₃ -(CH ₂) ₇ -CH=CH-(CH ₂) ₇ -COOH
41	27.6766	Oleic Acid	0.4085	CH ₃ -(CH ₂) ₇ -CH=CH-(CH ₂) ₇ -COOH
42	27.7438	Oleic Acid	0.4699	CH ₃ -(CH ₂) ₇ -CH=CH-(CH ₂) ₇ -COOH
43	27.7956	8-Hexadecenal, 14-methyl-, (Z)-	0.4778	C ₁₇ H ₃₂ O
44	27.8944	Oleic Acid	1.0437	CH ₃ -(CH ₂) ₇ -CH=CH-(CH ₂) ₇ -COOH
45	27.9648	Cis-Vaccenic acid	0.7333	C ₁₈ H ₃₄ O ₂
46	28.046	2-Methyl-E,E-3,13-octadecadien-1-ol	0.2723	C ₁₉ H ₃₆ O
47	28.1735	9-Octadecenoic acid, (E)-	1.1596	C ₁₈ H ₃₄ O ₂
48	28.4485	Disparlure	4.074	C ₁₉ H ₃₈ O
49	28.5622	Oleic Acid	3.1973	CH ₃ -(CH ₂) ₇ -CH=CH-(CH ₂) ₇ -COOH
50	28.7165	8-Hexadecenal, 14-methyl-, (Z)-	1.6674	C ₁₇ H ₃₂ O
51	28.8251	7,11-Hexadecadienal	2.1473	C ₁₆ H ₂₈ O
52	28.8821	2-Methyl-Z,Z-3,13-octadecadienol	1.9328	C ₁₉ H ₃₆ O
53	28.9787	7,11-Hexadecadienal	1.1991	C ₁₆ H ₂₈ O
54	29.1653	1-Octadecene	4.6336	C ₁₈ H ₃₆

55	29.2356	Oleic Acid	0.677	$\text{CH}_3-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{C}_{18}\text{H}_{32}\text{O}_2$
56	29.2608	cis-7,cis-11-Hexadecadien-1-yl acetate	0.5275	
57	29.2869	Oleic Acid	0.9154	$\text{CH}_3-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{C}_{18}\text{H}_{32}\text{O}_2$
58	29.3725	Oleic Acid	3.9393	$\text{CH}_3-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{C}_{19}\text{H}_{36}\text{O}$
59	29.474	Cyclopropaneoctanal, 2-octyl-	1.322	
60	29.6153	Oxacyclopentadecan-2-one	3.2977	$\text{C}_{14}\text{H}_{26}\text{O}_2$
61	29.6546	2-Methyl-Z,Z-3,13-octadecadienol	2.1783	$\text{C}_{19}\text{H}_{36}\text{O}$
62	29.7607	Octadecanal	4.2682	$\text{C}_{18}\text{H}_{36}\text{O}$
63	29.8906	Oleic Acid	1.8738	$\text{CH}_3-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{C}_{20}\text{H}_{40}$
64	29.9739	Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-	1.7717	
65	30.0277	Oleic Acid	2.1765	$\text{CH}_3-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{C}_{16}\text{H}_{30}\text{O}_2$
66	30.1751	9-Hexadecenoic acid	3.766	
67	30.2967	9-Octadecenoic acid, (E)-	6.6481	$\text{C}_{18}\text{H}_{34}\text{O}_2$
68	30.4079	1-Octadecene	7.8358	$\text{C}_{18}\text{H}_{36}$
69	30.8432	Oleic Acid	6.1692	$\text{C}_{18}\text{H}_{34}\text{O}_2$
70	32.378	Trimetozine	2.336	$\text{C}_{14}\text{H}_{19}\text{NO}_5$
71	32.9868	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	0.5519	$\text{C}_{21}\text{H}_{40}\text{O}_4$
72	33.1628	1-Octadecanesulphonyl chloride	0.3963	$\text{C}_{18}\text{H}_{37}\text{ClO}_2\text{S}$
73	34.3623	Oleic Acid	0.168	$\text{CH}_3-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{C}_{20}\text{H}_{34}\text{O}$
74	34.6303	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-	0.2845	
75	34.9348	9-Octadecenal	0.036	$\text{C}_{18}\text{H}_{34}\text{O}$
76	35.98	Tetratetracontane	2.694	$\text{C}_{44}\text{H}_{90}$
77	36.2924	Ricinoleic acid	0.1479	$\text{C}_{18}\text{H}_{34}\text{O}_3$
78	37.1895	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-	0.046	$\text{C}_{20}\text{H}_{40}$
79	37.241	Oleic Acid	0.006	$\text{CH}_3-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{C}_{18}\text{H}_{34}\text{O}_2$
80	37.3847	Oleic Acid	0.0701	$\text{CH}_3-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$ $\text{C}_{21}\text{H}_{40}\text{O}_4$
81	37.6661	(S)(+)-Z-13-Methyl-11-pentadecen-1-ol acetate	0.1097	
82	38.1874	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	0.4526	

Note: S/N: Serial number, RT: Retention Time

Fourier Transform Infra-Red spectroscopy (FTIR)

The authenticity and safety of plant components are crucial to the progress of any herbal product or contemporary medication. Since identifying and distinguishing the genuine plant is essential to solving the problems of adulterations and ensuring consumer safety, it is necessary to adopt a strategy or concept to this end (Abdulrahman et al. 2018b). The spectroscopy analytical technique known as fingerprinting is quick, easy, accurate, and non-destructive. It also doesn't require sample pre-treatment before analysis. The wide variety of medicinal plants makes them a gold mine for discovering novel compounds that can be utilized as drugs or as building blocks for developing new medicines with unique mechanisms (Oladunmoye et al. 2018). When taken singly or combined, many phytochemicals and secondary metabolites in plant extracts have anti-disease activities. Fourier transform infrared spectrometry, a physico-chemical analytical technique, is better used to identify the functional groups of the bioactive components in a plant or other related materials based on the peak value in the infrared radiation range (Oladunmoye et al. 2018). The "fingerprint" of the sample might be considered a biochemical or metabolic signature created by the FTIR method, which analyses the vibrations of bonds in chemical

functional groups (Alex et al. 2018). Therefore, by analyzing infrared spectrum absorption, the bond in the chemical can be identified (Bouyanfif et al. 2018). The spectra were captured using air-cooled deuterated triglycine sulphate and a Perkin Elmer Spectrum 400 Infra-Red (IR) spectroscope. Due to the numerical data generated by the noise, the instrumental noise region of the spectrum (below 400 cm^{-1}) was eliminated; furthermore, 11 spectra were obtained from *C. citriodora* essential oil preparations. The peaks of the extracts were sharp at 900 cm^{-1} , which represents C-H stretching (phenyl), 1400 correlates to C-O stretching vibration absorptions (mono-, oligo-, and carbohydrates), 2900 corresponds to the methoxy compounds of CH_3 and CH_2 from lipids, which have unique C-H stretching vibrations. Next, the spectral region of 3400 cm^{-1} represents the fingerprints of OH groups (from water, alcohols, phenols, carbohydrates, and peroxides) (Figure 4). Then, the vibration assignments for both stretching and bending were compared to information from the literature (Agatonovic-Kustrin et al. 2020). The information provided by the FTIR fingerprint helps enhance the community's economic and consumer health by indicating the prospect of finding different biological potentials in this plant to develop innovative functional herbals/drugs.

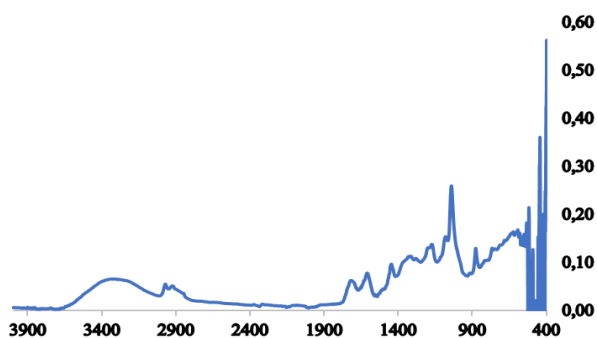


Figure 4. Overlay spectra of *Corymbia citriodora* leave

In conclusion, many ancient societies worldwide, particularly those of Africa, rely heavily on plant life for sustenance; traditional medicine places great value on using plants. Humans have used medicinal and aromatic plants for thousands of years to treat various ailments. Therefore, to the best of our knowledge, the chemical makeup of *C. citriodora* in northern Nigeria has not been documented in the literature. The plant has long been used to cure and manage diseases, including cancer, malaria, typhoid fever, and many others. However, little is known about the makeup of the oil collected from the plant, which is why the current study was necessary. This study describes the chemical composition of the essential oil extracted from northern Nigerian *C. citriodora* leaves. According to the data, monoterpene compounds (0.8137%), sesquiterpenoids (0.6568) and other compounds (95.7207% of the total) make up most of the total. Furthermore, 1-Octadecene (7.83%) was the most prevalent substance, followed by Oleic Acid, and 9-Octadecenoic acid, (E)-, (6.16%), Octadecanal, Disparlure, and 1-Octadecene (all of which were at or below 4%), and all other compounds. The *C. citriodora* essential oil yielded 11 spectra. The extracts had sharp peaks at 900 cm^{-1} (phenyl), 1400 (mono-, oligo-, and carbohydrates), and 2900 (lipid methoxy compounds of CH_3 and CH_2 , which have distinctive C-H stretching vibrations). OH, groups from water, alcohols, phenols, polysaccharides, and peroxides are fingerprinted at 3400 cm^{-1} . The research on bioactive substances has laid the groundwork for synthesizing new drugs and developing novel pharmaceutical formulations. Future research on the mentioned plant will be built on the foundation created by this study.

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Habitat characteristics, population structure, distribution, and regeneration status of *Taxus wallichiana* in South Vietnam

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Abstract. Hop NV, Xiong CC, Quy NV, Luong NT. 2023. Habitat characteristics, population structure, distribution, and regeneration status of *Taxus wallichiana* in South Vietnam. *Nusantara Bioscience* 15: 179-188. *Taxus wallichiana* Zucc. is an endangered plant species with high economic value, making it threatened with extinction. Biological information about this species is therefore essential for its conservation. This study is aimed to assess the habitat characteristics, population structure, distribution, and regeneration status of *T. wallichiana* in South Vietnam. Vegetation surveys were conducted from 2016 to 2021 in three regions in Lam Dong Province. The study revealed that it was distributed in regions with monsoon climate with a total extent of 379.57 ha, with habitats located on the hillside, narrow shelves, dissected terrain, high slope, on feralite soil with light brown to yellow, red, the soil layer thickness over 0.3 m and in clusters or streaks. The study recorded 634 trees with a diameter at breast height (DBH) ≥ 25 cm and 137 trees with 6 cm $\leq$ DBH < 25 cm. The trees were found primarily at 1401-1500 m above sea level in a mixed broadleaved-coniferous forest. The growth indicators of DBH and overall height (Hvn) developed strongly, but the stand volume was low owing to low tree density. The exponential function was the most suitable for simulating the relationship between Hvn and DBH. Seven thousand six hundred and seventy-three regenerating young individuals were recorded with low density. The density of the regenerating trees decreased as the tree height increased. The finding of this study suggests that *T. wallichiana* faces the danger of extinction, so solutions are required to conserve and develop this threatened species.

Keywords: Conservation, ecological, *Taxus wallichiana*, threatened plant, Vietnam

INTRODUCTION

The conservation of any species requires a deep understanding of its biological characteristics and life history, as well as the biotic and abiotic conditions of suitable habitats where the species can survive and thrive (Yousaf et al. 2022). This is especially prominent for rare plant species with conservation significance and importance to national, regional, and global biodiversity. The knowledge of ecology, growth, development, distribution, and regenerating status is necessary for conserving species from extinction. Conifers, in general, are a plant group with various benefits and values, yet they have restricted geographical distribution and habitat requirements (Yousaf et al. 2022). Therefore, conifers conservation and sustainable management require scientific understanding backed up with reliable data and information.

Taxus wallichiana Zucc. (Himalayan Yew) is a conifer species native to the Central Himalayas, China (Northwest Yunnan), Southeast Vietnam, Sumatra, Sulawesi, and the Philippines (Plants of the World Online 2023). This species is listed as endangered (EN) by the International Union for Conservation of Nature (The IUCN Red List of Threatened Species 2023). It has high conservation importance and significant economic values, including for timber and medicine purposes (Gajurel et al. 2014b; Bhardwaj 2023).

Previous studies have demonstrated that the leaves and bark of *T. wallichiana* produce taxol, which is the primary raw material of drugs to treat ovarian cancer, breast cancer, lung cancer, and melanoma (Paul et al. 2013; Iqbal et al. 2020; Bhardwaj 2023). The plant is essential in traditional medicine to treat common infections, colds, coughs, fever, and pain in several indigenous communities (Juyal et al. 2014; Sinha 2020; Bhardwaj 2023). This plant also provides livelihoods for local communities, such as timber and leaves for sale, to increase the income of local communities (Gajurel et al. 2014a). Such confirmed values have attracted and increased the pressures on this plant by increasing the risk of extinction.

Vietnam is a tropical country located in Southeast Asia. Vietnam's broad range of topography and climate has created many ecosystems, such as mangroves, dipterocarp forests, and semi-deciduous and evergreen forests (Luong et al. 2023). Furthermore, Vietnam is one of the centers of flora diversity worldwide, with about 12000 species (Ho, 1999). According to the Vietnam Red Data Book, 443 plant species were endangered, precious, and rare (Ban et al. 2007). Many threatened plant species face extinction due to natural and human factors, including *T. wallichiana*.

In Vietnam, two species of *Taxus* L. genus have been recorded, namely *Taxus chinensis* (Pilg.) Rehd. and *T. wallichiana*. The common name for *T. wallichiana* is

Thông Đỏ Lá Dài (Thông Đỏ Nam). This plant has a narrow distribution, with around 250 trees recorded in South Vietnam (Ban et al. 2007), and some trees have been discovered in North Vietnam (Trung 2014). This species is listed as vulnerable in the Vietnam Red Data Book (Ban et al. 2007) and belongs to group IIA of Decree 84/2021/ND-CP of the Government (Government of Vietnam 2021). The species is seriously threatened due to its limited distribution, while the number of individual trees is reduced due to illegal logging or harvesting of bark and leaves to be used for cancer drugs.

In South Vietnam, this plant species is still preserved with several mature individuals and some regenerating young trees. Moreover, some previous studies on *T. wallichiana* have been done by Hung and Nghia (2011) and Brum (2019), but they are incomplete and not systematic. Therefore, this study aimed to (i) determine habitat characteristics of *T. wallichiana* in the forest, (ii) determine density and distribution based on elevation, forest status, growth, and quality of mature trees, and (iii) assess the regeneration status of the existing population. This study provides a database of critical environmental factors, where *T. wallichiana* the distribution, growth, and reproduction. This can be utilized for developing conservation strategies for this species in South Vietnam.

MATERIALS AND METHODS

Study area and period

The study was conducted in Don Duong District (sub-district 324A, 324B, 324C, and 325A), Lac Duong District

(sub-district 125, 128, 130), and Duc Trong District (sub-zone 168, 277A, 278A), Lam Dong Province, South Vietnam during multiple field surveys from 2016 to 2021. The study area is geographically located at 114°7'48" - 120°2'50" North latitude and 108°25'30" - 108°39'53" East longitude (Figure 1). The populations of *T. wallichiana* were distributed on the Lam Vien Plateau. The studied area had three different vegetation types: Coniferous - broadleaved mixed forest, *Pinus kesiya* Royle ex Gordon natural forests, and shrubs. The site has two seasons, i.e., the rainy season from April to October and the dry season from November to April.

Data collection procedure

Interviews were performed before the field survey by distributing 35 questionnaires to experienced forest people, forestry staff, households, and individuals involved in forest protection. Based on the initial interview information, a field survey was conducted using the transect method. Nineteen transect lines were established for vegetation sampling with a distance of 500 m between the transects. At each transect line, two sampling plots were created and systematically arranged, totaling 38 sample plots, with the size of each sample plot being 500 m². At each transect line and sample plot, we collected information about coordinates, elevation, area and distribution, name of tree species, diameter at breast height (DBH), and overall height (Hvn) of all trees with (DBH) ≥ 6 cm and young trees. The young trees include DBH < 6 cm and Hvn < 6 m.

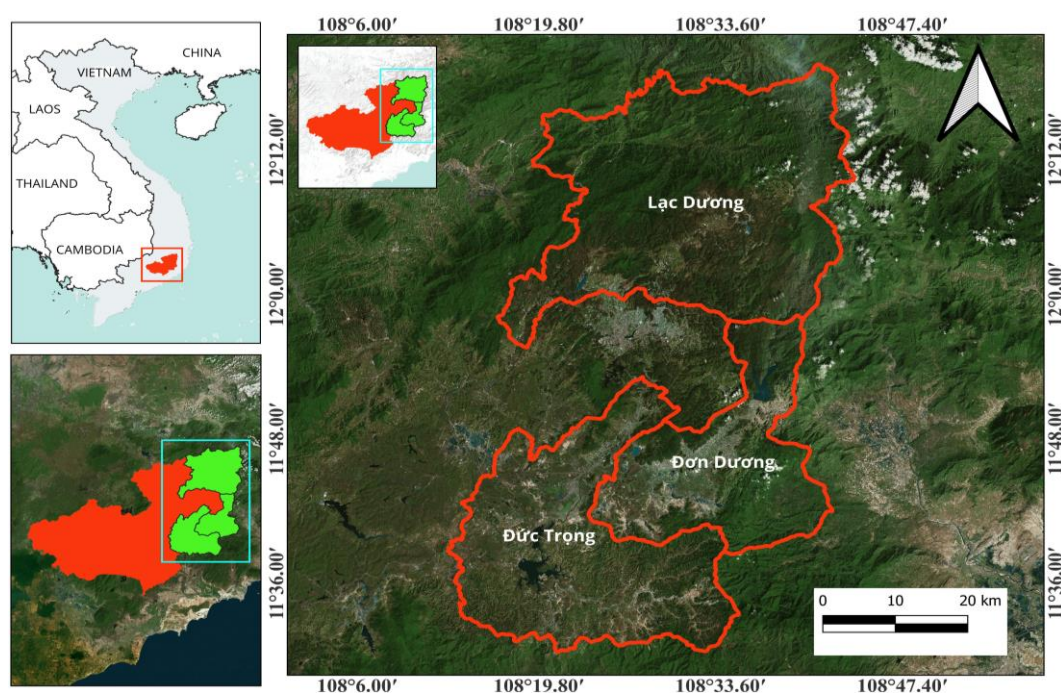


Figure 1. Map of the study area in Don Duong, Lac Duong and Duc Trong Districts, Lam Dong Province, South Vietnam

Data analysis

The species name was identified based on the morphological characteristics using An Illustrated Flora of Vietnam, volumes 1-3 (Ho 1999). The species' scientific name was corrected according to Plants of the World Online (2022). Climatic factors, i.e., temperature, humidity, and annual average rainfall, were collected from meteorological stations in the study areas. While slope was measured by Compass DC45-5C, rock mix ratio was determined by the estimator, and soil thickness was determined by soil profile. Trung (1999) stated that in the South of Vietnam, from above 1200 m, the temperature decreased from 0.5°C to 1°C when the altitude increased to 100 m. Field investigations showed that *T. wallichiana* was distributed in the 1200-1800m above sea level (asl) range. Therefore, to see the variation in altitude distribution, we divided the study area into six altitudinal ranges, namely: 1201-1300 m, 1301-1400 m, 1401-1500 m, 1501-1600 m, 1601-1700 m, 1701-1800 m.

Vegetation composition was measured using the Important Value Index (IVI) and calculated using the method by Daniel Marmillod as follows (Khanh 1996).

$$IVI (\%) = \frac{Ni\% + Gi\%}{2}$$

Where:

IVI is the Important Value of species i (%);

Ni is the percentage % of species (i) in the plant community;

Gi is the percentage % of the total basal area of species (i) in the plant community;

Daniel Marmillod states that tree species with $IVI \geq 5\%$ is ecologically significant species. According to Trung (1978), a group of tree species with $IVI > 50\%$ of the total number of individuals indicates that such a group is the dominant species group within a vegetation community. We, therefore, calculated the sum of the IVI of species with a value greater than 5%, ranked from high to low, and stopped when the total IVI reached 50%.

We also calculated the density of tree species as follows:

$$\text{Density} \left(\frac{\text{tree}}{\text{ha}} \right) = \frac{\text{Total number of trees of species in each sample plot (tree)} \times 10000}{\text{Area of sample plot (m}^2\text{)}}$$

The distribution of *T. wallichiana* was mapped by recording the coordinates of each tree encountered during the field survey using GPS64s and inputting them into spatial software (i.e., MapInfo ver. 15.0 and QGIS ver. 3.28.3). Statistical analysis was conducted using one-way

ANOVA (SPSS ver. 14.0) to compare the habitat characteristics, area, density, growth, and regeneration criteria.

RESULTS AND DISCUSSIONS

Abiotic characteristics of the habitat of *T. wallichiana*

Taxus wallichiana was observed in Don Duong, Duc Trong, and Lac Duong Districts, Lam Dong Province, Vietnam (Figure 2), primarily along hillsides and narrow terraces. The terrain was dissected by streams, northeast to southwest, with slopes ranging from 10°-30°. The species was mainly distributed on grey-brown to red-yellow feralite soil developed from granite, basalt, or acid. The soil's average depth is more than 0.3 m, with many exposed rocks, and the average proportion was more than 20% (Table 1).

In general, *T. wallichiana* was distributed in well-growing and well-developed forest vegetation communities. However, there were indications that humans moved some regenerated trees to make ornamental plants or encroached on forest land to grow coffee and fruit trees, which affects the distribution region and the number of regenerated trees in the areas.

Biotic characteristics of the habitat of *T. wallichiana*

Species composition

The species composition of the vegetation communities where *T. wallichiana* occurred was generally evergreen broad-leaved species and some conifer species. Especially in Lac Duong, *P. kesiya* predominated the vegetation. While in other areas, there were also conifers, although the number of individuals was limited and scattered, such as *Nageia wallichiana* (Presl) Kuntze, *Cephalotaxus mannii* Hook.f., *Podocarpus imbricatus* Blume, *Podocarpus neriifolius* D.Don, *Dacrydium elatum* (Roxb.) Wall. ex Loudon (Table 2).

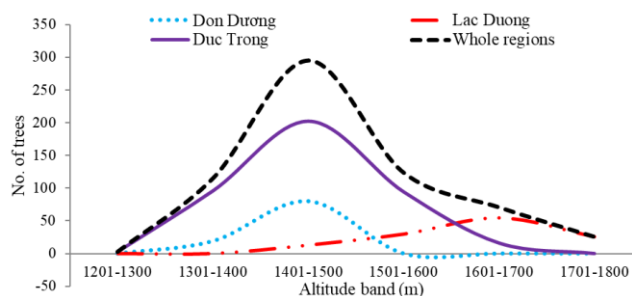
In Don Duong, the dominant species was *Syzygium wightianum* Wall. ex Wight & Arn., followed by *Rhodoleia championii* Hook. and *Schima superba* Gardner & Champ. In Lac Duong, *P. kesiya* was the dominant species, followed by *S. superba* and *Castanopsis echinocarpa* Miq. In Duc Trong, *Quercus braianensis* A.Camus predominated, followed by *Craibidendron vietnamense* Judd, *Nyssa javanica* (Blume) Wangerin, and *Lithocarpus silvicularum* (Hance) Chun (Table 2). The number of individuals of *T. wallichiana* in the sample plots in the study areas was not high. However, these individuals had a rather large DBH and height, so they appeared as an ecologically significant species in the vegetation community.

Table 1. Abiotic characteristics of the habitat of *Taxus wallichiana* in South Vietnam

Regions	Duc Trong	Don Duong	Lac Duong	Entire region
Soil type	Grey-brown feralite	Pale yellow feralite	Red yellow feralite	
The thickness of the soil layer (m)	> 0.8	> 0.8	0.3-0.4	> 0.3
Slope (°)	10-30	15-20	15-25	10-30
Mixed stone rate (%)	20	20	< 20	< 20
Supreme temperature (°C)	26	27	27	27
Lowest temperature (°C)	5	5	5	5
Average annual temperature (°C)	17.5	20	18	18.5
Average annual humidity (%)	85	80	83.5	83
Average annual rainfall (mm)	1600	1820	1800	1740

Table 2. Species with the highest Important Value Index (IV%) in the habitat of *Taxus wallichiana* in South Vietnam

Don Duong		Lac Duong		Duc Trong	
Species	IV%	Species	IV%	Species	IV%
<i>Syzygium wightianum</i>	14.57	<i>Pinus kesiya</i>	17.63	<i>Quercus braianensis</i>	13.16
<i>Rhodoleia championii</i>	9.86	<i>Schima superba</i>	11.38	<i>Craibidendron vietnamense</i>	12.52
<i>Schima superba</i>	9.76	<i>Castanopsis echinocarpa</i>	8.15	<i>Nyssa javanica</i>	8.67
<i>Taxus wallichiana</i>	7.16	<i>Lithocarpus truncatus</i>	7.24	<i>Lithocarpus silvicularum</i>	7.25
52 other species	58.65	<i>Taxus wallichiana</i>	5.82	<i>Taxus wallichiana</i>	6.19
		71 other species	49.78	60 other species	52.21

**Figure 3.** Distribution of *Taxus wallichiana* based on altitude**Table 3.** Distribution of *Taxus wallichiana* based on area extent (ha)

Regions	Tree with DBH $\geq$ 25 cm	Tree with 6 cm $\leq$ DBH < 25 cm
Don Duong	14.00 $\pm$ 4.08	3.00 $\pm$ 4.76
Lac Duong	32.67 $\pm$ 18.15	2.67 $\pm$ 4.62
Duc Trong	75.33 $\pm$ 83.43	2.67 $\pm$ 4.62
Remark	P-value<0.05	P-value>0.05

Distribution of *T. wallichiana* based on area extent

The total distribution area for *T. wallichiana* with DBH $\geq$ 25 cm was about 379.57 ha. However, the distribution area in each region was statistically different (P-value<0.05). It spanned from 14.00 $\pm$ 4.08 ha (Don Duong) to 75.33 $\pm$ 83.43 ha (Duc Trong) (Table 3).

For trees with 6 cm $\leq$ DBH<25 cm, the total distribution area was 28.36 ha, and the distribution area was not significantly different across the study regions (P-value>0.05). The largest extent was in Don Duong (3.00 $\pm$ 4.76 ha), while Lac Duong and Duc Trong were similar (2.67 $\pm$ 4.62 ha) (Figure 2). The two populations of *T. wallichiana* in Don Duong and Duc Trong are distributed closer together than in Lac Duong. Moreover, in these two regions, the distribution of *T. wallichiana* is more concentrated than that in Lac Duong (Figure 2). The distribution area between trees with DBH $\geq$ 25 cm and 6 cm $\leq$ DBH<25 cm had statistical differences (P-value<0.05). The results of the analysis showed that *T. wallichiana* was distributed in clusters.

Distribution of *T. wallichiana* based on altitude

The trees of *T. wallichiana* were found from 1276 m to 1750 m above sea level (Figure 3), but the number of trees

spread across altitudinal ranges differed. The pattern of distribution of trees by altitude in the Don Duong and Duc Trong areas was not significantly different.

In general, the number of trees rose at low altitudes, peaked at 1401-1500 m, then reduced at 1501-1600 m, except for the Lac Duong region, which peaked at 1601-1700 m and decreased at 1701-1800 m (Figure 3). The whole area data analysis showed that the number of individuals increased at 1201-1300 m, peaked at 1401-1500 m, and decreased at 1501-1600 m. Based on this result, the management strategy of *T. wallichiana* should prioritize conservation in the elevation range of 1401-1500 m (Figure 3), besides additional planting measures and promoting natural regeneration.

Distribution of *T. wallichiana* based on forest vegetation type

Most *T. wallichiana* trees were found in the mixed broadleaved-coniferous forest (Figure 4). This species was distributed in the dominant ecological layer, joining the grouping with *Nageia* sp., *Pinus krempfii* Lecomte, *P. imbricatus*, *P. neriifolius*, *Dacrydium* sp., *Magnolia* sp., *Garcinia* sp., *Cinnamomum* sp., *Schima* sp., *Syzygium* sp., and *Rhododendron* sp.

The plant likes light and moisture but needs shade to germinate and develop in the early years. In general, the mixed broadleaf-coniferous forest is the most suitable place for the growth and development of *T. wallichiana*. These results might be useful for proposing silvicultural solutions; for example, forest restoration and restocking of *T. wallichiana* can be conducted under the forest canopy to conserve and enhance the population of this species.

Stand structure and characteristics

Stand structure of trees with DBH $\geq$ 25 cm

The density of *T. wallichiana* trees with DBH>25 cm was very low, and there was no significant difference across the three regions (P-value>0.05). It ranged from 2.00 $\pm$ 1.00 (trees/ha) in Lac Duong to 3.00 $\pm$ 2.16 (trees/ha) in Don Duong. On the other hand, the growth size in terms of DBH and overall height (Hvn) were both high, and there was a substantial difference among the regions (P-value<0.05). Specifically, the DBH ranged from 43.33 $\pm$ 5.69 cm in Lac Duong to 76.67 $\pm$ 11.02 cm in Duc Trong, while the Hvn ranged from 20.67 $\pm$ 1.16 m (Lac Duong) to 22.67 $\pm$ 2.52 m (Duc Trong). The tree volume per hectare was low, ranging from 3.67 $\pm$ 4.04 (m³/ha) (Lac Duong) to 11.33 $\pm$ 2.08 (m³/ha) (Duc Trong), and there was

a statistically significant difference between these regions (P -value <0.05) (Table 4). Although the DBH and Hvn values were high, (V) was low due to low tree density. Thus, at Duc Trong, *T. wallichiana* grows and develops best. Therefore, this region should be prioritized for protecting and selecting seedlings for species restoration.

Stand structure of *T. wallichiana* trees with $6\text{ cm} \leq \text{DBH} < 25\text{ cm}$

The density of *T. wallichiana* trees with $6\text{ cm} \leq \text{DBH} < 25\text{ cm}$ ranged from 1.33 ± 0.58 (trees/ha) in Lac Duong to 4.25 ± 4.27 (trees/ha) in Don Duong, and there was no difference between regions (P -value >0.05). ANOVA analysis for DBH, Hvn, and V showed a statistically considerable difference among the three regions (P -value <0.05). The DBH ranged from $10.07 \pm 0.35\text{ cm}$ (Lac Duong) to $15.00 \pm 1.26\text{ cm}$ (Duc Trong), while the highest Hvn was at Duc Trong ($10.50 \pm 2.14\text{ m}$), and the lowest was Don Duong ($8.00 \pm 1.00\text{ m}$); and the volume, ranged from $0.06 \pm 0.03\text{ (m}^3\text{/ha)}$ (Lac Duong) to $0.19 \pm 0.08\text{ (m}^3\text{/ha)}$ (Duc Trong) (Table 5).

Correlation between overall height (Hvn) and diameter at breast height DBH

We employed polynomial, power, linear, and exponential functions to select the appropriate function to predict the correlation between Hvn and DBH (Table 6). The analysis showed that the exponential function was the most suitable to predict the Hvn growth DBH parameter with $R^2 = 0.42$ and P -value <0.5 .

Quality of mature trees

Overall, the quality across the three regions varied from good to medium to bad, with 40.85%, 38.49%, and 20.66%, respectively. This pattern was similar to that in Duc Trong (30.44%, 21.29%, and 13.09%, respectively) and slightly different in Don Duong and Lac Duong (Figure 5).

Regeneration status of *T. wallichiana*

The density of regenerating trees

A total of 7673 regenerating trees were recorded in the three regions, with the largest number of individuals found in Lac Duong (2038.00 ± 1440.95 trees) and the lowest number in Duc Trong (89.67 ± 15.57 trees). However, there was no statistically significant difference in the number of regenerating trees in these three regions (P -value >0.5). The regenerating trees were distributed over an area of about 379.57 ha, and there was a significant difference among the regions (P -value <0.05), which ranged from $14.00 \pm 4.08\text{ ha}$ (Don Duong) to $84.67 \pm 99.22\text{ ha}$ (Duc Trong). The comparison among the three areas showed that the regeneration density differed (P -value <0.05). Specifically, the density of regenerating trees was low in Don Duong, with 11.50 ± 3.70 (trees/ha) and high in Duc Trong, with 18.33 ± 2.89 (trees/ha) (Table 7). The tree likes light and moisture but needs shade to germinate and develop in the early years. As such, regenerating trees have a narrow distribution, small number, and low density, which increases the risk of extinction caused by the increasing human impacts on forest resources.

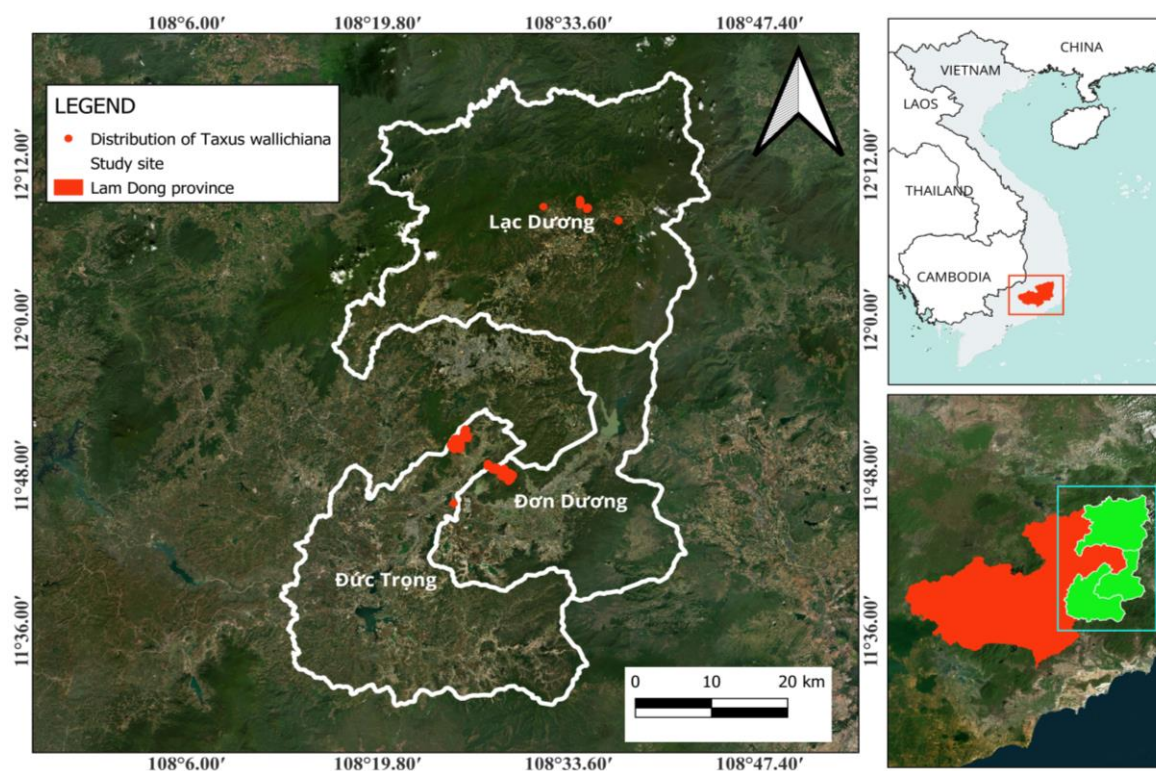


Figure 2. Distribution map of *Taxus wallichiana* in Don Duong, Lac Duong and Duc Trong Districts, Lam Dong Province, South Vietnam

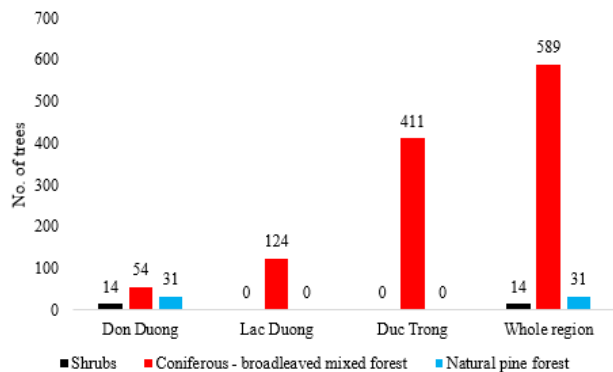


Figure 4. The abundance of *Taxus wallichiana* based on forest vegetation type

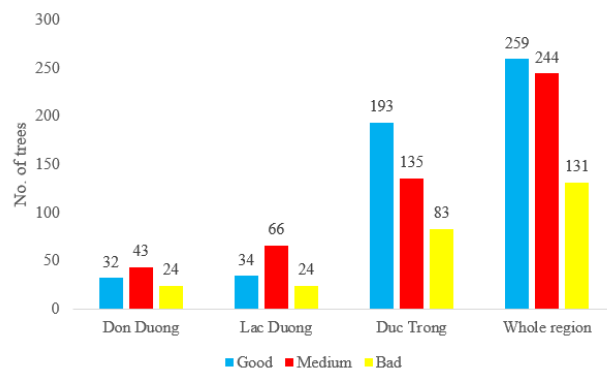


Figure 5. Quality of mature trees of *Taxus wallichiana*

Table 4. Stand structure of *Taxus wallichiana* with DBH ≥ 25 cm

Regions	D $\pm$ SD (tree/ha)	DBH $\pm$ SD (cm)	Hvn $\pm$ SD (m)	V $\pm$ SD (m ³ /ha)
Don Duong	3.00 $\pm$ 2.16	60.75 $\pm$ 14.71	18.50 $\pm$ 1.00	6.00 $\pm$ 2.71
Lac Duong	2.00 $\pm$ 1.00	43.33 $\pm$ 5.69	20.67 $\pm$ 1.16	3.67 $\pm$ 4.04
Duc Trong	2.33 $\pm$ 0.58	76.67 $\pm$ 11.02	22.67 $\pm$ 2.52	11.33 $\pm$ 2.08
Remark	P-value>0.05	P-value<0.05	P-value<0.05	P-value<0.05

Note: D: Density (tree/ha); N: Number of the tree; V: Volume (m³/ha); SD: Std. Deviation

Table 5. Stand structure of *Taxus wallichiana* with 6 cm $\leq$ DBH<25 cm

Regions	D $\pm$ SD (tree/ha)	DBH $\pm$ SD (cm)	Hvn $\pm$ SD (m)	V $\pm$ SD (m ³ /ha)
Don Duong	4.25 $\pm$ 4.27	12.58 $\pm$ 0.48	8.00 $\pm$ 1.00	0.10 $\pm$ 0.05
Lac Duong	1.33 $\pm$ 0.58	10.07 $\pm$ 0.35	9.67 $\pm$ 1.45	0.06 $\pm$ 0.03
Duc Trong	3.00 $\pm$ 3.46	15.00 $\pm$ 1.26	10.50 $\pm$ 2.14	0.19 $\pm$ 0.08
Remark	P-value>0.05	P-value<0.05	P-value<0.05	P-value<0.05

Note: D: Density (tree/ha), V: Volume (m³/ha); SD: Std. Deviation

Table 6. Correlation analysis between overall height (Hvn) and diameter at breast height (DBH) of *Taxus wallichiana*

Function	Don Duong	Lac Duong	Duc Trong	Entire regions
Polynomial	$H_{vn} = -0.04(DBH)^2 + 4.47(DBH) - 7.56$ (R ² = 0.32)	$H_{vn} = 0.35(DBH)^2 - 10.37(DBH) + 104.29$ (R ² = 0.53)	$H_{vn} = 0.16(DBH)^2 - 2.88(DBH) + 51.00$ (R ² = 0.38)	$H_{vn} = 0.20(DBH)^2 - 4.36(DBH) + 60.23$ (R ² = 0.41)
Power	$H_{vn} = 4.64(DBH)^{0.85}$ (R ² = 0.35)	$H_{vn} = 0.50(DBH)^{1.46}$ (R ² = 0.50)	$H_{vn} = 1.57(DBH)^{1.19}$ (R ² = 0.37)	$H_{vn} = 1.07(DBH)^{1.29}$ (R ² = 0.38)
Linear	$H_{vn} = 2.81(DBH) + 7.18$ (R ² = 0.31)	$H_{vn} = 3.65(DBH) - 31.07$ (R ² = 0.45)	$H_{vn} = 4.22(DBH) - 24.06$ (R ² = 0.36)	$H_{vn} = 4.34(DBH) - 31.08$ (R ² = 0.37)
Exponential	$H_{vn} = 21.83e^{0.05(DBH)}$ (R ² = 0.35)	$H_{vn} = 8.01e^{0.08(DBH)}$ (R ² = 0.54)	$H_{vn} = 16.45e^{0.06(DBH)}$ (R ² = 0.40)	$H_{vn} = 13.40e^{0.07(DBH)}$ (R ² = 0.42)
Remark	$H_{vn} = 21.83e^{0.05(DBH)}$ (R ² = 0.35)	$H_{vn} = 8.01e^{0.08(DBH)}$ (R ² = 0.54)	$H_{vn} = 16.45e^{0.06(DBH)}$ (R ² = 0.40)	$H_{vn} = 13.40e^{0.07(DBH)}$ (R ² = 0.42)

Table 7. Regeneration status of *Taxus wallichiana*

Regions	A $\pm$ SD (ha)	N $\pm$ SD (tree)	D $\pm$ SD (tree/ha)
Don Duong	14.00 $\pm$ 4.08	322.50 $\pm$ 125.80	11.50 $\pm$ 3.70
Lac Duong	32.67 $\pm$ 18.15	2038.00 $\pm$ 1440.95	15.00 $\pm$ 10.39
Duc Trong	84.67 $\pm$ 99.22	89.67 $\pm$ 15.57	18.33 $\pm$ 2.89
Remark	P-value<0.05	P-value>0.05	P-value<0.05

Note: A: Area (ha); N: Number of trees (tree); D: Density (tree/ha); SD: Std. Deviation

Table 8. The density of *Taxus wallichiana* based on height level

Height level/regions	Don Duong D±SD (tree/ha)	Lac Duong D±SD (tree/ha)	Duc Trong D±SD (tree/ha)	Conclusion
<0.5 (m)	7.25±6.55	6.33±4.93	9.00±1.00	P-value>0.05
0.6-1.0 (m)	2.25±1.71	4.33±2.08	4.67±1.53	P-value>0.05
1.1-1.5 (m)	0.50±1.00	1.67±1.53	1.67 ±1.53	P-value>0.05
1.6-2.0 (m)	1.25 ±1.26	0.33±0.58	1.67 ±2.08	P-value>0.05
2.1-3.0 (m)	-	1.67±2.08	1.00±1.00	P-value>0.05
3.1-5.0 (m)	0.25±0.50	0.67±1.16	0.33±0.58	P-value>0.05
<6 (m)	4.00±0.25	-	-	P-value>0.05
Whole height level	1.68±0.50	2.14±1.48	2.62±0.41	P-value>0.05

Note: D: Density (tree/ha); SD: Std. Deviation; P-value>0.05: no statistically significant difference

Height level of regenerating trees

The height levels of regenerating trees were not substantially different among the three regions (P-value>0.05). The tree density decreased as the height level increased, with the highest density found at a height of <0.5 m and the lowest at a height of <6 m. This indicated that the regeneration ability of the species was low, especially for the individuals with height levels >6 m (Table 8). Regenerating trees appeared in sites with little litter, ground herbs, and gaps with plenty of light. This can be a suggestion to propose solutions to improve regeneration capacity in the study regions.

Discussion

Taxus wallichiana (Himalaya Yew) is an endangered native species distributed in some subtropical regions in South Vietnam with high humidity and strongly dissected terrain. In Bidoup-Nui Ba National Park, Vietnam, under the canopy of *T. wallichiana* populations, there were many broad-leaved trees typical of humid subtropical Fagaceae, Lauraceae, and Magnoliaceae forests. In addition, plants indicating high humidity, such as *Illicium griffithii* Hook.fil. & Thomson, *Rhododendron* sp., ferns, mosses, and orchid species were found (Brum 2019). In North Vietnam, *T. wallichiana* is distributed in mixed evergreen broadleaved-coniferous forests on rocky terrain, belonging to the dominant ecological stratum (A2), where there are tree species from the genera *Pterocarpus* Jacq., *Syzygium* Gaertn., *Cinnamomum* Schaeff., *Acer* L., *Nageia* Gaertn., and the Fagaceae family (Trung 2014). Species co-occurring with *T. wallichiana* observed in Central Nepal were *Lyonia ovalifolia* (Wall.) Drude, *Daphniphyllum himalayense* (Benth.) Müll.Arg., *Rhododendron arboretum* Sm., *Symplocos ramosissima* Wall., *Quercus semecarpifolia* Sm., *Lindera pulcherrima* (Nees) Benth., *Eurya cerasifolia* (D.Don) Kobuski, *Mahonia nepalensis* DC. ex Dippel, *Viburnum erubescens* Wall., *Litsea cubeba* (Lour.) Pers. (Shreshta 2018). *T. wallichiana* was distributed at a depth of litter ranging from 3.2 to 32 cm, where the soil pH ranged from 4.84 to 6.48, indicating the soil's acidic property (Shreshta 2018).

The species grows in mixed broad-leaved and coniferous forests, on red-brown to red-yellow basalt soils, pH 4.08 to 4.56, in the midland terrain of the mountainside where it was difficult to follow the ravines, streams with exposed rocks, stand forests with 2-3 forest canopy layers (Hung and Nghia 2011). It occurs with an average monthly temperature of 21°C, rainfall of 135.3 mm, humidity of 80%, and number of sunny hours of 194 hours (Hung and Nghia 2011) and slope ranges of 10°-45°, consistent with the results of the

present study. While in Northwestern Himalaya, the observed slope ranges from 10°-60° (Pant and Samant 2008).

In this study, *T. wallichiana* was found to have a narrow distribution in the alpine subtropical forest belt, where the altitude ranged from 1200 m to 1800 m above sea level. Previous studies have shown this species has a wide ecological range from tropical to temperate regions, with altitudes from 900 m to 3800 m asl (Gajurel et al. 2014a; Dissanayake et al. 2023). In Bidoup Nui Ba National Park, Vietnam, these populations were narrowly distributed based on altitude (100 m) between 1400-1500 m asl, with a soil layer thickness of 20-40 cm (Brum 2019). Hung and Nghia (2011) found that it was distributed at elevations from 1298-1770 m, where adult populations and regenerating individuals predominate. The study conducted by Trung (2014) in North Vietnam found it at a lower altitude than that in South Vietnam.

Moreover, the amplitude of variation in altitude is also narrower than that of South Vietnam (1178-1300 m against 1200-1800 m). This result was quite similar to the altitude range that *T. wallichiana* distributes in Central Nepal, where the range of elevation variation was 300 m (2200-2500 m) (Shreshta 2018). Some reports in Asia have also shown that this plant species was distributed at higher altitudes than in South Vietnam. In India, it is distributed in the temperate and subalpine zones of the Himalayan region between 1800-3300 m asl in the hills of Meghalaya and Manipur (Nazir et al. 2020). In comparison, Nimasow et al. (2016) found it in the humid temperate regions of the Eastern Himalayas at altitude distribution range from 1600 to 2700 m asl. Yousaf et al. (2022) found in different parts of Pakistan's Abbottabad, Mansehra, Tor Ghar, and Battagram Districts, between altitude ranges from 2100 to 3400 m. In Nepal, this species is found in the wild between 2200-3800 m asl, mainly at 2600 m (Gajurel et al. 2014a). When modeling the distribution of *T. wallichiana* based on climate variables, Gajurel et al. (2014b) also revealed that the most suitable altitudinal range was between 2600-3000 m als, where most populations are recorded (Table 9). These statistics show significant differences in the elevation pattern of *T. wallichiana* between the present study and other regions of Asia. Caused by geographical latitudes, there are differences in climatic conditions between regions: Vietnam belongs to the equatorial region characterized by a tropical monsoon climate, where this species is distributed with subtropical characteristics, while other ecoregions belong to temperate climates. Furthermore, this species grows and develops mainly in temperate biomes (Plants of the World Online 2022).

Table 9. Comparison of elevation distribution of *Taxus wallichiana* in some locations across the world

Location	Elevation (m asl)	References
South Vietnam	1200-1800	<i>This study</i>
Da Lat, Vietnam	1298-1770	Hung and Nghia (2011)
Bidoup-Nui Ba, Vietnam	1400-1500	Brum (2019)
North Vietnam	1178-1300	Trung (2014)
Himalaya	900-3800	Gajurel et al. (2014a), Dissanayake et al. (2023)
Himalayan, India	1800-3300	Nazir et al. (2020)
Eastern Himalaya	1600-2700	Nimasow et al. (2016)
Himalaya	2100-3400	Yousaf et al. (2022)
Nepal	2200-3800	Gajurel et al. (2014a)
Nepal Himalaya	2600-3000	Gajurel et al. (2014b)
Central Nepal	2200-2500	Shreshta (2018)

Taxus wallichiana is rare in the wild. On the surveyed transect line, only two mature trees were recorded in Phia Oac-Phia Den National Park, North Vietnam (Trung 2014). Shreshta (2018) found that the population of *T. wallichiana* in Central Nepal with the number was less, ranging from 12-110 trees. At the same time, there were only 181 adults found in different parts of Pakistan's Abbottabad, Mansehra, Tor Ghar, and Battagram districts (Yousaf et al. 2022). Altogether, 812 individuals were recorded in the six valleys of the Nepal Himalayas (Gajurel et al. 2014b). The size class distribution of this species recorded in the plots based on the circumference at breast height suggested limited regeneration. Over 42% of *T. wallichiana* were around 90 cm in circumference. While nearly 18% were 71~90 cm, almost 11% were 51~70 cm, and almost 30% were 30.1~50 cm (Pant and Samant 2008).

Brum (2019) reported that *T. wallichiana* composes the main canopy layer (A1), and some individuals are in the adjacent layer (A2) following the vertical structure of the stand. Hung and Nghia (2011) reported that the logarithmic function was the most suitable to simulate the correlation between DBH and Hvn with the coefficient of determination $R^2 = 0.7383$ (P-value <0.5). Hung and Nghia (2011) have established a correlation equation to determine Hvn through DBH, applied to the whole regions as follows: $y = -1.63831 + 8.4648 \ln(\text{DBH}) + 2.72973x' + 0.31215x' \ln(\text{BH})$, for Ho Tien: $\text{Hvn} = 1.09142 + 8.77696 \ln(\text{DBH})$, and Nui Voi: $\text{Hvn} = -1.63831 + 8.46481 \ln(\text{DBH})$.

The plant community composition consists of significant species such as *Cinnamomum* sp., *Lithocarpus* sp., *Castanopsis* sp., *Quercus* sp., *Magnolia* sp., *Syzygium* sp., *C. mannii*, *D. elatum*, *Keteleeria evelyniana* Mast. and *T. wallichiana* forming the dominant stratum of the forest (Hung and Nghia, 2011). It often occurs with subtropical plants such as *Abies pindrow* (Royle ex D.Don) Royle, *Q. semecarpifolia*, *Quercus floribunda* Lindl. ex A.Camus, *Quercus leucotrichophora* A.Camus, *Betula utilis* D.Don, *Acer caesium* Wall. ex Brandis, *Pinus wallichiana* A.B.Jacks., *R. arboretum* and *Betula alnoides* Buch.-Ham. ex D.Don (Singh and Singh 1992). In the Arunachal Himalaya, it was found in plant communities of broadleaf and gymnosperm species such as *Castanopsis* sp., *Cinnamomum* sp., *I. griffithii*, *Michelia* sp., *Quercus* sp., *Rhododendron arizelum* Balf.fil. & Forrest, *Abies* spp., *P. wallichiana*, etc. (Paul et al. 2013).

This study found that the population density of *T. wallichiana* (2-3 trees/ha) was lower than in previous studies in South Vietnam. Hung and Nghia (2011) found that the density ranged from 2.5-16 (trees/ha). At the same time, other study reports recorded density fluctuations from 16-64 trees/ha (Brum 2019). Studies in different locations in Asia have shown that the density of *T. wallichiana* is higher in South Vietnam. A report in a temperate mixed conifer forest of western Himalaya showed even lower densities of *T. wallichiana*, with 72 trees/ha and present under the canopy of *Picea smithiana* (Wall.) Boiss. (Uniyal 2013). The *T. wallichiana* population found in the Arunachal Himalaya had a density of 52 individuals per ha-1 with a baseline area of 15.21 m²/ha (Paul et al. 2013). Yousaf et al. (2022) discovered that *T. wallichiana* was rarely distributed, with only 181 adults in different parts of Abbottabad, Mansehra, Tor Ghar and Battagram districts, Pakistan.

The natural regeneration of *T. wallichiana* was not uniform among the study sites. Hung and Nghia (2011) could not find regenerating trees in 2 of 5 surveyed sites. The absence can be caused by several factors, including seeds that have a dormant physiological period, seeding regeneration under the canopy not happening due to the thick layer of litter, the regeneration period falling in the dry season, invasion of *P. kesiya* (Hung and Nghia 2011). Brum (2019) confirmed that the regenerating trees of *T. wallichiana* have a low percentage in the species composition. The density of regenerating trees recorded in Bidoup-Nui Ba National Park ranged from 500 to 3000 trees/ha (Brum 2019). The study by Trung (2014) in North Vietnam showed that there were 2 of 15 locations where mature, regenerating trees were found. Only 1 of 15 sites recorded two regenerated individuals with a height of 3-3.5 cm, 2-3 m from the parent tree (Trung 2014). So, it regenerates poorly from seed. After the seeds fall in September - October, they remain dormant for almost eighteen months. It has also been observed that this species is sparsely distributed. Only one seedling was recorded, while no saplings were observed at the Arunachal Himalaya (Paul et al. 2013). Lander et al. (2010) reported that it grows very slowly, with poor natural regeneration mainly due to low seed yield and production rate. The effects of overuse and habitat degradation are exacerbated by the species' relative intolerance to fire, drought, and

poor regeneration. Its habitat has also been degraded by deforestation and human land use (Samant et al. 1999). Brum (2019) has determined that the East and Southeast regions have a higher regeneration rate than others. Furthermore, canopy cover negatively correlates with the density and rate of regenerated trees (Brum 2019). Our study shows that light is an essential factor in the regeneration ability of *T. wallichiana*.

Brum (2019) reported the distribution of the number of regenerating trees by height class and showed agreement with the present study. This study found that regenerating trees at a height lower than 1 m accounted for the most significant proportion (26.19%) and gradually decreased at the higher height level due to competition for nutrient space with other species and shrub vegetation (Brum 2019). The *T. wallichiana* seedlings and saplings were in relatively low abundance compared to the other associated species of the communities. Its seedlings were not found in *A. pindrow*-*Cedrus deodara* (Lamb.) G. Don mixed, *A. pindrow*, and *Q. semecarpifolia* communities. Neither seedlings nor saplings were recorded in the *Q. semecarpifolia* community. The low abundance of *T. wallichiana* compared to other associated species indicates either a competitive disadvantage compared to other species, selective exploitation, poor regeneration from seed, or all three effects. Given this trend, the species may soon be extirpated from the sanctuary.

Through field investigation, the present study shows that illegal logging because of the economic value of this species is the most important cause of population size reduction. Besides, the unlawful collection of young trees for ornamental purposes by local people is the cause of the decrease in the number of regenerated trees (Brum 2019). The loss of forest habitat, where *T. wallichiana* is distributed, is caused by the encroachment of households on forest land for agricultural cultivation, such as tea, coffee, and pepper cultivation. The sanctions to curb illegal exploitation are insufficient to protect against the decrease in the habitat size of this endangered, precious, and rare plant species. There is a growing demand for wood, firewood, and medicinal purposes; illegal cutting and smuggling of trees and conversion of forests for farming lead to habitat loss and environmental degradation (Haq et al. 2022). Yousaf et al. (2022) argued that the cause of the decline in the population size of *T. wallichiana* was habitat loss due to human activities, such as mining, which reduce the occurrence and hinder the regeneration of species. Other threats include the exploitation for medicinal purposes for taxol extraction, fuelwood collection, timber used for shingles and climate change (Yousaf et al. 2022). Local communities' over-exploitation of tree bark and leaves for medicinal purposes and trampling by herbivores are essential causes of poor regeneration, especially at lower altitudes. In addition to human factors, natural factors also significantly affect the growth and development of regenerated plants. Therefore, developing and implementing protection laws, policies, and appropriate management is necessary to conserve and develop this plant species (Yousaf et al. 2022).

In conclusion, 614 mature and 7673 regenerating young trees of *T. wallichiana* were recorded in three locations in South Vietnam. The species distribution area was characterized by a monsoon climate with an average annual temperature, humidity, and rainfall of 18.5°C, 83%, and 1740 mm, respectively. The species was distributed within about 379.57 ha, with the largest in Duc Trong and the smallest in Don Duong. The tree density was low, and no apparent difference existed between the studied regions. *T. wallichiana* was found in the mixed broadleaved-coniferous forest from 1276-1750 m, mostly at 1401-1500 m. Tree with DBH trees ≥ 25 cm had good dimensional growth in diameter and height. Still, they had low volume per hectare due to the low density, which showed significant differences between the three regions. Meanwhile, trees with $6 \text{ cm} \leq \text{DBH} < 25 \text{ cm}$ were scattered with low volume per hectare. The exponential function was the most suitable for forecasting Hvn through DBH. Most of the trees were of good and medium quality. The regeneration density was low, and there were significant regional differences. While there was no obvious difference in regeneration by height class, mainly at the low height level ($< 0.5 \text{ m}$), there was primarily a shortage of regenerative trees at the class of 3.1-6 m. The low number of mature and regenerated trees, low-density hectare, poor regeneration ability, lack of promising regenerative tree class, clustered distribution, and narrow range show that it may soon become extinct from its scope. In the future, the habitat of *T. wallichiana* populations will face drastic changes due to disturbances caused by human activities and climate change. Therefore, this species is difficult to spread and regenerate naturally. Only with solid habitat protection and supported species conservation programs can these species be sustained under climate change.

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The link of socioeconomic importance to the conservation status of the Mediterranean endemic plants in Egypt

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Abstract. Shaltout K, Bedair H, El-Khalafy MM, Keshta A, Halmy MWA. 2023. The link of socioeconomic importance to the conservation status of the Mediterranean endemic plants in Egypt. *Nusantara Bioscience* 15: 189-211. Mediterranean Basin is the second world's richest hotspot and one of the most critical spots on the planet for endemic species. Therefore, the present study aimed at screening the Mediterranean endemics and evaluating the uses of recorded taxa quantitatively through developing new indices and updating the existing ones. Indeed, Semi-structured interviews were used with 43 informants. Relative Cultural Importance (RCI), Species Conservation Importance (SCI), and Relative Medicinal Importance (RMI) indices were applied to determine the most valuable taxa. A preliminary list of 65 Mediterranean endemic species belonging to 49 genera and 22 families in Egypt was compiled from the available literature. Fifty-seven (87.7%) species have at least one aspect of the potential or actual economic value. The most represented use category was the medicinal value (39 taxa: 69.6%). *Thymbra capitata* (L.) Cav. was mentioned in the highest use categories (NU: 5). *Veronica syriaca* Roem. & Schult. attained the maximum ethnobotanical Relative Importance (RI) index value. It is crucial to shed light on the cultural value of Mediterranean endemics to guide future management planning to support the conservation and sustainable use of these critical species.

Keywords: Conservation index, Cultural Importance Index, endemism, Mediterranean Basin, quantitative ethnobotany

INTRODUCTION

Lopez-Alvarado and Farris (2022) reported that the Mediterranean region is the world's second-largest biodiversity hotspot. It includes more than 25,000 species of flowering plants worldwide (Zahran 2010). Being the meeting ground between Europe, Asia, and Africa makes the Mediterranean basin surrounded by temperate, arid, and tropical biogeographical regions in comparison to the more homogeneous areas to the north and south (Zahran 2010). Consequently, the Mediterranean basin's edaphic, climatic, and topographical intricacy makes it exceptionally rich in biodiversity (Thompson 2020). Many Mediterranean species are restricted to a single or few localities in sandy areas, isolated mountain ranges, or islands of unusual soils or rocky grounds; thus, this region is characterized by a high degree of endemism compared to other regions (Zahran 2010). Generally, coastal sectors and subsectors are more affluent in plant endemism than inland areas in Egypt (Abdelaal et al. 2020).

Endemism is an integral component in the Mediterranean region of plant diversity (Fois et al. 2022). A characteristic element of this endemism is that of all the species endemic to the Mediterranean region, 60% are narrow endemic species, i.e., they have a single well-defined area distribution restricted within a small part of the Mediterranean region (Thompson 2020). The

Mediterranean flora is thus replete with narrow endemic taxa. This region's spatial isolation is a characteristic feature, with several islands, peninsulas, and high mountains (Vargas 2020; Bedair et al. 2023a). Across the Mediterranean Basin, over 10,000 islands and islets are distributed. The largest islands in the region are Sicily and Sardinia, followed by Cyprus, Crete, Aegean Islands, Corsica, and Balearic Islands (Médail 2022).

Species extinction has increased tremendously in the last few decades. Efforts for plant species conservation have been the focus of many scientists to combat species extinction and to control invasive species. Integrating local indigenous knowledge was among various methods for the sustainable conservation of natural resources (Hanazaki et al. 2018)-especially for plants with medicinal uses and applications. Uses and applications of traditional folk medicine are widely common worldwide and among rural populations of Mediterranean regions (Emre et al. 2021). The knowledge collection on plant species and their various uses is of great importance for preserving cultural heritage and conserving plant diversity (Janačković et al. 2022). The culture of using wild plants for medicinal purposes in the Mediterranean area is well established, especially among rural communities. Preserving the traditional knowledge of using wild plants for medicinal application has been a top priority in Mediterranean and European countries (Varga et al. 2019; Bedair 2023).

Indeed, the term quantitative ethnobotany was coined for the first time by (Prance et al. 1987). It focuses on measuring the usefulness of plants and vegetation to humans. Different approaches to determining the importance of plants have been developed by various researchers (Halmy and Salem 2015; Heneidy et al. 2017; Ahmed et al. 2023; Shaltout et al. 2023). To measure the importance of plant species for humans, (Reyes-García et al. 2006) recommended using an integrated index termed cultural value. It considers the frequency of citation and versatility of the species, and its formula is further explained in this study. Therefore, this study aims to provide an updated dynamic checklist of the Mediterranean endemics distributed along the Mediterranean coast of Egypt and to apply the cultural indices to investigate folk uses and classification of useful Mediterranean endemics to highlight the significance of these endangered taxa to help in management a conservation plan of them in the near future.

MATERIALS AND METHODS

Study area

Delimitation of the Mediterranean floristic region in this study was mainly assessed according to the system of Good (1974). In Egypt, the Mediterranean territory extends about 970 km from Rafah on the Egyptian-Palestinian border to Sallum on the Egyptian-Libyan border, with an average width of 15-20 km in a north-south direction and a limited area of 16,500 km². (Facciola 1990) extended the Mediterranean territory in Egypt from Rafah District on the Egyptian-Palestinian border to Sallum on the Egyptian-Libyan border and distinguished between three subsectors

for the Mediterranean coastal land: (i) Western (the Mareotis, extending for 550 km between Sallum and Alexandria District, with annual rainfall between 220-150 mm), (ii) middle (Deltaic extending for 180 km between Alexandria and Port Said District with about 12 km wide), and (iii) east (Sinaitic, extending for 220 km between Rafah and Port Said, with annual rainfall between 150-100 mm) (Figure 1).

List justification

In this study, a taxon was considered a Mediterranean endemic if its distribution was confined to the Mediterranean floristic region determined by Good (1974). Undoubtedly, in this study, we focused on only Mediterranean endemics distributed along the Mediterranean coast of Egypt. In this study, we depended on the preliminary list of Mediterranean endemics in Egypt prepared by Bedair et al. (2020).

Additional data were collected from field visits and the herbaria of Real Jardín Botánico de Madrid, Madrid, Spain; Tanta University (TANE), Tanta, Egypt; Alexandria University (ALEX), Alexandria, Egypt; Cairo University (CAI), Cairo, Egypt; Agricultural Research Center (CAIM), Egypt; Desert Research Center (CAIH), Cairo, Egypt; Assiut University (ASTU), Assiut, Egypt; and National Research Centre (CAIRC), Cairo, Egypt.

The recorded taxa were arranged alphabetically according to the Angiosperm Phylogeny Group system (APG IV) (2016). The accepted names are followed by World Flora Online (WFO 2022), International Plant Names Index (IPNI 2022), and World Checklist of Selected Plant Families (WCSP 2022).

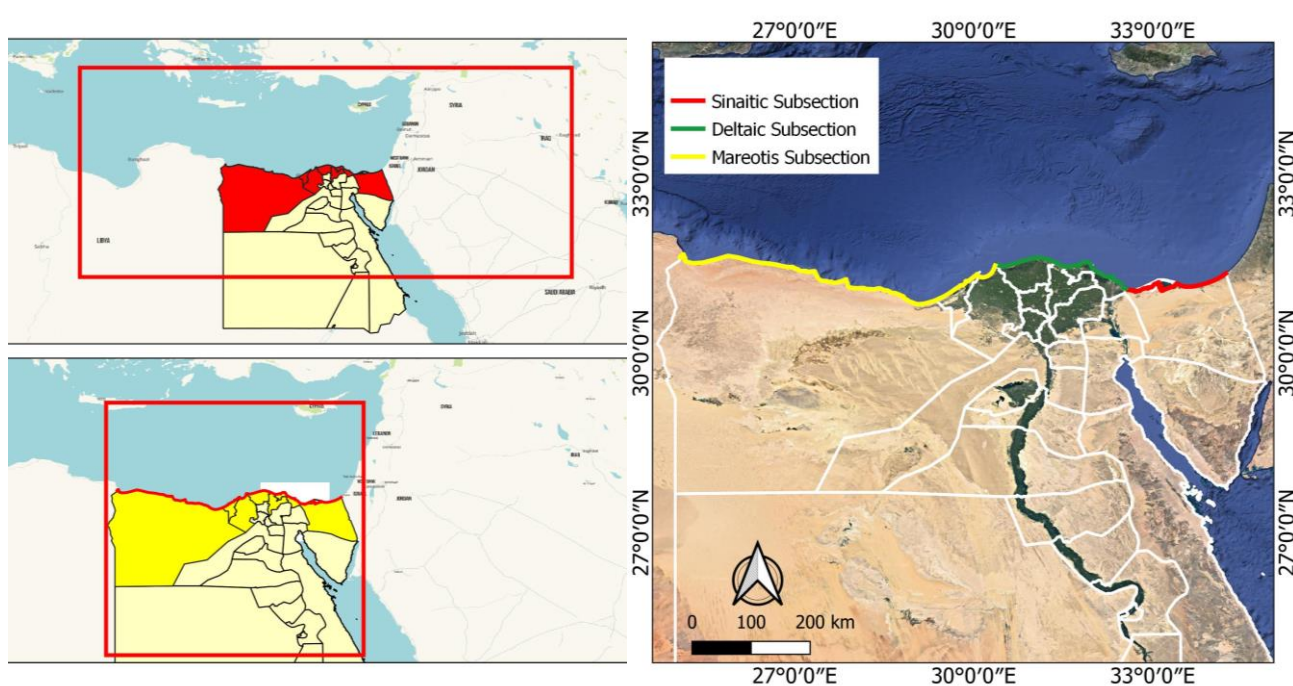


Figure 1. Map of the study area in the Mediterranean territory of Egypt from Rafah to Sallum

Ethnographic data

Data in this study were collected through four different sources:

Literature reviews: included collecting data from (Rizk 1986; Al-Eiswi and Takruri 1989; Rizk and Al-Nowaihi 1989; Belal and Springuel 1996; Chevallier 1996; Ayyad 1998; Ahmed 2009; Shaltout et al. 2010; Bedair 2020; Bidak et al. 2021; Bedair et al. 2022a; Bedair et al. 2022b; Shaltout and Bedair 2022; Shaltout et al. 2023).

Datasets: were collected from (PFAF 2022; Plants 2022; PlantUse 2022).

Open and semi-structured interviews (questionnaires): These interviews were administered to herbalists and local inhabitants, including pre-prepared data sheets of vernacular plant names from the preliminary list. Also, open discussions included some informal questions about the plant's uses, how often it is used, and the best time for using it. Live plants, voucher specimens, and pictures of plant species were provided when possible. They were also asked to fill out the questionnaire identified in Table S1.

Experts: Information was also gathered from researchers and experts familiar with the Mediterranean flora.

Quantitative assessment of the ethnobotanical importance

Indices used for the quantitative assessment of the ethnobotanical, conservation, and medicinal importance of the Mediterranean are described in Table 1. The correlation among the cultural indices was tested using Spearman's simple correlation (r) test. Statistics were computed with the R software (R Core Team 2013) by the "corrgram" package. Both two-way indicator species analysis (TWINSPAN) and non-metric multidimensional scaling (non-metric MDS) were carried out for estimations of the 14 phytochemical classes recorded in 30 Mediterranean endemics (Gauch Jr. and Whittaker 1981) to divide the classes into groups. All the analysis was done using the Community Analysis Package Statistics program (CAP 4).

Table 1. Indices used for the quantitative assessment of the ethnobotanical and medicinal importance of the Mediterranean endemics

Index	Formula	Description/Source
Use-reports (UR _s)	$UR_s = \sum_{u=u_1}^{u_{NC}} \sum_{i=i_1}^{i_N} UR_{ui}$	It can be calculated in 2 steps: (i) Number of informants who mentioned each use category for the species, and (ii) Sum of all URs of each use category (Kufer et al. 2005).
Relative Frequency of Citation (RFC _s)	$RFC_s = \frac{FC_s}{N} = \frac{\sum_{i=i_1}^{i_N} UR_i}{N}$	It is obtained by dividing the number of informants who reported the use of a species (frequency of citation FC), or the sum of the UR of all informants interviewed for the species without considering the use category, by the total number of informants (Tardío and Pardo-de-Santayana 2008).
Relative Importance (RI _s)	$RI_s = \frac{RFC_{s(max)} + RNU_{s(max)}}{2}$	RFC _{s(max)} are obtained by dividing FC _s by the maximum value in all the species [FC _s /max (FC)]. RNU _{s(max)} are obtained by dividing the number of uses of the species by the maximum value in all the species of the survey [NU _s /max (NU)] (Pardo-de-Santayana et al. 2006).
Cultural Value (CV _s)	$CV_s = \left[\frac{NU_s}{NC} \right] \times \left[\frac{FC_s}{N} \right] \times \left[\frac{\sum_{u=u_1}^{u_{NC}} \sum_{i=i_1}^{i_N} UR_{ui}}{N} \right]$	NU _s = number of different uses reported for the species. NC = Total use categories reported in the study (Reyes-García et al. 2006).
Cultural Importance (CI _s)	$CI_s = \sum_{u=u_1}^{u_{NC}} \sum_{i=i_1}^{i_N} UR_{ui} / N$	Summation of the proportion of informants that mention each species use (Tardío and Pardo-de-Santayana 2008).
Species Conservation Importance (SCI)	$SCI = \frac{\sum_{i=1}^n W_i C_i}{10}$	This approach is a function of six criteria (conservation status, abundance, life form, goods, services, and geographic distribution of each species), each scored out of 10, and the weight factor that was given 2 for the conservation status and 1 for the rest of criteria (Halmy and Salem 2015).
Relative Medicinal Importance (RI _i)	$RI_i = \left(\frac{\frac{nCS_i}{nCS_m} + \frac{nPI_i}{nPI_m}}{2} \right) \times 100$	nCS _i refers to the number of human body systems treated by species i, nCS _m is the maximum number of body systems treated by the most widely used species m; and nPI _i refers to the number of healing properties attributed to species i, while nPI _m is the maximum number of healing properties attributed to the most widely used species m; (Monteiro et al. 2011).

RESULTS AND DISCUSSION

Taxonomic diversity

There are 73 taxa recorded in this study as Mediterranean endemics in Egypt (a total of 65 species) belonging to 49 genera and 22 families. The *Allium* L. and *Fumaria* L. (4 taxa), *Bellevia* Lapeyr are the most represented genera. (4 taxa), *Muscari* Mill., *Centaurea* L., and *Limonium* Mill. (2 taxa). Any taxon does not represent gymnosperms, while 3 clades represent angiosperms. Monocot families are 4 (Amaryllidaceae, Asparagaceae, Poaceae, and Posidoniaceae), represented by 10 genera and 18 species, while only Papaveraceae represents Eudicots. Core Eudicots are the most represented by 17 families (5 Superrosids and 12 Superasterids). The highly represented families are Asteraceae (13 species), Fabaceae (10 species), Asparagaceae (8 species), and Amaryllidaceae (5 species). On the other hand, 12 families are represented by only one species (Posidoniaceae, Convolvulaceae, Euphorbiaceae, Resedaceae, Apiaceae, Caprifoliaceae, Caryophyllaceae, Rubiaceae, Santalaceae, Solanaceae and Scrophulariaceae) (Table 2).

Ethnobotanical analysis

A total number of 43 informants cited plant uses. Fifty-seven (87.7%) species of the recorded Mediterranean endemics have at least one aspect of the potential or actual economic goods. The offered goods could be arranged in descending order as follows: medicinal (39 taxa: 69.6%), human food (13 taxa: 23.2%), grazing (11 taxa: 19.6%), other uses (11 taxa: 19.6%), timber (6 taxa: 10.7%), then fuel (4 taxa: 7.1%) (Figure 2). Further, fourteen Mediterranean endemics (21.5% of the total taxa) have at

least one aspect of the environmental services. The services of the recorded species could be arranged descendingly as follows: soil fertility (8 taxa), sand accumulation (8 taxa), nitrogen fixation (6 taxa), esthetic concerns (3 taxa), wind-breaking (2 taxa), water storage and phytoremediation (one taxon each) (Figure 3). Two Mediterranean endemics have only one service (14.3% of the total environmentally important taxa), 9 taxa have two services (64.3%), and 3 taxa have three services (21.4%) (Figure 4).

The relation between the number of Mediterranean endemics and the number of use categories (NU) they offer approximates an inverse J-shape distribution. Thirty-six taxa have been mentioned in one use category (64.3%) and 16 in two use categories (28.6%), while 3 taxa have been mentioned in three use categories (5.4%). Whereas, *Thymbra capitata* (L.) Cav. has been mentioned in the highest use categories (NU: 5) (Figure 5, Table 3).

The folk species of *T. capitata* (known as *Za'tar* in Egypt) is the most culturally significant according to the CI index (3.6), followed by *Kharshouf* (*Cynara cornigera* Lindl.), which has a CI index value of 1. While 16 species have a low CI index value of 0.02 (e.g., *Centaurea aegialophila* Wagenitz, 1974; *Scilla peruviana* L.; *Silene fruticosa* L.; and *Valerianella petrovitchii* Asch. ex Rohlfs (Table 3). The 16 species with the highest CI index value are perennials. Although the various indices in Table 4 yield appreciable differences in species ranking, all four indices put *T. capitata* in the first ranking. *Allium* sp. (known as *Basal* in Egypt), *T. capitata*, and *C. cornigera* have the highest citation frequency (FC: 43), which means that all the informants mentioned the usefulness of these species.

Table 2. Major taxonomic groups of the Mediterranean endemics in Egypt

Taxonomic clade	Family (F)	Genus (G)	Species (S)	Subspecies (Sub)	Variety (V)	Sub/S	S/G	G/F
Monocots	4	10	18	0	0	0	1.8	2.5
Eudicots	1	1	3	1	0	0.3	3	1
Core Eudicots								
Superrosids	5	13	14	2	0	0.1	1.1	2.6
Superasterids	12	25	30	3	2	0.1	1.2	2.1
Total	22	49	65	6	2	0.5	7.1	8.2

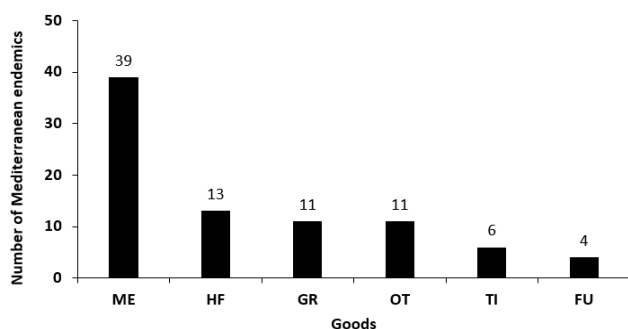


Figure 2. Descending arrangement of the use categories of the Mediterranean endemics in Egypt. Use categories are coded as ME: Medicinal, OT: Other uses, GR: Grazing, FU: Fuelwood, HF: Human food, and TI: Timber

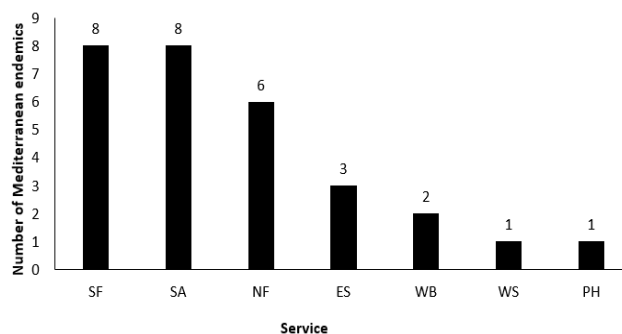


Figure 3. Some Mediterranean endemics in Egypt are concerned with their environmental services. Services are coded as SF: Soil fertility, SA: Sand accumulation, NF: Nitrogen fixation, ES: Esthetic concerns, WB: Wind-breaking, WS: Water storage, and PH: Phytoremediation

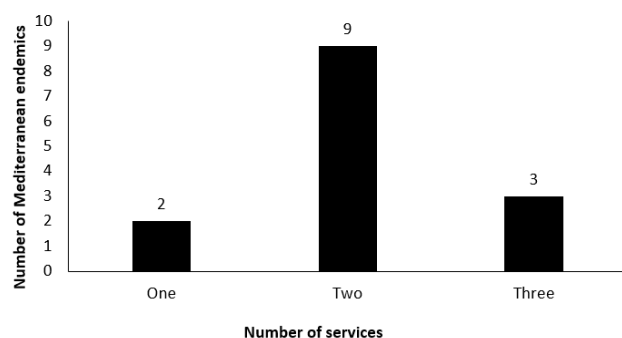


Figure 4. Number of Mediterranean endemics in Egypt concerning the number of ecosystem services

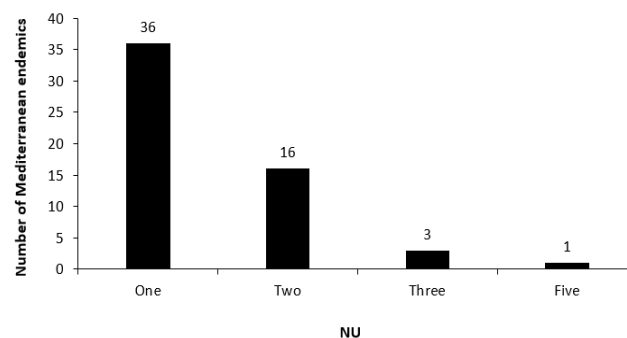


Figure 5. Number of Mediterranean endemics in Egypt concerning the number of use-categories (NU)/goods a species offers

The correlation analysis revealed a strong association between the combinations of the assessed indices (Figure 6). A very high correlation exists between FC and RFC, UR, and CI indices ($r = 1$). Indeed, CV and NU have the highest correlation with the number of use reports (0.91 and 0.72, respectively). In general, the more multilateral the Mediterranean endemic, the more it has widespread knowledge of usefulness and the more it is mentioned by the informants (Figure 7).

Regarding the species conservation importance (SCI) index, only 2 species attained a value of more than 0.75 (*Ebenus armitagei* Schweinf. & Taub. and *T. capitata*, about 60% of the recorded species are important (attained values of 0.50-0.75). In addition, 6 species have moderate importance (they are *Herniaria cyrenaica* F.Herm., *Muscari albiflorum* (Täckh. & Boulos) Hosni, *C. aegialophila*, *Cynosurus coloratus* Lehm. ex Steud., *Euphorbia parvula* Delile and *Veronica syriaca* Roem. & Schult.). Interestingly, no species has a value lower than 0.25, indicating the importance of Mediterranean endemics in Egypt (Table 5, Table S2).

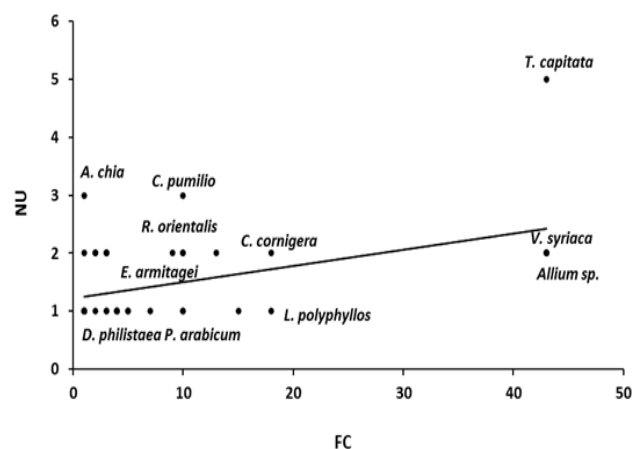


Figure 7. The number of use categories for each species (NU) concerning the frequency of citation (FC) of the useful Mediterranean endemics. Each dot represents one taxon, but only the most useful ones are labeled

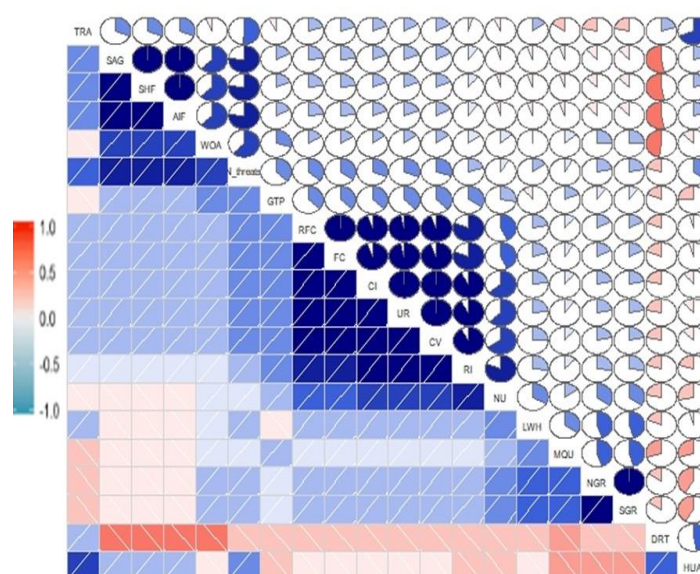


Figure 6. A correlogram representing the relation between the cultural indices and types of threats on the recorded Mediterranean endemics. Threats are abbreviated as HUA: Housing and Urban Areas; TRA: Tourism and Recreation Areas; GTP: Gathering Terrestrial Plants; WOA: Work and Other Activities; DRT: Droughts; LWH: Logging and Wood Harvesting; SAG: Shifting Agriculture; SHF: Small-holder Farming; AIF: Agro-industry Farming; NGR: Nomadic Grazing; SGR: Small-holder Grazing; Ranching or Farming; MQU: Mining & Quarrying

Table 3. Cultural Importance Index (CI) of the 56 useful Mediterranean endemics in Egypt, with the CI component of each use category

Scientific name	Goods						Total CI	Services						Total CI	
	ME	HF	GR	OT	FU	TI		SF	SA	NF	ES	WB	WS		PH
<i>Thymbra capitata</i>	1	1	0.81	0.09	0.7		3.6		0.12		0.07	0.12			0.31
<i>Cynara cornigera</i>	1	1					2								
<i>Allium barthianum</i>	0.23	1					1.23								
<i>Allium blomfieldianum</i>	0.23	1					1.23								
<i>Allium mareoticum</i>	0.23	1					1.23								
<i>Allium trifoliatum</i> subsp. <i>hirsutum</i>	0.23	1					1.23								
<i>Veronica syriaca</i>	1	0.23					1.23								
<i>Lotus polyphyllus</i>	0.42		0.42				0.84	0.58	0.23			0.07			0.88
<i>Lycium schweinfurthii</i> var. <i>aschersonii</i>	0.58	0.23		0.12			0.58								
<i>Ebenus armitagei</i>	0.3		0.12				0.42	0.07	0.07						0.14
<i>Pancratium arabicum</i>	0.42						0.42		0.23						0.23
<i>Anthemis microsperma</i>	0.2		0.16				0.36		0.07						0.07
<i>Desmazeria philistaea</i>			0.35				0.35								
<i>Centaurea pumilio</i>	0.23				0.07		0.3		0.07		0.07				0.14
<i>Reseda orientalis</i>	0.23			0.02			0.25								
<i>Posidonia oceanica</i>	0.23						0.23								
<i>Trifolium argutum</i>	0.23						0.23	0.07	0.07						0.14
<i>Trigonella berythea</i>		0.16					0.16	0.07	0.07						0.14
<i>Fumaria microstachys</i>	0.07			0.07			0.14								
<i>Hyoseris radiata</i> subsp. <i>graeca</i>	0.07		0.07				0.14		0.07				0.07		0.14
<i>Hyoseris scabra</i>	0.07		0.07				0.14		0.07		0.07				0.14
<i>Lathyrus marmoratus</i>	0.07		0.07				0.14								
<i>Lotus cytisoides</i>	0.12						0.12	0.23	0.07						0.3
<i>Thesium humile</i> var. <i>maritima</i>			0.12				0.12								
<i>Crepis libyca</i>	0.05	0.05					0.1								
<i>Vicia sinaica</i>	0.05	0.05					0.1	0.07	0.07						0.14
<i>Bellevalia mauritanica</i>	0.09						0.09								
<i>Bellevalia salah-eidii</i>	0.09						0.09								
<i>Bellevalia sessiliflora</i>	0.09						0.09								
<i>Bellevalia warburgii</i>	0.09						0.09								
<i>Leopoldia bicolor</i>	0.09						0.09								
<i>Muscari albiflorum</i>	0.09						0.09								
<i>Muscari parviflorum</i>	0.09						0.09								
<i>Verbascum letourneuxii</i>	0.09						0.09								
<i>Bupleurum nanum</i>	0.05			0.02			0.07								
<i>Fumaria judaica</i> subsp. <i>judaica</i>	0.07						0.07								
<i>Limonium sinuatum</i> subsp. <i>romanum</i>	0.05			0.02			0.07								

<i>Anthemis chia</i>	0.02	0.02	0.02	0.06				
<i>Heliotropium hirsutissimum</i>	0.05			0.05				
<i>Echinops taeckholmianus</i>	0.02		0.02	0.04				
<i>Centaurea aegialophila</i>	0.02			0.02				
<i>Coronilla repanda</i>	0.02			0.02	0.07	0.07		0.14
<i>Crepis aculeata</i>			0.02	0.02				
<i>Cynosurus coloratus</i>			0.02	0.02				
<i>Euphorbia parvula</i>	0.02			0.02				
<i>Sulla spinossima</i>			0.02	0.02	0.07	0.07		0.16
<i>Helianthemum crassifolium</i> subsp. <i>sphaerocalyx</i>			0.02	0.02				
<i>Helichrysum orientale</i>			0.02	0.02				
<i>Herniaria cyrenaica</i>	0.02			0.02				
<i>Linaria joppensis</i>			0.02	0.02				
<i>Scilla peruviana</i>			0.02	0.02				
<i>Silene fruticosa</i>	0.02			0.02				
<i>Taraxacum minimum</i>		0.02		0.02				
<i>Teucrium brevifolium</i>	0.02			0.02				
<i>Trisetaria koelerioides</i>			0.02	0.02				
<i>Valerianella petrovichii</i>			0.02	0.02				

Note: Goods are coded as ME: Medicinal; OT: Other uses; GR: Grazing; FU: Fuel; HF: Human food; and TI: timber. Services are coded as SF: Soil fertility; SA: Sand accumulation, NF: Nitrogen fixation; ES: Esthetic concerns; WB: Wind-breaking; WS: Water storage; and PH: Phytoremediation

Table 4. Evaluation of useful Mediterranean endemics in Egypt using four quantitative indices and species ranking based on each index

Scientific name	Basic values				Indices			Ranking			
	FC	UR	NU	CI	RFC	RI	CV	CI	RFC	RI	CV
<i>Thymra capitata</i>	43	155	5	3.6	1	1	2.99	1	1	1	1
<i>Cynara cornigera</i>	43	86	2	2	1	0.7	0.66	2	1	2	2
<i>Allium barthianum</i>	43	53	2	1.23	1	0.7	0.41	3	1	2	3
<i>Allium blomfieldianum</i>	43	53	2	1.23	1	0.7	0.41	3	1	2	3
<i>Allium mareoticum</i>	43	53	2	1.23	1	0.7	0.41	3	1	2	3
<i>Allium trifoliatum</i>	43	53	2	1.23	1	0.7	0.41	3	1	2	3
<i>Veronica syriaca</i>	43	53	2	1.23	1	0.7	0.41	3	1	2	3
<i>Lotus polyphyllus</i>	18	36	2	0.84	0.42	0.41	0.12	4	2	4	4
<i>L. schweinfurthii</i> var. <i>aschersonii</i>	10	25	3	0.58	0.23	0.42	0.07	5	4	3	5
<i>Ebenus armitagei</i>	13	18	2	0.42	0.42	0.41	0.06	6	2	4	6
<i>Pancreatium arabicum</i>	18	18	1	0.42	0.42	0.31	0.03	6	2	6	7
<i>Anthemis microsperma</i>	9	16	2	0.36	0.2	0.3	0.02	7	5	7	8
<i>Desmazeria philistaea</i>	15	15	1	0.35	0.35	0.28	0.02	8	3	8	8
<i>Centaurea pumilio</i>	10	13	2	0.3	0.23	0.32	0.02	9	4	5	8
<i>Reseda orientalis</i>	10	11	2	0.25	0.23	0.32	0.02	10	4	5	8
<i>Posidonia oceanica</i>	10	10	1	0.23	0.23	0.22	0.009	11	4	11	9
<i>Trifolium argutum</i>	10	10	1	0.23	0.23	0.22	0.009	11	4	11	9
<i>Trigonella berythea</i>	7	7	1	0.16	0.16	0.18	0.004	12	6	13	10
<i>Fumaria microstachys</i>	3	6	2	0.14	0.07	0.24	0.003	13	9	9	11
<i>Hyoseris radiata</i> subsp. <i>graeca</i>	3	6	2	0.14	0.07	0.24	0.003	13	9	9	11
<i>Hyoseris scabra</i>	3	6	2	0.14	0.07	0.24	0.003	13	9	9	11
<i>Lathyrus marmoratus</i>	3	6	2	0.14	0.07	0.24	0.003	13	9	9	11
<i>Lotus cytisoides</i>	5	5	1	0.12	0.12	0.16	0.002	14	7	14	12
<i>Thesium humile</i> var. <i>maritima</i>	5	5	1	0.12	0.12	0.16	0.002	14	7	14	12
<i>Vicia sinaica</i>	2	4	2	0.1	0.05	0.23	0.002	15	10	10	12
<i>Crepis libyca</i>	2	4	2	0.1	0.05	0.23	0.001	15	10	10	13
<i>Bellevia mauritanica</i>	4	4	1	0.09	0.09	0.15	0.001	16	8	15	13
<i>Bellevia salah-eidii</i>	4	4	1	0.09	0.09	0.15	0.001	16	8	15	13
<i>Bellevia sessiliflora</i>	4	4	1	0.09	0.09	0.15	0.001	16	8	15	13
<i>Bellevia warburgii</i>	4	4	1	0.09	0.09	0.15	0.001	16	8	15	13
<i>Muscari albiflorum</i>	4	4	1	0.09	0.09	0.15	0.001	16	8	15	13
<i>Muscari parviflorum</i>	4	4	1	0.09	0.09	0.15	0.001	16	8	15	13
<i>Verbascum letourneuxii</i>	4	4	1	0.09	0.09	0.15	0.001	16	8	15	13
<i>Leopoldia bicolor</i>	4	4	1	0.09	0.09	0.15	0.0006	16	8	15	14
<i>Bupleurum nanum</i>	2	3	2	0.07	0.05	0.23	0.001	17	10	10	13
<i>Fumaria judaica</i> subsp. <i>judaica</i>	3	3	1	0.07	0.07	0.09	0.001	17	9	18	13
<i>Limonium sinuatum</i> subsp. <i>romanum</i>	3	3	1	0.07	0.07	0.09	0.001	17	9	18	13
<i>Anthemis chia</i>	1	3	3	0.06	0.02	0.31	0.001	18	11	6	13
<i>Heliotropium hirsutissimum</i>	2	2	1	0.05	0.05	0.13	0.0004	19	10	16	15
<i>Echinops taekholmianus</i>	1	2	2	0.04	0.02	0.21	0.0003	20	11	12	16
<i>Centaurea aegialophila</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Coronilla repanda</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Crepis aculeata</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Cynosurus coloratus</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Euphorbia parvula</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Hedysarum spinosissimum</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>H. crassifolium</i> subsp. <i>sphaerocalyx</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Helichrysum orientale</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Herniaria cyrenaica</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Linaria joppensis</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Scilla peruviana</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Silene fruticosa</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Taraxacum minimum</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Teucrium brevifolium</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Trisetaria koelerioides</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17
<i>Valerianella petrovichii</i>	1	1	1	0.02	0.02	0.11	0.0001	21	11	17	17

Note: Basic values are coded as FC: Frequency of citation; UR: Number of use-reports; and NU: Number of uses. Quantitative indices are coded as CI: Cultural importance; RFC: Relative frequency of citation; RI: Relative importance; and CV: Cultural value

Table 5. The Species Conservation Index (SCI) values, the corresponding conservation importance category, and the number of species recorded in each category (Halmy and Salem 2015)

SCI index	Conservation Importance Category	Number of species
0.20-0.25	Low importance	0
0.25-0.50	Moderate importance	6
0.50-0.75	Important	48
0.75-1.00	High Importance	2

The medicinal plants were well established for more than 150 therapeutic uses and for treating many human diseases (Table S3). The maximum number of healing properties (NP_m) was attributed to *V. syriaca* (15), followed by *T. capitata* (12) and *Allium* spp. (11). The maximum ethnobotanical relative importance (RI) index value was attained by *T. capitata*, while the minimum value of 11.7 was attained by seven species, namely, *Fumaria judaica* subsp. *judaica*, *Heliotropium hirsutissimum* Grauer, *Hyoseris radiata* subsp. *graeca* Halácsy, *Limonium sinuatum* subsp. *romanum* Täckh. & Boulos, *Lycium schweinfurthii* var. *aschersonii* (Dammer) Feinbrun, *M. albiflorum* and *M. parviflorum*.

Applying TWINSpan to 14 phytochemical classes recorded in 30 medicinal species led to the recognition of 7 groups at the fifth classification level (Table 6, Figure 8. A). The application of non-metric MDS on the same data set indicates reasonable segregation among these groups (Figure 8. B). The groups are named after the species with the highest number of phytochemical classes: the I- *Taraxacum minimum* Heldr. ex Nyman, 1879 group. This group is constituted of the terpenes class. The highest species are *Echinops taeckholmianus* Amin, *S. fruticosa*, and *H. hirsutissimum*; the II- *E. taeckholmianus* group.

This group is constituted of propanoids, lipids, and alkaloids. The highest species are *Pancratium arabicum* Sickenb. and *Posidonia oceanica* (L.) Delile; the III- *H. radiata* subsp. *graeca* group comprised of polyphenols and flavonoids. This group contains 93% of the recorded species. The highest species are *V. syriaca*, *C. cornigera*, and *L. schweinfurthii* var. *aschersonii*; the IV- *P. arabicum* group comprises glycosides, tannins, resins, and carbohydrates. The highest species are *Bellevallia* spp., *F. judaica* subsp. *judaica*, and *Verbascum letourneuxii* Asch. & Schweinf.; V- *P. oceanica* group constituted of steroids. The highest species are *P. arabicum*, *T. minimum*, and *V. syriaca*; the VI- *S. fruticosa* group is the only species in this group and is constituted of vitamins; and the VII- *Allium* species group, which is constituted of saponins and organophosphorus compounds. The highest species are *Allium* species and *P. arabicum*.

The application of TWINSpan to 10 treatment body systems led to the recognition of 3 groups at the second level of classification: I- This group is constituted of immune, circulatory and cardiovascular, Nervous systems and general pain; II- This group is constituted of respiratory, digestive urinary and renal and exocrine systems; III- This group is constituted of the only reproductive system (Figure 9, Table 7).

Indeed, the arrow length of the CANOCO ordination expresses the relative importance of the phytochemical class. Polyphenols, alkaloids, and organophosphorus compounds are the most effective classes in the recorded Mediterranean endemics. The urinary system (Ur) correlated highly with steroids. Respiratory system (Rs) correlated with a high occurrence of propanoids. The nervous system (Ne) and immune system (Im) correlated with tannins, resins, and carbohydrates (Figure 10).

Table 6. The phytochemical classes were recorded in Mediterranean endemics in Egypt

Scientific name	Po	Fl	Pr	St	Te	Sa	Or	Li	Al	Gl	Ta	Re	Ca	Vi	Total
<i>A. barthianum</i>		1				1	1								3
<i>A. blomfieldianum</i>		1				1	1								3
<i>A. mareoticum</i>		1				1	1								3
<i>A. trifoliatum</i>		1				1	1								3
<i>A. chia</i>	1	1													2
<i>B. mauritanica</i>	1														1
<i>B. salah-eidii</i>	1														1
<i>B. sessiliflora</i>	1														1
<i>B. warburgii</i>	1														1
<i>C. aegialophila</i>					1										1
<i>C. pumilio</i>					1										1
<i>C. cornigera</i>	1	1													2
<i>E. taekholmianus</i>	1	1	1		1			1	1						6
<i>E. parvula</i>					1										1
<i>F. judaica</i> subsp. <i>judaica</i>									1						1
<i>H. hirsutissimum</i>	1	1			1			1							4
<i>H. radiata</i> subsp. <i>graeca</i>	1	1													2
<i>L. sinuatum</i> subsp. <i>romanum</i>	1	1													2
<i>L. polyphyllus</i>	1														1
<i>L. schweinfurthii</i> var. <i>aschersonii</i>	1	1													2
<i>M. albiflorum</i>	1														1
<i>M. parviflorum</i>	1														1
<i>P. arabicum</i>		1		1		1	1	1	1	1	1	1	1		10
<i>P. oceanica</i>	1		1	1				1							4
<i>S. fruticosa</i>		1		1	1	1								1	5
<i>T. minimum</i>	1	1		1	1	1				1					6
<i>T. brevifolium</i>					1										1
<i>T. capitata</i>	1														1
<i>V. letourneuxii</i>					1					1					2
<i>V. syriaca</i>	1	1	1	1											4
Total	18	15	3	5	9	7	5	4	3	3	1	1	1	1	

Note: The phytochemical classes are coded as Po: Polyphenols; Fl: Flavonoids; Pr: Propanoids; St: Steroids; Te: Terpenes; Sa: Saponins; Or: Organophosphorus compounds; Li: Lipids; Al: Alkaloids; Gl: Glycosides; Ta: Tannins; Re: Resins; Ca: Carbohydrates; Vi: Vitamins

Table 7. The body systems that Mediterranean endemics treat

Scientific name	Im	Ci	Rs	Di	Mu	Ne	Ur	Rp	Ge	Ex	Total
<i>A. barthianum</i>	1	1				1			1		4
<i>A. blomfieldianum</i>	1	1				1			1		4
<i>A. mareoticum</i>	1	1				1			1		4
<i>A. trifoliatum</i>	1	1				1			1		4
<i>A. chia</i>									1		1
<i>B. mauritanica</i>									1		1
<i>B. salah-eidii</i>									1		1
<i>B. sessiliflora</i>									1		1
<i>B. warburgii</i>									1		1
<i>C. aegialophila</i>									1		1
<i>C. pumilio</i>									1	1	2
<i>C. cornigera</i>										1	1
<i>E. taekholmianus</i>			1					1	1		3
<i>E. parvula</i>			1	1		1			1	1	5
<i>F. judaica</i> subsp. <i>judaica</i>						1					1
<i>H. hirsutissimum</i>									1		1
<i>H. radiata</i> subsp. <i>graeca</i>									1		1
<i>L. sinuatum</i> subsp. <i>romanum</i>									1		1
<i>L. polyphyllus</i>									1		1
<i>L. schweinfurthii</i> var. <i>aschersonii</i>									1		1
<i>M. albiflorum</i>									1		1
<i>M. parviflorum</i>									1		1
<i>P. arabicum</i>		1				1			1		3
<i>P. oceanica</i>		1		1					1	1	4
<i>S. fruticosa</i>	1						1		1		3
<i>T. minimum</i>							1		1		2
<i>T. brevifolium</i>									1		1
<i>T. capitata</i>		1	1	1			1		1	1	6
<i>V. letourneuxii</i>									1		1
<i>V. syriaca</i>	1		1	1			1		1	1	6
Total	6	7	4	4		7	4	1	28	6	

Note: Body systems are coded as Im: Immune; Ci: Circulatory and cardiovascular; Rs: Respiratory; Di: Digestive; Mu: Muscular and skeletal; Ne: Nervous; Ur: Urinary and renal; Rp: Reproductive; Ge: General pain; and Ex: Exocrine

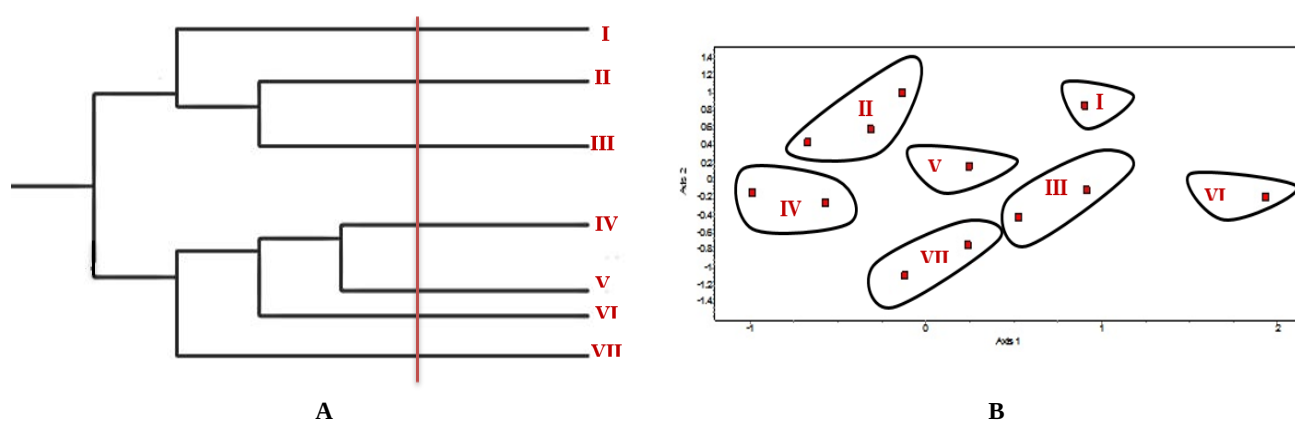


Figure 8. A. Dendrogram of the 7 groups derived after application of TWINSpan classification technique at the fifth level, B. Cluster segregation of the 7 groups using non-metric MDS

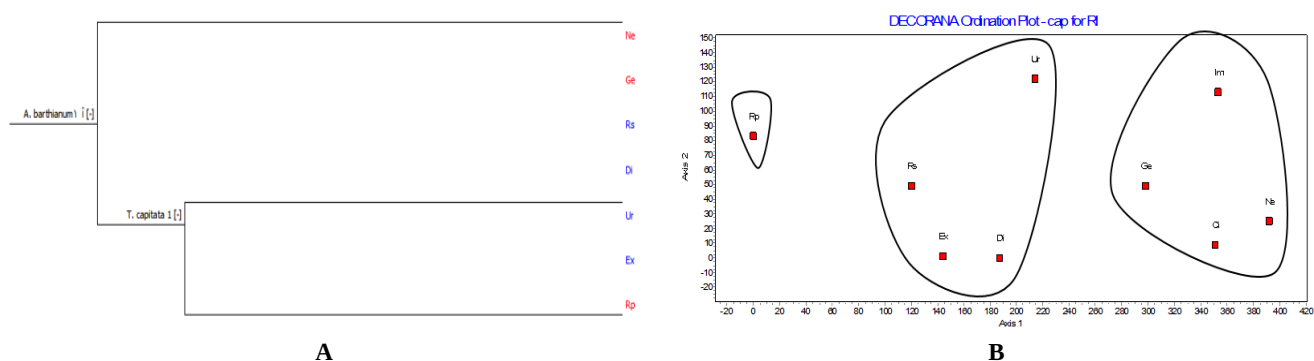


Figure 9. A. Dendrogram of the 3 groups derived after application of TWINSpan classification technique at the second level, B. Cluster segregation of the 3 groups using DECORANA. Body systems are coded as Im: Immune, Ci: Circulatory and cardiovascular, Rs: Respiratory, Di: Digestive, Mu: Muscular and skeletal, Ne: Nervous, Ur: Urinary and renal, Rp: Reproductive, Ge: General pain and Ex: Exocrine

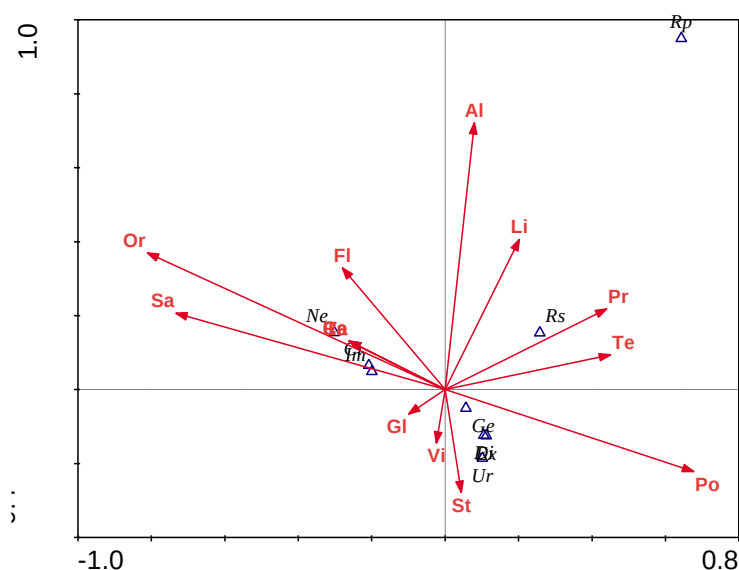


Figure 10. The CCA ordination plot for the phytochemical classes was recorded in Mediterranean endemics (represented by arrows) and their effect on treating different body systems. The phytochemical classes are coded as Po: Polyphenols, Fl: Flavonoids, Pr: Propanoids, St: Steroids, Te: Terpenes, Sa: Saponins, Or: Organophosphorus compounds, Li: Lipids, Al: Alkaloids, Gl: Glycosides, Ta: Tannins, Re: Resins, Ca: Carbohydrates and Vi: Vitamins. Body systems are coded as Im: Immune, Ci: Circulatory and cardiovascular, Rs: Respiratory, Di: Digestive, Mu: Muscular and skeletal, Ne: Nervous, Ur: Urinary and renal, Rp: Reproductive, Ge: General pain and Ex: Exocrine

Discussion

The present study has proved the ethnobotanical uses of Mediterranean endemics in Egypt. The region suffers from huge human impact and is threatened due to the extensive climatic change projections. Consequently, conservation plans are required to conserve these restricted endemic taxa and plant diversity in the Egyptian Mediterranean territory. Many Mediterranean species are restricted to a single or few localities in sandy areas, isolated mountain ranges, or islands of unusual soils or rocky grounds; thus, this region is characterized by a high degree of endemism compared to other regions (Zahran 2010; Radha and Khwarahm 2022). According to UNEP (2022), the Mediterranean is one of the most vulnerable regions in the world to the impacts of global warming. The Mediterranean region's temperature is warming 20% faster than the global average; a 2°C global warming will reduce precipitation by ~10 to 15%.

Further, water temperature is expected to rise between 1.8 and 3.5°C by 2100, with hotspots in the Eastern and northern parts of the Western Mediterranean. Sea level is expected to rise between 0.43 and 2.5 m by 2100. Consequently, these events will result in increased heat waves, coastal erosion, fires, invasive species, floods, acidification of the sea, and the risk of extinction of certain species in the region (UNEP 2022). Unfortunately, over 100 kilometers of Egypt's Mediterranean territory have already been lost due to climate change and human activities, especially the establishment of tourist summer resorts. As a result, it is not easy to find any traces of the natural vegetation reported earlier in this region (El-Hadidi and Hosni 2000; Halmy 2012, 2019). The Mediterranean is vulnerable to climate change impacts in several ways. Water scarcity, the widespread and long-known challenge in many Mediterranean countries, will likely increase in the following decades due to anticipated climate change (Cramer et al. 2019; Bedair et al. 2023b, c; Hama and Khwarahm 2023). In ethnobotanical studies, quantitative analyses serve as a technique for gathering data comparable to that from other studies and drawing valid conclusions from the data gathered. The Mediterranean region in Egypt typically has high rates of human impact, which speeds up the loss of agricultural tradition and ethnobotanical expertise. There is a medium- and long-term danger of irreversible knowledge loss since local inhabitants often learn about ethnobotany from their elderly neighbors and parents (or grandparents). Mediterranean flora offers vast benefits for human beings. About 70% of the recorded taxa have medicinal values. For instance, the marine macrophyte *P. oceanica* was initially intended to treat skin diseases but is now used to treat acne, leg discomfort, colitis, diabetes, hypertension, and respiratory infections (Rizk 2017). In addition, (Farid et al. 2018) assumed that the leaf extract has anticancer activity, while the ball extract showed antiviral activity against the H5N1 virus. Herbalists in the region utilize and sell the plant as a medical plant (e.g., in the Egyptian Center for Herbs and Cross in Alexandria and Tahreer Street in Matrouh District, Egypt) (Bidak et al. 2021). Leaves and flowering branches of *T. capitata* were proven to treat stomach diseases,

cough, and throat inflammation (Seif El-Nasr and Bidak 2005).

Moreover, *Fumaria* species have anti-inflammatory, antibacterial, antifungal, antinematode, and antinociceptive effects (Gupta et al. 2012). Common garlic and onions, two economically major *Allium* crop species, play a significant role in the daily diet as vegetables and for therapeutic purposes. Antiviral, antibacterial, antifungal, antidiabetic, anticarcinogenic, antiplatelet, antispasmodic, antiseptic, antihelminthic, antithrombotic, anti-asthmatic, anticarminative, antioxidant, anti-inflammatory, antihypertensive, hypoglycemic, hypotensive, lithontriptic, and hypocholesterolemic properties are just a few of the health benefits that *Allium* species reported providing (Najeebullah et al. 2021). Further, roots and bulbs of *Bellevia* species are stated to have anti-rheumatic and anti-inflammatory effects (Savio et al. 2019). *Centaurea pumilio* Hill, 1762, has antimicrobial and antioxidant activities (Naeim et al. 2020). The shoot system extract of *C. cornigera* has been proven to have antimicrobial, antioxidant, and antidiabetic substances. Moreover, in folk medicine in various Mediterranean nations, *C. cornigera* is used to treat several illnesses, including hepatitis, hyperlipidemia, hepatobiliary dysfunction, digestive complaints, irritable bowel syndrome, hyperlipoproteinemia, obesity, dyspeptic disorder, diabetes mellitus, liver complaints, and improving liver regeneration after partial hepatectomy (Ozdenefe et al. 2022).

Moreover, the Phenolic compounds represented in this study by the two most important groups, polyphenols and flavonoids, represent the largest category of phytochemicals and are most widely distributed in the recorded ethnopharmacological species. They have excellent antioxidant activities against radical-mediated disease processes. They also have anti-cancerous, antimicrobial cytotoxicants and vasodilating activities. Terpenoids are the second largest secondary metabolites category in the present study. They comprise nine Mediterranean endemics, such as *T. minimum* and *S. fruticosa*. Terpenoids can have medical effects such as anticarcinogenic, anti-malarial, anti-ulcer, hepatocidal, antimicrobial, or diuretic activity. Examples include perilla alcohol, the anti-malarial medication artemisinin, and the diterpenoid anticancer medicine taxol.

Further, many of them are commercially valuable because of their use as flavors and fragrances in foods and cosmetics (Koche et al. 2016). These results agree with (Koche et al. 2016), who elucidated that phenolics and terpenoids are the two major phytochemical classes occurring as natural products by 45% and 27%, respectively. Interestingly, 7 species, including the 4 recorded *Allium* species, contain Saponins as part of their defense system. They have antifungal and antiviral activities as well as promising agents for the human and animal immune systems (Traore et al. 2000). Furthermore, 5 Mediterranean endemics contain Steroids (*P. arabicum*, *P. oceanica*, *S. fruticosa*, *T. minimum*, and *V. syriaca*). This phytochemical group has antimicrobials, detoxifying agents, strengthners, anti-rheumatics, and anti-malarial, hepatocidal activities (Koche et al. 2016). Indeed,

the reason for the linkage between Tannins, Carbohydrates, and Resins in *P. arabicum* is that they can form reversible and irreversible complexes. Plant extracts with tannins are used as astringents to treat and prevent diarrhea, as well as diuretics that are effective against stomach and duodenal cancers and pharmaceuticals that are anti-inflammatory, antiseptic, antioxidant, and homeostatic pharmaceuticals (Koche et al. 2016). Similar to many plant species of the genus *Echinops* L., *E. taeckholmianus* is well known for many plant products (e.g. essential oils) that have wide variety of traditional medical applications, including pain, inflammation, respiratory diseases, aphrodisiac, and removal of renal stones (Bitew and Hymete 2019).

The RI values represented the number of indications assigned to each species, indicating the significance of each species in traditional medicine. The more therapeutic uses for a species, the more significant it is to the local population's overall therapeutic framework and the higher the RI index value. Numerous species had reached RI values above 50.0, indicating an enormous contribution of these species to the regional pharmacopeia. The RI index evaluates the adaptability of medicinal plants in regional pharmacopeia. It can thus be used to compare the function of particular species in the regional pharmacopeia. It can assist in tracking changes in the long-term utilization of significant medicinal species. The RI index can be used to track the evolution of conventional knowledge through time (Bennett and Prance 2000; Monteiro et al. 2011). The *V. syriaca* is the only species with an RI of 100 % due to its broad therapeutic uses in treating many body systems. It treats pharyngitis and laryngitis, respiratory disorders like influenza, digestive and urinary disorders, gout, and rheumatics. It is also applied externally in impaired healing of wounds and skin disorders. In addition, it has antioxidant, anti-inflammatory, analgetic, antimicrobial, cytotoxic, and chologogic activities (Witkowska-Banaszczak et al. 2016).

Fruits, flowers, and vegetative and underground parts of some Mediterranean endemics provide food sources for the local inhabitants. For example, *Trigonella berythea* Boiss. & C.I.Blanché is a food additive (PFAF 2022). Interestingly, *T. capitata* is widely utilized as a food source and was cited for many edible uses. It is used as a condiment. Spanish oregano oil is an extracted oil from the plant used for flavoring. The leaves are also used as a food additive or for aromatic tea (Facciola 1990). The *C. cornigera* is a wild edible plant in many Mediterranean countries such as Egypt, Libya, and Cyprus. It can be used as a vegetable or boiled with meat (Ozdenefe et al. 2022). Further, the *T. minimum* is a food source (Ahmed 2009). *Vicia sinaica* Boulos is a source of pulse proteins for the human diet. Moreover, *L. schweinfurthii* var. *aschersonii* is a good food source (Bedair 2020).

Some Mediterranean endemics are grazed and browsed by the domestic and wild animals in the region. There is evidence that halophytes (e.g., *Limonium echinoides* (L.) Mill.) are preferred by livestock in salt marshes, especially in the dry seasons, for their high water content. Camels, sheep, and goats are the main grazing animals that forage on the species inhabiting salt marshes (Shaltout and Ahmed

2012). The *Lotus polyphyllus* E.D.Clarke and *C. pumilio* are consumed by livestock in the Mediterranean dunes in Egypt (Ahmed et al. 2014). The *T. berythea* and *Sulla spinosissima* (L.) B.H.Choi & H.Ohashi are used as fodder plants because they are rich in high percentages of proteins and vitamins (Haouala et al. 2009). It was reported that the seeds and pods of *E. armitagei* are heavily eaten by insects, especially bruchid beetles *Callosobruchus maculatus* (Fabricius, 1775) (Shaltout et al. 2018). Mediterranean perennials such as *C. pumilio*, *C. cornigera*, *T. capitata*, *S. fruticosa*, and *L. schweinfurthii* var. *aschersonii* are used as timber and fuel. On the other hand, some species have other uses, such as *Reseda orientalis* (Müll.Arg.) Boiss., for manufacturing dyes and perfumes (PFAF 2022). The essential oils obtained from *Helichrysum orientale* (L.) Gaertn. are used in perfume blending (PFAF 2022). In comparison, *Anthemis* L. species is utilized in cosmetics (PFAF 2022). *Fumaria microstachys* (Haukskn.) Pugsley, *Linaria joppensis* Bornm., *V. petrovitchii*, and *L. sinuatum* subsp. *romanum* have esthetic concerns (PFAF 2022). Moreover, *S. peruviana* is an ornamental plant (PFAF 2022). Further, *T. capitata* and *Bupleurum nanum* Poir. have aromatic values and are widely used as good sources of essential oils. Extract of *L. schweinfurthii* var. *aschersonii* can be used as insecticide (Bedair 2020).

The Cultural Importance (CI) index revealed that the *Za'tar* (*T. capitata*) and *Kharshouf* (*C. cornigera*) are the informants in the survey who highly cited the most culturally significant species as their uses. Remarkably, all the informants have emphasized these species' medicinal and nutritional values, which can be attributed to the widespread uses for which the local inhabitants utilize them. The local inhabitants in Mersa Matrouh District collect *Za'tar* and sell it as a food recipe. In addition, it is widely used in folk medicine to treat influenza, cough, diarrhea, respiratory disorders, flu, and asthma. It was proven to have antidiabetic, antimicrobial, antioxidant, antiviral, antiparasitic, and hepatoprotective properties (Bouyahya et al. 2020). Furthermore, four species of *Basal* (*Allium* sp.) were mentioned by all the informants as being used in food and folk medicine.

Interestingly, the 16 species with the highest CI index are all perennials. This can be attributed to the availability of these species in the different seasons, revealing the relation between the prevalence of use, the number of uses for which a species can be utilized, and the availability of the species throughout the year. Consequently, species with higher longevity would have more chance to be widespread, and known to informants is much higher than annuals that die off after only one season. This observation agrees to some extent with (Bennett and Prance 2000; Pardo-de-Santayana et al. 2006; Tardío and Pardo-de-Santayana 2008; Berlin 2014), who assumed that perennials, especially woody perennials, are more likely to be beneficial since they have more number of distinct parts (leaves, roots, flowers, fruits, seeds, bark, wood) that might have a variety of uses.

The *T. capitata* came in the first ranking in all the indices. This result is logical because this species is common and predominant in the western Mediterranean

coastal strip and was mentioned by all the informants. There are noticeable differences in species ranking according to all the used indices. For example, RFC puts *P. oceanica* in the 4th position, decreasing to the 11th position based on CI and RI and the 9th rank based on cultural value. The same rankings are obtained for *Trifolium argutum* Banks & Sol. Some extensively used Mediterranean endemics, such as *Allium* sp. and *C. cornigera* (mentioned by all the surveyed 43 informants), but with few uses (folk medicine and human food), are undervalued when using the CV, RI, and CI indices, rising to the first ranking with the RFC index.

The Cultural Value (CV) index gives redundant weight to the diversity of use (Tardío and Pardo-de-Santayana 2008) as it is obtained by multiplying the Relative Frequency of Citation (RFC), Cultural Importance (CI) and Number of Uses (NU/NC). The Relative Importance (RI) index is the mean between the positive correlation of the maximum relative NU and FC ($r = 0.5$); hence, it overweighs the diversity of uses. The strong correlation between FC and RFC is logical because RFC is obtained by dividing FC by the number of informants participating in the survey. CI index is also strongly correlated with the use reports (UR: $r = 1$). Therefore, it can be stated that those indices, especially the CI index, are more objective than those depending on use categories.

Further, we can assure that most useful Mediterranean endemics in Egypt have few uses (less than three); only 4 taxa have three uses or more. The positive correlation between FC and NU assures that the more multilateral the Mediterranean endemic, the more it has widespread knowledge of usefulness and the more it is mentioned by the informants. Similar results were obtained by (Tardío and Pardo-de-Santayana 2008) in Northern Spain and by (Shaheen et al. 2017) in Pakistan.

Indeed, ecosystem services are what people obtain from the ecosystem as a whole (Reid et al. 2005). About 21.5% of the recorded endemics offer at least one aspect of environmental services. In the present study, there are 8 sand accumulators (*P. arabicum*, *Anthemis microsperma* Boiss. & Kotschy, *Hyoseris scabra* L., *H. radiata* subsp. *graeca*, *C. pumilio*, *T. capitata*, *L. polyphyllus* and *S. spinosissima*). Most of them are perennials, a finding supported by Seif El-Nasr and Bidak (2005), who elucidated that phanerophytes and chamaephytes can decrease sand drift and increase soil sedimentation effectively. On the other hand, there are 2 windbreaks (*T. capitata* and *L. polyphyllus*), which can reproduce by seeds or running roots (Shaltout and Ahmed 2012). Spectacularly, 8 taxa have a role in soil fertility. All of them belong to the family Fabaceae (*E. armitagei*, *L. polyphyllus*, *L. cytisoides*, *S. spinosissima*, *V. sinaica*, *Coronilla repanda* (Poir.) Guss., *T. argutum* and *T. berythea*). They form a symbiotic association with some species of bacteria (e.g., *Rhizobium* Frank, 1889 and *Bradyrhizobium* Jordan, 1982 species). These bacteria consume nitrogen from the air and fix it into usable form to the legume plant. This fixation occurring in nodules, a specialized roots structures on the plant. After the legume plant roots died and the nodules decompose, nitrogen that

the bacteria fixed will be released and become available for subsequent crops (Kebede 2021).

In this study, only *H. radiata* subsp. *graeca* has water storage capability (Shaltout et al. 2010). Water storage has a role in drought avoidance, decreasing competition, and increasing productivity (Hartzell et al. 2017). Moreover, this species is consumed as a vegetable side dish in many countries of the Mediterranean basin. Furthermore, *S. spinosissima* can accumulate toxic metals and thus be used in the phytoremediation of pollutants. According to the data provided in a recent study (Oubohssaine et al. 2022), *S. spinosissima* growing in heavy metal soils is linked to various active bacterial communities that likely support plant growth and tolerance under the current stressful conditions. The superior strains found are excellent candidates for use in rehabilitation programs for chosen plants in contaminated habitats.

The considerable proportion of species (about 60%) attaining SCI values between 0.50-0.75 indicates that the Egyptian flora is rich in critical Mediterranean endemics. The value of the Species Conservation Index reflects the importance of each species not only in terms of its economic and ecological uses but also in terms of its conservation status, rarity, life span, and geographical distribution (Halmy and Salem 2015). Indeed, all species recorded in this study occur in only one global floristic region (i.e., Mediterranean endemics); geographic distribution does not significantly impact the index in this study. Hence, efforts are needed to reduce the pressure on the species in the region by controlling the local inhabitants' grazing and over-collection activities. In addition, controlling the vast habitat loss due to summer resort construction on the western Mediterranean coastal strip is a must. More studies are critically needed for sound conservation management for endemics in the Mediterranean region, where the paucity of information about species composition and vegetation structures curtails conservation efforts. Research that assesses the combined impact of climate change and other human-induced changes, such as land use/land cover changes, will also be valuable.

Limitations of the applied indices

Even though quantification is a relatively old idea in ethnobotany (de Albuquerque and Hanazaki 2009), quantitative methodologies have recently received more attention (Galeano 2000). A quantitative approach is needed to address issues such as threatened species, pressure over natural resources, and impacts of the use of plants. Consequently, the popularity of ethnobotany appears to be linked to recognizing its ethical, biological, and social implications (de Albuquerque and Lucena 2005). These factors have transformed the importance and significance of local knowledge, placing ethnobotany in a privileged position in searching for "solutions to very complex social or environmental problems" (Alexiades 2003). In addition, the lack of appreciation of previous literature is one of the principal weaknesses of many recently published works. Authors often present exciting ideas and describe situations similar to those encountered

in other studies, but they do not place their work in the context of or discuss it in terms of the available literature. This may be partly explained by the lack of books/texts on ethnobotany that synthesize current ethnobotanical trends. Indeed, the limitations in building a unifying theory are another weakness of numerous ethnobotanical studies (de Albuquerque and Hanazaki 2009).

In conclusion, In this study, we highlighted the importance of the Mediterranean endemics in Egypt that are critically under threat of extinction and have been neglected for many years. Our findings revealed that the most important species in the region are *T. capitata*, *Allium* spp., *C. cornigera*, and *V. syriaca*. In addition, we performed a multivariate analysis that revealed that polyphenols, flavonoids, and terpenes are the phytochemical classes with the highest occurrence in these Mediterranean endemics. This study is an alarm for the informants, researchers, and biodiversity conservation organizations of the ethnobiological importance of these threatened taxa. Consequently, taking action to manage a plan for their conservation is crucial.

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Table S1. Example of inquiry about the goods of Mediterranean endemics in Egypt. Goods are coded as: ME: Medicinal, HF: Human food, GR: Grazing, FU: Fuel, TI: Timber, and OT: Other

Name or number: Age: Region:

Specialty: Date:

Scientific name	Common name	Goods					
		ME	HF	GR	OT	FU	TI
<i>Thymbra capitata</i>	زعتر						
<i>Cynara cornigera</i>	خرشوف						
<i>Allium barthianum</i>	بصل						
<i>Allium blomfieldianum</i>	بصل						
<i>Allium mareoticum</i>	بصل						
<i>Allium trifoliatum</i> subsp. <i>hirsutum</i>	بصل						
<i>Veronica syriaca</i>	حبق, زهرة الحواشي السورية						
<i>Lycium schweinfurthii</i> var. <i>aschersonii</i>	جسوع						
<i>Pancratium arabicum</i>	سوسن						
<i>Anthemis microsperma</i>	أريبيان						
<i>Desmazeria philistaea</i>	حلفا						
<i>Centaurea pumilio</i>	مرير						
<i>Reseda orientalis</i>	نبيل الخروف						
<i>Posidonia oceanica</i>	ثنتنارة						
<i>Trifolium argutum</i>	قضايب						
<i>Trigonella berythea</i>	حنذقوق						
<i>Lathyrus marmoratus</i>	حبرحد						
<i>Thesium humile</i> var. <i>maritima</i>	حب الكريس						
<i>Vicia sinaica</i>	دحريج						
<i>Bellevalia mauritanica</i>	بصيل						
<i>Bellevalia salah-eidii</i>	بصيل						
<i>Bellevalia sessiliflora</i>	بيلوش						
<i>Bellevalia warburgii</i>	بصيل						
<i>Leopoldia bicolor</i>	بصيل						
<i>Muscari albiflorum</i>	بصيل						
<i>Muscari parviflorum</i>	بصيل						
<i>Anthemis chia</i>	أريبيان						
<i>Echinops taeckholmianus</i>	خشروف						
<i>Centaurea aegialophila</i>	مرير						
<i>Euphorbia parvula</i>	لبين						
<i>Herniaria cyrenaica</i>	أم لبيد						

Table S2. Species Conservation Importance (SCI) of the recorded Mediterranean endemics in the Egyptian flora. IUCN Regional Conservation Status is coded as follows: CR: Critically Endangered, EN: Endangered, RE: Regionally Extinct, EX: Extinct, RA: Rare, IN: Indeterminate, and DD: Data Deficient. Life span is coded as AN: Annual and PE: Perennial. Abundance categories are coded as cc: Very common, c: Common, r: Rare, and rr: Very rare. Goods are abbreviated as GR: Grazing, ME: Medicinal, HF: Human food, FU: Fuel, TI: Timber, and OT: Other uses. Services are coded as WB: Wind-breaking, SA: Sand accumulation, SF: Soil fertility, WS: Water storage, ES: Esthetic concerns, NF: Nitrogen fixation, and PH: Phytoremediation

Binomial	Family	Common name	Regional conservation status	Life span	Abundance	Goods	Services	SCI
<i>Thymbra capitata</i> (L.) Cav.	Lamiaceae Martimov	Mediterranean Wild Thyme, Conehead Thyme	EN	PE	c	ME, HF, GR, FU, OT	SA, WB, ES	0.864
<i>Ebenus armitagei</i> Schweinf. & Taub.	Fabaceae Lindl.	-	EN	PE	rr	GR, ME	SF, NF	0.752
<i>Hyoseris scabra</i> L.	Asteraceae	Annual Hyoseris	CR	AN	rr	GR, ME	SA, ES	0.72
<i>Lotus cytisoides</i> L.	Fabaceae Lindl.	Grey Bird's-foot-trefoil	EN	PE	rr	ME	SF, NF	0.72
<i>Lotus polyphyllos</i> E.D.Clarke	Fabaceae	-	EN	PE	c	GR, ME	SA, WB, SF	0.72
<i>Lycium schweinfurthii</i> var. <i>aschersonii</i> (Dammer) Feinbrun	Solanaceae Juss.	Schweinfurth's Tea-tree	EN	PE		ME, HF, OT (insecticide)		0.72
<i>Sulla spinosissima</i> (L.) BH.Choi & H.Ohashi	Fabaceae Lindl.	Spiny Sulla; Dorniger Sussklee	EN	AN	r	GR	SA, SF, PH	0.72
<i>Vicia sinaica</i> Boulos	Fabaceae Lindl.	-	EX	AN	rr	HF, ME	SF, NF	0.72
<i>Lathyrus marmoratus</i> Boiss. & Blanche	Fabaceae Lindl.	-	EN	AN		HF, ME		0.704
<i>Allium barthianum</i> Asch. & Schweinf.	Amaryllidaceae J.St.-Hil.	Garlic	EN	PE	r	HF, ME		0.688
<i>Allium blomfieldianum</i> Asch. & Schweinf.	Amaryllidaceae J.St.-Hil.	Onions	EN	PE	rr	ME, HF		0.688
<i>Allium mareoticum</i> Bornm. & Gauba	Amaryllidaceae J.St.-Hil.	Onions	EN	PE	rr	ME, HF		0.688
<i>Allium trifoliatum</i> Cirillo	Amaryllidaceae J.St.-Hil.	Pink Garlic, Three-leaved Garlic	RE	PE	rr	HF, ME		0.688
<i>Centaurea pumilio</i> Hill, 1762	Asteraceae Bercht. & J.Presl	-	EN	PE	c	GR, ME, FU	SA, ES	0.688
<i>Coronilla repanda</i> (Poir.) Guss.	Fabaceae Lindl.	Curly Crown Vetch	CR	AN	rr	ME	SF, NF	0.688
<i>Crepis libyca</i> (Pamp.) Bab.	Asteraceae	-	CR	PE	rr	ME, HF		0.688
<i>Cynara cornigera</i> Lindl.	Asteraceae Bercht. & J.Presl	White Artichoke	CR	PE	r	ME, HF		0.688
<i>Echinops taekholmianus</i> Amin	Asteraceae Bercht. & J.Presl	-	CR	PE	r	GR, ME		0.688
<i>Limonium sinuatum</i> subsp. <i>romanum</i> Täckh. & Boulos	Plumbaginaceae Juss.	Wavyleaf sea lavender	CR	PE	rr	ME, OT (Esthetic)		0.688
<i>Pancratium arabicum</i> Sickenb.	Amaryllidaceae J.St.-Hil.	-	EN	PE	r	ME	SA	0.688
<i>Trifolium argutum</i> Banks & Sol.	Fabaceae Lindl.	Sharp-tooth Clover	CR	AN	rr	ME	SF, NF	0.688
<i>Trigonella berythea</i> Boiss. & C.I.Blanche	Fabaceae Lindl.	-	EN	AN	rr	HF	SF, NF	0.688
<i>Bellevallia mauritanica</i> Pomel	Asparagaceae	-	EN	PE	rr	ME		0.656
<i>Bellevallia salah-eidii</i> Täckh. & Boulos	Asparagaceae Juss.	-	EX	PE	rr	ME		0.656
<i>Bellevallia sessiliflora</i> (Viv.) Kunth	Asparagaceae Juss.	-	EN	PE	rr	ME		0.656
<i>Fumaria microstachys</i> (Hausskn.) Pugsley	Papaveraceae Juss.	-	CR	AN	rr	ME, OT (Esthetic)		0.656
<i>Helichrysum orientale</i> (L.) Gaertn.	Asteraceae Bercht. & J.Presl	Eastern Strawflower	CR	PE	rr	OT (perfume blending)		0.656
<i>Hyoseris radiata</i> subsp. <i>graeca</i> Halácsy	Asteraceae Bercht. & J.Presl	PE Hyoseris	EN	PE	c	GR, ME	SA, WS	0.656

<i>Muscari parviflorum</i> Desf.	Asparagaceae Juss.	Lesser Grape Hyacinth	CR	PE	rr	ME	0.656
<i>Reseda orientalis</i> (Müll.Arg.) Boiss.	Resedaceae Marti×v	-	CR	AN	rr	ME, OT (dyes and perfumes)	0.656
<i>Scilla peruviana</i> L.	Asparagaceae Juss.	Peru squill	CR	PE	rr	OT (ornamental plant)	0.656
<i>Silene fruticosa</i> L.	Caryophyllaceae	-	CR	PE	rr	ME	0.656
<i>Taraxacum minimum</i> Heldr. ex Nyman, 1879	Asteraceae Bercht. & J.Presl	-	CR	PE	rr	HF	0.656
<i>Teucrium brevifolium</i> Schreb.	Lamiaceae Marti×v	Shortleaf Germander	CR	PE	rr	ME	0.656
<i>Verbascum letourneuxii</i> Asch. & Schweinf.	Scrophulariaceae Juss.	-	EN	PE	r	ME	0.656
<i>Crepis aculeata</i> Boiss.	Asteraceae Bercht. & J.Presl	-	CR	AN	rr	GR	0.624
<i>Desmazeria philistaea</i> (Boiss.) H.Scholz	Poaceae Barnhart	-	CR	AN	rr	GR	0.624
<i>Fumaria judaica</i> subsp. <i>judaica</i>	Papaveraceae Juss.	Judean Fumitory	EN	AN	r	ME	0.624
<i>Helianthemum crassifolium</i> subsp. <i>sphaerocalyx</i> (Gaub. & Janch.) Maire	Cistaceae Juss.	-	EN	PE		OT (wildcard)	0.624
<i>Linaria joppensis</i> Bornm.	Plantaginaceae Juss.	-	CR	AN	rr	OT (Esthetic)	0.624
<i>Thesium humile</i> var. <i>maritima</i> (N.D.Simpson) F.M.Saad	Santalaceae R.Br.	Low Bastard Toadflax	EN	AN	r	GR	0.624
<i>Trisetaria koelerioides</i> (Bornm. & Hack.) Melderis	Poaceae Barnhart	-	CR	AN	rr	GR	0.624
<i>Valerianella petrovitchii</i> Asch. ex Rohlf	Caprifoliaceae Juss.	-	CR	AN	rr	OT (Esthetic)	0.624
<i>Anthemis microsperma</i> Boiss. & Kotschy	Asteraceae Bercht. & J.Presl	-	EN	AN	c	GR, ME SA	0.592
<i>Heliotropium hirsutissimum</i> Grauer	Boraginaceae Juss.	Hairy Heliotrope	CR	AN		ME	0.592
<i>Bellevalia warburgii</i> Feinbrun	Asparagaceae Juss.	-	RE	PE	rr	ME	0.576
<i>Leopoldia bicolor</i> (Boiss.) Eig & Feinbrun	Asparagaceae Juss.	Tacel Hyacinth	RE	PE	rr	ME	0.576
<i>Bupleurum nanum</i> Poir.	Apiaceae	-	EN	AN	c	ME, OT (Oil)	0.56
<i>Anthemis chia</i> L.	Asteraceae	Chios Chamomile	DD	AN	rr	ME, HF, OT (cosmetics)	0.528
<i>Posidonia oceanica</i> (L.) Delile	Posidoniaceae Vines	Neptune Grass	EN	PE	cc	ME	0.528
<i>Herniaria cyrenaica</i> F.Herm.	Caryophyllaceae Juss.	-	DD	PE	rr	ME	0.496
<i>Muscari albiflorum</i> (Täckh. & Boulos) Hosni	Asparagaceae Juss.	-	DD	PE	rr	ME	0.496
<i>Centaurea aegialophila</i> Wagenitz, 1974	Asteraceae Bercht. & J.Presl	-	DD	AN	rr	ME	0.464
<i>Cynosurus coloratus</i> Lehm. ex Steud.	Poaceae Barnhart	-	DD	AN	rr	GR	0.464
<i>Euphorbia parvula</i> Delile	Euphorbiaceae Juss.	-	DD	AN	rr	ME	0.464
<i>Veronica syriaca</i> Roem. & Schult.	Plantaginaceae Juss.	The Syrian speedwell	DD	AN	rr	ME	0.464

Table S3. Medicinal plants recorded in the study area, their ethnobotanical relative importance (RI), therapeutic uses, parts used, and supporting references

Scientific name	RI	Therapeutic uses	Part used	Major chemical constituents	Reference
<i>Veronica syriaca</i> Roem. & Schult.	100	It treats respiratory disorders like influenza, pharyngitis and laryngitis, digestive and urinary disorders, gout, and rheumatics. It is also applied externally in skin disorders and impaired healing of wounds. It has antioxidant, anti-inflammatory, analgetic, antimicrobial, cytotoxic, and cholagogues activity	Aerial parts	Flavonoid compounds, mainly derivatives of luteolin, apigenin, scutellarein, and isoscutellarein; phenolic compounds, such as derivatives of arbutin, salidroside, and verbascoside; phenylpropanoids, numerous iridoids, derivatives of aucubin and catalpol, as well as phenolic acids and steroid saponosides.	(Witkowska-Banaszczak et al. 2016)
<i>Thymbra capitata</i> (L.) Cav.	90	Heart, liver, respiratory diseases, urinary digestive system, inflammation, Antidiabetic, cough, cold, stomachache, cardiovascular system, eye diseases, infusion as analgesic and vomiting	Aerial parts	Carvacrol, Eucalyptol (1,8-Cineole), β -Caryophyllene, p-Cymene, γ -Terpinene, Borneol, α -Terpineol,	(Gupta et al. 2012)
<i>Euphorbia parvula</i> Delile	71.7	Anticancer, respirational infections, body and skin irritations, digestion complaints, inflammatory infections, body pain, microbial illness, snake or scorpion bites, pregnancy, and sensory disorders.	Whole plant	Polycyclic and macrocyclic diterpenes	(Kemboi et al. 2020)
<i>Allium barthianum</i> Asch. & Schweinf.	70	Chronic diseases, anticancer, preventive cardiovascular and heart diseases, anti-inflammation, antiobesity, antidiabetes, antioxidants, antimicrobial activity, neuroprotective and immunological effects	Leaves	Organosulfur compounds, quercetin, flavonoids, saponins, and others	(Zeng et al. 2017)
<i>Allium blomfieldianum</i> Asch. & Schweinf.	70	Chronic diseases, anticancer, preventive cardiovascular and heart diseases, anti-inflammation, antiobesity, antidiabetes, antioxidants, antimicrobial activity, neuroprotective and immunological effects	Leaves	Organosulfur compounds, quercetin, flavonoids, saponins, and others	(Zeng et al. 2017)
<i>Allium mareoticum</i> Bornm. & Gauba	70	Chronic diseases, anticancer, preventive cardiovascular and heart diseases, anti-inflammation, antiobesity, antidiabetes, antioxidants, antimicrobial activity, neuroprotective and immunological effects	Leaves	Organosulfur compounds, quercetin, flavonoids, saponins, and others	(Zeng et al. 2017)
<i>Allium trifoliatum</i> Cirillo	70	Chronic diseases, anticancer, preventive cardiovascular and heart diseases, anti-inflammation, antiobesity, antidiabetes, antioxidants, antimicrobial activity, neuroprotective and immunological effects	Leaves	Organosulfur compounds, quercetin, flavonoids, saponins, and others	(Zeng et al. 2017)

<i>Posidonia oceanica</i> (L.) Delile	66.7	Treat inflammation and irritation, but also as a remedy for acne, lower limb pain, colitis, sore throat, diabetes, hypertension, and skin problems, prevent respiratory infections	Leaves	Chicoric acid, caftaric, gentisic, chlorogenic, caffeic, ferulic, cinnamic, gallic, p-coumaric acids, quercetin, myricetin, kaempferol, and isorhamnetin. Phenolics such as phloroglucinol, pyrocatechol, pyrogallol, vanillin aldehyde, 4-hydroxybenzaldehyde, 3,4-dihydroxy benzaldehyde, benzoic acid, p-hydroxybenzoic acid, p-anisic acid, vanillic acid, syringic acid, proanthocyanidins, and calchones (as phloretin and phloridzin). Lipids such as palmitic, palmitoleic, oleic, and linoleic acids, as well as the phytosteroids campesterol, stigmasterol, and β -sitosterol	(Vasarri et al. 2021)
<i>Echinops taeckholmianus</i> Amin	45	Anti-inflammation, pain, and fever; ailments related to the respiratory tract, including cough and sore throat; uterus tumor and leucorrhoea	Whole plant	Thiophenes and terpenes. Flavonoids and other phenolic compounds, alkaloids, lipids, and phenylpropanoids	(Bitew and Hymete 2019)
<i>Taraxacum minimum</i> Heldr. ex Nyman, 1879	43.3	Treat cystitis, liver and gastric ailments, hepatic and renal detoxification, and diabetes as an anti-inflammatory and anticarcinogenic, antimicrobial, and antiviral agent	Leaves and roots	Phenolics terpenoids, triterpenoids, steroids, coumarins, phenols, saponins, flavonoids, flavones, flavonols, chalcones, phlobatannins, and cardiac glycosides	(Valenzuela et al. 2018)
<i>Panocratium arabicum</i> Sickenb.	41.7	Reduces blood pressure, treats asthma and malaria, has anticancer, cardiotonic, and hallucinogenic effects	Leaves and bulb	Sterols, tanning chlorides, sulfates, reducing sugars, flavonoids, alkaloids, resins, saponins, carbohydrates, glycosides, and traces of volatile oils	(Morsi et al. 2000)
<i>Silene fruticosa</i> L.	41.7	Antimicrobial, Antiviral, antioxidant, Antitumor, Phagocytic, Immunomodulatory, Hepatoprotection	Whole plant	Phytoecdysteroids, triterpene saponins, terpenoids, benzenoids, flavonoids, N-containing compounds, sterols, vitamins, and others	(Mamadalieva et al. 2014)
<i>Centaurea pumilio</i> Hill, 1762	23.3	Antibacterial and skin diseases	Root and aerial parts	Butanoic acid-2-methyl, 2-methyl butyl, Hexyl isovalerate, 5-Methylhexyl 2-Methylbutanoate, α -copaene, β -Caryophyllene, Isogermacrene D, Octacosane	(Mostafa et al. 2019)
<i>Centaurea aegialophila</i> Wagenitz, 1974	21.7	As a diuretic, to treat fever and diabetes, anti-inflammatory activity	Root and aerial parts		(Demir et al. 2011)
<i>Anthemis chia</i> L.	18.3	Antioxidant, antibacterial, antidiabetic activities	Flowers	Chlorogenic acid, quercetin, hyperoside, vanillic acid, protocatechuic acid, caffeic acid, apigenin 7-glucoside and luteolin 7-glucoside	(Sarikurkcü, 2020)
<i>Bellevia mauritanica</i> Pomel	18.3	Antioxidant, antibacterial, and anticholinesterase activities	Bulbs-roots sh	The ethyl acetate fraction, quinic acid, gallic acid, fumaric acid, malic acid, caffeic acid and others	(Ouelbani et al. 2020)
<i>Bellevia salah-eidii</i> Täckh. & Boulos	18.3	Antioxidant, antibacterial, and anticholinesterase activities	Bulbs-roots sh	The ethyl acetate fraction, quinic acid, gallic acid, fumaric acid, malic acid, caffeic acid and others	(Ouelbani et al. 2020)
<i>Bellevia sessiliflora</i> (Viv.) Kunth	18.3	Antioxidant, antibacterial, and anticholinesterase activities	Bulbs-roots sh	The ethyl acetate fraction, quinic acid, gallic acid, fumaric acid, malic acid, caffeic acid and others	(Ouelbani et al. 2020, Abdelsalam et al. 2023)
<i>Bellevia warburgii</i> Feinbrun	18.3	Antioxidant, antibacterial, and anticholinesterase activities	Bulbs-roots sh	The ethyl acetate fraction, quinic acid, gallic acid, fumaric acid, malic acid, caffeic acid and others	(Ouelbani et al. 2020)
<i>Cynara cornigera</i> Lindl.	18.3	Antibacterial, antioxidant, Antidiabetic activity	Shoot system	Polyphenols, flavonoids	(Ozdenefe et al. 2022)

<i>Lotus polyphyllus</i> E.D.Clarke	15	Anti-inflammatory and cytotoxic activities	Whole plant	3,4-Caffeic acid dimethyl ether and methyl ferulate	(Osman et al. 2015)
<i>Teucrium brevifolium</i> Schreb.	15	Anti-inflammatory and antitumor activities	Aerial parts	Sesquiterpenes include spathulenol, d-cadinene, caryophyllene, caryophyllene oxide, a-humulene, and torreyol	(Menichini et al. 2009)
<i>Verbascum letourneuxii</i> Asch. & Schweinf.	15	Antioxidant and cytotoxic activities	Aerial parts	Catalpol and its 6-O-(α -l-rhamnopyranopranosyl) derivative, aucubin, 6-O-syringoyl ajugol, harpagoside, eukovoside, martynoside, dehydrodiconferyl alcohol-9-O-d-glucopyranoside and its methyl derivative, syringin and coniferin	(Farid et al. 2020)
<i>Fumaria judaica</i> subsp. <i>judaica</i>	11.7	Acts as an Acetylcholinesterase (ache) inhibitor and has therapeutic applications in Alzheimer's disease	Whole plant	Anticholinesterase alkaloids, such as physostigmine and galantamine	(Mukherjee et al. 2007)
<i>Heliotropium hirsutissimum</i> Grauer	11.7	Antimicrobial activity	Whole plant	Essential oils (especially thymol), flavonoids (antioxidants), triterpenoids, and other compounds of a phenolic nature or free hydroxyl group, which are classified as active antimicrobial compounds	(Jaradat et al. 2014)
<i>Hyoseris radiata</i> subsp. <i>graeca</i> Halácsy	11.7	Antioxidant activities	Whole plant	Phenols, flavonoids	(Shaltout et al. 2010)
<i>Limonium sinuatum</i> subsp. <i>romanum</i> Täckh. & Boulos	11.7	Antioxidant activities	Flower		(Xu et al. 2017)
<i>Lycium schweinfurthii</i> var. <i>aschersonii</i> (Dammer) Feinbrun	11.7	Antioxidant activity against DPPH and ABTS+ free radicals	Whole plant	Phenolics and flavonoids	(Mamdouh and Smetanska 2022; Shaltout and Bedair 2023)
<i>Muscari albiflorum</i> (Täckh. & Boulos) Hosni	11.7	Antioxidant activities	Flowers and leaves	Phenolics	(Mammadov et al. 2012)
<i>Muscari parviflorum</i> Desf.	11.7	Antioxidant activities	Flowers and leaves	Phenolics	(Mammadov et al. 2012)

Avifaunal diversity of Northwestern Himalayas (Jammu and Kashmir) and Trans Himalayas (Ladakh) of India

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Abstract. Yousuf P, Parvaiz S, Zehbi N, Altaf S, Zargar BN, Rather R, Tantray J, Lateef M. 2023. Avifaunal diversity of Northwestern Himalayas (Jammu and Kashmir) and Trans Himalayas (Ladakh) of India. *Biodiversitas* 24: 212-231. The Himalayas are home to various plants and animals, making it one of the world's most biodiverse regions. The northwestern Himalayan region of the trans-Himalayan Region of Ladakh and Jammu and Kashmir, India are home to various bird species due to varied topography and temperature. In this study, we collected the checklists and field data for 3 years based on compiling data from published and unpublished scientific literature, print and electronic, currently accessible in research articles, papers, books, websites, web pages, and field notes. The trans-Himalayas (Ladakh) and Northwestern Himalayas (Jammu and Kashmir) are home to 677 bird species, which belong to 89 families; there are approximately 107 families in the Indian sub-continent, equals to 6.77% of total bird species (>10,000) found globally and 49.2% of total bird species (1,376) in the Indian subcontinent. The family Muscicapidae (53 or 7.8%) has the most species, followed by Accipitridae (41 or 6%), and so on. Furthermore, the threatened species number is 54 (8%), which includes 23 (3.40%), 22 (3.25%), 6 (0.8%), and 3 (0.44%) under Vulnerable, Near Threatened, Endangered, and critically Endangered categories. Moreover, among 677 species, 228 species (33.77%) are found in all the 3 regions, 77 (11.4%) in only Jammu & Kashmir but not Ladakh, 24 (3.55%) in Jammu & Ladakh but not in Kashmir, 152 (22.5%) only in Jammu but not in Kashmir & Ladakh, 80 (11.85%) in Kashmir & Ladakh but not in Jammu, 26 (3.84%) only in Kashmir but not in Jammu & Ladakh, 71 (10.51%) only in Ladakh but not in Jammu & Kashmir. This information could provide these regions first-of-its-kind comprehensive data on bird diversity.

Keywords: Avifauna, bird diversity, birds, Himalayas, Jammu and Kashmir, Ladakh

Abbreviations: J&K: Jammu and Kashmir, UT: Union Territory, IUCN: International Union for the Conservation of Nature, NT: Near Threatened, VU: Vulnerable, EN: Endangered, CR: Critically Endangered, R: Resident, P: Passage Migrant, S: Summer Visitor, W: Winter Visitor, J: Jammu, K: Kashmir, L: Ladakh

INTRODUCTION

The Himalayas, one of the most remarkable mountain ranges on Earth, play a vital role in supporting the lives and livelihoods of approximately one-fifth of the global population. This vast region is a majestic landscape and a hotbed of biodiversity, with its unique ecosystems and rich flora and fauna (Dar and Khuroo 2013; Ara et al. 2019). Among the Himalayan regions, Jammu and Kashmir (J&K) State stands out as a significant area of immense ecological importance. Nestled in the northwestern Himalayas, J&K State encompasses a vast expanse characterized by remarkable elevational and topographical diversity, ranging from the subtropical lowlands of Jammu to the temperate Kashmir valley and extending to the frigid desert of Ladakh, which now holds the status of a separate Union Territory.

The ecological significance of J&K State becomes even more apparent when considering its pivotal role in bridging the Palearctic and Oriental regions of the planet. These two distinct biogeographic realms converge in this region, resulting in a rich blend of diverse species and habitats.

The J&K State is home to approximately 50% of the Himalayan flora and fauna in India, making it a critical conservation area for preserving this unique biodiversity. The state's vastness serves as a picture of the entire Himalayan range, encapsulating the incredible natural variations and adaptations that occur within the region.

The avian diversity of J&K State exemplifies the exceptional diversity found in this Himalayan region. The state inhabits an impressive variety of bird species, including endemic species that are exclusively found in higher elevations and those that are also distributed throughout the rest of the Indian subcontinent within the tropics and subtropics (Tariq 2018). Among the chordates, avifauna holds a significant position in terms of endemism in J&K State. While earlier studies have documented the avian population in the state, such as "Breeding Birds of Kashmir," "The Valley of Kashmir," and "Birds and Mammals of Ladakh," a comprehensive and up-to-date checklist of avifaunal diversity in J&K State remains absent. Recent contributions, including "Floral and Faunal Analysis of Chatlam Wetland Reserve: A Detailed Analysis" and "Birds of Jammu and Kashmir including

Ladakh" (Yousuf 2023), have shed light on specific aspects of the avian biodiversity in the region. Bird diversity has been documented in some local areas in Jammu, but not the entire region (Sharma et al. 2018; Sohil and Sharma 2019; Malik and Sheikh 2020; Sohil and Sharma 2020a; Sohil and Sharma 2020b; Wani 2020).

Similarly, some local wetlands and cities in Kashmir Valley have previously been documented for bird diversity (Kait et al. 2014; Noor et al. 2014; Dar et al. 2017; Ul Haq et al. 2019). However, the need for comprehensive and consolidated documentation of avifauna in Jammu and Kashmir State is still evident. Therefore, this research paper aims to bridge this knowledge gap and provide a comprehensive overview of the avian biodiversity in J&K State, including Ladakh. By collating existing data and incorporating recent studies, we aim to create a complete checklist encompassing the diverse bird species in this Himalayan region.

This article will also serve as a valuable resource for various stakeholders, including birdwatchers, ornithologists, and avian biologists. The comprehensive checklist presented herein offers them an up-to-date and reliable reference, aiding in identifying, studying, and conserving avian species in J&K State. Furthermore, by highlighting the range and conservation status of the featured taxa, this paper will contribute to the broader understanding of the ecological significance of this Himalayan region and the urgent need for its preservation. Additionally, we will discuss the significance of J&K State as a transit point for migratory bird populations, elucidating its critical role in the Central Asian and East Africa-West Asia Flyways.

MATERIALS AND METHODS

The results presented in this study are based on a comprehensive collection of data and a meticulous compilation of scientific literature, both published and unpublished. The study area included the Northwestern Himalayas (Jammu and Kashmir) and Trans Himalayas (Ladakh), as shown in Figure 1. The data collection process spanned two years, during which various sources were consulted. These sources included published materials such as research articles, papers, books, field notes, and electronic resources available in print and online formats. The primary published materials utilized in this study include the works of renowned ornithologists and researchers. These sources include the seminal works of Grewal (2016), Ahmad et al. (2017), and Suhail (2020). These publications provided valuable insights into the avifaunal diversity of the Jammu and Kashmir State. In addition to published literature, various online sources were consulted to augment the dataset. These online sources included reputable platforms such as eBird (<https://ebird.org>), Cornell Lab of Ornithology (<https://www.birds.cornell.edu>), Birds of the World (<https://birdsoftheworld.org>), Wildlife Conservation Fund (<https://www.wcf.org.in>), as well as several Facebook groups dedicated to birdwatching and wildlife conservation in the Jammu and Kashmir region. These Facebook groups included "J&K Birdwatchers' Club-WCF," "Save Wildlife of Kashmir," "Captivating Nature," "Kashmir Birdwatch," "Birds of Kashmir," "J&K Birdlife," and others. The information and observations shared by members of these groups provided valuable firsthand accounts and contributed to the completeness of the checklist.

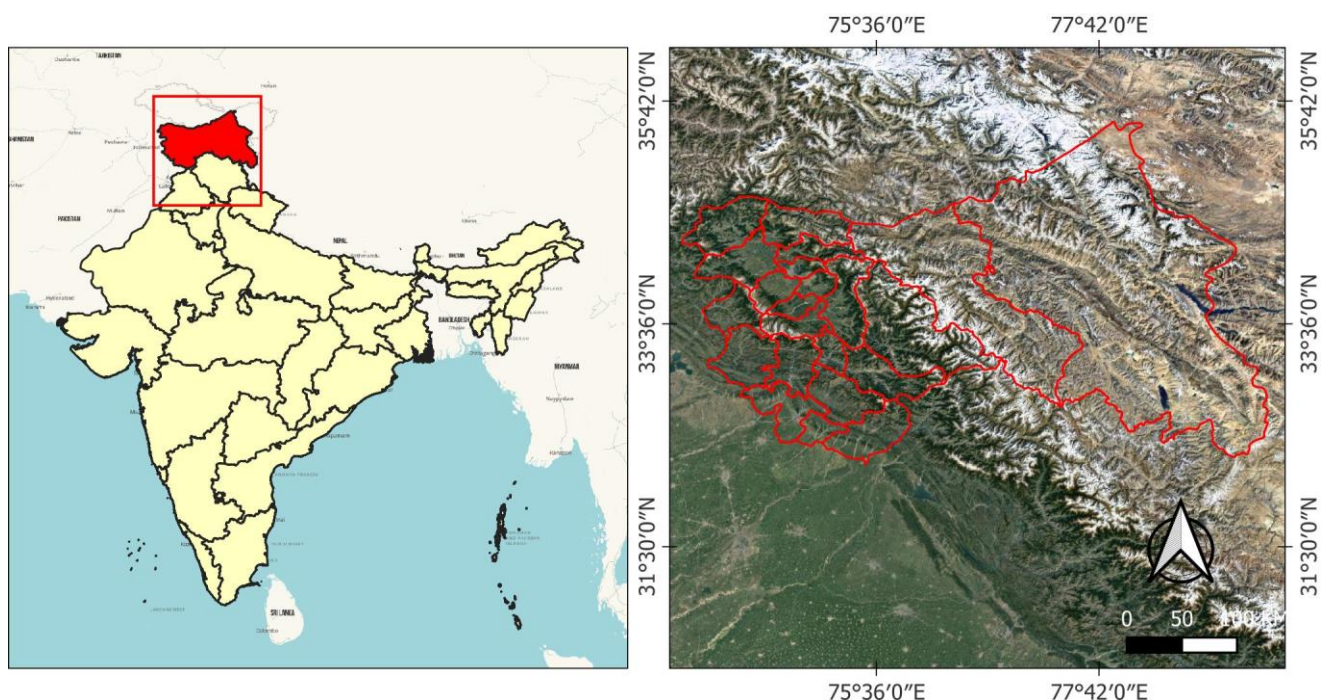


Figure 1. Map of Northwestern Himalayas (Jammu and Kashmir) and Trans Himalayas (Ladakh), India

To ensure the inclusion of the most up-to-date information, the authors incorporated their extensive field notes accumulated over more than two decades of birdwatching expeditions in the region. These unpublished records served as a valuable supplement to the published literature and online sources, providing additional data on bird species occurrences within Jammu and Kashmir State. The resulting checklist presented in this paper encompasses all species credibly recorded within the state until 2023. Each species in the checklist is assigned a conservation status based on the International Union for the Conservation of Nature's (IUCN) Red List categories. The study uses the conservation status categories as follows: CR (Critically Endangered), EN (Endangered), NT (Near Threatened), VU (Vulnerable), and LC (Least Concern). This classification helps to understand the conservation priorities and the potential risks faced by various bird species in the region. Furthermore, the checklist categorizes the bird species into four classes based on their distribution within the Jammu and Kashmir State borders. These categories are as follows: Resident (R), representing species that can be observed throughout the year; Passage Migrant (P), representing species that visit the region for a short period during migration; Summer Visitor (S), denoting species that are primarily observed during the summer breeding season; and Winter Visitor (W), indicating species that are predominantly observed during the winter months.

Therefore, to provide a more localized perspective, the checklist also presents information on the distribution of each species within the three regions of Jammu, Kashmir, and Ladakh. This breakdown allows for a better understanding of the regional distribution patterns and variations in species composition within the state. Thus, this research paper's comprehensive data collection and compilation process has resulted in a robust checklist of avifaunal diversity in Jammu and Kashmir State. The inclusion of published literature, online sources, and the authors' field observations ensure that the checklist provides an up-to-date and reliable reference for birdwatchers, ornithologists, and avian biologists interested in the avian biodiversity of this remarkable Himalayan region.

RESULTS AND DISCUSSION

The avifaunal diversity in Jammu and Kashmir, including Ladakh, is remarkable, with 677 bird species belonging to 89 families recorded in the region. This accounts for approximately 6.77% of the total bird species (>10,000) found globally and 49.2% of the total bird species (1,376) found in the Indian subcontinent (Table 1). The significant bird species richness can be attributed to the diverse climates in the three regions of Jammu, Kashmir, and Ladakh. Kashmir exhibits a temperate climate, Jammu a sub-tropical climate, and Ladakh a cold desert-type climate. When comparing the bird families found in Jammu and Kashmir, including Ladakh, to global and subcontinental figures, the region is home to a

substantial proportion of avian families. Out of the 249 families found worldwide, 89 are in Jammu and Kashmir, representing approximately 35.74% of the total families found globally; similarly, out of the 107 bird families in the Indian subcontinent, Jammu and Kashmir harbor 83.17% of these families (Figure 2). This high representation of bird families further highlights the region's significance in terms of avian diversity.

Among the families in Jammu and Kashmir, several are particularly diverse regarding species richness. The dominant families include Muscicapidae (flycatchers), Accipitridae (hawks, kites, and eagles), Fringillidae (finches), Anatidae (ducks, geese, and swans), Scolopacidae (snipes, sandpipers, and other waders), Phylloscopidae (leaf warblers), Motacillidae (wagtails and pipits), Strigidae (owls), Corvidae (jays, magpies, and crows), Phasianidae (partridges and pheasants), Laridae (gulls and terns), Rallidae (crakes and rails), Emberizidae (buntings), Columbidae (doves and pigeons), Turdidae (thrushes), Alaudidae (larks), Charadriidae (plovers and lapwings), Ardeidae (egrets, herons, and bitterns), Picidae (woodpeckers), Cuculidae (cuckoos), Passeridae (sparrows and snow finches), Cisticolidae (prinias and tailorbirds), Acrocephalidae (reed warblers), Hirundinidae (swallows and martins), and Paridae (tits).

The number of species within each of these dominant families varies, with Muscicapidae having 53 species (7.8% of the total species in Jammu and Kashmir), Accipitridae with 41 species (6%), Fringillidae and Anatidae with 33 species each (4.87% each), Scolopacidae with 26 species (3.8%), Phylloscopidae with 21 species (3.1%), Motacillidae with 19 species (2.8%), Strigidae with 18 species (2.6%), Corvidae and Phasianidae with 17 species each (2.5% each), Laridae and Rallidae with 16 species each (2.37% each), Emberizidae and Columbidae with 15 species each (2.22% each), and Turdidae, Alaudidae, and Charadriidae with 14 species each (2.07% each). Other families such as Ardeidae, Picidae, Cuculidae, Passeridae, Cisticolidae, Acrocephalidae, Hirundinidae, and Paridae also contribute to the overall avian diversity in the region (Figure 2).

These findings highlight the impressive variety of bird species and families in Jammu and Kashmir, including Ladakh. The diverse ecosystems, ranging from temperate forests to sub-tropical landscapes and cold desert regions, provide habitats that support a wide range of avian life. Understanding the region's distribution and richness of bird families and species is crucial for effective conservation strategies and management efforts. By preserving and protecting the diverse avifauna of Jammu and Kashmir, we can ensure the long-term survival of these remarkable species and contribute to the conservation of global avian biodiversity.

Threatened bird species

The conservation status of bird species in Jammu and Kashmir, including Ladakh, is of paramount importance. Previous studies on threatened birds in the region have estimated a total of 24 species falling into four categories: Near Threatened (NT), Vulnerable (VU), Endangered

(EN), and Critically Endangered (CR). However, it is important to note that limited research has been conducted specifically on threatened bird species in Jammu and Kashmir. In our study, we revisited the list of threatened bird species in Jammu and Kashmir, including Ladakh, and found that the number has significantly increased. According to the International Union for Conservation of Nature (IUCN) assessment, the number of threatened bird species in the region now stands at 54, accounting for approximately 8% of the total bird species (Table 2). These threatened species encompass a range of conservation categories, with 22 species classified as Near Threatened (3.25%), 23 as Vulnerable (3.40%), 6 as Endangered (0.8%), 3 as Critically Endangered (0.44%) and 1 as Data Deficient (0.15%). This highlights the urgent need for conservation efforts to protect these species and their habitats.

The Western Himalaya, classified as the Endemic Bird Area 128 by BirdLife International, harbors various important habitats for bird species. The main habitat types within this area include subalpine forests, montane grasslands, and temperate coniferous or broad-leaved forests (Rahmani et al. 2014). Within these habitats, there are eleven bird species with extremely limited distributions. Remarkably, 9 of these 11 species (81.8%) are endemic to Jammu and Kashmir (Stattersfield et al. 1998; Birdlife International 2001). The species endemic to Jammu and Kashmir include the Kashmir Flycatcher (*Ficedula subrubra*), Spectacled Finch (*Callacanthis burtoni*), Western Tragopan (*Tragopan melanocephalus*), Cheer Pheasant (*Catreus wallichii*), Tytler's Leaf Warbler (*Phylloscopus tytleri*), Brook's Leaf Warbler (*Phylloscopus subviridis*), Kashmir Nuthatch (*Sitta cashmirensis*), White-throated Tit (*Aegithalos nivergularis*), and Orange Bullfinch (*Pyrrhula aurantiaca*) (Khah et al. 2012). These species are of particular conservation concern due to their limited range and vulnerability (Bhat et al. 2020).

Additionally, within the Western Himalayas, six of these species are endemic to the region, which means they are found exclusively within the Western Himalayas, while one species, the Kashmir Flycatcher, is known to breed only in the forests of Kashmir. These endemic and range-restricted species are highly significant in terms of conservation priorities, as their restricted distributions make them more susceptible to habitat loss, fragmentation, and other threats. Identifying and conserving these endemic and range-restricted bird species within Jammu and Kashmir, including Ladakh, are crucial for maintaining the region's avian diversity and ecological integrity. By focusing conservation efforts on protecting their habitats and addressing the threats they face, we can contribute to preserving these unique species and their associated ecosystems. Furthermore, conserving these endemic species can have broader implications for the overall conservation of the Western Himalayan region and its avifauna.

Discussion

In this study, we have documented 677 bird species in Jammu and Kashmir, including Ladakh, representing a diverse array of avian life. These species are distributed among 89 families, highlighting the region's richness and complexity of avifaunal diversity. Among these species, most 622 (91.88%) fall under the least concern category, indicating a relatively stable population and a lower risk of extinction. However, it is crucial to recognize that 54 species (8%) are classified as threatened according to the latest IUCN assessment (Figure 3). These threatened species require urgent attention and targeted conservation efforts to mitigate the factors contributing to their decline. Examining the distribution of bird species within the three regions of Jammu, Kashmir, and Ladakh reveals interesting patterns. Moreover, 228 species (33.77%) are found in all three regions, indicating a considerable overlap in avian diversity.

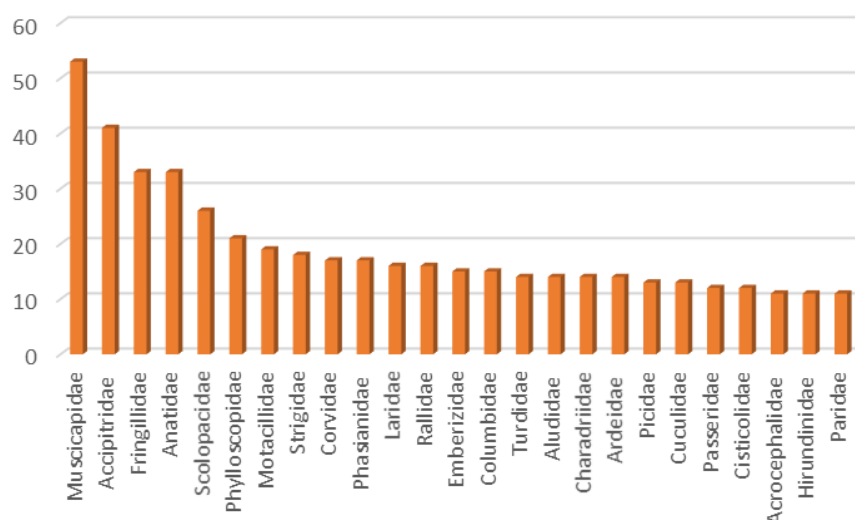


Figure 2. Bird families with the most number of species

Table 1. A complete checklist of bird species found in Jammu (J), Kashmir (K), and Ladakh (L) in India

Common Name	Scientific Name	Distribution	Status	IUCN Status
Phasianidae (partridges and pheasants)				
Chukar	<i>Alectoris chukar</i>	JKL	R	LC
Snow Partridge	<i>Lerwa lerwa</i>	JKL	R	LC
Hill Partridge	<i>Arborophila torqueola</i>	J	R	LC
Tibetan Partridge	<i>Perdix hodgsoniae</i>	L	R	LC
Grey Francolin	<i>Ortygornis pondicerianus</i>	J	R	LC
Black Francolin	<i>Francolinus francolinus</i>	J	R	LC
Cheer Pheasant	<i>Catreus wallichii</i>	JKL	R	VU
Kalij Pheasant	<i>Lophura leucomelanos</i>	JK	R	LC
Koklass Pheasant	<i>Pucrasia macrolopha</i>	JK	R	LC
Common Quail	<i>Coturnix coturnix</i>	KL	S	LC
Jungle Bush-quail	<i>Perdica asiatica</i>	J	R	LC
Himalayan Monal	<i>Lophophorus impejanus</i>	JK	R	LC
Tibetan Snowcock	<i>Tetraogallus tibetanus</i>	L	R	LC
Himalayan Snowcock	<i>Tetraogallus himalayensis</i>	KL	R	LC
Indian Peafowl	<i>Pavo cristatus</i>	JK	R	LC
Red Junglefowl	<i>Gallus gallus</i>	J	R	LC
Western Tragopan	<i>Tragopan melanocephalus</i>	JK	R	VU
Turnicidae				
Barred Buttonquail	<i>Turnix suscitator</i>	J	R	LC
Anatidae (ducks, geese, and swans)				
Gadwall	<i>Mareca strepera</i>	JKL	W/P	LC
Eurasian Wigeon	<i>Mareca Penelope</i>	JKL	W/P	LC
Northern Shoveller	<i>Spatula clypeata</i>	JKL	W/P	LC
Northern Pintail	<i>Anas acuta</i>	JKL	W/P	LC
Indian Spot-billed Duck	<i>Anas poecilorhyncha</i>	JL	W	LC
Mallard	<i>Anas platyrhynchos</i>	JKL	S/W	LC
White-headed Duck	<i>Oxyura leucocephala</i>	K	W	EN
Tufted-Duck	<i>Aythya fuligula</i>	JKL	W/P	LC
Ferruginous Duck	<i>Aythya nyroca</i>	JKL	W/P	NT
Common Goldeneye	<i>Bucephala clangula</i>	K	W	LC
Common Pochard	<i>Aythya ferina</i>	JKL	W/P	VU
Red-crested Pochard	<i>Netta rufina</i>	JKL	W/P	LC
Long-tailed Duck	<i>Clangula hyemalis</i>	JKL	W/P	VU
Lesser Whistling-Duck	<i>Dendrocygna javanica</i>	J	S	LC
Knob-billed Duck	<i>Sarkidiornis melanotos</i>	J	W	LC
Ruddy Shelduck	<i>Tadorna ferruginea</i>	JKL	W/P	LC
Common Shelduck	<i>Tadorna tadorna</i>	JKL	P	LC
Marbled Teal	<i>Marmaronetta angustirostris</i>	JK	W	VU
Green-winged Teal	<i>Anas crecca</i>	JKL	W/P	LC
Baikal Teal	<i>Sibirionetta formosa</i>	JK	W	LC
Garganey	<i>Spatula querquedula</i>	JKL	P	LC
Common Merganser	<i>Mergus merganser</i>	JKL	W/P	LC
Cotton Pygmy-geese	<i>Nettapus coromandelianus</i>	JK	W	LC
Greater White-fronted Goose	<i>Anser albifrons</i>	JKL	W/P	LC
Lesser White-fronted Goose	<i>Anser erythropus</i>	JK	V/W	VU
Bar-headed Goose	<i>Anser indicus</i>	JKL	S/W	LC
Greylag Goose	<i>Anser anser</i>	JKL	W/P	LC
Tundra Bean-Goose	<i>Anser serrirostris</i>	JK	W	LC
Whooper Swan	<i>Cygnus cygnus</i>	K	W	LC
Mute Swan	<i>Cygnus olor</i>	L	W	LC
Tundra Swan	<i>Cygnus columbianus</i>	K	W	LC
Greater Scaup	<i>Aythya marila</i>	L	W	LC
Smew	<i>Mergellus albellus</i>	K	V	LC
Podicipedidae (grebes)				
Great Crested Grebe	<i>Podiceps cristatus</i>	JKL	S/P	LC
Little Grebe	<i>Tachybaptus ruficollis</i>	JKL	S/R	LC
Eared Grebe	<i>Podiceps nigricollis</i>	JKL	V	LC
Red-necked Grebe	<i>Podiceps grisegena</i>	L	W	LC
Horned Grebe	<i>Podiceps auritus</i>	L	P	VU
Ardeidae (egrets, herons and bitterns)				
Little Egret	<i>Egretta garzetta</i>	JKL	R/P	LC
Great Egret	<i>Ardea alba</i>	JKL	R	LC

Intermediate Egret	<i>Ardea intermedia</i>	JL	R	LC
Cattle Egret	<i>Bubulcus ibis</i>	JKL	R	LC
Grey Heron	<i>Ardea cinerea</i>	JKL	R/P	LC
Purple Heron	<i>Ardea purpurea</i>	JL	R	LC
Indian Pond-heron	<i>Ardeola greyii</i>	JKL	R/W	LC
Striated Heron	<i>Butorides striata</i>	J	P	LC
Black-crowned Night-heron	<i>Nycticorax nycticorax</i>	JKL	R/S	LC
Little Bittern	<i>Ixobrychus minutus</i>	KL	S/P	LC
Black Bittern	<i>Ixobrychus flavicollis</i>	JKL	W	LC
Yellow Bittern	<i>Ixobrychus sinensis</i>	J	R	LC
Cinnamon Bittern	<i>Ixobrychus cinnamomeus</i>	KL	S/P	LC
Great Bittern	<i>Botaurus stellaris</i>	JL	W/V	LC
Ciconiidae (storks)				
Painted Stork	<i>Mycteria leucocephala</i>	J	W	NT
Asian Woolly-necked Stork	<i>Ciconia episcopus</i>	J	W	NT
Black Stork	<i>Ciconia nigra</i>	JKL	P	LC
Black-necked Stork	<i>Ephippiorhynchus asiaticus</i>	L	S	NT
Asian Openbill	<i>Anastomus oscitans</i>	J	P	LC
Glossy Ibis	<i>Plegadis falcinellus</i>	JKL	P/V	LC
Black-headed Ibis	<i>Threskiornis melanocephalus</i>	J	W	NT
Red-naped Ibis	<i>Pseudibis papillosa</i>	J	W	LC
Threskiornithidae				
Eurasian Spoonbill	<i>Platalea leucorodia</i>	J	P	LC
Phalacrocoracidae (cormorants)				
Little Cormorant	<i>Microcarbo niger</i>	JL	R/V	LC
Indian Cormorant	<i>Phalacrocorax fuscicollis</i>	J	W	LC
Great Cormorant	<i>Phalacrocorax carbo</i>	JKL	P	LC
Anhingidae				
Oriental Darter	<i>Anhinga melanogaster</i>	J	W	NT
Pandionidae (osprey)				
Osprey	<i>Pandion haliaetus</i>	JKL	S/P	LC
Accipitridae (hawks, kites, and eagles)				
Black-winged Kite	<i>Elanus caeruleus</i>	JL	R/V	LC
Red Kite	<i>Milvus milvus</i>	L	V	LC
Black Kite	<i>Milvus migrans</i>	J	R	LC
Brahminy Kite	<i>Haliastur indus</i>	JKL	S	LC
Montagu's Harrier	<i>Circus pygargus</i>	JKL	P	LC
Pallid Harrier	<i>Circus macrourus</i>	JL	W/P	NT
Hen Harrier	<i>Circus cyaneus</i>	JKL	W/P	LC
Eurasian Marsh-Harrier	<i>Circus aeruginosus</i>	JKL	W/P	LC
Besra	<i>Accipiter virgatus</i>	JK	R	LC
Shikra	<i>Accipiter badius</i>	JK	R/S	LC
Northern Goshawk	<i>Accipiter gentilis</i>	JKL	W/P	LC
Eurasian Sparrowhawk	<i>Accipiter nisus</i>	JKL	R/P	LC
Rough-legged Hawk	<i>Buteo lagopus</i>	L	R	LC
White-eyed Buzzard	<i>Butastur teesa</i>	J	R	LC
Common Buzzard	<i>Buteo buteo</i>	JKL	W	LC
Himalayan Buzzard	<i>Buteo refectus</i>	JKL	P	LC
Long-legged Buzzard	<i>Buteo rufinus</i>	JKL	R/W/P	LC
Upland Buzzard	<i>Buteo hemilasius</i>	JKL	W/S	LC
Oriental Honey-buzzard	<i>Pernis ptilorhynchus</i>	JKL	R/P	LC
Crested Serpent-eagle	<i>Spilornis cheela</i>	J	R	LC
Short-toed Snake-Eagle	<i>Circaetus gallicus</i>	JL	R/P	LC
Booted Eagle	<i>Hieraaetus pennatus</i>	JKL	W/S/P	LC
Bonelli's Eagle	<i>Aquila fasciata</i>	JK	R	LC
Mountain Hawk-eagle	<i>Nisaetus nipalensis</i>	JKL	R	LC
Changeable Hawk-Eagle	<i>Nisaetus cirrhatus</i>	K	V	LC
Black Eagle	<i>Ictinaetus malaiensis</i>	J	R	LC
Indian Spotted Eagle	<i>Clanga hastata</i>	J	R	VU
Greater Spotted Eagle	<i>Clanga clanga</i>	JKL	W/P	VU
Tawny Eagle	<i>Aquila rapax</i>	JK	V	VU
Steppe Eagle	<i>Aquila nipalensis</i>	JKL	W/P	EN
Imperial Eagle	<i>Aquila heliaca</i>	JKL	W/P	VU
Golden Eagle	<i>Aquila chrysaetos</i>	JKL	R	LC
Pallas's Fish-Eagle	<i>Haliaeetus leucoryphus</i>	JKL	S/P	EN
White-tailed eagle	<i>Haliaeetus albicilla</i>	JK	P	LC
Egyptian Vulture	<i>Neophron percnopterus</i>	JKL	R/P	EN

Bearded Vulture	<i>Gypaetus barbatus</i>	JKL	R	NT
White-rumped Vulture	<i>Gyps bengalensis</i>	JK	R	CR
Slender-billed Vulture	<i>Gyps tenuirostris</i>	J	R	CR
Eurasian Griffon	<i>Gyps fulvus</i>	JKL	R	LC
Himalayan Griffon	<i>Gyps himalayensis</i>	JKL	R	NT
Cinereous Vulture	<i>Aegypius monachus</i>	JL	W	NT
Falconidae (falcons)				
Merlin	<i>Falco columbarius</i>	JL	W/P	LC
Lesser Kestrel	<i>Falco naumanni</i>	JKL	P	LC
Eurasian Kestrel	<i>Falco tinnunculus</i>	JKL	W/S	LC
Eurasian Hobby	<i>Falco Subbuteo</i>	JKL	W/S	LC
Peregrine Falcon	<i>Falco peregrinus</i>	JKL	W/P	LC
Laggar Falcon	<i>Falco jugger</i>	J	R	NT
Saker Falcon	<i>Falco cherrug</i>	JL	W/P	EN
Red-necked Falcon	<i>Falco chicquera</i>	J	P	NT
Amur Falcon	<i>Falco amurensis</i>	JL	P	LC
Red-footed Falcon	<i>Falco vespertinus</i>	L	P	VU
Rallidae (crakes, rails)				
Water Rail	<i>Rallus aquaticus</i>	JKL	S	LC
Red-legged Crake	<i>Rallina fasciata</i>	K	V	LC
Baillon's Crake	<i>Zapornia pusilla</i>	JKL	W/S	LC
Spotted Crake	<i>Porzana porzana</i>	JKL	W/P	LC
Ruddy-breasted Crake	<i>Zapornia fusca</i>	JK	R	LC
Corn Crake	<i>Crex crex</i>	L	P	LC
Brown Crake	<i>Zapornia akool</i>	J	R	LC
White-breasted Waterhen	<i>Amaurornis phoenicurus</i>	JL	R	LC
Grey-headed Swampphen	<i>Porphyrion popiliocephalus</i>	JKL	R	LC
Common Moorhen	<i>Gallinula chloropus</i>	JKL	R/P	LC
Eurasian Coot	<i>Fulica atra</i>	JKL	R/W/P	LC
Black-necked Crane	<i>Grus nigricollis</i>	L	S	VU
Common Crane	<i>Grus grus</i>	JK	W	LC
Demoiselle Crane	<i>Anthropoides virgo</i>	KL	P	LC
Sarus Crane	<i>Antigone Antigone</i>	J	W	VU
Siberian Crane	<i>Leucogeranus leucogeranus</i>	L	P	CR
Burhinidae (thicknee)				
Great Thick-knee	<i>Esacus recurvirostris</i>	J	R	NT
Eurasian Thick-knee	<i>Burhinus oedicnemus</i>	K	V	LC
Indian Thick-knee	<i>Burhinus indicus</i>	J	W	LC
Ibidorhynchidae (Ibisbill)				
Ibisbill	<i>Ibidorhyncha struthersii</i>	KL	R	LC
Recurvirostridae (stilts and avocets)				
Black-winged Stilt	<i>Himantopus Himantopus</i>	JKL	W/P	LC
Pied Avocet	<i>Recurvirostra avosetta</i>	JKL	W	LC
Charadriidae (plovers and lapwings)				
Red-wattled Lapwing	<i>Vanellus indicus</i>	JKL	R	LC
Yellow-wattled Lapwing	<i>Vanellus malabaricus</i>	JK	W/S	LC
White-tailed Lapwing	<i>Vanellus leucurus</i>	J	W	LC
Northern Lapwing	<i>Vanellus vanellus</i>	JKL	W/P	LC
River Lapwing	<i>Vanellus duvaucelii</i>	JL	W/S	NT
Grey-headed Lapwing	<i>Vanellus cinereus</i>	J	W/S	LC
Black-bellied Plover	<i>Pluvialis squatarola</i>	L	P	LC
Pacific Golden-Plover	<i>Pluvialis fulva</i>	JKL	P	LC
Greater Sand-Plover	<i>Charadrius leschenaultia</i>	L	W	LC
Lesser Sand-Plover	<i>Charadrius mongolus</i>	JKL	P/S	LC
Common Ringed Plover	<i>Charadrius hiaticula</i>	L	W	LC
Little Ringed Plover	<i>Charadrius dubius</i>	JKL	R/S	LC
Kentish Plover	<i>Charadrius alexandrinus</i>	JKL	P	LC
Caspian Plover	<i>Charadrius asiaticus</i>	J	W/S	LC
Jacaniidae (Jacanas)				
Pheasant-tailed Jacana	<i>Hydrophasianus chirurgus</i>	JKL	S	LC
Scolopacidae (snipes, sandpipers and waders)				
Eurasian Curlew	<i>Numenius arquata</i>	KL	P	NT
Whimbrel	<i>Numenius phaeopus</i>	KL	P	LC
Black-tailed Godwit	<i>Limosa limosa</i>	KL	P	NT
Terek Sandpiper	<i>Xenus cinereus</i>	KL	P	LC
Wood Sandpiper	<i>Tringa glareola</i>	JKL	P	LC
Green Sandpiper	<i>Tringa ochropus</i>	JKL	W/P	LC

Common Sandpiper	<i>Actitis hypoleucos</i>	JKL	W/S	LC
Marsh Sandpiper	<i>Tringa stagnatilis</i>	JKL	P	LC
Broad-billed Sandpiper	<i>Calidris falcinellus</i>	L	W/S	LC
Curlew Sandpiper	<i>Calidris ferruginea</i>	KL	P	NT
Sharp-tailed Sandpiper	<i>Calidris acuminata</i>	L	W/S	VU
Sanderling	<i>Calidris alba</i>	L	W/S	LC
Common Greenshank	<i>Tringa nebularia</i>	JKL	W/P	LC
Common Redshank	<i>Tringa tetanus</i>	JKL	W/P/S	LC
Spotted Redshank	<i>Tringa erythropus</i>	KL	W	LC
Ruddy Turnstone	<i>Arenaria interpres</i>	KL	P	LC
Ruff	<i>Calidris pugnax</i>	KL	W/S	LC
Dunlin	<i>Calidris alpina</i>	JKL	P	LC
Temminck's Stint	<i>Calidris temminckii</i>	JKL	W/P	LC
Little Stint	<i>Calidris minuta</i>	JKL	P	LC
Red-necked Phalarope	<i>Phalaropus lobatus</i>	KL	P	LC
Common Snipe	<i>Gallinago gallinago</i>	JKL	W/P	LC
Jack Snipe	<i>Limnocyptes minimus</i>	JKL	W	LC
Pin-tailed Snipe	<i>Gallinago stenura</i>	JKL	P	LC
Solitary Snipe	<i>Gallinago solitaria</i>	KL	R/P	LC
Eurasian Woodcock	<i>Scolopax rusticola</i>	KL	S	LC
Rostratulidae (painted snipe)				
Greater Painted-Snipe	<i>Rostratula benghalensis</i>	JKL	R	LC
Glareolidae (coursers and pratincoles)				
Collared Pratincole	<i>Glareola pratincole</i>	L	P	LC
Oriental Pratincole	<i>Glareola maldivarum</i>	J	P	LC
Small Pratincole	<i>Glareola lactea</i>	J	R	LC
Stercorariidae				
Parasitic Jaeger	<i>Stercorarius parasiticus</i>	L	V	LC
Laridae (gulls, terns)				
Pallas's Gull	<i>Ichthyaeetus ichthyaeetus</i>	KL	P	LC
Brown-headed Gull	<i>Chroicocephalus brunnicephalus</i>	JKL	W/S	LC
Black-headed Gull	<i>Chroicocephalus ridibundus</i>	KL	W	LC
Little Gull	<i>Hydrocoloeus minutus</i>	L	P	LC
Common Gull	<i>Larus canus</i>	K	W/S	LC
Lesser Black-backed Gull	<i>Larus fuscus</i>	L	W/P	LC
Caspian Gull	<i>Larus cachinnans</i>	L	P	LC
Little Tern	<i>Sterna albifrons</i>	KL	P	LC
Common Tern	<i>Sterna hirundo</i>	JKL	S	LC
Gull-billed Tern	<i>Gelochelidon nilotica</i>	JKL	P	LC
River Tern	<i>Sterna aurantia</i>	JKL	R/S	VU
Black-bellied Tern	<i>Sterna acuticauda</i>	K	W/S	EN
Arctic Tern	<i>Sterna paradisaea</i>	KL	W/S	LC
Black Tern	<i>Chlidonias niger</i>	L	S	LC
Whiskered Tern	<i>Chlidonias hybrida</i>	KL	S/P	LC
White-winged Tern	<i>Chlidonias leucopterus</i>	L	P	LC
Pteroclididae (sandgrouse)				
Tibetan Sandgrouse	<i>Syrrhaptes tibetanus</i>	L	R	LC
Chestnut-bellied Sandgrouse	<i>Pterocles exustus</i>	J	R	LC
Black-legged Kittiwake	<i>Rissa tridactyla</i>	L	R	VU
Columbidae (pigeons and doves)				
Rock Pigeon	<i>Columba livia</i>	JKL	R	LC
Hill Pigeon	<i>Columba rupestris</i>	KL	R	LC
Yellow-eyed Pigeon	<i>Columba eversmanni</i>	KL	W/V	VU
Snow Pigeon	<i>Columba leuconota</i>	JKL	R	LC
Common Wood-Pigeon	<i>Columba palumbus</i>	JKL	R	LC
Speckled Wood-Pigeon	<i>Columba hodgsonii</i>	KL	R	LC
Yellow-footed Green Pigeon	<i>Terron Phoenicopterus</i>	JL	R	LC
Wedge-tailed Green-pigeon	<i>Treron sphenurus</i>	JK	R	LC
Oriental Turtle-dove	<i>Streptopelia orientalis</i>	JKL	R/S	LC
Laughing Dove	<i>Spilopelia senegalensis</i>	JKL	R/P	LC
Red Collared-dove	<i>Streptopelia tranquebarica</i>	J	S	LC
Spotted Dove	<i>Spilopelia chinensis</i>	JKL	R/S	LC
Eurasian Collared-dove	<i>Streptopelia decaocto</i>	JKL	R/S	LC
European Turtle-Dove	<i>Streptopelia turtur</i>	L	P	VU
Asian Emerald Dove	<i>Chalcophaps indica</i>	J	S	LC
Psittaculidae (parrots and parakeets)				
Slaty-headed Parakeet	<i>Psittacula himalayana</i>	JKL	R	LC

Plum-headed Parakeet	<i>Psittacula cyanocephala</i>	JKL	R	LC
Red-breasted Parakeet	<i>Psittacula alexandri</i>	J	R	NT
Rose-ringed Parakeet	<i>Psittacula krameria</i>	JKL	R	LC
Alexandrine Parakeet	<i>Psittacula eupatria</i>	JKL	R	NT
Cuculidae (cuckoos)				
Grey-bellied Cuckoo	<i>Cacomantis passerines</i>	JL	S	LC
Banded Bay Cuckoo	<i>Cacomantis sonneratii</i>	J	V	LC
Square-tailed Drongo-Cuckoo	<i>Surniculus lugubris</i>	J	S	LC
Jacobin Cuckoo	<i>Clamator jacobinus</i>	JKL	S	LC
Asian Koel	<i>Eudynamis scolopacea</i>	JKL	R/S	LC
Indian Cuckoo	<i>Cuculus Micropterus</i>	J	S	LC
Common Cuckoo	<i>Cuculus canorus</i>	JKL	S	LC
Himalayan Cuckoo	<i>Cuculus saturates</i>	JKL	S	LC
Lesser Cuckoo	<i>Cuculus poliocephalus</i>	JK	S	LC
Large Hawk-Cuckoo	<i>Hierococcyx sparveriioides</i>	J	S	LC
Common Hawk-cuckoo	<i>Hierococcyx varius</i>	JK	R	LC
Sirkeer Malkoha	<i>Taccocua leschenaultii</i>	J	R	LC
Greater Coucal	<i>Centropus sinensis</i>	J	R	LC
Tytonidae (Barn Owls)				
Barn Owl	<i>Tyto alba</i>	K	R	LC
Strigidae (owls)				
Brown Fish-owl	<i>Ketupa zeylonensis</i>	JK	V	LC
Pallid Scops-Owl	<i>Otus brucei</i>	L	V	LC
Indian Scops-Owl	<i>Otus bakkamoena</i>	J	R	LC
Eurasian Scops-Owl	<i>Otus scops</i>	JL	S	LC
Collared Scops-Owl	<i>Otus lettia</i>	J	R	LC
Mountain Scops-Owl	<i>Otus spilocephalus</i>	J	R	LC
Oriental Scops-Owl	<i>Otus sunia</i>	J	R	LC
Brown boobook	<i>Ninox scutulata</i>	J	R	LC
Eurasian Eagle-owl	<i>Bubo bubo</i>	KL	R	LC
Rock Eagle-Owl	<i>Bubo bengalensis</i>	J	R	LC
Long-eared Owl	<i>Asio otus</i>	JKL	W/S	LC
Short-eared Owl	<i>Asio flammeus</i>	JKL	W/P	LC
Tawny Owl	<i>Strix aluco</i>	K	R	LC
Brown Wood-owl	<i>Strix leptogrammica</i>	J	V	LC
Little Owl	<i>Athene noctua</i>	L	R	LC
Spotted Owlet	<i>Athene brama</i>	J	R	LC
Collared owlet	<i>Taeniopteryx brodiei</i>	JKL	R	LC
Asian Barred Owlet	<i>Glaucidium cuculoides</i>	JK	R	LC
Caprimulgidae (nightjars)				
European Nightjar	<i>Caprimulgus europaeus</i>	KL	S/P	LC
Indian Nightjar	<i>Caprimulgus asiaticus</i>	J	V	LC
Large-tailed Nightjar	<i>Caprimulgus macrurus</i>	J	R	LC
Grey Nightjar	<i>Caprimulgus jotaka</i>	J	R	LC
Savanna Nightjar	<i>Caprimulgus affinis</i>	J	R	LC
Sykes's Nightjar	<i>Caprimulgus mahrattensis</i>	J	R	LC
Apodidae (swifts)				
Little Swift	<i>Apus affinis</i>	J	R	LC
Common Swift	<i>Apus apus</i>	JKL	S	LC
Pacific Swift	<i>Apus pacificus</i>	KL	S	LC
Alpine Swift Apus melba	<i>Tachymarptis melba</i>	JKL	S	LC
Blyth's Swift	<i>Apus leuconyx</i>	KL	S	DD
House Swift	<i>Apus nipalensis</i>	L	S	LC
White-throated Needletail	<i>Hirundapus caudacutus</i>	JK	S	LC
Coraciidae (rollers)				
European Roller	<i>Coracias garrulus</i>	JKL	P/S	LC
Indian Roller	<i>Coracias benghalensis</i>	JK	R	LC
Alcedinidae (kingfishers)				
White-throated Kingfisher	<i>Halcyon smyrnensis</i>	JKL	R	LC
Crested Kingfisher	<i>Megaceryle lugubris</i>	JK	R	LC
Pied Kingfisher	<i>Ceryle rudis</i>	JK	R	LC
Common Kingfisher	<i>Alcedo atthis</i>	JKL	R/S	LC
Meropidae (bee-eaters)				
European Bee-eater	<i>Merops apiaster</i>	JKL	S/P	LC
Blue-cheeked Bee-eater	<i>Merops persicus</i>	L	P	LC
Blue-tailed Bee-eater	<i>Merops philippinus</i>	J	S	LC
Asian Green Bee-eater	<i>Merops orientalis</i>	JK	R	LC

Chestnut-headed Bee-eater	<i>Merops leschenaulti</i>	J	S	LC
Upupidae (hoopoe)				
Eurasian Hoopoe	<i>Upupa epops</i>	JKL	R/S	LC
Bucerotidae (hornbills)				
Indian Grey Hornbill	<i>Ocyrceros birostris</i>	JK	R	LC
Megalaimidae (barbets)				
Coppersmith Barbet	<i>Psilopogon haemacephalus</i>	J	R	LC
Great Barbet	<i>Psilopogon virens</i>	JK	R	LC
Blue-throated Barbet	<i>Psilopogon asiaticus</i>	JK	R	LC
Brown-headed Barbet	<i>Psilopogon zeylanicus</i>	J	R	LC
Lineated Barbet	<i>Psilopogon lineatus</i>	J	S	LC
Picidae (woodpeckers)				
Eurasian Wryneck	<i>Jynx torquilla</i>	JKL	S/P	LC
Speckled Piculet	<i>Picumnus innominatus</i>	JK	R	LC
Yellow-crowned Woodpecker	<i>Psilopogon lineatus</i>	J	R	LC
Brown-fronted Woodpecker	<i>Dendrocopos auriceps</i>	JK	R	LC
Rufous-bellied Woodpecker	<i>Dendrocopos hyperythrus</i>	J	R	LC
Fulvous-breasted Woodpecker	<i>Dendrocopos macei</i>	J	S	LC
Grey-capped Pygmy Woodpecker	<i>Yungipicus canicapillus</i>	J	R	LC
Himalayan Woodpecker	<i>Dendrocopos himalayensis</i>	JKL	R	LC
Scaly-bellied Woodpecker	<i>Picus squamatus</i>	JKL	R	LC
Lesser Yellownape	<i>Picus chlorolophus</i>	J	R	LC
Grey-headed Woodpecker	<i>Picus canus</i>	JK	R	LC
Streak-throated Woodpecker	<i>Picus xanthopygaeus</i>	J	S	LC
Black-rumped Flameback	<i>Dinopium benghalense</i>	J	R	LC
Tephrodornithidae (woodshrikes)				
Common Woodshrike	<i>Tephrodornis pondicerianus</i>	J	R	LC
Aegithinidae (loras)				
Common lora	<i>Aegithina tiphia</i>	J	R	LC
Laniidae (shrikes)				
Long-tailed Shrike	<i>Lanius schach</i>	JKL	R/S	LC
Lesser Grey Shrike	<i>Lanius minor</i>	L	P	LC
Great Grey Shrike	<i>Lanius excubitor</i>	KL	S	LC
Iberian Grey Shrike	<i>Lanius meridionalis</i>	L	V	VC
Grey-backed Shrike	<i>Lanius tephronotus</i>	JKL	P	LC
Isabelline Shrike	<i>Lanius isabellinus</i>	JKL	W/P	LC
Bay-backed Shrike	<i>Lanius vittatus</i>	J	R	LC
Red-backed Shrike	<i>Lanius collurio</i>	L	P	LC
Brown Shrike	<i>Lanius cristatus</i>	J	R	LC
Campephagidae (cuckooshrikes and minivets)				
Black-winged Cuckooshrike	<i>Lalage melaschistos</i>	J	V	LC
Black-headed Cuckooshrike	<i>Lalage melanoptera</i>	JL	S	LC
Large Cuckooshrike	<i>Coracina macei</i>	J	R	LC
Long-tailed Minivet	<i>Pericrocotus ethologus</i>	JKL	S	LC
Scarlet Minivet	<i>Pericrocotus speciosus</i>	J	R	LC
Small Minivet	<i>Pericrocotus cinnamomeus</i>	J	R	LC
Rosy Minivet	<i>Pericrocotus roseus</i>	JK	V	LC
Oriolidae (orioles)				
Eurasian Golden Oriole	<i>Oriolus oriolus</i>	JKL	S	LC
Dicruridae (Drongos)				
Australian Spangled-Drongo	<i>Dicrurus bracteatus</i>	J	V	LC
Black Drongo	<i>Dicrurus macrocerus</i>	JKL	R/V	LC
Ashy Drongo	<i>Dicrurus leucophaeus</i>	JKL	S	LC
Bronzed Drongo	<i>Dicrurus aeneus</i>	J	S	LC
Hair-crested Drongo	<i>Dicrurus hottentottus</i>	J	W	LC
Corvidae (Jays, magpies, and crows)				
Kashmir Nutcracker	<i>Nucifraga multipunctata</i>	JK	R	LC
Eurasian Jackdaw	<i>Corvus monedula</i>	KL	R/S	LC
Red-billed Chough	<i>Pyrrhocorax pyrrhocorax</i>	JKL	R	LC
Yellow-billed Chough	<i>Pyrrhocorax graculus</i>	KL	R	LC
Rook	<i>Corvus frugilegus</i>	JKL	W	LC
Carion Crow	<i>Corvus corone</i>	KL	S/R	LC
House Crow	<i>Corvus splendens</i>	JKL	R/V	LC
Hooded Crow	<i>Corvus cornix</i>	L	R	LC
Large-billed Crow	<i>Corvus macrorhynchos</i>	KL	R	LC
Common Raven	<i>Corvus corax</i>	JKL	R/W	LC
Eurasian Magpie	<i>Pica pica</i>	L	R	LC

Eurasian Jay	<i>Garrulus glandarius</i>	JK	R	LC
Black-headed Jay	<i>Garrulus lanceolatus</i>	JKL	R	LC
Yellow-billed Blue Magpie	<i>Urocissa flavirostris</i>	JK	R	LC
Red-billed Blue-Magpie	<i>Urocissa erythroryncha</i>	J	S	LC
Rufous Treepie	<i>Dendrocitta vagabunda</i>	J	R	LC
Grey Treepie	<i>Dendrocitta formosae</i>	JK	R	LC
Remizidae				
White-crowned Penduline Tit	<i>Remiz coronatus</i>	L	V	LC
Paridae (tits)				
Ground Tit	<i>Pseudopodoces humilis</i>	L	R	LC
Fire-capped Tit	<i>Cephalopyrus flammiceps</i>	JKL	S	LC
Rufous-vented Tit	<i>Periparus rubidiventris</i>	JK	R	LC
Rufous-naped Tit	<i>Periparus rufonuchalis</i>	JKL	R	LC
Coal Tit	<i>Periparus ater</i>	JKL	R	LC
Grey-crested Tit	<i>Lophophanes dichrous</i>	J	R	LC
Yellow-browed Tit	<i>Sylviparus modestus</i>	KL	V	LC
Cinereous Tit	<i>Parus cinereus</i>	JKL	R/S	LC
Green-backed Tit	<i>Parus monticolus</i>	JKL	R	LC
Himalayan Black-lored Tit	<i>Macholophus xanthogenys</i>	J	R	LC
Azure Tit	<i>Cyanistes cyanus</i>	L	P	LC
Aegithalidae				
White-browed Tit Warbler	<i>Leptopoecile sophiae</i>	L	R	LC
Black-throated Tit	<i>Aegithalos concinnus</i>	JK	R	LC
White-throated Tit	<i>Aegithalos niveogularis</i>	JK	R	LC
White-cheeked Tit	<i>Aegithalos leucogenys</i>	L	P	LC
Aludidae (larks)				
Bengal Bushlark	<i>Mirafra assamica</i>	J	R	LC
Indian Bushlark	<i>Mirafra erythroptera</i>	J	P	LC
Ashy-crowned Sparrow Lark	<i>Eremopterix griseus</i>	J	R	LC
Oriental Skylark	<i>Alauda gulgula</i>	JKL	S	LC
Eurasian Skylark	<i>Alauda arvensis</i>	JKL	W	LC
Crested Lark	<i>Galerida cristata</i>	JKL	R/P	LC
Bimaculated Lark	<i>Melanocorypha bimaculate</i>	L	P	LC
Tibetan Lark	<i>Melanocorypha maxima</i>	L	S	LC
Horned Lark	<i>Eremophila alpestris</i>	KL	R	LC
Desert Lark	<i>Ammomanes deserti</i>	J	V	LC
Greater Short-toed Lark	<i>Calandrella brachydactyla</i>	JKL	P	LC
Asian Short-toed Lark	<i>Alaudala cheleensis</i>	L	V	LC
Hume's Short-toed Lark	<i>Calandrella acutirostris</i>	L	S	LC
Sand Lark	<i>Alaudala raytal</i>	J	R	LC
Pycnonotidae (bulbuls)				
Red-vented Bulbul	<i>Pycnonotus cafer</i>	J	R	LC
Himalayan Bulbul	<i>Pycnonotus leucogenys</i>	JKL	R	LC
Black Bulbul	<i>Hypsipetes leucocephalus</i>	JK	R	LC
Red-whiskered Bulbul	<i>Pycnonotus jocosus</i>	J	R	LC
Hirundinidae (swallows and martins)				
Plain Martin	<i>Riparia paludicola</i>	JKL	R/P	LC
Pale Martin	<i>Riparia diluta</i>	KL	S	LC
Grey-throated Martin	<i>Riparia chinensis</i>	JL	P	LC
Eurasian Crag-Martin	<i>Ptyonoprogne rupestris</i>	KL	S	LC
Asian House-Martin	<i>Delichon dasypus</i>	KL	S	LC
Common House-Martin	<i>Delichon urbicum</i>	KL	S	LC
Bank Swallow	<i>Riparia riparia</i>	JKL	S	LC
Streak-throated Swallow	<i>Petrochelidon fluvicola</i>	J	R	LC
Barn Swallow	<i>Hirundo rustica</i>	JKL	S/P	LC
Red-rumped Swallow	<i>Cecropis daurica</i>	JKL	S/P	LC
Wire-tailed Swallow	<i>Hirundo smithii</i>	JKL	S/P	LC
Cisticolidae (prinias and tailorbirds)				
Ashy Prinia	<i>Prinia socialis</i>	J	R	LC
Grey-breasted Prinia	<i>Prinia hodgsonii</i>	JKL	R	LC
Jungle Prinia	<i>Prinia sylvatica</i>	J	R	LC
Yellow-bellied Prinia	<i>Prinia flaviventris</i>	J	R	LC
Plain Prinia	<i>Prinia inornate</i>	JK	R	LC
Rufous-fronted Prinia	<i>Prinia buchanani</i>	J	R	LC
Zitting Cisticola	<i>Cisticola juncidis</i>	J	R	LC
Golden-headed Cisticola	<i>Cisticola exilis</i>	J	P	LC
Himalayan Prinia	<i>Prinia crinigera</i>	JK	R	LC

Graceful Prinia	<i>Prinia gracilis</i>	J	R	LC
Common Tailorbird	<i>Orthotomus sutorius</i>	J	R	LC
Delicate Prinia	<i>Prinia lepida</i>	J	P	LC
Acrocephalidae (reed warblers)				
Clamorous Reed Warbler	<i>Acrocephalus stentoreus</i>	KL	S	LC
Black-browed Reed Warbler	<i>Acrocephalus bistrigiceps</i>	L	V	LC
Blyth's Reed Warbler	<i>Acrocephalus dumetorum</i>	JKL	P	LC
Moustached Warbler	<i>Acrocephalus melanopogon</i>	J	W	LC
Paddyfield Warbler	<i>Acrocephalus Agricola</i>	JKL	P	LC
Blunt-winged Warbler	<i>Acrocephalus concinens</i>	K	S	LC
Sedge Warbler	<i>Acrocephalus schoenobaenus</i>	L	V	LC
Great Reed Warbler	<i>Acrocephalus arundinaceus</i>	L	V	LC
Sykes's Warbler	<i>Iduna rama</i>	JKL	P	LC
Booted Warbler	<i>Iduna caligata</i>	KL	P	LC
Thick-billed Warbler	<i>Arundinax aedon</i>	JK	W	LC
Scotocercidae (bush warblers)				
Brownish-flanked Bush Warbler	<i>Horornis fortipes</i>	JKL	S	LC
Grey-sided Bush Warbler	<i>Cettia brunnifrons</i>	JK	R	LC
Aberrant Bush Warbler	<i>Horornis flavolivaceus</i>	K	W	LC
Locustellidae (locustella, warblers, and grassbirds)				
Long-billed Bush Warbler	<i>Locustella major</i>	KL	S	NT
Common Grasshopper Warbler	<i>Locustella naevia</i>	JL	P	LC
Striated Grassbird	<i>Cincloramphus palustris</i>	J	P	LC
Bristled Grassbird	<i>Schoenicola striatus</i>	J	R	VU
Sylviidae (sylvia warblers)				
Barred Warbler	<i>Curruca nisoria</i>	L	P	LC
Asian Desert Warbler	<i>Curruca nana</i>	J	P	LC
Garden Warbler	<i>Sylvia borin</i>	L	P	LC
Greater whitethroat	<i>Curruca communis</i>	JKL	R	LC
Lesser Whitethroat	<i>Curruca curruca</i>	JKL	S	LC
Hume's Whitethroat	<i>Sylvia althaea</i>	KL	S	LC
Phylloscopidae (leaf warblers)				
Common Chiffchaff	<i>Phylloscopus collybita</i>	JKL	W/P	LC
Mountain Chiffchaff	<i>Phylloscopus sindianus</i>	KL	P/S	LC
Plain Leaf-warbler	<i>Phylloscopus neglectus</i>	KL	V	LC
Dusky Warbler	<i>Phylloscopus fuscatus</i>	L	P	LC
Sulphur-bellied Warbler	<i>Phylloscopus griseolus</i>	JKL	S	LC
Tickell's Leaf Warbler	<i>Phylloscopus affinis</i>	JKL	S	LC
Tytler's Leaf Warbler	<i>Phylloscopus tytleri</i>	JKL	P/S	LC
Greenish Warbler	<i>Phylloscopus trochiloides</i>	JKL	P/S	LC
Green Warbler	<i>Phylloscopus nitidus</i>	J	P	LC
Wood Warbler	<i>Phylloscopus sibilatrix</i>	L	R	LC
Yellow-browed Warbler	<i>Phylloscopus inornatus</i>	L	R	LC
Large-billed Leaf Warbler	<i>Phylloscopus magnirostris</i>	KL	S	LC
Blyth's Leaf Warbler	<i>Phylloscopus reguloides</i>	K	S	LC
Western Crowned Warbler	<i>Phylloscopus occipitalis</i>	JKL	S/P	LC
Hume's Warbler	<i>Phylloscopus humei</i>	JKL	S/P	LC
Brooks's Leaf Warbler	<i>Phylloscopus subviridis</i>	KL	W	LC
Buff-barred Warbler	<i>Phylloscopus pulcher</i>	K	S	LC
Lemon-rumped Warbler	<i>Phylloscopus chloronotus</i>	JKL	S	LC
Ashy-throated Warbler	<i>Phylloscopus maculipennis</i>	J	R	LC
Grey-hooded Warbler	<i>Phylloscopus xanthoschistos</i>	JK	R	LC
Whistler's Warbler	<i>Phylloscopus whistleri</i>	JKL	S	LC
Sylviidae				
Yellow-eyed Babbler	<i>Chrysomma sinense</i>	J	R	LC
Timaliidae				
Black-chinned Babbler	<i>Cyanoderma pyrrhops</i>	J	R	LC
Rusty-cheeked Scimitar-babbler	<i>Erythrogonys erythrogonys</i>	J	R	LC
White-browed Scimitar-Babbler	<i>Pomatorhinus schisticeps</i>	J	R	LC
White-browed Shrike-babbler	<i>Pteruthius aeralatus</i>	JK	V	LC
Puff-throated Babbler	<i>Pellorneum ruficeps</i>	J	V	LC
Chestnut-tailed Minla	<i>Actinodura strigula</i>	J	R	LC
Vireonidae				
Green Shrike-Babbler	<i>Pteruthius xanthochlorus</i>	J	R	LC
Pellorneidae				
Rufous-vented Grass Babbler	<i>Laticilla burnesii</i>	J	R	NT

Leiothrichidae					
Common Babbler	<i>Argya caudata</i>	JK	R	LC	
Striated Babbler	<i>Argya earlei</i>	J	R	LC	
Jungle Babbler	<i>Argya striata</i>	J	R	LC	
Red-billed Leiothrix	<i>Leiothrix lutea</i>	JK	R	LC	
Rufous Sibia	<i>Heterophasia capistrata</i>	JK	R	LC	
Streaked Laughingthrush	<i>Trochalopteron lineatum</i>	JKL	R	LC	
Variegated Laughingthrush	<i>Trochalopteron variegatum</i>	JK	R	LC	
White-crested Laughingthrush	<i>Garrulax leucolophus</i>	J	R	LC	
White-throated Laughingthrush	<i>Pterorhinus albogularis</i>	J	P	LC	
Chestnut-crowned Laughingthrush	<i>Trochalopteron erythrocephalum</i>	J	W	LC	
Pnoepyidae					
Scaly-breasted Cupwing	<i>Pnoepyga albiventer</i>	J	R	LC	
Zosteropidae (white-eyes)					
Whiskered Yuhina	<i>Yuhina flavicollis</i>	JK	R	LC	
Indian White-eye	<i>Zosterops palpebrosus</i>	JKL	R	LC	
Regulidae (goldcrest)					
Goldcrest	<i>Regulus regulus</i>	JKL	R/P	LC	
Troglodytidae (wren)					
Eurasian Wren	<i>Troglodytes troglodytes</i>	JKL	R	LC	
Sittidae (nuthatches)					
Kashmir Nuthatch	<i>Sitta cashmirensis</i>	K	R	LC	
White-cheeked Nuthatch	<i>Sitta leucopsis</i>	JKL	R	LC	
Chesnut-bellied Nuthatch	<i>Sitta cinnmoventris</i>	J	R	LC	
Tichodromidae					
Wallcreeper	<i>Tichodroma muraria</i>	JKL	W/R	LC	
Certhiidae (treecreepers)					
Bar-tailed Treecreeper	<i>Certhia himalayana</i>	JKL	W/R	LC	
Hodgson's Treecreeper	<i>Certhia hodgsoni</i>	JKL	R	LC	
Sturnidae (starlings and mynas)					
European Starling	<i>Sturnus vulgaris</i>	JKL	W/S	LC	
Spot-winged Starling	<i>Saroglossa spilopetra</i>	K	V	LC	
Brahminy Starling	<i>Sturnia pagodarum</i>	JKL	S	LC	
Chestnut-tailed Starling	<i>Sturnia malabarica</i>	J	W	LC	
Rosy Starling	<i>Pastor roseus</i>	JKL	W/P	LC	
Indian Pied Starling	<i>Gracupica contra</i>	JL	R	LC	
Bank Myna	<i>Acridotheres ginginianus</i>	J	R	LC	
Common Myna	<i>Acridotheres tristis</i>	JK	R	LC	
Jungle Myna	<i>Acridotheres fuscus</i>	J	R	LC	
Turdidae (thrushes)					
Black-throated Thrush	<i>Turdus atrogularis</i>	JKL	W	LC	
Red-throated Thrush	<i>Turdus ruficollis</i>	JKL	W	LC	
Dusky Thrush	<i>Turdus eunomus</i>	L	V	LC	
Tickell's Thrush	<i>Turdus unicolor</i>	JKL	S	LC	
Mistle Thrush	<i>Turdus viscivorus</i>	JKL	R	LC	
Tibetan Blackbird	<i>Turdus maximus</i>	KL	V/R	LC	
Grey-winged Blackbird	<i>Turdus boulboul</i>	JK	R	LC	
Chestnut Thrush	<i>Turdus rubrocanus</i>	KL	R	LC	
Alpine Thrush	<i>Zoothera mollissima</i>	KL	R	LC	
Scaly Thrush	<i>Zoothera dauma</i>	JKL	S	LC	
Orange-headed Thrush	<i>Geokichla citrina</i>	JK	S	LC	
Song Thrush	<i>Turdus philomelos</i>	L	R	LC	
Naumann's Thrush	<i>Turdus naumanni</i>	L	R	LC	
Grandala	<i>Grandala coelicolor</i>	KL	R	LC	
Muscicapidae (flycatchers)					
Blue Whistling-thrush	<i>Myiophonus caeruleus</i>	JKL	R	LC	
Chestnut-bellied Rock-Thrush	<i>Monticola rufiventris</i>	J	R	LC	
Blue Rock-Thrush	<i>Monticola solitarius</i>	KL	R	LC	
Blue-capped Rock-Thrush	<i>Monticola cinclorhyncha</i>	JK	R	LC	
Rufous-tailed Rock-Thrush	<i>Monticola saxatilis</i>	L	P	LC	
Himalayan Bluetail	<i>Tarsiger rufilatus</i>	JKL	R	LC	
Indian Blue Robin	<i>Larvivora brunnea</i>	JK	S	LC	
Golden bush-robin	<i>Tarsiger chrysaesus</i>	K	S	LC	
Oriental Magpie-Robin	<i>Copsychus saularis</i>	JKL	R	LC	
Rufous-tailed Scrub-Robin	<i>Cercotrichas galactotes</i>	L	S	LC	
Indian Robin	<i>Copsychus fulicatus</i>	J	R	LC	
Bluethroat	<i>Luscinia svecica</i>	JKL	W/P	LC	

Himalayan Rubythroat	<i>Calliope pectoralis</i>	JKL	P/S	LC
Siberian Rubythroat	<i>Calliope calliope</i>	J	W	LC
Chinese Rubythroat	<i>Calliope tschebaiewi</i>	L	R	LC
Brown Rock-chat	<i>Oenanthe fusca</i>	JK	R	LC
Little Forktail	<i>Enicurus scouleri</i>	KL	R	LC
Spotted Forktail	<i>Enicurus maculatus</i>	JK	R	LC
White-bellied Redstart	<i>Luscinia phaenicuroides</i>	KL	R	LC
Black Redstart	<i>Phoenicurus ochruros</i>	JKL	P/S	LC
Rufous-backed Redstart	<i>Phoenicurus erythronotus</i>	JKL	W	LC
Blue-capped Redstart	<i>Phoenicurus coeruleocephala</i>	JKL	R/S	LC
Blue-fronted Redstart	<i>Phoenicurus frontalis</i>	JKL	R/S	LC
White-winged Redstart	<i>Phoenicurus erythrogaster</i>	KL	W/S	LC
Common Redstart	<i>Phoenicurus phoenicurus</i>	KL	P	LC
Plumbeous Water-redstart	<i>Phoenicurus fuliginosus</i>	JKL	R	LC
White-capped Redstart	<i>Phoenicurus leucocephalus</i>	JKL	R/S	LC
Isabelline Wheatear	<i>Oenanthe isabelline</i>	L	P	LC
Desert Wheatear	<i>Oenanthe deserti</i>	JKL	W/S	LC
Pied Wheatear	<i>Oenanthe pleschanka</i>	KL	S	LC
Variable Wheatear	<i>Oenanthe picata</i>	JKL	W/S	LC
Hume's Wheatear	<i>Oenanthe albonigra</i>	L	V	LC
Red-tailed Wheatear	<i>Oenanthe chrysopygia</i>	J	W	LC
Northern Wheatear	<i>Oenanthe oenanthe</i>	L	R	LC
Pied Bushchat	<i>Saxicola caprata</i>	JKL	S	LC
African Stonechat	<i>Saxicola torquatus</i>	JKL	S/P	LC
White-tailed Stonechat	<i>Saxicola leucurus</i>	JK	W/S	LC
Grey Bushchat	<i>Saxicola ferreus</i>	JK	R	LC
Dark-sided Flycatcher	<i>Muscicapa sibirica</i>	JKL	S	LC
Spotted Flycatcher	<i>Muscicapa striata</i>	KL	P	LC
Asian Brown Flycatcher	<i>Muscicapa dauurica</i>	JKL	S/V	LC
Rusty-tailed Flycatcher	<i>Ficedula ruficauda</i>	JKL	S	LC
Ultramarine Flycatcher	<i>Ficedula supercilialis</i>	JK	S	LC
Kashmir Flycatcher	<i>Ficedula subrubra</i>	K	S	VU
Red-breasted Flycatcher	<i>Ficedula parva</i>	JKL	W/P	LC
Rufous-gorgeted Flycatcher	<i>Ficedula strophilata</i>	J	R	LC
Taiga Flycatcher	<i>Ficedula albicilla</i>	JKL	W	LC
Slaty-blue Flycatcher	<i>Ficedula tricolor</i>	JK	W/S	LC
Little Pied Flycatcher	<i>Ficedula westermanni</i>	K	S	LC
Tickell's Blue Flycatcher	<i>Cyornis tickelliae</i>	JK	R	LC
Blue-throated Blue Flycatcher	<i>Cyornis rubeculoides</i>	JKL	S	LC
Rufous-bellied Niltava	<i>Niltava sundara</i>	JK	W	LC
Verditer Flycatcher	<i>Eumyias thalassinus</i>	JK	S	LC
Stenostiridae (fantail, canary flycatcher)				
Yellow-bellied Fairy-Fantail	<i>Chelidorhynch hypoxantha</i>	JK	W	LC
Grey-headed Canary-Flycatcher	<i>Culicicapa ceylonensis</i>	JK	S	LC
Monarchidae (monarch flycatchers)				
Asian paradise-Flycatcher	<i>Terpsiphone paradisi</i>	JKL	S	LC
Black-naped Monarch	<i>Hypothymis azurea</i>	J	P	LC
Rhipiduridae (fantails)				
White-browed Fantail	<i>Rhipidura aureola</i>	J	R	LC
White-throated Fantail	<i>Rhipidura albicollis</i>	J	W	LC
Cinclidae (dippers)				
Brown Dipper	<i>Cinclus pallasii</i>	JKL	R	LC
White-throated Dipper	<i>Cinclus cinclus</i>	KL	R	LC
Dicaeidae (flowerpeckers)				
Thick-billed Flowerpecker	<i>Dicaeum agile</i>	J	R	LC
Fire-breasted Flowerpecker	<i>Dicaeum ignipectus</i>	J	R	LC
Pale-billed Flowerpecker	<i>Dicaeum erythrorhynchos</i>	J	R	LC
Nectariniidae (sunbirds and spiderhunters)				
Purple Sunbird	<i>Cinnyris asiaticus</i>	JL	S	LC
Crimson Sunbird	<i>Aethopyga siparaja</i>	J	R	LC
Mrs. Gould's Sunbird	<i>Aethopyga gouldiae</i>	J	S	LC
Pittidae family				
Indian Pitta	<i>Pitta brachyura</i>	J	R	LC
Eurylaimidae				
Long-tailed Broadbill	<i>Psarisomus dalhousiae</i>	J	W/S	LC
Bombycillidae				
Bohemian Waxwing	<i>Bombycilla garrulus</i>	K	S	LC

Ploceidae (weavers)					
Baya Weaver	<i>Ploceus philippinus</i>	J	R	LC	
Streaked Weaver	<i>Ploceus manyar</i>	J	R	LC	
Black-breasted Weaver	<i>Ploceus benghalensis</i>	J	R	LC	
Estrildidae (avadavats and munias)					
Red Avadavat	<i>Amandava amandava</i>	J	R	LC	
Indian Silverbill	<i>Euodice malabarica</i>	J	R	LC	
Scaly-breasted Munia	<i>Lonchura punctulate</i>	JK	R/S	LC	
Tricoloured Munia	<i>Lonchura malacca</i>	J	R	LC	
Prunellidae (accentors)					
Altai Accentor	<i>Prunella himalayana</i>	KL	W	LC	
Alpine Accentor	<i>Prunella collaris</i>	KL	R	LC	
Robin Accentor	<i>Prunella rubeculoides</i>	KL	R	LC	
Rufous-breasted Accentor	<i>Prunella strophia</i>	JKL	R/S	LC	
Black-throated Accentor	<i>Prunella atrogularis</i>	JKL	W/P	LC	
Brown Accentor	<i>Prunella fulvescens</i>	KL	R	LC	
Motacillidae (wagtails and pipits)					
Forest Wagtail	<i>Dendronanthus indicus</i>	JKL	P	LC	
Western Yellow Wagtail	<i>Motacilla flava</i>	KL	P	LC	
Eastern Yellow Wagtail	<i>Motacilla tschutschensis</i>	J	P	LC	
Grey Wagtail	<i>Motacilla cinerea</i>	JKL	S	LC	
Citrine Wagtail	<i>Motacilla citreola</i>	JKL	S	LC	
White Wagtail	<i>Motacilla alba</i>	JKL	S	LC	
White-browed Wagtail	<i>Motacilla maderaspatensis</i>	JL	R/V	LC	
Olive-backed Pipit	<i>Anthus hodgsoni</i>	JKL	V	LC	
Tree Pipit	<i>Anthus trivialis</i>	JKL	P	LC	
Red-throated Pipit	<i>Anthus cervinus</i>	KL	P	LC	
Rosy Pipit	<i>Anthus roseatus</i>	JKL	W/S/P	LC	
Water Pipit	<i>Anthus spinoletta</i>	JKL	P	LC	
Paddyfield Pipit	<i>Anthus rufulus</i>	JKL	R	LC	
Tawny Pipit	<i>Anthus campestris</i>	J	W	LC	
Long-billed Pipit	<i>Anthus similis</i>	JK	P/S	LC	
Upland Pipit	<i>Anthus sylvanus</i>	JK	R	LC	
Richard's Pipit	<i>Anthus richardi</i>	K	W	LC	
American Pipit	<i>Anthus rubescens</i>	JL	P	LC	
Meadow Pipit	<i>Anthus pratensis</i>	L	R	LC	
Passeridae (sparrows and snowfinches)					
House Sparrow	<i>Passer domesticus</i>	JKL	R	LC	
Russet Sparrow	<i>Passer cinnamomeus</i>	JKL	R	LC	
Spanish Sparrow	<i>Passer hispaniolensis</i>	KL	V	LC	
Sind Sparrow	<i>Passer pyrrhonotus</i>	J	R	LC	
Eurasian Tree Sparrow	<i>Passer montanus</i>	L	R	LC	
Rock Sparrow	<i>Petronia petronia</i>	L	V	LC	
Yellow-throated Sparrow	<i>Gymnoris xanthocollis</i>	J	S	LC	
Tibetan Snowfinch	<i>Montifringilla henrici</i>	L	R	LC	
Blanford's Snowfinch	<i>Pyrgilauda blanfordi</i>	L	R	LC	
Rufous-necked Snowfinch	<i>Montifringilla ruficollis</i>	L	P	LC	
White-rumped Snowfinch	<i>Montifringilla taczanowskii</i>	L	R	LC	
Black-winged Snowfinch	<i>Montifringilla adamsi</i>	KL	R	LC	
Fringillidae (finches)					
Common chaffinch	<i>Fringilla coelebs</i>	K	W	LC	
Brambling	<i>Fringilla montifringilla</i>	KL	W	LC	
Plain Mountain-finch	<i>Leucosticte nemoricola</i>	JKL	W/R	LC	
Black-headed Mountain Finch	<i>Leucosticte brandti</i>	L	R	LC	
Eurasian Linnet	<i>Linaria cannabina</i>	L	P	LC	
Twite	<i>Linaria flavirostris</i>	KL	R	LC	
Eurasian Siskin	<i>Spinus spinus</i>	KL	P	LC	
Red Crossbill	<i>Loxia curvirostra</i>	KL	R	LC	
Desert Finch	<i>Rhodospiza obsoleta</i>	L	P	LC	
Crimson-winged Finch	<i>Rhodopechys sanguineus</i>	L	R	LC	
Trumpeter Finch	<i>Bucanetes githagineus</i>	L	R	LC	
Yellow-breasted Greenfinch	<i>Chloris spinoides</i>	JKL	S	LC	
Fire-fronted Serin	<i>Serinus pusillus</i>	KL	R	LC	
European Goldfinch	<i>Carduelis carduelis</i>	KL	R/S	LC	
Spectacled Finch	<i>Callacanthus burtoni</i>	K	R	LC	
Mongolian Finch	<i>Bucanetes mongolicus</i>	L	R	LC	
Common Rosefinch	<i>Carpodacus erythrinus</i>	JKL	P/S	LC	

Pink-browed Rosefinch	<i>Carpodacus rodochroa</i>	JKL	S	LC
Red-mantled Rosefinch	<i>Carpodacus rhodochlamys</i>	KL	R/S	LC
Red-fronted Rosefinch	<i>Carpodacus puniceus</i>	JKL	R	LC
Streaked Rosefinch	<i>Carpodacus rubicilloides</i>	L	R	LC
Great Rosefinch	<i>Carpodacus rubicilla</i>	L	R	LC
Himalayan White-browed Rosefinch	<i>Carpodacus thura</i>	KL	R/S	LC
Blyth's Rosefinch	<i>Carpodacus grandis</i>	KL	R	LC
Sillem's Rosefinch	<i>Carpodacus sillemi</i>	L	W/S	LC
Pale Rosefinch	<i>Carpodacus stoliczkae</i>	L	W/S	LC
Dark-breasted Rosefinch	<i>Procarduelis nipalensis</i>	JK	S	LC
Orange Bullfinch	<i>Pyrrhula aurantiaca</i>	K	R	LC
Red-headed Bullfinch	<i>Pyrrhula erythrocephala</i>	J	P	LC
Hawfinch	<i>Coccothraustes coccothraustes</i>	JKL	V	LC
Black-and-yellow Grosbeak	<i>Mycerobas icterioides</i>	JK	R	LC
White-winged Grosbeak	<i>Mycerobas carnipes</i>	JKL	R	LC
Collared Grosbeak	<i>Mycerobas affinis</i>	K	W	LC
Emberizidae (buntings)				
Crested Bunting	<i>Emberiza lathami</i>	JKL	S	LC
Yellowhammer	<i>Emberiza citrinella</i>	KL	W	LC
Common Reed Bunting	<i>Emberiza schoeniclus</i>	KL	W	LC
Little Bunting	<i>Emberiza pusilla</i>	KL	P	LC
White-capped Bunting	<i>Emberiza stewartia</i>	JKL	P/S	LC
Chestnut-eared Bunting	<i>Emberiza fucata</i>	KL	R	LC
Rock Bunting	<i>Emberiza cia</i>	JKL	R/S	LC
Pine Bunting	<i>Emberiza leucocephalos</i>	JKL	W	LC
Ortolan Bunting	<i>Emberiza hortulana</i>	L	P	LC
Red-headed Bunting	<i>Emberiza bruniceps</i>	JKL	P	LC
Black-headed Bunting	<i>Emberiza melanocephala</i>	J	P	LC
Yellow-breasted Bunting	<i>Emberiza melanocephala</i>	JK	S/W	LC
Grey-necked Bunting	<i>Emberiza buchanani</i>	KL	P	LC
Rustic Bunting	<i>Emberiza rustica</i>	L	P	VU
Striolated Bunting	<i>Emberiza striolata</i>	JK	S/W	LC
Otididae				
Little Bustard	<i>Tetrax tetrax</i>	L	P	NT

Furthermore, 77 species (11.4%) occur in Jammu and Kashmir but not Ladakh, highlighting the unique species composition in these regions. Similarly, 24 species (3.55%) are restricted to Jammu and Ladakh, while 80 species (11.85%) are exclusive to Kashmir and Ladakh. Moreover, 152 species (22.5%) are found solely in Jammu, reflecting the distinct avifauna present in this region. Additionally, 26 species (3.84%) are confined to Kashmir, and 71 species (10.51%) are exclusively found in Ladakh (Figure 4). These distribution patterns emphasize the importance of considering the ecological nuances and habitat variations across the three regions when implementing conservation strategies.

Notably, the bird diversity of Jammu surpasses that of Kashmir and Ladakh. This discrepancy can be attributed to the sub-tropical climate prevalent in Jammu, which is more similar to other Indian states. Consequently, many of the bird species found in Jammu can also be observed in other parts of India. On the other hand, the unique climates of Kashmir and Ladakh create conditions conducive to the presence of species not commonly found in other regions of the country. Therefore, bird enthusiasts and researchers must visit Kashmir and Ladakh to witness the distinct avian fauna that thrives in these areas. Some bird species in these regions are depicted in Figure 5. Despite the remarkable avian diversity in Jammu and Kashmir, the state lags behind the rest of the country regarding avian research. The

scientific investigation of birds in the region has been limited, with only a few studies focusing on specific species or groups. This knowledge gap hampers our understanding of various bird species' ecology and conservation needs, particularly those facing significant threats.

Furthermore, it is crucial to prioritize comprehensive and systematic scientific research on a wide range of bird species to address this gap and improve our understanding of the region's avian ecology. Such research endeavors should encompass various aspects, including species distribution, habitat preferences, breeding behavior, migratory patterns, and responses to environmental changes. By conducting rigorous scientific studies, we can gain valuable insights into the ecology of birds in Jammu and Kashmir and develop targeted conservation strategies tailored to the specific needs of different species. Furthermore, effective bird conservation requires collaborative efforts among researchers, conservationists, policymakers, and local communities. It is essential to raise awareness about the significance of avian diversity, promote responsible birdwatching practices, and involve local communities in conservation initiatives. The involvement of stakeholders from diverse backgrounds is crucial for successfully implementing conservation measures and preserving bird species in Jammu and Kashmir.

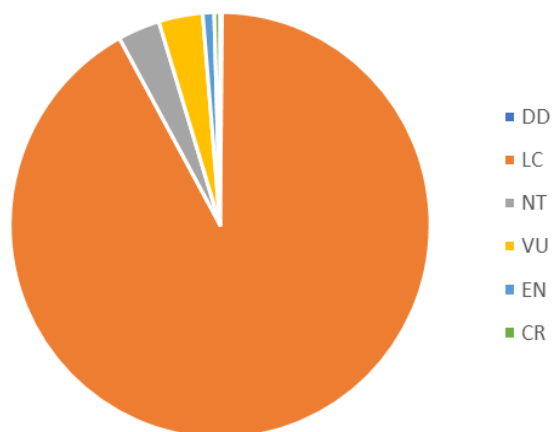


Figure 3. Graph showing the number of birds under different IUCN categories

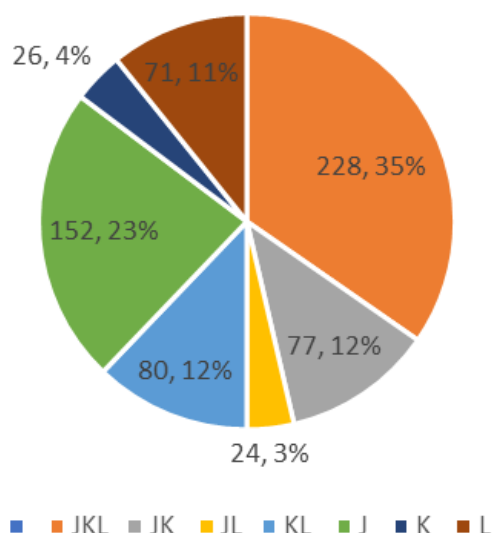


Figure 4. Number of bird species found in different regions of Jammu, Kashmir and Ladakh (India)

Therefore, our study highlights the diverse avian fauna in Jammu and Kashmir, including Ladakh, with 677 bird species distributed among 89 families. While most species fall under the Least Concern category, it is important to address the threats faced by the 54 threatened species to ensure their survival. The varying distributions of bird species across the three regions emphasize the need to consider regional differences in ecological conditions when designing conservation strategies. Additionally, the state's limited scientific research on birds underscores the urgency to conduct comprehensive studies to fill knowledge gaps and guide conservation efforts. By prioritizing scientific research, fostering collaboration, and engaging local communities, we can enhance our understanding of avian ecology, promote effective conservation measures, and safeguard the remarkable avifaunal diversity of Jammu and Kashmir, including Ladakh, for future generations.

Table 2. Threatened birds of Jammu, Kashmir and Ladakh, India

Common Name	Scientific Name
Near Threatened	
Ferruginous Duck	<i>Aythya nyroca</i>
Painted Stork	<i>Mycteria leucocephala</i>
Asian Woolly-necked Stork	<i>Ciconia episcopus</i>
Black-necked Stork	<i>Ephippiorhynchus asiaticus</i>
Black-headed Ibis	<i>Threskiornis melanocephalus</i>
Oriental Darter	<i>Anhinga melanogaster</i>
Pallid Harrier	<i>Circus macrourus</i>
Bearded Vulture	<i>Gypaetus barbatus</i>
Himalayan Griffon	<i>Gyps himalayensis</i>
Cinereous Vulture	<i>Aegypius monachus</i>
Laggar Falcon	<i>Falco jugger</i>
Red-necked Falcon	<i>Falco chicquera</i>
Great Thick-knee	<i>Esacus recurvirostris</i>
River Lapwing	<i>Vanellus duvaucelii</i>
Eurasian Curlew	<i>Numenius arquata</i>
Black-tailed Godwit	<i>Limosa limosa</i>
Curlew Sandpiper	<i>Calidris ferruginea</i>
Red-breasted Parakeet	<i>Psittacula alexandri</i>
Alexandrine Parakeet	<i>Psittacula eupatria</i>
Long-billed Bush Warbler	<i>Locustella major</i>
Rufous-vented Grass Babbler	<i>Laticilla burnesii</i>
Little Bustard	<i>Tetrax tetrax</i>
Vulnerable	
Cheer Pheasant	<i>Catreus wallichii</i>
Western Tragopan	<i>Tragopan melanocephalus</i>
Common Pochard	<i>Aythya ferina</i>
Long-tailed Duck	<i>Clangula hyemalis</i>
Marbled Teal	<i>Marmaronetta angustirostris</i>
Lesser White-fronted Goose	<i>Anser erythropus</i>
Horned Grebe	<i>Podiceps auritus</i>
Indian Spotted Eagle	<i>Clanga hastata</i>
Greater Spotted Eagle	<i>Clanga clanga</i>
Tawny Eagle	<i>Aquila rapax</i>
Imperial Eagle	<i>Aquila heliaca</i>
Red-footed Falcon	<i>Falco vespertinus</i>
Black-necked Crane	<i>Grus nigricollis</i>
Sarus Crane	<i>Antigone Antigone</i>
Sharp-tailed Sandpiper	<i>Calidris acuminata</i>
River Tern	<i>Sterna aurantia</i>
Black-legged Kittiwake	<i>Rissa tridactyla</i>
Yellow-eyed Pigeon	<i>Columba eversmanni</i>
European Turtle-Dove	<i>Streptopelia turtur</i>
Bristled Grassbird	<i>Schoenicola striatus</i>
Kashmir Flycatcher	<i>Ficedula subrubra</i>
Rustic Bunting	<i>Emberiza rustica</i>
Endangered	
White-headed Duck	<i>Oxyura leucocephala</i>
Steppe Eagle	<i>Aquila nipalensis</i>
Pallas's Fish-Eagle	<i>Haliaeetus leucoryphus</i>
Egyptian Vulture	<i>Neophron percnopterus</i>
Saker Falcon	<i>Falco cherrug</i>
Black-bellied Tern	<i>Sterna acuticauda</i>
Critically Endangered	
White-rumped Vulture	<i>Gyps bengalensis</i>
Slender-billed Vulture	<i>Gyps tenuirostris</i>
Siberian Crane	<i>Leucogeranus leucogeranus</i>



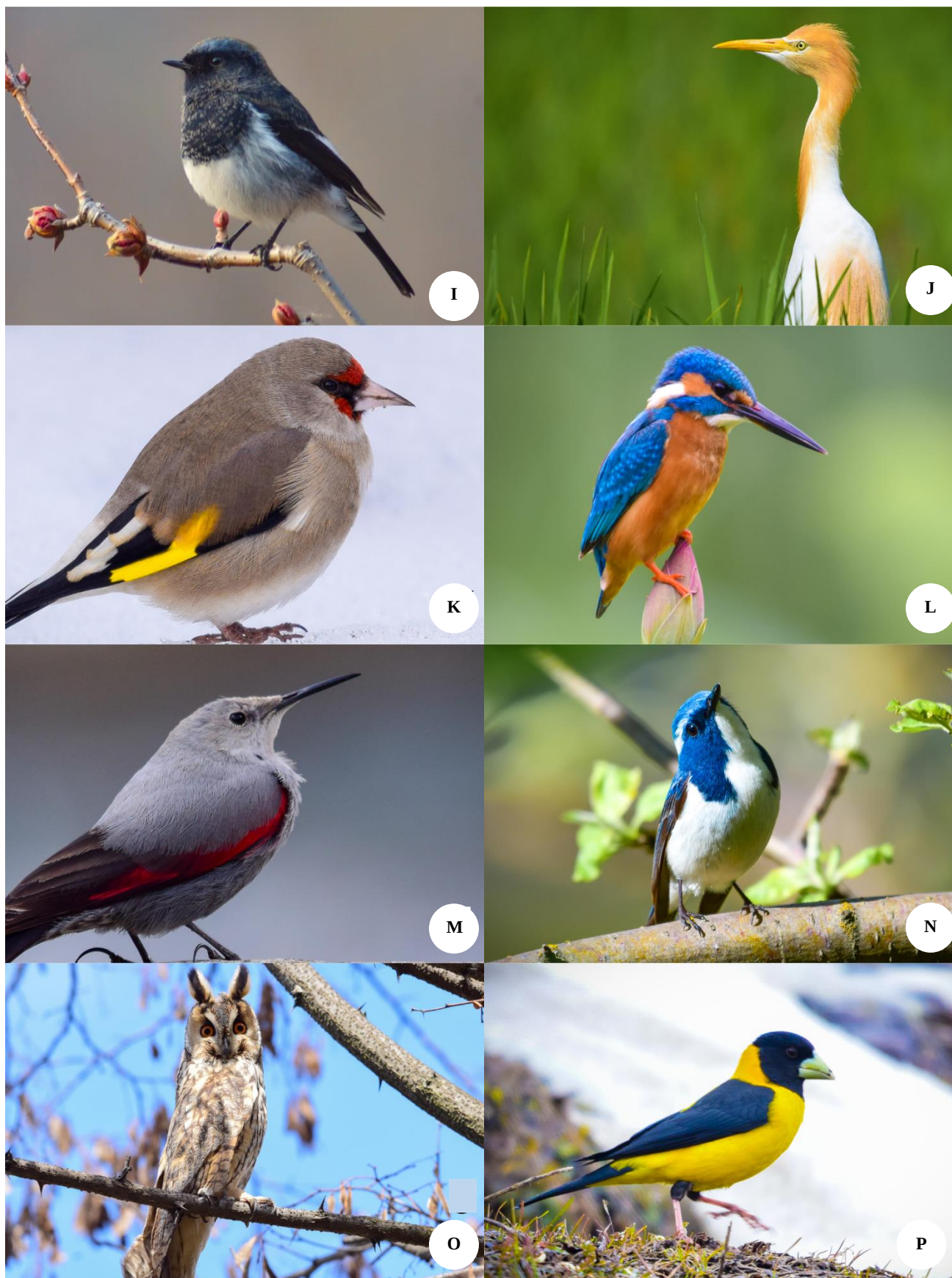


Figure 5. Some birds found in Jammu, Kashmir and Ladakh (India): A. Kashmir Nuthatch, B. Orange Bullfinch, C. Ibis Bill, D. Asian Paradise Flycatcher, E. Kashmir Flycatcher, F. Tawny Owl, G. Demoiselle Crane, H. Himalayan Rubythroat, I. Blue-Capped Redstart, J. Cattle Egret, K. Himalayan Goldfinch, L. Common Kingfisher, M. Wallcreeper, N. Ultramarine Flycatcher, O. Long-Eared Owl, P. Black-And-Yellow Grosbeak. Picture credits: Rouf Rather, Bilal Nasir Zargar, Ovais Shafi, and Sheikh Haris

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The initial complete chloroplast genome of *Ludisia discolor* (Orchidaceae) in Vietnam

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Abstract. Ho VT, Nguyen MP, Trinh TH. 2023. The initial complete chloroplast genome of *Ludisia discolor* (Orchidaceae) in Vietnam. *Nusantara Bioscience* 15: 232-237. *Ludisia discolor* (Ker Gawl.) Blume is traditionally utilized as an effective herb to treat various diseases. Due to its high medicinal value, this herb has been overharvested and faces extinction in a wild population. Morphological characterization to DNA barcode-based techniques are applied to accurately identify this plant for conservation and breeding purposes; nevertheless, they show limitations. This study aimed to characterize complete chloroplast (cp) sequences for effectiveness in plant species' classification and phylogenetic analysis. The cp genome of *L. discolor* collected from Vietnam was initially sequenced, annotated, and compared with published cp genomes from NCBI GenBank to understand its genetic makeup better. The results reveal that the complete cp genome of *L. discolor* is 159,043 bp, consisting of 96 protein-coding genes, 12 rRNA, and 33 tRNA genes. Moreover, 53 simple sequence repeats (SSR) were detected in this genome, most of which are polyA and polyT. Phylogenetic analysis also revealed a significant distance between the cp genomes of *L. discolor* from Vietnam with other *L. discolor* accessions from China. These findings could provide valuable insights into the taxonomy, plant identification, breeding, protection, and conservation programs of *L. discolor* in Vietnam.

Keywords: Chloroplast genome, *Ludisia discolor*, medicinal plant, next-generation sequencing, simple sequence repeat

Abbreviations: LSC: Large Single Copy, NGS: Next-Generation Sequencing, SSC: Small Single Copy, SSR: microsatellite

INTRODUCTION

Ludisia discolor (Ker Gawl.) Blume, an enchanting jewel orchid distributed in East and Southeast Asia, is rich in medicinal compounds used to treat several diseases, such as tuberculosis, hemoptysis, and loss of appetite (Liu et al. 2021). The increasing demand and inadequate management practices have led to its scarcity due to overharvesting (Poobathiy et al. 2019). Consequently, wild *L. discolor* is dying out due to overexploitation in recent years and has been reported in several countries, such as China (Su et al. 2017), Malaysia (Burkhan et al. 2022), and Vietnam (Nguyen and Phung 2023). Moreover, the powder of *L. discolor* in the market was mixed with powders of other jewel orchid species, such as *Anoectochilus roxburghii*, *A. formosanus*, or *Goodyera schlechtendaliana* leading the reduced effectiveness of treatment efficacy (Chai et al. 2022).

Traditionally, the morphological identification of jewel orchids has been employed for conservation purposes. Nonetheless, this approach has limitations, particularly when closely related species share similar morphological characteristics, and the morphological identification is mainly based on personal experience and unproducible due to several jewel orchids possessing resemble features (Ho et al. 2021). Furthermore, different jewel orchid species display a high degree of morphological feature overlap, thereby, the reliability of morphological identification in

question, especially for closely related species sharing similar morphological characteristics.

Numerous researchers applied DNA barcodes to identify several jewel orchid species based on standardized DNA regions to compensate for the drawback of morphological identification. Huynh et al. (2019) applied nine DNA barcode regions to identify six jewel orchid accessions belonging to three species collected from Vietnam. Their obtained data show only ITS 1 and ITS2 regions are applicable to discriminate *Anoectochilus* and *Lusidia* species (Huynh et al. 2019). After screening about 7,000 sequences of 380 medicinal orchid species in Asia, ITS is the best barcode, and the combination of ITS and matK will produce higher discrimination results (Raskoti and Ale 2021). Nevertheless, DNA barcode also shows drawbacks since this technique only applies short DNA sequences, limiting the discrimination power. Gao et al. (2009) reported that ITS sequence could not determine species level in the genus *Anoectochilus* since it is highly assembled to *L. discolor*. Chen and Shiao (2015) reported that ITS and matK did not effectively distinguish *L. discolor* from its hybrid cultivars. The accuracy of this method is also highly dependent on the availability of corresponding sequences in the database.

In recent years, the widespread adoption and increased affordability of next-generation sequencing (NGS) technology have greatly facilitated the comprehensive characterization of the complete chloroplast (cp) genome in

numerous plant species in the family Orchidaceae, such as *Dendrobium nobile* (Konhar et al. 2019), *Paphiopedilum delenatii* (Vu et al. 2020), and *Vanda coerulea* (Wang et al. 2020). The high discrimination power of complete cp genomes comes from significant variation within and between plant species in their structure and sequence (Daniell et al. 2016). This technique has proven to be highly effective in identifying and classifying jewel orchids, as it enables thorough examination of the entire cp sequences. Therefore, Oh et al. (2019a) found the effectiveness in phylogeographic and phylogenetic studies of species in the genus *Goodyera* using NGS. Despite the wide distribution of *L. discolor* across East and Southeast Asia, only a limited number of cp genomes from this plant in China have been sequenced (Yu et al. 2019). In this study, we sequenced, annotated, and compared the cp genome of *L. discolor* originating from Vietnam with published cp sequences. The obtained data could provide valuable information for taxonomy and conservation programs concerning this plant species in Vietnam.

MATERIALS AND METHODS

Plant materials

Ludisia discolor samples were provided by the Biotechnology Center of Ho Chi Minh City (HCMBIOTECH), Vietnam. The remaining cloned plants continue to be cultivated for further studies and conservation.

Procedures

Five grams of fresh leaves were utilized to extract total DNA using the Isolate II Plant DNA Kit (Bioline, UK), following the manufacturer's protocol. The quality and quantity of DNA were assessed through 1% gel electrophoresis and Nanodrop (ThermoScientific, Delaware, USA), respectively.

For library construction, 100–1,000 ng of DNA was fragmented using Covaris S220 through acoustic disruption. The fragmented DNA underwent final repair, dA tailing, adapter ligation, and purification before being selected for library construction. Before sequencing, the effective concentration of each sample library in the library mixture was determined using qPCR to ensure accurate sample concentration and reliable sequencing data. Base calling was performed using the RTA software integrated with the sequencer, which converted the four fluorescence signals obtained from the Charge-Coupled Device (CCD) into binary bcl data in real-time. The bcl data were subsequently transformed into a fastq file using bcl2fastq (v.2.17), a part of the software package provided by Illumina. Concurrent demultiplexing of the data was carried out based on the index information. Primary analysis was conducted using the built-in High-Content Screening (HCS) sequencer software to determine whether the read data passed the chastity filter based on the signal quality of the first 25 cycles. To assess the quality of the raw reads, FastQC was initially used in the Galaxy portal (<http://usegalaxy.org>). Subsequently, the raw reads were

submitted to the Sequence Read Archive (SRA) database in NCBI under the PRJNA965922 project.

Data analysis

The Geseq program (<https://chlorobox.mpimp-golm.mpg.de/geseq.html>) was utilized for gene annotation and localization in the chloroplast genomes (Tillich et al. 2017). Simple sequence repeat (SSR) motifs were identified using MISA (accessible at <http://pgrc.ipk-gatersleben.de/misa/misa.html>) with default parameters (Beier et al. 2017). In addition, to identify the location of extended repetitive regions, commonly known as minisatellites. In DNA sequences, the REPuter software (<http://bibiserv.cebitec.uni-bielefeld.de/reputer>) was with default settings employed as described by Kurtz et al. (2001). This software detected long repeat regions with Hamming distance = 3, a minimum size of 30 base pairs, and a minimum identity of 90%. It categorized the repeats into four types, namely forward (F), reverse (R), complement (C), and palindromic (P).

The MAFFT program (accessible at <https://mafft.cbrc.jp/alignment/server/>) was employed to align the obtained cp sequence with three other cp sequences of *L. discolor* downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/nucleotide/>) with default parameters (Kato et al. 2019). The alignment was then utilized to determine the phylogenetic relationship among the genomes. Phylogenetic trees of the obtained cp sequence and three cp sequences of *L. discolor* downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/nucleotide/>) consisting of MN317571.1, MN880628.1, and NC_030540.1 were constructed using the Neighbor-Joining (NJ) method, a distance-based approach, by using MEGA X software (accessible at <https://www.megasoftware.net/>) with 500 bootstrap replicates and default parameters (Kang et al. 2017). The chloroplast genome of *Dendrobium sinense* (OM792979.1), a common orchid species for ornamental purposes in Vietnam and China, was used as an outgroup.

RESULTS AND DISCUSSION

Chloroplast genomes own notable advantages in phylogenetic and taxonomic analysis, such as moderate rate of nucleotide replacement, significant variation in coding and non-coding genes, moderate genome size, and collinear properties among different plant species make these genomes an important source for genomic comparison (Su et al. 2020). This study generated a dataset of 7.01 GB of 150 bp pair-end reads, resulting in 19,634,198 raw reads. These reads had a Phred score of approximately 35. From this dataset, 19,388,976 clean reads (98.75% of the total) were selected for further analysis. The assembly of the cp genome map revealed a conserved circular structure including four typical structures, namely Large Single Copy (LSC), Small Single Copy (SSC), and two inverted repeats (IRA and IRB) with a total length of 159,043 bp. The genes located in cp genome are depicted inside and outside the cp map (Figure 1), with those outside being

transcribed in a clockwise direction and those inside in a counterclockwise direction. The functions of genes are described in different colors and explained in figure legend. The size of this cp genome is slightly different from three previously published complete cp genomes in this species, namely MN317571.1, MN880628.1, and NC_030540.1, with cp genome lengths of 153,324 bp, 153,373 bp, and 153,054 bp, respectively. So far, the sizes of sequenced cp genomes range from 120 kb to 160 kb, and different explanations for the variation in cp genomes have been proposed, such as variation in intergenic regions within a genus, variation in inverted repeat regions, and gene loss (Zheng et al. 2017). Park et al. (2020) reported the different cp genome sizes between two *Viburnum erosum* accessions from Korea. By analyzing genomes of 17 accessions in *Aegilops tauschii* species collected across large geographic areas from Turkey, Georgia, Iran, Turkmenistan, Kazakhstan, Tajikistan, Afghanistan, Pakistan, and India to Xinjiang, Shannxi and Henan Provinces in China, Su et al. (2020) found that the cp genome length of these accessions

varies from 135,551 to 136,009 bp. Although these genomes were found to be relatively conserved, several structure variations were also noticed among these cp genomes, and this difference was mostly detected in non-coding regions. The variations in cp genome size have also been reported in other jewel orchid species, such as *Goodyera schlechtendalia* (Oh et al. 2019a). The variations in cp genome size were also reported in two accessions of this species collected from China and Korea by Kim and Kim (2022) and in peach (Amar et al. 2019).

Microsatellites, also known as simple sequence repeats (SSRs), are distributed with high frequency in the cp genomes and are considered to play important roles in the evolution and adaptation of plants. These repeat motifs are commonly employed to detect genomic variations among species. This study applied the MISA program to identify the presence and abundance of SSRs within the obtained cp genome. The program utilized default parameters, focusing on tandem repeat sequences comprising 1-6 nucleotide repeat units.

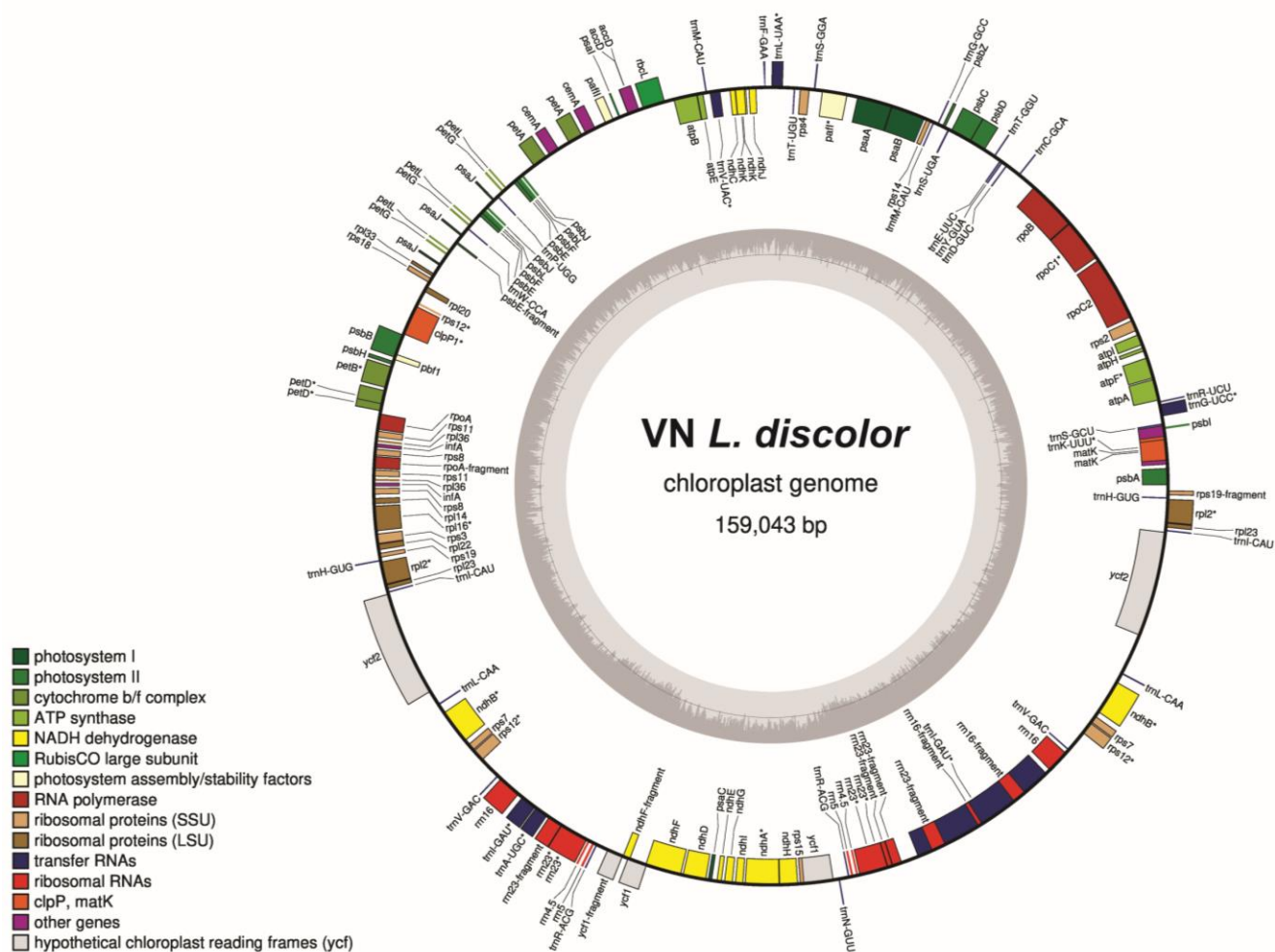


Figure 1. The circular representation of *L. discolor* chloroplast (cp) genome from Vietnam. The genes are depicted inside and outside the circle, with those outside being transcribed in a clockwise direction and those inside in a counterclockwise direction. The inner circle is divided into two regions, with the dark gray color indicating the GC content and the light gray color representing the AT content. Furthermore, the genes are color-coded based on their functional groups.

Moreover, 53 SSRs were detected in the cp genome (Table 1). These SSR motifs encompassed five types: A, T, C, AT, and TA. Among them, mononucleotide motifs consisting of T and A were the most prevalent, accounting for 69.8% (37 SSRs) and 28.3% (15 SSRs) of the total, respectively. Conversely, the genome contained only one PolyC in NC_030540.1, while no polyG was detected. Interestingly, only *L. discolor* from Vietnam shows the presence of the TA motif. It is worth noting that short polyA and polyT repeats are commonly observed in cp genomes, while polyG or polyC repeats are relatively rare (Lei et al. 2016). The different SSR patterns within the cp genome of a plant species have been reported previously. *In silico* comparative analysis, up to polymorphic 49 SSR loci were detected among cp genomes of four *Liriodendron chinense* accessions (Li et al. 2020). The difference in the number and types of SSR motifs was also reported recently in different accessions of *Tetrastigma hemsleyanum* in the family Vitaceae (Dong et al. 2022). Furthermore, this study also reported the variation of SSR numbers corresponding to the regions samples collected. Microsatellites in the cp genome are inherited from a single parent and are frequently employed as molecular markers in evolutionary studies, including investigations of genetic diversity and species identification. The TA motif identified in this study has the potential for evolutionary research on *L. discolor* from Vietnam and helps identify and conserve different species within this genus.

The REPuter tool applied in this study shows the possibility of identifying long repeated regions, known as minisatellites, containing 10 to 100 nucleotides in the cp genome and being proven suitable for analyzing repeat motifs in complete genomes (Kurtz et al. 2001). The difference in length of long repeats is commonly found while comparing cp genomes of different plant species. However, this software obtained slight variation in the number and types of long repeat elements among four cp genomes within one species (Table 1). Most of these motifs are in two types consisting of "forward" and "palindromic"; only the MN317571.1 sequence shows the presence of motifs in "complement" and "reverse" regions. This data is in line with previous studies when almost long repeats are forward and palindromic repeats in *Paphiopedilum delenatii* (Vu et al. 2020), *Corydalis temulifolia* and *C. adunca* (Huang et al. 2022), *Dipterygium glaucum*, *Cleome chrysantha*, *Cleomella lutea*, and *Tarenaya hassleriana* (Alzahrani et al. 2021). The abundance of repeats in forward regions was also reported in *Cypripedium* species

(Zhang et al. 2022). The different numbers and locations of these long repeats could come from the architecture of studied cp genomes and display the influence of evolution on cp genomes. Identifying long repeats is important in understanding plant genomes' reorganization, rearrangement, and phylogeny (Song et al. 2022).

The outcomes of the phylogenetic analysis on the four chloroplast (cp) genomes revealed a strong relationship among four *L. discolor* accessions. Three *L. discolor* accessions from China were grouped into a single cluster, whereas *L. discolor* accession from Vietnam was separated into another branch (Figure 2). The clustering pattern of this phylogenetic tree is reliable due to the high bootstrap value indicated on the tree. The phylogenetic tree is considered stable and reliable when this bootstrap value is greater than 75, and the higher the value, the more consistent the relationship in evolutionary analysis (Xue et al. 2019). Specifically, the three accessions from China, MN317571.1, MN880628.1, and NC_030540.1, exhibited a closer relationship and clustered within a monophyletic group. On the other hand, the accession from Vietnam formed a relatively distinct branch, indicating a separation from the Chinese accessions.

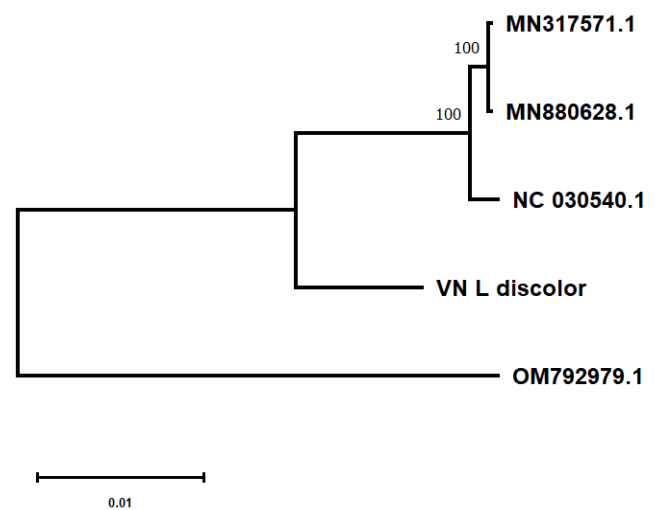


Figure 2. Phylogenetic tree of four cp genomes of *Ludisia discolor* orchid accessions. The chloroplast sequences of *Dendrobium sinense* (OM792979.1) were used as an outgroup. Numbers near branches are bootstrap values.

Table 1. The different repeat motifs and location of long repeat types in the chloroplast genomes of *Ludisia discolor* accessions

Accession	Country of origin	Repeat motifs					Total	Location of long repeat types				Total
		A	T	C	AT	TA		Forward	Complement	Reverse	Palindromic	
VN_L_discolor	Vietnam	15	37	0	0	1	53	21	0	0	27	48
MN317571.1	China	22	38	0	1	1	62	22	1	2	24	47
MN880628.1	China	23	38	0	1	1	63	24	0	0	26	50
NC_030540.1	China	22	30	1	1	1	55	22	0	0	27	49

Phylogenetic analysis based on complete cp genomes has increased power in plant taxonomy. After characterizing two cp genomes consisting of one common accession of *Goodyera schlechtendaliana* collected from China and another *G. schlechtendaliana* accession collected from Korea, both of them a highly similar in morphology and just different in the lateral appendages of the column in their flowers in the later. Oh et al. (2019b) found that the Korean accession is a sister of China's accession and could be a new species. Seventeen accessions from *Aegilops tauschii* were relatively divided into three main clusters corresponding to collection sites (Su et al. 2020). Also applying NGS method, the oolong tea (*Phoenix dancong*) from China was detected with a complicated genetic relationship with different tea species in the genus *Camellia*. Among 75 complete cp genomes, cp genomes of five Fujian oolong tea species are most similar to that of *Phoenix dancong* and form a monophyletic cluster (Liu et al. 2022). These results prove invaluable effectiveness of NGS in determining the origin and classification of plants which could facilitate the effective utilization and management of plant's genetic resources.

In conclusion, our study involved the sequencing and characterization of the complete chloroplast (cp) sequence of *L. discolor*, a jewel orchid species collected from Vietnam. By comparing the newly sequenced cp genome with existing sequences, we identified distinct features such as variations in genome size and sequence repeat motifs among different cp genomes. These findings contribute to understanding cp genomes' typical structure and content, specifically in Vietnam's *L. discolor*. The observed differences among cp genomes also provide insights into the genetic structure within the genus *Ludisia*. Furthermore, the unique TA repeat motifs and highly divergent regions identified in the Vietnam *L. discolor* cp genome have the potential for developing molecular markers, which could be utilized in future investigations related to this valuable herb's taxonomy and conservation efforts in Vietnam.

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Screening for endophytic bacteria from *Ambon Banana* (*Musa paradisiaca*) as biocontrol agent of anthracnose (*Colletotrichum gloeosporioides*) on bananas fruit

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Abstract. Pangastuti A, Pratiwi H, Setyaningsih R. 2023. Screening for endophytic bacteria from *Ambon Banana* (*Musa paradisiaca* L.) as biocontrol agent of anthracnose (*Colletotrichum gloeosporioides*) on bananas fruit. *Nusantara Bioscience* 15: 238-244. Post-harvest *Ambon Bananas* (*Musa paradisiaca* L.) are sensitive to anthracnose disease, caused by *Colletotrichum gloeosporioides*, and causes the fruit to rot quickly. Thus, chemical fungicides are employed, damaging living organisms and the environment. One solution is biocontrol using endophytic microorganisms as antagonistic agents against the anthracnose fungus that causes anthracnose disease. This study aimed to obtain potential endophytic bacteria from the *Ambon Banana* plant that had inhibitory activity against the growth of the pathogenic fungus *C. gloeosporioides* that causes anthracnose in bananas. Endophytic bacteria were recovered from *Ambon Banana* roots by crushing plant components. Therefore, bacterial isolates were tested for antagonistic interactions with pathogenic fungi using the dual culture approach. The 16S rRNA gene sequence analysis was used to identify bacterial isolates with the most significant inhibitory potential. According to the findings of this study, seven isolates of endophytic bacteria, A2-1, A2-2, A5-2, A6-2, A6-3, A8-1, and A9-1, can limit the growth of the pathogenic fungus *C. gloeosporioides*. The strain A6-3, identified as *Pseudomonas pseudoalcaligenes*, exhibited the greatest potential as a biocontrol agent against the pathogenic fungus *C. gloeosporioides*. It achieved the highest inhibition rate of 43.59%, resulting in an extended fruit shelf life and reduced harm susceptibility.

Keywords: Anthracnose, banana, biocontrol agent, *Colletotrichum gloeosporioides*, endophytes

INTRODUCTION

Ambon Banana (*Musa paradisiaca* L.) is an Indonesian tropical horticulture plant commonly used for its fruits, either directly or as a variety of meals. Because of its sweet flavor, this fruit is popular among the general people. Banana fruit farmers, on the other hand, regularly face various challenges, notably during the post-harvest, storage, and transportation procedures. The *Ambon* fruit banana is affected by pathogenic fungal infestation, which decreases fruit quality and leads it to degrade quickly. One of the pathogens is the fungus *Colletotrichum gloeosporioides*, which causes anthracnose in banana plants (Abd-Elsalam et al. 2010; Gautam 2014a; Gautam 2014b). Anthracnose attacks on various plant organs often start with the appearance of minute black or dark brown spots, which then grow and consolidate to cause damage to these organs (Bele et al. 2018). The pathogenic fungi can infect plants in the field and become inactive; after the banana fruit is harvested, *C. gloeosporioides* begin to grow again as the ripen banana fruit and cause damage. This fungal infection spreads faster in wounded tissue and ripe fruit (Unnithan et al. 2018).

Therefore, to achieve optimal agricultural yields, it is essential to carefully analyze plants' growth and defensive mechanisms throughout their developmental stages. Farmers depend on chemical fertilizers and pesticides to enhance productivity, while infections are treated with

antibiotics or antifungals. The long-term consequences of chemical substance overabundance in the cultivation system can lead to detrimental outcomes (Ayesha et al. 2021). Synthetic fungicides, namely thiabendazole, prochloraz, benomyl, and carbendazim, are commonly employed in agriculture for their fungicidal properties. Those substances mentioned above are chemical fungicides that possess the ability to inhibit or mitigate the occurrence of anthracnose in bananas. Spray or dips can be applied to banana fruits before or after harvesting.

Previous studies have documented that fungicides have a greater propensity to elicit adverse impacts on non-target soil microbial communities when compared to insecticides and herbicides, potentially compromising soil fertility (Muñoz-Leoz et al. 2013). In addition, fungicides have a significant influence on several non-target beneficial organisms, such as antagonistic organisms, predators, and parasites that play a crucial role in controlling harmful insects. Certain fungicides can undergo leaching, thereby infiltrating the deeper soil layers and contaminating the groundwater table. This contamination poses a significant risk to human health (Pathak et al. 2022). In addition, pathogens can acquire resistance due to the indiscriminate application of fungicides, leading to more aggressive strains (Goswami et al. 2018).

Moreover, with the many losses connected with chemical fungicides mentioned above, biocontrol is considered a more environmentally sustainable and less

hazardous approach for mitigating anthracnose infestation in banana crops (Fu et al. 2017; Vieira et al. 2017). Endophytic bacteria are found in plants and can be used to treat pathogenic fungi because they produce compounds that inhibit or kill pathogenic germs. Endophytic bacteria live in the same ecological niche as plant pathogens. As a result, they are more effective at treating plant infections (Hong and Park 2016). Moreover, it can be argued that endophyte bacteria exhibit more excellent ecological friendliness and sustainability than chemical fungicides. These entities do not contribute to environmental pollution, soil degradation, or residue-related issues (Xu et al. 2021). In addition, these substances do not harm advantageous creatures, including pollinators, predators, and decomposers. Endophytes can produce bioactive compounds with antimicrobial, antifungal, or antiviral activity, such as siderophores, antibiotics, hydrolytic enzymes, organic acids, and volatile organics (Alam et al. 2021). Endophytic bacteria, such as *Bacillus* CA8 and *Pseudomonas* PK5, have been widely reported to suppress various plant diseases, including blood disease in banana plants (Nawangsih 2007). The presence of endophytic bacteria within the roots of plants leads to the production of chitinase and protease enzymes, which possess the ability to impede the growth and development of pathogenic fungus (Pradana et al. 2016). This study aimed to obtain potential endophytic bacteria from the *Ambon Banana* root that have inhibitory activity against the pathogenic fungus *C. gloeosporioides* that causes anthracnose in bananas. Endophytic bacteria derived from the same host are expected to have more potential to be applied as biocontrol agents for anthracnose disease on bananas.

MATERIALS AND METHODS

Isolation of endophyte bacteria

The roots of the *Ambon Banana* plant from Sukoharjo District in Central Java, Indonesia were utilized to obtain endophytic bacteria. The banana roots from 5 individual plants were thoroughly cleaned with running water, dried with a paper towel, and weighed up to 1 gram. The roots were surface sterilized by immersing them in 70% alcohol for 30 seconds, followed by 2 minutes in a 2% NaOCl solution. Next, using sterile distilled water, the roots were cleaned four times. The surface-sterilized roots were mashed in a mortar with 9 mL of distilled water. The filter paper (Whatman grade 1 qualitative filter papers) filters the root solution. To each petri dish containing Luria Agar (LA) medium, 100 µL of root solution was spread with dry galski rods. The inoculated media were incubated for 72 hours. The formed single colonies were then isolated and purified.

Fungus culture

The *C. gloeosporioides* isolates were collected from the Indonesian Culture Collection (InaCC) LIPI. The fungus was rejuvenated using a Potato Dextrose Agar (PDA) medium. PDA medium was prepared by dissolving 3.9 grams of PDA powder (Oxoid, England) in 100 mL of

distilled water and autoclaved for 15 minutes at 121°C. Petri dishes were filled with PDA medium and left to cool. PDA medium was inoculated with the fungus *C. gloeosporioides* using a culture needle at the center. The mixture was then cultured for 5-7 days to allow the fungus to grow (Mayadianti et al. 2020).

In vitro antagonistic assay

The dual culture approach using PDA media was used to observe bacterial isolates' ability to inhibit the growth of *C. gloeosporioides*. The test was conducted for 10 pure bacterial isolates from the previous process. Fungal mycelium was taken as an agar plug using the cork borer. A fungal agar plug was placed in the center of each Petri dish. Bacteria were scratched along a 2 cm line beside the plug; a 3 cm distance separated bacteria and fungus. In addition, as a control, a petri dish containing only the fungus *C. gloeosporioides* was placed in the center of the petri dish. The inoculated Petri dishes were incubated for 7 days at 25°C (Hamaoka et al. 2021). Inhibitory measurement data is calculated to determine the percentage of inhibition using the formula:

$$P = \frac{R1 - R2}{R1} \times 100\%$$

Note: P= inhibition percentage; R1 = average diameter of the pathogenic fungal colonies in the control; R2 = average diameter of pathogenic fungal colonies in the treatment

Chitinase assay

Colloidal chitin was prepared by dissolving 2 grams of powdered chitin into 20 mL of 12 M HCL. The mixture was added to 400 mL of cold distilled water and incubated at 4°C for 24 hours. Colloidal chitin was washed with water until the pH was 7. Solid chitin media was made with the composition of colloidal chitin 3 g, K₂HPO₄ 0.105 g, MgSO₄ 0.075 g, KH₂PO₄ 0.045 g, FeSO₄ 0.0015, agar 2.25 g, and 150 mL distilled water. All ingredients were stirred using a magnetic stirrer until dissolved and put into an Erlenmeyer flask. The chitin media solution was then sterilized. The chitin medium was poured into a petri dish and allowed to harden, and the bottom of the petri dish was divided into four regions. Bacterial isolates were inoculated on the specified area. The petri dish was incubated at 30°C for 16-48 hours. A clear zone around the bacteria was observed (Lau et al. 2020). The chitinolytic index of each isolate was calculated. A chitinolytic index is a ratio between the clear zone's and bacterial colonies' diameter. According to Suryadi et al. (2013), the formula for determining the Chitinolytic Index (CI) is:

$$CI = \frac{\text{Clear zone diameter}}{\text{Colony diameter}}$$

Morphological characterization of selected bacterial isolates

The bacteria that demonstrated the ability to inhibit the growth of *C. gloeosporioides* were selected for further characterization. Luria Agar (LA) media was used

to characterize bacterial isolates morphology. The LA media composition consisted of 3.75 g of Luria-Bertani broth (HiMedia, India) and 2.25 g of Bacto agar (Oxoid, England) mixed with 150 mL of distilled water. The media was sterilized with autoclaving at a temperature of 121°C for 15 minutes, transferred into petri dishes, and subsequently allowed to solidify. The bacterial isolate was aseptically streaked in a quadrant on an agar plate. The sample was subjected to incubation for 24-48 hours until the emergence of a solitary colony. The single bacterial colonies formed were observed for various characteristics, including color, shape (round or spread), fringe (regular or irregular), and elevation (rising, convex, or flat) (Sanjaya et al. 2019).

Gram stain

A single loop of bacterial culture was deposited onto the surface of a glass slide and afterward submerged in sterile distilled water. Subsequently, the isolate was fixed on the surface of the slide by passing it over an open flame. The isolate was subjected to a 1-minute exposure to crystal violet, followed by a thorough washing and subsequent drainage. The isolate was subjected to a 2-minute iodine treatment followed by a subsequent washing process for the next step. The isolate was then subjected to a treatment involving the application of 96% ethanol, followed by a subsequent washing and draining process. Last, the bacterial isolates were subjected to a 30-second safranin treatment followed by a next washing step.

Bacteria identification

Identification of selected bacterial isolates was carried out at PT. Genetika Science Indonesia, based on the gene sequences encoding 16S rRNA (Stackebrandt and Goebel 1994). The genomic DNA of the isolates was extracted with Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005) and then amplified using a 16S RNA PCR primer (27F/1492R) with MyTaq HS Red Mix (2X) (Bioline, BIO-25048). The PCR products were sequenced, and then the sequences were compared with the database using BLAST.

RESULTS AND DISCUSSION

Results

A total of ten isolates of endophytic bacteria were acquired from the roots of *Ambon Banana* plants. The

isolates were assigned alphanumeric identifiers A1-1, A2-1, A2-2, A4-1, A5-2, A6-1, A6-2, A6-3, A8-1, A9-1. Furthermore, 7 isolates can prevent the growth of the pathogenic fungus *C. gloeosporioides* (Figure 1). The isolate exhibiting the highest percentage of inhibition was A6-3, which had an inhibition index of 43.59% (Table 1). In comparison to earlier experiments, the isolate exhibited low levels of antifungal activity. None of the isolates showed chitinolytic activity, which suggested that the mechanism of inhibition of fungi from these bacterial isolates was not through the production of chitinase enzymes that damage the cell walls of fungi.

Endophytic bacteria selected had different morphological characteristics in color, shape, periphery, and colony elevation (Table 2). Root endophytes encompass a wide array of taxonomic groups, each contributing distinct functional attributes to the plant-microbe association. This diversity manifests in various forms, from taxonomic richness to functional versatility, impacting nutrient acquisition, pathogen suppression, and overall plant fitness. Most of the bacterial isolates belong to the group of gram-negative bacteria. Based on the results of identification using the gene marker encoding 16S rRNA, isolate A6-3, which had the highest inhibition against the growth of the fungus *C. gloeosporioides*, was *Pseudomonas pseudoalcaligenes*.

Table 1. Inhibition index of fungal growth of *C. gloeosporioides* by endophytic bacteria from *Ambon Banana* roots with two replicates

Bacterial Isolate	Inhibition Index Against Fungus <i>C. gloeosporioides</i> (%)	Chitinolytic Activity
A1-1	0	-
A2-1	17.95	-
A2-2	16.67	-
A4-1	0	-
A5-2	30.77	-
A6-1	0	-
A6-2	7.69	-
A6-3	43.59	-
A8-1	21.79	-
A9-1	11.54	-
Control	0	-

Table 2. Characteristics of selected endophytic bacteria isolates from *Ambon Banana* roots

Isolate Code	Gram Stain	Colony Color	Form	Periphery	Elevation	Identification (based on 16S rRNA gene sequence)
A2-1	+	White	Round	Regular	Convex	na
A2-2	+	Cloudy white	Round	Regular	Rising	na
A5-2	-	Clear white	Round	Regular	Flat	na
A6-2	+	White	Round	Regular	Flat	na
A6-3	-	White	Spread	Irregular	Flat	<i>Pseudomonas pseudoalcaligenes</i>
A8-1	-	White	Round	Irregular	Flat	na
A9-1	-	Yellowish	Round	Regular	Flat	na

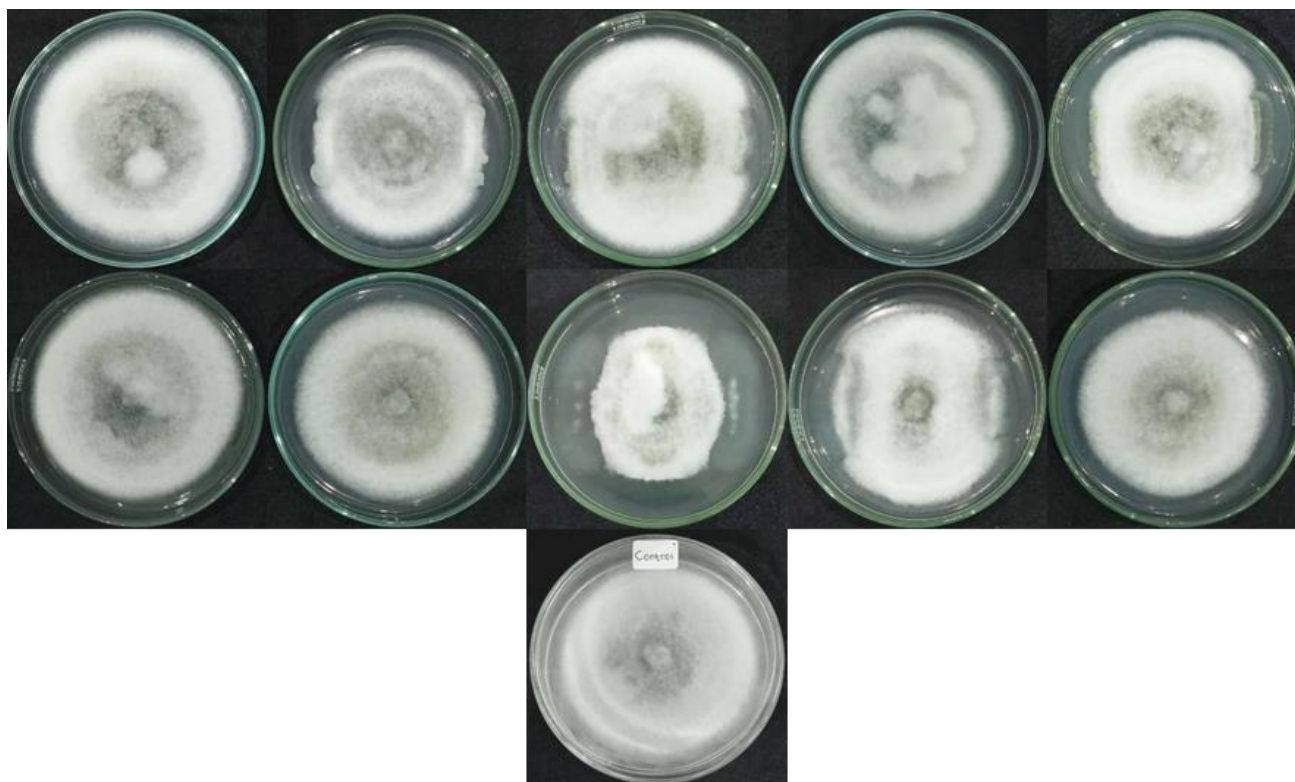


Figure 1. Inhibition of the growth of pathogenic fungi *C. gloeosporioides* by endophytic bacteria of Ambon Banana roots *in vitro*: from left to right, top: A1-1, A2-1, A2-2, A4-1, A5- 2; middle: A6-1, A6-2, A6-3, A8-1, A9-1, below; control *C. gloeosporioides*

Discussion

This study successfully obtained several endophytic bacterial isolates from the roots of banana ambon that showed inhibitory activity of the fungus *C. gloeosporioides*. However, the fungal inhibitory activity of these bacterial isolates was still relatively low to be applied as a biocontrol agent. The effectiveness of endophytic bacteria in inhibiting the pathogenic fungus is considered adequate if it exceeds 60%. Nevertheless, it is advisable to employ endophytic bacteria with an inhibitory activity surpassing 90% to suppress pathogenic fungi in agricultural settings successfully (Sanjaya et al. 2019). Isolate A6-3 with an inhibition index of less than 60% was classified as less effective if it was to be applied as a biocontrol agent. Suboptimal growth conditions may contribute to diminished antifungal effectiveness by impeding the production of bioactive antifungal compounds. In order to increase this isolate's antifungal activity, it is necessary to characterize the ideal conditions for growth and metabolite production. The symbiotic relationship between endophyte and host plants affects the production of secondary metabolites, such as antifungal compounds (Alam et al. 2021), so obtaining antifungal activity outside the host requires specialized strategies. Pathogenic infections cause the host to produce certain compounds, such as salicylic acid, jasmonic acid, and ethylene, that can trigger the production of antifungal compounds in bacterial endophytes (Ryan et al. 2008). This may be fine if the endophyte is applied to banana plants because it comes from the same host, so it can produce an antifungal

compound when the pathogenic fungi infect the host. Therefore, further tests are needed to apply this bacterium on banana plants to prevent *C. gloeosporioides* infections.

None of the isolates had chitinolytic activity on the chitin agar media. Chitinase is one of the antifungal mechanisms of bacteria. Endophytes employ various enzymes that facilitate the sequential hydrolysis of the plant cell wall to establish colonization on plants' surfaces. These enzymes play a role in the indirect reduction of phytopathogens and contribute to the destruction of fungal cell walls. However, the expression of endophytic bacterial chitinase enzymes also requires induction from the host. Some bacterial endophytes may produce this enzyme constitutively, while others may produce them only under certain conditions, such as nutrient availability, environmental stress, or signal molecules from the host (Mushtaq et al. 2023). Thus, if the bacteria are outside the host, the chitinase enzyme will not be produced due to the absence of signals from the host. There is also the possibility that these bacterial isolates inhibit fungal growth by other enzymes or metabolites. Besides chitin, glucans are also found in the cell walls of fungi and some bacteria, and glucanases can inhibit fungi by breaking down their cell wall structure and integrity (Kulshreshtha and Sharma 2022). Several enzymes produced by bacteria can also degrade the cell walls of pathogenic fungi, such as proteases and cellulases (Boiu-Sicuia et al. 2023). Other non-enzymatic antibacterial and antifungal compounds, such as siderophores, antibiotics, organic acids, and volatile organics, can be produced by bacterial endophytes (Digra

and Nonsom 2023). These compounds inhibit fungal pathogens' growth, metabolism, or cell integrity.

Based on the sequence of the 16S rRNA gene, isolate A6-3, which had the highest inhibition against the growth of the fungus *C. gloeosporioides*, was identified as *P. pseudoalcaligenes*, a species of bacteria that belongs to the genus *Pseudomonas*, which is known for producing various secondary metabolites with antimicrobial activities. One study reported that an isolate of *P. pseudoalcaligenes* (FP-2) from the root nodules of *Vicia faba* L. showed antifungal activity against *Fusarium* spp. These fungal pathogens cause plant wilt disease (Eshetu and Tesfaye 2020). According to the research of Pliego et al. (2007), *P. pseudoalcaligenes* strain AVO110 was able to control white root rot disease in avocados caused by the pathogen *Rosellinia necatrix* by reducing disease progression to 45% compared to controls. The mechanism of inhibition by *P. pseudoalcaligenes* strain AVO110 against the pathogen *R. necatrix* is a competition for nutrients and niches (Pliego et al. 2019). The *P. pseudoalcaligenes* also produce siderophores, which mediate iron competition with pathogenic fungi (Gamit and Tank 2019).

Some species of *Pseudomonas* were also reported to be able to produce volatile compounds. The Volatile Organic Compounds (VOCs) emitted by *Pseudomonas* sp., such as acetophenone, 1,3-butadiene, 2-undecanone, benzaldehyde, 1,2-benzisothiazol-3(2H)-one, dimethyl trisulfide, dimethyl disulfide, benzothiazole, nonanal, N,N-dimethyldodecylamine, 3,5,5-trimethyl-1-hexanol, isovaleric acid, cyclohexanol, 2-ethyl 1-hexanol, n-decanal, decyl alcohol, have been documented for their antagonistic properties (Naz et al. 2022). These compounds have been found to induce resistance in host plants against various bacterial and fungal pathogens. Dimethyl disulfide is one of the volatile compounds produced by *Pseudomonas fluorescens* that can inhibit the growth of the fungus *Penicillium italicum*, which causes green mold disease in citrus, which is characterized by changes in spore morphology, spore collapse, hyphae rupture, and shrinks. Mycelium is broken without any attached conidia (Wang et al. 2021). Schulz-Bohm et al. (2017) investigated the VOCs produced by *Pseudomonas putida* KT2440 and their effects on *Fusarium oxysporum* f. sp. *lycopersici*, a fungus that causes wilt disease in tomato plants. They found that 2-phenylethanol was the most effective VOC in inhibiting fungal growth and spore germination and that it induced plant defense responses and enhanced plant resistance to the fungus.

VOCs produced by bacteria can inhibit the fungus *C. gloeosporioides* and overcome anthracnose disease on fruit. According to research by Huang et al. (2014) and Reyes-Perez et al. (2019), *Bacillus atrophaeus* and *Stenotrophomonas rhizophila* produced volatile compounds that interfered with hyphae development and spore germination of the pathogenic fungus *C. gloeosporioides*. This happens because volatile compounds can destroy the primary function consistency and degrade fungal cell walls, causing cell damage and leakage of intracellular substances, resulting in bending and morphological changes in fungal hyphae and spores (Tenorio-Salgado et

al. 2013). Further research is needed on whether VOCs are the compounds responsible for inhibiting *C. gloeosporioides* by *P. pseudoalcaligenes* A6-3 isolates.

Previous studies have reported using endophytic bacteria as biocontrol agents for anthracnose disease on bananas or other fruits. Damasceno et al. (2019) reported the isolation and identification of endophytic and rhizosphere bacteria from sisal plants (*Agave sisalana*) that showed antagonistic activity against *C. musae*, the causal agent of anthracnose in bananas. The most effective strains belonged to the genera *Pseudomonas*, *Bacillus*, and *Paenibacillus*. They also showed plant growth promotion and induced systemic resistance in banana plants. The utilization of endophytic bacteria as biocontrol agents has been demonstrated to exhibit greater efficacy compared to chemical fungicides. The efficacy of *S. rhizophila* in treating mangoes for anthracnose was found to surpass that of synthetic fungicides, establishing the bacteria as a superior, cost-effective, and environmentally benign alternative for managing *C. gloeosporioides* (Reyes-Perez et al. 2019).

Several advantages are associated with using endophytes as biocontrol agents for suppressing host diseases. Endophytic microorganisms can establish residence within the internal tissues of plants and engage in competitive interactions with phytopathogens, impeding their growth and development (Fadiji and Babalola 2020). In addition to producing various antimicrobial compounds, endophytes can augment plants' resilience by initiating and preparing the host's defensive responses, including synthesizing phytoalexins, phenolics, and enzymes (Tewari et al. 2019). Endophytic microorganisms enhance plant development and improve stress tolerance by many mechanisms, such as hormone production, mineral solubilization, nitrogen fixation, and pollutant detoxification. *Pseudomonas* spp. produce plant hormones, such as auxins, cytokinins, gibberellins, and ethylene, that regulate plant growth and development. For example, *P. fluorescens* can produce Indole-3-Acetic Acid (IAA), an auxin type that stimulates root elongation and branching in maize and wheat plants (dos Santos et al. 2020). *Pseudomonas* spp. is also known as a producer of organic acids, siderophores, and chelating agents that dissolve insoluble forms of these minerals and make them accessible to plants (de Andrade et al. 2023). The *P. stutzeri* form nodules on the roots of legumes and non-legumes and fix atmospheric nitrogen into ammonia, a form that plants can assimilate. Therefore, the *P. pseudoalcaligenes* A6-3 that we successfully isolated can be used as a biocontrol agent for anthracnose disease on banana fruit while assisting the growth of the host. The advantage of this bacterium is that it was isolated from banana plants, so it is expected to colonize banana fruit and inhibit the growth of pathogens that cause anthracnose disease that damages post-harvest banana fruit.

The occurrence of anthracnose disease on bananas is classified as a post-harvest disease. Consequently, applying biocontrol strategies to mitigate this disease typically involves post-harvest protection techniques. These strategies aim to ensure the safety of the fruit from transit

and storage (Morales-Cedeño et al. 2021). Most practices for applying bacteria as a biocontrol agent during the post-harvest phase involve spraying a bacterial suspension onto the specific fruit or vegetable being targeted. Numerous research studies have investigated using post-harvest biocontrol techniques on fruit, employing diverse application means. Damasceno et al. (2019) applied the endophytic bacterial suspension onto bananas post-harvest using spraying, as the bacteria obtained were determined to be non-pathogenic to humans. So far, *P. pseudoalcaligenes* is not considered pathogenic to humans or plants, making it safe to apply as a biocontrol agent. In the future, *P. pseudoalcaligenes* A6-3 may be used by applying a bacterial suspension to the root of banana plants or onto post-harvest *Ambon Bananas*. Further research is still needed to apply this bacterium in the field.

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Effect of phosphate solubilizing and nitrogen-fixing bacteria on Pontianak siam citrus (*Citrus ×nobilis* var. *microcarpa*) seed germination

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Abstract. *Adhyaningtyas RFI, Rahmawati, Mukarlina. 2023. Effect of phosphate solubilizing and nitrogen-fixing bacteria on Pontianak siam citrus (Citrus ×nobilis var. microcarpa) seed germination. Nusantara Bioscience 15: 245-250.* West Kalimantan is one of Indonesia's largest producing areas for Siam citrus (*Citrus ×nobilis* var. *microcarpa* Hassk.). It is necessary to carry out the proper treatments within the early stages of planting, which increases the speed of germination of citrus seeds to quickly increase the number of seedlings. The biological agents that play a role in promoting plant growth are a group of Plant Growth-Promoting Rhizobacteria (PGPR), consisting of Phosphate-Solubilizing Bacteria (PSB) and Nitrogen-Fixing Bacteria (NFB). This study aims to determine the effect of a combination treatment or a single treatment of phosphate-solubilizing and nitrogen-fixing bacteria, which is known to obtain the best results in the germination and growth of Siam citrus seeds. The study was conducted in a nonfactorial, Completely Randomized Design (CRD). The given treatments were control (without bacterial inoculum), nitrogen-fixing bacteria (NFB P1), phosphate solubilizing bacteria (PSB P2), a combination of NFB and PSB bacteria with a ratio of 1:1 (P3), a combination of NFB and PSB bacteria with a ratio of 2:1 (P4), and the combination of NFB and PSB bacteria with a ratio of 1:2 (P5). Observations were made for 4 weeks after planting. A single treatment with phosphate-solubilizing bacteria (P2) gave the best result on almost all parameters, such as germination percentage, emergence time of germination, root length, number of leaves and fresh weight of Siam citrus seedlings.

Keywords: Germination, nitrogen-fixing bacteria, phosphate-solubilizing bacteria, Pontianak Siam citrus

INTRODUCTION

Pontianak Siam citrus (*Citrus ×nobilis* var. *microcarpa* Hassk.) is a horticultural crop with a high production rate in Indonesia, among 160 other citrus cultivars (Dharmawan et al. 2009). West Kalimantan is one of the largest citrus-producing areas in Indonesia. It is known by its trade name, Pontianak citrus, which refers to the type of Siam citrus. Siam citrus production in West Kalimantan continues to increase yearly, reaching 1,190,770 quintals produced in 2018. The Pontianak Siam citrus is a type of citrus that is consumed widely and has a distinctive sweet taste (Sa'adah et al. 2022). Siam citrus also shows a sweet aroma and taste, thinned skin, and high water content (Wahibah et al. 2011). Siam citrus is a vitamin C source and contains various compounds such as glycemic and non-glycemic carbohydrates, potassium, folate, calcium, thiamin, niacin, vitamin B6, phosphorus, magnesium, riboflavin and other nutritional content (Ahmed and Saeid 2021).

Propagation of citrus plants is done in generative and vegetative methods. The generative method uses seeds as a propagation agent, while the vegetative method uses the vegetative parts of plants for plant propagation. Citrus growers commonly use the vegetative method by grafting two parts of the plant, scion and rootstock. Rootstocks are produced through certified source seeds or from planting seeds from trees to be propagated (Singh et al. 2018; Albrecht et al. 2019).

The rootstock production through seeds sown begins with the germination process on the growth media. Generally, the seeds begin to germinate perfectly on day 15, 18 to 45 days, depending on the type of citrus seeds sown (Neto et al. 2016; Prajapati et al. 2017). Several experiments were conducted to accelerate the germination of citrus seeds. Qessaoui et al. (2020) conducted research using *Pseudomonas* Migula 1894 to accelerate germination time within 14 days of germination. Bacteria that can increase plant growth are known as the Plant Growth Promoting Bacteria (PGPB), commonly found in the rhizosphere area, also known as Plant Growth-Promoting Rhizobacteria (PGPR). PGPR generally consists of bacteria that can provide plant nutrients, such as nitrogen-fixing, phosphate-solubilizing, and other nutrient-providing bacteria (Noumavo et al. 2013).

PGPR treatment with single kind action of bacteria has been shown to increase several growth parameters such as root length, seedling height, dry and fresh weight, germination percentage, number of leaves and number of flowers that tested on sorghum plants (Widawati and Suliasih 2018), *Vicia faba* L. beans (Demissie et al. 2013), and rice (Bandeppa 2022). Wahyuni et al. (2020) conducted similar research using Banjar siam citrus, the same genus of orange, in different locations, yet resulting insignificantly in effect on plant growth. Therefore, nitrogen-fixing bacteria and phosphate-solubilizing bacteria were chosen in the Pontianak Siam seed germination test to

obtain good quality rootstock with shorter germination time.

MATERIALS AND METHODS

Preparation of bacterial inoculum

The isolates of Phosphate-Solubilizing Bacteria (PSB) and Nitrogen-Fixing Bacteria (NFB) used in this experiment were obtained from the Microbiology Laboratory, Department of Biology, Universitas Tanjungpura, Pontianak District, Indonesia. This isolate was isolated from the rhizosphere of citrus plants in Siam citrus plantations in the Singkawang area, West Kalimantan, Indonesia. The isolates were first propagated in the Nutrient Broth (NB) media and grown until the cell density reached 10^8 - 10^9 cells/mL or equivalent to 0.5 Optical Density (OD).

Seed extraction

Seeds were obtained from Siam citrus selected directly from Siam citrus plantations. Seeds were taken aseptically from the pulp using sterile tweezers. The removed seeds are then washed in water until there is no mucus covering the seeds. During this immersion, the seed viability was also selected with the criteria that the seeds were not flat, hollow, or floating. Then, the seeds were soaked in water at 52°C to prevent *Phytophthora* sp. growth for 10 min.

Preparation of planting media

The planting medium used in the germination and growth of Siam citrus seeds is peat soil, which has been neutralized for its pH to 6.5 with dolomite. The peat soil was then sterilized in an autoclave at 121°C for 15 min. Then, the soil was divided into 500 grams for each pot.

Making PSB and NFB cultures and treatment applications

The treatments given to the germination of Siam citrus seeds were P0 (control); P1 (NFB 100%); P2 (PSB 100%); P3 (NFB: PSB = 1:1); P4 (NFB: PSB = 2:1); and P5 (NFB: PSB = 1:2). One treatment was carried out in 10 replications, each replication contains 4 seeds. The inoculum made for one treatment was 5 mL. Therefore, each seed was inoculated with 0.125 mL culture with a cell density of 10^8 cells/mL or equivalent to 0.5 OD (Rahman et al. 2015) on nutrient broth media. Applying treatment to seeds is carried out every three days (Widyaningsih et al. 2020). Plant nurture was carried out daily by watering and weeding the weeds on the soil. Planting and observations were carried out 4 weeks after planting (WAP).

Seedling germination, growth, and parameters observation

Parameters observed in this experiment were germination percentage, sprout time emergence, the height of Siam citrus seeds, number of leaves, root length, and fresh weight. Parameters of seedling height, the number of leaves, root length, and wet weight were carried out at the end of the observation, which was four weeks after

planting. Germination percentage and time of emergence were observed every day until the end of planting. The data of these two parameters are calculated by the equation below (Guragain et al. 2021).

$$\text{Sprouts time emergence} = \frac{N1T1 + N2T2 + \dots + NxTx}{\text{Total sprouting seeds}}$$

Information:

N : Number of sprouts that appear in a certain time unit

T : Day of observation

$$\text{Germination Percentage (\%)} = \frac{\text{Total sprouts}}{\text{Total potted seeds}}$$

Data analysis

The data obtained from observations during 4 WAP on the Siam citrus plants were then analyzed using Analysis of Variance (ANOVA) to determine the effect of the treatment of suspension of phosphate-solubilizing and nitrogen-fixing bacteria on the germination of Siam citrus seeds. Results that have an effect will be followed by Duncan's test with a level of 5% in differentiating several treatments.

RESULTS AND DISCUSSION

Germination percentage

Germination percentage with the treatment of phosphate-solubilizing and nitrogen-fixing bacteria in single or combined forms was not significantly different from the control ($P \geq 0.05$). Between treatments, P2 (PSB 100%) and P0 (Control) had a similar and highest percentage value among the other treatments. The combination treatment (P3, P4, and P5) and the single treatment of 100% NFB (P1) were not able to increase the germination percentage on the last day of observation (Table 1).

Time of emergence of sprouts

The time of emergence of sprouts with the treatment of phosphate-solubilizing bacteria and nitrogen-fixing bacteria in treatment P1 (100% NFB) gave significantly different results at the 5% confidence level ($P \leq 0.05$) for all treatments with an average germination day of 10, 45 days. Single treatment of phosphate solubilizing bacteria (P2) and combination treatment (P3 and P4) had a higher average germination day than P0 (Control), which was 14 days. The P5 treatment (NFB 1: PSB 2) took longer for sprouts to emerge than all treatments (Table 2).

Seedling height

Parameters of seedling height with the administration of phosphate-solubilizing bacteria and nitrogen-fixing bacteria either in single or combined treatments could not give a significant difference at the 5% confidence level ($P \geq 0.05$) to the Control treatment (P0). Based on the values resulting from each treatment, it was shown that treatment P1 (NFB 100%) was the seed with the longest height among the

other treatments, which was 6.28 cm, followed by treatment P4 (NFB 2 : PSB 1), which had a seed length of 6.16cm. Treatments P5 (NFB 1 : PSB 2) and P0 (Control) were included in the lowest average length of seedlings (Table 3).

Number of leaves

The average number of leaves in treatment P2 (PSB 100%) was significantly different from treatments P1 (NFB 100%) and P3 (NFB 1 : PSB 1) but not significantly different from treatment P0 (Control), P4 (NFB 2 : PSB 1), and P5 (NFB 1 : PSB 2). Among the P0 treatment (Control) with the treatment of phosphate-solubilizing bacteria and nitrogen-fixing bacteria, both in a single form and in combination, the average value of the highest number of leaves sequentially was P2 (PSB 100%) as many as 15.1 strands, then followed by the treatment P0 (control) was 14.0 strands, P4 (NFB 2 : PSB 1) was 13.4 strands, and in P5 (NFB 1 : PSB 2) was 13.0 strands. The lowest average number of leaves was shown in treatment P1 (100% NFB), with 12.0 leaves and in treatment P3 (NFB 1 : PSB 1), with 11.4 leaves (Table 4).

Table 1. Germination percentage (%) Siam citrus seeds

Treatment	Germination Percentage (%)
P0 (Control)	100.00 ± 0.00 ^b
P1 (NFB)	90.00 ± 12.90 ^a
P2 (PSB)	100.00 ± 0.00 ^b
P3 (NFB 1 : PSB 1)	97.50 ± 7.90 ^{ab}
P4 (NFB 2 : PSB 1)	92.50 ± 12.07 ^{ab}
P5 (NFB 1 : PSB 2)	97.50 ± 7.90 ^{ab}

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level through Duncan's test

Table 2. The average time of emergence (days) of Siam citrus seeds

Treatment	Average Time of Sprouts Emergence (Days)
P0 (Control)	15.72 ± 5.06 ^a
P1 (NFB)	10.45 ± 1.62 ^b
P2 (PSB)	14.90 ± 3.58 ^a
P3 (NFB 1 : PSB 1)	14.50 ± 3.97 ^a
P4 (NFB 2 : PSB 1)	14.48 ± 3.69 ^a
P5 (NFB 1 : PSB 2)	16.18 ± 3.27 ^a

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level through Duncan's test

Table 3. Average seed height (cm) Siam citrus

Treatment	Average Height of Siam Citrus Seeds (cm)
P0 (Control)	5.84 ± 0.87 ^a
P1 (NFB)	6.28 ± 0.69 ^a
P2 (PSB)	5.91 ± 1.03 ^a
P3 (NFB 1 : PSB 1)	5.93 ± 0.85 ^a
P4 (NFB 2 : PSB 1)	6.16 ± 0.82 ^a
P5 (NFB 1 : PSB 2)	5.82 ± 0.61 ^a

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level via Duncan's test

Root length

The root length parameter from the results of the analysis of variance with a confidence level of 5% in treatment P1 (NFB 100%) was significantly different from treatment P3 (NFB 1 : PSB 1) and P5 (NFB 1 : PSB 2), but not significantly different from treatment P0 (Control), P2 (PSB 100%), and P4 (NFB 2 : PSB 1) ($P \leq 0.05$). When compared based on the root length value of each treatment, the highest root length value was found in treatment P1 (100% NFB), which was 6.34 cm, and P2 (100% PSB) was 6.04 cm (Table 5).

Fresh weight

All treatments showed that either single or combined treatment of phosphate-solubilizing bacteria showed no significant difference ($P \geq 0.05$). Based on a comparison of the average wet weight of Siam citrus seedlings, P2 (PSB 100%), P4 (NFB 2 : PSB 1), and P5 (NFB 1 : PSB 2) treatments were the highest wet weight. The lowest average wet weight was shown by treatment P0 (Control) and P3 (NFB 1 : PSB 1) (Table 6).

Table 4. Average number of leaves (strands)

Treatment	Average Number of Leaves (Strands)
P0 (Control)	14.0 ± 2.53 ^{ab}
P1 (NFB)	12.0 ± 2.70 ^a
P2 (PSB)	15.1 ± 2.60 ^b
P3 (NFB 1 : PSB 1)	11.4 ± 1.83 ^a
P4 (NFB 2 : PSB 1)	13.4 ± 4.22 ^{ab}
P5 (NFB 1 : PSB 2)	13.0 ± 2.78 ^{ab}

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level via Duncan's test

Table 5. Average root length (cm)

Treatment	Average Root Length (cm)
P0 (Control)	5.92 ± 0.97 ^{ab}
P1 (NFB)	6.34 ± 0.51 ^b
P2 (PSB)	6.04 ± 1.22 ^{ab}
P3 (NFB 1 : PSB 1)	5.15 ± 0.76 ^a
P4 (NFB 2 : PSB 1)	5.53 ± 0.92 ^{ab}
P5 (NFB 1 : PSB 2)	5.30 ± 1.07 ^a

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level via Duncan's test

Table 6. Average height, number of leaves, root length, and wet weight of Siam citrus seeds

Treatment	Average Wet Weight of Siam Citrus Seeds (g)
P0 (Control)	0.22 ± 0.06 ^a
P1 (NFB)	0.23 ± 0.36 ^a
P2 (PSB)	0.26 ± 0.56 ^a
P3 (NFB 1 : PSB 1)	0.22 ± 0.56 ^a
P4 (NFB 2 : PSB 1)	0.25 ± 0.34 ^a
P5 (NFB 1 : PSB 2)	0.24 ± 0.47 ^a

Note: Numbers followed by different letters indicate that there is a significant difference at the 5% level via Duncan's test

Discussion

Germination of seeds generally depends on several environmental factors: seed quality, growing media, temperature, light and moisture of the surroundings. The moisture content of the planted seeds must not be lower than 6% to make it not inhibitory to germinate. The seed must be kept moist by using the seeds as soon as possible after extracting it from the host fruit. Seed requires a humid environment to germinate; hence, the first thing to concern is the storage of the seeds. The germination will be delayed if the seeds are left dry (Orbović et al. 2013). The other factor, growing media, determines the germination's moisture level. The germination process needs water to end the dormancy and start imbibition (Haj Sghaier et al. 2022). The proper growing media that keeps the moisture content could increase the seed's and seedlings' germination percentage and plant height (Handayani and Yuzammi 2019). Temperature, light intensity and other limiting factors carried the germination process then impacted the quantity of seedlings' height, root length, number of leaves, and fresh weight.

Treatment of 100% phosphate-solubilizing bacteria (P2) gave the best results in the parameter of germination percentage, number of leaves, and fresh weight of seedlings. The results obtained in the P2 treatment indicated the presence of P content in the organic and inorganic soil, which had been dissolved by phosphate-dissolving bacterial agents from the rhizosphere of Siam citrus. Phosphate content in the growing media becomes available and can be used by plants as a supply of macronutrients needed, thus increasing plant biomass distributed in plant parts such as crowns, roots, and leaves. Phosphate solubilizing bacteria cause the availability of P to be absorbed by plants by producing several compounds that play a role in dissolving P that is not dissolved in the soil. Several compounds produced by phosphate-solubilizing bacteria, such as organic acids, exopolysaccharides, and other enzymes, will make P available to plants (Sharma et al. 2013). Soil contains phosphate, which binds to cations such as Ca^{2+} , Al^{3+} and Fe^{3+} . This cation forms a chelate with the carboxyl and hydroxyl groups of organic acids produced by phosphate-dissolving bacteria and releases phosphate bonds into a dissolved form (Kalayu 2019). The roots will absorb the available P element. Element P is channeled through the stem to the leaves to help the photosynthesis process in the leaves. Hormones produced by bacteria also influence increased growth by phosphate-solubilizing bacteria. Plant roots produce exudates in the form of tryptophan, which acts as a signaling molecule to produce IAA (Tariq and Ahmed 2022). Phosphate-solubilizing bacteria *Bacillus* Cohn 1872 and *Pseudomonas* produced the auxin, increasing plant mass and plant root growth (Kudoyarova et al. 2017). In another study, *Bacillus* sp. produces different types of growth hormones: IAA, jasmonic acid, and gibberellic acid (Mažlytė et al. 2022). In addition, ACC deaminase, the key enzyme synthesized by phosphate-solubilizing bacteria, hydrolyzed the precursor of ethylene (ACC), plant growth inhibitory agents (Zaidi et al. 2009).

The success of a single treatment of phosphate-solubilizing bacteria was shown by the research of Viruel et al. (2014), which can increase the germination parameters and plant height of corn. This was also shown in the increase in the number of leaves by the 100% PSB treatment, which was caused by the available P absorption activity. The increase in wet weight by adding phosphate-solubilizing bacteria has also been proven in the research by Ehsan et al. (2014), who tested on wheat plants. Demissie et al. (2013) tested seed germination of *V. faba* bean with the addition of phosphate-solubilizing bacteria, resulting in increased germination. Gholami et al. (2009) suggested that these bacteria can synthesize a hormone that stimulates seed germination, the gibberellin hormone. Gibberellin hormone triggers the activity of specific enzymes that support germination, α -amylase, which increases starch assimilation.

Treatment of 100% nitrogen-fixing bacteria (P1) can produce the best parameter values on the time of sprout emergence, seedling height, and root length. The average germination time by this treatment gave the best value among the other treatments, which was 10.45 DAP. It is reported that citrus seed's germination time is 18 and 22 days on paper substrate (Ragagnin et al. 2022). Similar results were also found in the study of Naing and Zin (2013), which utilized nitrogen-fixing bacteria from the genus *Azotobacter* Beijerinck 1901 and *Azospirillum* Tarrand et al. 1979 in rice plants, resulting in an increase in plant height growth on the 30th day after planting, root length on the 40th day after planting, and wet weight. Plants on the 20th day after planting compared to the control treatment.

The combined treatment consisting of P3 (NFB and PSB 1:1), P4 (NFB and PSB 2:1) and P5 (NFB and PSB 1:2) could not give the best results for all parameters. This is thought to be caused by several conditions, namely competition between bacteria in different growth media, differences in elemental composition found between peat soil media and adapted bacterial growth media, and differences in the growth phases of two different bacteria. The results in this study are contrary to the findings of Li et al. (2020), which resulted in increased yields for all growth parameters in a consortium treatment of phosphate-solubilizing bacteria and nitrogen-fixing bacteria in wheat plants. This difference is thought to cause competition between the bacterial consortia in the new substrate, although the synergism test results show that the two isolates can grow together in Nutrient agar media. This statement is supported by Deng and Wang (2016) that differences in substrate composition will affect bacterial interactions in their research using bacteria grown on two different types of media, but in one of the media, it causes competition between bacteria. The competitive result between bacteria and host plants for the source of needed elements, such as nitrogen, has been recorded by Kuzyakov and Xu (2013). It stated that one of the factors of competition between microorganisms and plants is the different stages of nutrient demand of each organism. When the plant is on a high level of N demand, it will decrease the microorganism uptake of N nutrients.

In conclusion, some growth parameters were affected by the single treatment, P1 (nitrogen-fixing bacteria) and P2 (phosphate-solubilizing bacteria). P2, which consists of singular phosphate-solubilizing bacteria, gives the best results on citrus seed germination and growth, especially on germination percentage, sprout time emergence, number of leaves, and fresh weight. P1, consisting of singular nitrogen-fixing bacteria, only gives the best seedling height and root length results. This means that singular treatment was better than three variations of combined treatment. Chemical and molecular tests can identify those potential bacteria (PSB and NFB) and could be used to make biofertilizers to utilize the bacterial function.

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The potency of Berastagi sweet orange (*Citrus sinensis*) peel extract for obesity treatment: An experimental study in male rats

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Abstract. Batubara WRP, Susilawati TN, Indarto D. 2023. The potency of Berastagi sweet orange (*Citrus sinensis*) peel extract for obesity treatment: A study in the rats. Nusantara Bioscience 15: 251-257. Obesity is an abnormal accumulation of body fat and is a trigger factor for various degenerative diseases. Obese continues to grow every year in the world. This study aimed to investigate the effects of Orange Peel Extract (OPE) on Body Weight (BW), obesity index (Lee Index), Body Fat Percentage (BFP) and visceral fat in obese-model rats. Thirty male Wistar rats were divided into 5 groups: controls (negative/NC given aquadest and positive/PC given orlistat 12.3 mg/kgBW/day) and interventions (OPE 1-3) given OPE 250, 500 and 750 mg/kgBW/day, respectively. All rats were fed a high-fat, high-fructose (HFHFr) diet for 28 days. Statistical analysis was performed using ANOVA and Friedman test with $p < 0.05$. The average BW in all groups increased significantly after 14 and 28 days of the intervention ($p = 0.005$). A significant decrease in the obesity index average was found in the OPE 2 and 3 groups ($p = 0.028$ and $p = 0.034$) compared to the NC group. The OPE 1-3 and PC group significantly decreased in average BFP compared with the NC group on the 28th day of intervention ($p < 0.05$). In conclusion, OPE can reduce obesity induced by HFHFr. Future research could be directed to evaluate the OPE side effects for obesity treatment.

Keywords: Berastagi sweet orange, *Citrus sinensis* L., obesity, orange, orange peel extract

INTRODUCTION

Obesity is one of the epidemic diseases that has continued to increase over the years (Haththotuwa et al. 2020). It had been predicted that by 2022, 38% of the world's population aged >5 years will be obese with a Body Mass Index (BMI) ≥ 25 kg/m² (Lobstein et al. 2023). Obesity in men is characterized by body fat percentage (BFP) $>25\%$ and in women $>35\%$ (Chusyd et al. 2016). Obesity is a condition of abnormal body fat accumulation caused by unbalanced energy intake (Chusyd et al. 2016; Panuganti et al. 2023). In addition, several other factors cause obesity, including genetic factors, hormonal and psychological factors, gut microbiota, diseases and drugs, chemical exposure and the environment (Safaei et al. 2021). Not only changing body image (Godoy-Izquierdo et al. 2020; Gow et al. 2020), obesity is also the cause of several chronic diseases such as diabetes mellitus, dyslipidemia, cancer and heart disease (Ramón-Arbués et al. 2019; Friedenreich et al. 2021). Fat accumulation in the body can occur when caloric intake exceeds the amount of calories burned. Fat accumulation in subcutaneous adipose tissue, skeletal muscle, liver, and other metabolic organs can increase Body Weight (BW) (Frank et al. 2019). The accumulation of visceral fat can also reduce adiponectin levels resulting in reduced oxidation of triglycerides in adipose tissue and liver, called adiposopathy. This causes an increase in body weight beyond normal limits, which over time can become obesity (Fonseca et al. 2020).

Obesity treatment involves pharmacologic therapy, non-pharmacologic therapy, and bariatric surgery (Wolfe et al. 2016). Commonly used non-pharmacological treatments involve various dietary methods such as *kenyang* diets (Isyofi et al. 2022), and low macronutrient diets, such as low fat or low carbohydrate diets combined with exercise. Non-pharmacological treatments are usually effective but often fail because patients do not follow the program consistently (Higuera-Hernández et al. 2018). Pharmacological therapy with drugs is also less effective because it usually requires higher costs and prolonged treatment (Kosmalski et al. 2023). At the same time, bariatric surgery can be performed if the BMI criteria is ≥ 40 kg/m². However, the surgery costs a lot of money and poses side effects such as causing anaemia (Fink et al. 2022). Those are why other alternatives are needed to treat obesity, and hopefully, they will be more effective and efficient than other treatments. Therefore, the use of phytochemicals in natural materials should be considered.

Citrus fruits belonging to the Rutaceae family are among the most abundant fruits in the world (Zayed et al. 2021). Berastagi sweet orange (*Citrus sinensis* L.) is one of the oranges that are easy to find and spread in almost all regions in Indonesia. Previous researchers worldwide have found that citrus peels have a variety of substances (vitamins, minerals, and phytochemicals) that have good health benefits. Still Berastagi orange peel has not yet been investigated regarding its potential in reducing variable obesity. However, citrus processing produces a large

amount of waste every year. It is known that orange peel is the main waste of citrus fruit, causing serious environmental pollution if not handled properly. Sweet orange peel waste is one of the natural materials that contain various nutrients that are not healthy. Nutrients found in sweet orange peel extract include vitamin C and magnesium (Batubara et al. 2023) and the phytochemical substances in flavonoid glycosides, phenolic compounds, pectin, and essential oils (Li et al. 2019; Huang et al. 2020), which are thought to have beneficial effects for the treatment of obesity. This study aimed to investigate the effects of sweet Orange Peel Extract (OPE) administration on BW, Lee Index, BFP and visceral fat in rats on a high-fat, high-fructose (HFHFr) diet.

MATERIALS AND METHODS

Extraction of sweet orange peel

Sweet orange peels were extracted from previous research (Batubara et al. 2023). The sweet orange used was originally from Berastagi, Sumatera, Indonesia and the fruits were obtained from a traditional market in Surakarta, Central Java, Indonesia. Berastagi sweet orange has the following specifications; round-shaped orange fruit with an average weight of 190-490 grams; the diameter of the fruit is 7.2-10.3 cm; the surface color is yellow, yellowish green and orange; the skin tends to be shiny; the surface texture is slightly smooth to rough; the skin is relatively thick 6.2-7.75 mm and more difficult to peel. Other characteristics include smooth skin surface, fragrant aroma, and sweet taste. The preparation of OPE using the maceration method carried out at the Phytochemistry Laboratory of Universitas Setia Budi had been performed previously (Depari et al. 2021) with minor modifications. Fresh sweet orange peels were cut and dried at room temperature for five days. About 1500 grams of sweet orange peel is crushed to produce about 1360 grams of simplicia. The simplicia was then dissolved in 96% ethanol (Shisam Mas Chemical Pharmacy) in a ratio of 1:10 for two days with occasional stirring. The ethanol as a solvent was referred to previous research, which stated that nutritional content from orange peel, such as vitamin C and magnesium dissolved in ethanol solvent (Uçak and Khalily 2022). The simplicia was then filtered using filter paper and put into a rotary evaporator (IKA RV 8 series with semi-automatic model and power intensity of 24 V made in USA) with a temperature of 70°C and a rotation of 70 rpm to evaporate the ethanol in the extract and then, put into oven blower (Binder WTB FD 56 made in Indonesia) to get a thick extract. The extract was weighed with an analytical balance, and the yield (%) was calculated. The OPE was stored in glass bottles and kept at 2-4°C before further analysis.

Animals and experimental design

The laboratory of Universitas Sebelas Maret, Central Java, Indonesia, provided laboratory experimental research using male albino rats. The samples in this study were 30 white male Wistar rats (*Rattus norvegicus*) aged 4-5 weeks,

weighed 100-200 g, in good health, with normal activity and behavior, and no anatomical abnormalities. The number of samples was calculated using the formula of Arifin and Zahiruddin (2017) $n=DF/(k+1)$, with DF represents minimum (10) or maximum (20), k number of groups, and n is the number of samples in each group, then the maximum value plus 20% reserved. Based on the formula, the number of samples in this study was 6 rats/group. The selected male rats were randomly divided into 5 groups: Negative Control (NC), Positive Control (PC), and three intervention groups (OPE 1-3). Animal maintenance was carried out in the Technical Implementation Unit of The Integrated Laboratory of Universitas Sebelas Maret. All rats were housed in one room with a 12-hour light-dark cycle at a room temperature of 23±2°C and humidity of 50±10%. All rats were acclimatized by feeding standard comfeed BR-2 (PT. Japfa Comfeed Indonesia Tbk.) and drinking ad-libitum for seven days before treatment. The Research Ethics Committee of the Faculty of Medicine, Universitas Sebelas Maret, approved the research protocol with Ethical Clearance No. 89/UN27.06.11/KEP/EC/2023.

OPE intervention

Preparation of HFHFr feed referred to previous research (Sundari et al. 2022). HFHFr was prepared in the Technical Implementation Unit of The Integrated Laboratory of Universitas Sebelas Maret from a mixture of 32 grams BR-2 feed, 28 grams duck egg yolk, 40 grams beef tallow, 12 grams chicken liver, and 4 grams butter and it consisted of 54.64% fat and 10% fructose. All rats were fed HFHFr (10% body weight) for 28 days. After the rats were obese (Lee Index >300), each group underwent further experiment for 28 days; i.e.: OPE groups 1-3 were given OPE 250, 500 and 750 mg/kgBW/day, respectively; the PC group was given orlistat (Novell Pharmaceutical Laboratories) 12.3 mg/kgBW/day, and NC group was given aquadest. All test materials were diluted in 2 mL of aquadest and then given to the test animals orally using a blunt needle sonde every morning. The dose of OPE given refers to research conducted by Depari et al. (2021) with an effective dose of 1000 mg/kgBW in reducing sugar levels in obese rats accompanied by diabetes mellitus, besides that, the dose of orlistat is a standard dose from humans converted to rats (Ardiansyah et al. 2018).

Measurement of BW, Lee Index, BFP, and visceral fat

The BW and naso-anal length of rats were measured and recorded weekly. The measurements of BW and naso-anal length were taken using digital scales (Joil accuracy rate of 0.1 gr, PT Arta Joil Tappa, Indonesia) and tape measure (Onemed PT. Jayamas Medica Industri Tbk Indonesia). Lee Index and BFP measurements were obtained with the following formula (Kora et al. 2000; Lee et al. 2011):

$$\begin{aligned} \text{Lee Index (Obesity index)} &= \{ \text{Body weight (g)}^{1/3} / \text{Naso-anal length (cm)} \} \times 102 \\ \text{TM index} &= \{ \text{Body weight (g)}^{1/3} / \text{Naso-anal length (cm)} \} \\ \text{Body fat content} &= 0.581 \times \text{TM index} - 22.03 \end{aligned}$$

Visceral fat measurement was performed on the last day of the intervention. Rats were sacrificed, then dissected, and wet fat on the abdominal part of the rat was taken and weighed using digital scales (Joil accuracy rate of 0.1 gr, PT Arta Joil Tappa, Indonesia).

Statistical analysis

The data obtained were processed using SPSS (Statistical Program for Social Science) version 22 and presented as mean $\pm$ Standard Deviation (SD). Normal distributed data such as Mean BW and BFP between groups were analyzed using a one-way Analysis of Variance (ANOVA) and followed by a Least Significance Different (LSD) test. The duration of OPE intervention within groups was measured using Repeated Measure ANOVA followed by Tukey's Post Hoc test. For data that is not normally distributed, such as the mean Lee Index and Visceral Fat non-parametric test, the Friedman test was used, followed by the Willcoxon and Mann Whitney test. A p -value of <0.05 means that there was a significant difference between groups.

RESULTS AND DISCUSSION

Effect of OPE administration on BW

There were significant ($p=0.005$) BW changes in all groups after 0, 14, and 28 days of HFHFr diet (Figure 1.A). Figure 1.B shows that the average BW of rats increased at the end of the intervention period, and only the OPE2 group decreased statistically significantly ($p>0.05$).

Effect of OPE administration on Lee Index

Figure 2.A showed that the administration of HFHFr diet significantly increased the Lee Index of rats, and at the end of modeling, the whole group was declared obese (Lee Index higher than 300 g/cm^3). The OPE administration

significantly reduced the rats' Lee Index at the end of the intervention. A significant decrease in the mean Lee Index was found in the OPE2 and OPE3 groups ($p=0.028$ and $p=0.034$) compared to the NC group from day 0 to day 28 of the intervention (Figure 2.B).

Effect of OPE administration on BFP

Mean BFP in all groups changed significantly on 0, 14, and 28 days of intervention ($p<0.05$) (Figure 3.A). Figure 3.B shows that OPE administration significantly decreased the mean BFP in all intervention groups (OPE 1-3) and the PC group compared to the NC group on day 28 of the intervention ($p<0.05$). The mean $\pm$ SD BFP values in the intervention groups and PC group were as follows (3.32 ± 1.69), (2.51 ± 1.92), (2.33 ± 1.54) and (3.26 ± 1.4).

Effect of OPE administration on visceral fat mass

There was no significant difference ($p>0.05$) in the visceral fat mass of rats in the control and the intervention groups (Table 1). Although there was no statistically significant difference between the control and the intervention groups, the average visceral fat weight in the intervention group (OPE 1-3) was lower than that in the PC group.

Table 1. Mean differences in visceral fat mass of rats with obesity with or without OPE administration

Groups	Mean $\pm$ SD (g)	P
	After 28 days of the intervention	
OPE1	1.54 ± 0.49	0.756
OPE2	1.30 ± 1.09	
OPE3	1.83 ± 0.63	
PC	1.85 ± 0.89	
NC	1.30 ± 0.47	

Note: Group differences were tested using Kruskal-Wallis ($p<0.05$)

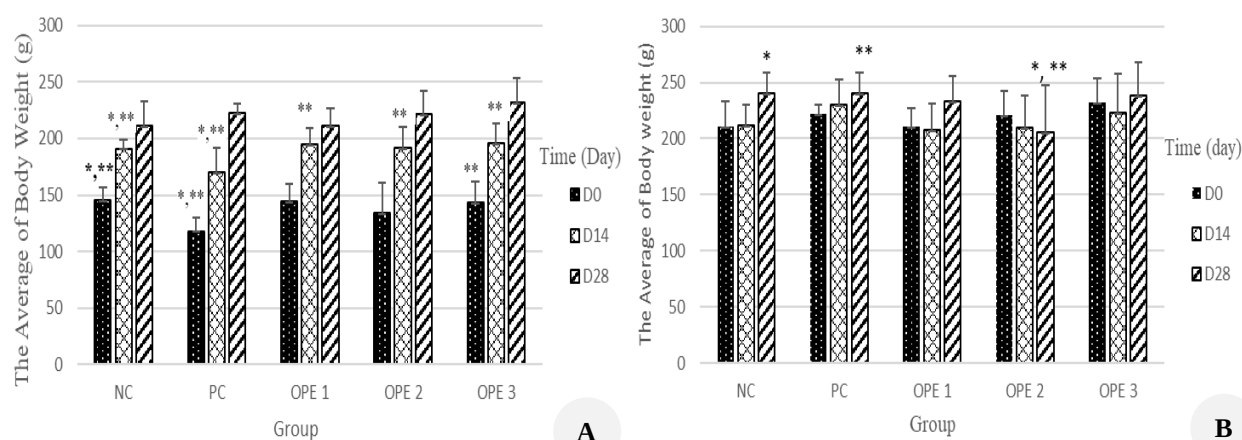


Figure 1. BW changes among all groups. A. Comparative effects (in each group) of HFHFr administration on body weight changes from days 0, 14 and 28 obesity induction. B. Comparative effect (in each groups) of OPE administration on BW from day 0, 14 to 28 of the intervention. *: significance of the change in average body weight compared to NC, **: significance of the change in average body weight compared to PC. ($p<0.05$)

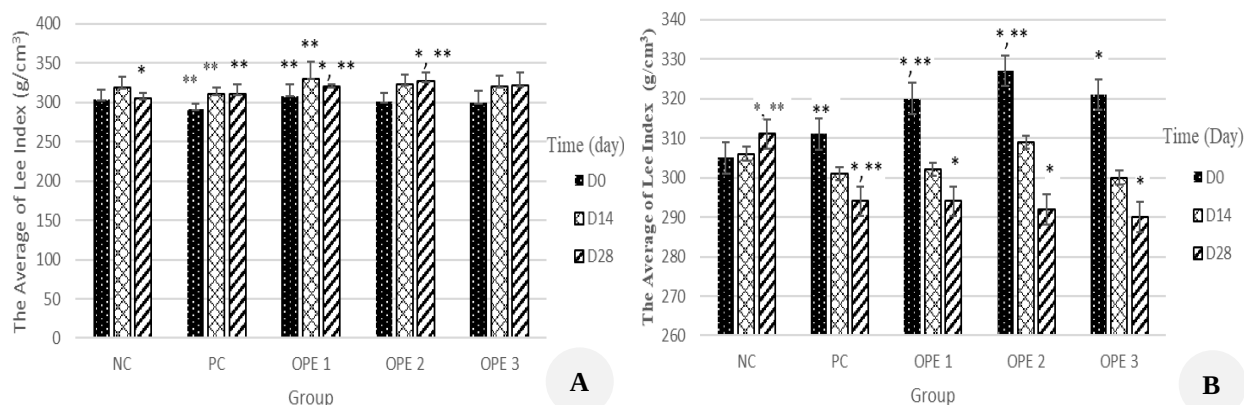


Figure 2. Lee Index changes among groups: A. Comparative effect of OPE administration among male rats with HFHF on day 0, 14, and 28 obesity induction. B. Comparative effect of OPE administration within male rats' groups on Lee Index from day 0, 14 to 28 interventions. *: significance of the change in average body weight compared to NC, **: significance of the change in average body weight compared to PC. ($p < 0.05$)

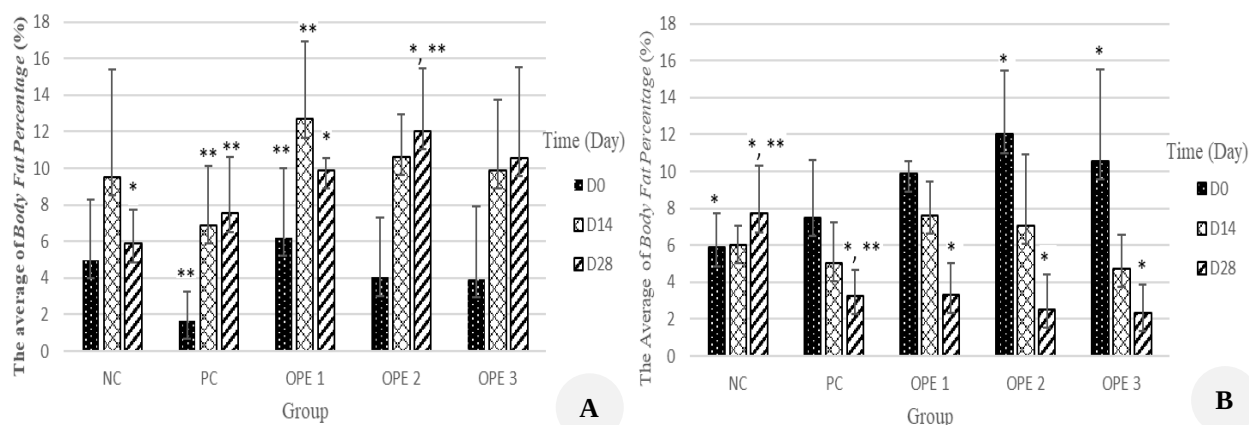


Figure 3. BFP changes among groups: A. Comparative effect of OPE administration among male rats with HFHF on day 0, 14, and 28 obesity induction. B. Comparative effect of OPE administration within male rats' groups on BFP from day 0, 14 to 28 interventions. *: significance of the change in average body weight compared to NC, **: significance of the change in average body weight compared to PC. ($p < 0.05$)

Discussion

Our study investigated the impact of OPE on BW, Lee Index, BFP, and visceral fat in obesity-induced male rats. Berastagi sweet orange is one of the Washington Navel Orange varieties grown in Berastagi Area, North Sumatra Province, Indonesia. Berastagi sweet orange is spread in almost all regions in Indonesia at a relatively affordable price. In general, only the pulp is consumed and the peel becomes waste. Preliminary research has been conducted to determine the nutritional content of OPE and it was found that OPE contains 1556.6 mg/100 g vitamin C and 2397.45 mg/kg magnesium which has beneficial effects in reducing obesity variables (Batubara et al. 2023).

Determining the Lee Index is a relatively easy and inexpensive way to determine obesity status by comparing BW with the naso-anal length of rats. Body mass index is influenced by various factors such as age, gender, and demographics (Purnell 2015). In this study, after being given OPE for 28 days, BW increased significantly. The Lee Index and BFP of rats decreased significantly, but

there was no significant difference in visceral fat between groups. The average BW of all groups increased significantly on 14 and 28 days of the intervention, and OPE 2 was a significant difference between the NC and PC groups. This study aligns with research that found that OPE enriched with essential oils caused weight gain in test animals (Montalbano et al. 2019). Although all of OPE (172) did not cause weight loss, the weight gain in the intervention groups was lower than that in the NC group. Hesperidin is one of the phytochemical substances from the flavonoid glycoside group commonly found in citrus peels (da Silva et al. 2019). Hesperidin combined with hesperetin, has been proven to stimulate the release of cholecystokinin (CCK) in the intestinal endocrine cell STC-1, which is responsible for stimulating the digestion of fat and protein in the intestine. CCK is a peptide hormone that regulates appetite and satiety by signaling to the brain to reduce food intake. Therefore, stimulating CCK secretion by hesperidin and hesperetin can suppress appetite and reduce food intake, helping to control body

weight and treat obesity (Kim et al. 2013; Gouveia et al. 2022). In the process of weight loss, there is a possibility that there will be an increase in body weight after a few days of treatment called the phenomenon of weight fluctuation and can occur for several reasons, including; the presence of fluid retention, hormonal changes, and the weight of undigested food and waste in the digestive system (Lean and Malkova 2016; Conti 2018; Livovsky et al. 2020). However, this weight fluctuation is normal and temporary, so a longer treatment time is needed to lose weight. This is in line with the previous research, which proved that administering Pacitan sweet orange peel extract at 500 mg/kg BW and 750 mg/kg BW for six weeks can significantly reduce BW (Samsudin et al. 2017). In addition, the increase in body weight in all groups was directly proportional to the increase in body length in mice, so the researchers concluded that the determination of obesity in this study could not only focus on BW variables but needed to be compared with body length (Lee Index).

In this study, we found that there was a decrease in Lee Index and BFP in the intervention group, which also align with previous studies showing that OPE was effective in reducing Lee Index and BFP, but at the same time, visceral fat in the intervention group was also significantly reduced (Gosslau et al. 2018; Huang et al. 2022; Lee 2023). The vitamins and minerals (vitamin C and magnesium) contents in OPE are thought to cause of the decrease in the Lee and BFP indices in this study. Ascorbic acid or vitamin C has a good effect on treating visceral obesity because it reduces BW, visceral adipose tissue and visceral adipocyte size (Liu et al. 2017) and with hesperidin, can activate peroxisome proliferator-activated receptor- α (PPAR α), the target enzyme responsible for the β -oxidation of fatty acids in visceral adipose tissue and liver (Lee et al. 2019; Xiong et al. 2019). When activated, PPAR α regulates the expression of genes involved in peroxisomal oxidation of α and β very long-chain fatty acids (VLCFA) and branched-chain fatty acids (Pettersen et al. 2019) as well as inhibits pyruvate oxidation by increasing pyruvate dehydrogenase kinase-4 (PDK4), increasing fatty acid and glycerol oxidation (Zhang et al. 2014) and may contribute to weight loss by increasing fatty acid oxidation and decreasing glucose oxidation. With an intracellular distribution of 99%, Mg is the fourth most abundant mineral in the human body (Asbaghi et al. 2021). Magnesium is important in various bodily functions, including energy metabolism, glucose utilization, protein synthesis, fatty acid synthesis and breakdown, muscle contraction, and overall cellular function (Fiorentini et al. 2021). Magnesium is also important in weight loss as it is involved in the production of ATP through oxidative phosphorylation in the mitochondria (Schwalfenberg and Genuis 2017). Studies have shown that adequate magnesium intake, a healthy diet, and intense exercise can help lose weight. Magnesium may also help reduce stress and improve sleep (Zhang et al. 2022), indirectly contributing to weight loss.

Visceral fat metabolizes faster than subcutaneous fat, which means it is harder to target with exercise alone or diet alone, so it can take a long time to reduce visceral fat if only relying on one treatment (Liu et al. 2017). This aligns

with a previous study, which mentioned that it took at least 6 weeks to see a significant difference in visceral fat between the control and the treatment groups given fermented sweet orange peel (Liu et al. 2017). OPE can reduce obesity, but this study has several limitations that should be considered; the intervention period was only 4 weeks, which is relatively short compared to most intervention trials on obesity. The weight loss effect could be even greater if the OPE treatment is given over a longer observation period. In addition, there is a need to analyze the potential of other nutrients in OPE that may strengthen the effect of OPE in reducing obesity.

In conclusion, there's a discrepancy between the results of previous studies and this study regarding visceral fat. This is because visceral fat is the type of fat stored deep in the abdominal cavity, encasing organs including the liver and intestines and is considered dangerous as it is associated with a higher risk of type 2 diabetes, insulin resistance, heart disease and certain cancers (Covassin et al. 2018; Chait and den Hartigh 2020). This study reinforces the beneficial effects of OPE to ameliorate HFHFr diet-induced obesity. However, further studies are needed to identify other anti-obesity components and comprehensively understand the cellular and molecular mechanisms of OPE effects in obesity. This study demonstrates the potential of OPE to be developed into a functional food for managing HFHFr diet-induced obesity.

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Population genetic structure of *Kawakawa* (*Euthynnus affinis* Cantor, 1849) in Malaysian waters based on COI gene

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Abstract. Binashikhbubkr K, Setyawan AD, Naim DM. 2023. Population genetic structure of *Kawakawa* (*Euthynnus affinis* (Cantor, 1849)) in Malaysian waters based on COI gene. *Nusantara Bioscience* 15: 258-268. *Kawakawa* (*Euthynnus affinis* Cantor, 1849) is widely distributed in the subtropical and tropical waters of the Indo-Pacific region. Still, insufficient data about its stock, management, and protection in Malaysia and nearby waters raises concerns about overfishing and depletion. Therefore, to ensure effective and successful management of a species, it is imperative to conduct a molecular-based assessment of the stock structure. The present study investigated the population genetic structure of *E. affinis* in Malaysian waters using the mtDNA COI gene. Furthermore, the 632 bp segment of the COI region was sequenced in 372 individuals from 19 distinct populations in Malaysian waters. The results revealed that the genetic divergence varied from low to high. The average Haplotype diversity (H_d) and nucleotide diversity (π) were calculated to be 0.5401 and 0.0045, respectively. Examining haplotype distribution unveiled the presence of 22 unique haplotypes within the COI gene of *E. affinis*. The analysis of the Neighbor Joining (NJ) tree and the Minimum Spanning Network (MSN) revealed the formation of three distinct clades among *E. affinis* samples. Analysis of Molecular Variance (AMOVA) showed a significant genetic structure among the 19 populations of *E. affinis* [$F_{ST} = 0.5354$ ($P < 0.05$)]. The neutrality test and mismatch distribution analysis indicated that the specimens underwent a period of population expansion. This study is a significant milestone, providing the first comprehensive documentation of the genetic structure of *E. affinis* in Malaysia.

Keywords: COI, *Euthynnus affinis*, fisheries stock management, mitochondrial DNA, population genetics

INTRODUCTION

Assessing animal population distribution and genetic structure is important in developing efficient conservation strategies, particularly for species that have undergone overexploitation. Maintaining species biodiversity and populations and safeguarding them against commercial exploitation are crucial objectives (Turan et al. 2005; Ha et al. 2020). The stock composition of a species holds significance, as its geographical distribution and genetic structure influence it. Hence, comprehending this framework will yield numerous advantages, including recognizing populations with distinct ecological, genetic, and management attributes. Furthermore, acquiring this knowledge is important in developing suitable conservation and management strategies tailored to various demographic cohorts.

Additionally, it is advantageous to pinpoint a region characterized by substantial genetic diversity, as this is a crucial attribute to safeguard the enduring welfare of a species (Khan et al. 2012). Utilizing this information will facilitate the evaluation of interrelatedness and genetic exchange between populations, thereby enabling a comprehensive evaluation of genetic well-being. Overall, the comprehension of species stock structure is important in management, conservation, and species' sustained

survival and resilience (Cronin-Fine et al. 2013; El Mghazli et al. 2021).

Researchers have proposed various techniques to ascertain the presence of marine and freshwater fish stocks. The earlier mentioned techniques encompass morphometric techniques (Griffiths et al. 2017), geometric morphometrics (Imtiaz and Naim 2018; Mohammadi-Sarpiri et al. 2021; Binashikhbubkr et al. 2022), meristic analysis (Gain et al. 2017), otolith shape analysis (Wujdi et al. 2022), and DNA analysis techniques, specifically focusing on mitochondrial genes (Kumar et al. 2012; Johnson et al. 2016; Kasim et al. 2020; Jamaludin et al. 2022). DNA coding, particularly utilizing the COI gene in mitochondrial DNA, has been widely used since the early 2000s in various research, including forensics, population, ecology, phylogenetics, and biodiversity (Hürkan 2020). These genes are useful in several research objectives, such as ensuring seafood safety, managing fisheries resources, finding new species, and documenting animal biodiversity (Seyhan and Turan 2016). The conservation of COI genes in mitochondrial genomes is important for classifying and identifying different animal species (Hebert et al. 2009; Kamal et al. 2020). Recent studies on marine fish have proven that using COI genes can be an effective tool for species identification, population genetic analysis, and fisheries management (Kunal and Kumar 2013; Halim et al. 2022).

Kawakawa, scientifically known as *Euthynnus affinis* (Cantor, 1849), is a type of tuna found in the Indo-Pacific region, particularly in warm seas. It is a crucial species for Malaysia and nearby waters but faces threats from overfishing and illegal fishing activities (Samsudin et al. 2015; Mardlijah et al. 2022). To ensure its sustainable use, strong surveillance and management strategies are essential. Despite these challenges, *E. affinis* remains significant for Malaysia's fishing industry and local economy. Mitochondrial DNA (mtDNA) methods have been used in previous studies in Malaysia and nearby waters to understand the genetic diversity and stock structure of *E. affinis*. Santos et al. (2010) used DNA markers from the control region (specifically D-loop) to study 35 *E. affinis* samples from the Philippines and 13 samples from Peninsular Malaysia (Pangkor Island and Penang Island). Another study by Masazurah et al. (2012) focused on the population structure of *E. affinis* in two states in Peninsular Malaysia (Perlis and Penang) using cytochrome b (Cyt b) marker. These investigations revealed a lack of understanding of the stock structure, genetic diversity, and phylogenetic connections of *E. affinis* in Malaysian waters. Therefore, this research is crucial for fisheries managers to create effective regional and international policies to address this fish resource's decline. Additionally, the findings can contribute to better management and conservation strategies for the *E. affinis* population in Malaysian waters.

This study evaluated the genetic variability and organization of 19 distinct populations of Malaysian *E. affinis* by utilizing the mitochondrial cytochrome oxidase I (COI) marker. This study represents a pioneering effort in utilizing the COI gene to examine the genetic makeup and population composition of *E. affinis* in Malaysian waters, thereby yielding significant insights.

MATERIALS AND METHODS

Sampling and study area

The specimens belonging to the species *E. affinis* were collected from November 2019 to September 2021. The collection encompassed a total of 19 distinct commercial fish landing sites. The sites mentioned above comprised a total of 11 landing sites located in Peninsular Malaysia and 8 landing sites situated in Malaysian Borneo (specifically Sabah and Sarawak). These landing sites are distributed across four main geographical regions: Straits of Malacca, South China Sea, Sulu Sea, and Celebes Sea. In this study, samples of *E. affinis* were obtained from 5 distinct landing sites situated in the Straits of Malacca: Batu Maung (Penang), Lumut (Perak), Sungai Besar and Kuala Selangor (Selangor), and Kukup (Johor). In addition, a total of 10 landing sites have been designated in the South China Sea, namely Pasir Puteh and Tok Bali (Kelantan), Pulau Tenggol and Pantai Kijal (Terengganu), Kuantan (Pahang), Endau (Johor), Bintawa, Pulau Buit, and Mukah (Sarawak), and Kota Kinabalu (Sabah); 2 landing sites from the Sulu Sea: Kudat and Sandakan (Sabah), and 2 landing sites from the Celebes Sea: Lahad Datu and Tawau (Sabah) (Table 1, Figure 1). All samples were morphologically identified and validated based on Collette and Nauen (1983). The specimens were relocated to the Molecular Ecology Laboratory at Universiti Sains Malaysia's School of Biological Sciences. Therefore, to enhance visibility, the fish samples underwent rinsing with running water, gentle tapping, and subsequent placement on the left side of a flat surface featuring a black background. For each specimen obtained, a segment measuring approximately 1-2 cm from the right pectoral fin was excised and stored in 95% ethanol for DNA extraction.

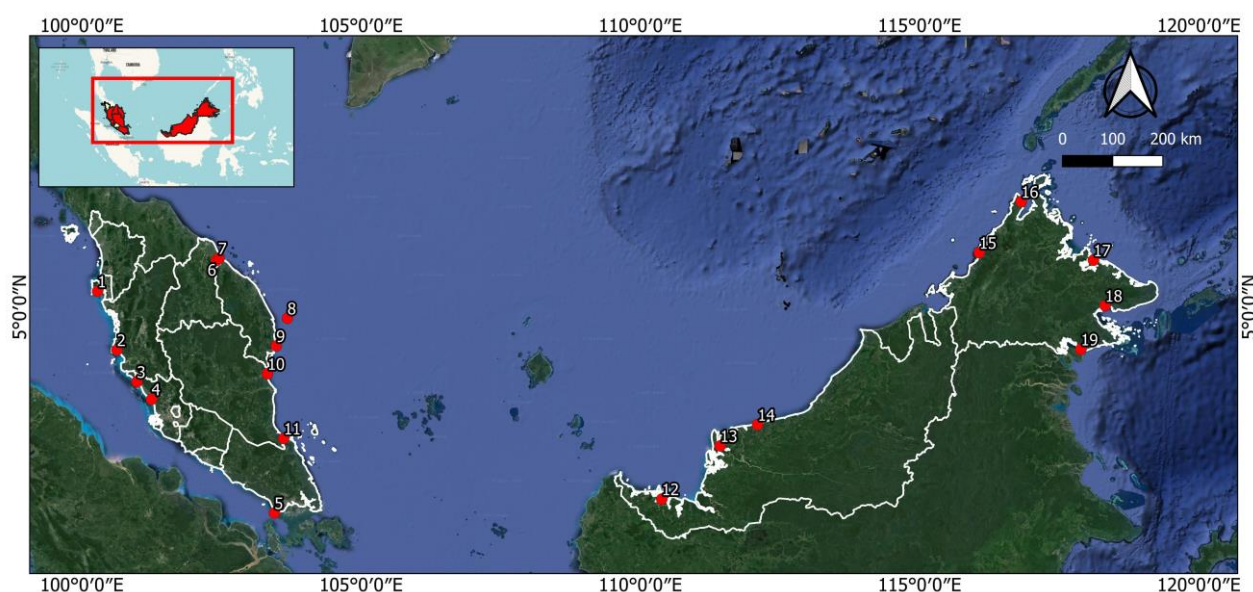


Figure 1. The specimen sampling areas of *Euthynnus affinis* obtained across the Straits of Malacca (M), South China Sea (SCS), Sulu Sea (SS), and Celebes Sea (CS) are as follows: 1. Batu Maung, 2. Lumut, 3. Sungai Besar, 4. Kuala Selangor, 5. Kukup, 6. Pasir Puteh, 7. Tok Bali, 8. Pulau Tenggol, 9. Pantai Kijal, 10. Kuantan, 11. Endau, 12. Bintawa, 13. Pulau Buit, 14. Mukah, 15. Kota Kinabalu, 16. Kudat, 17. Sandakan, 18. Lahad Datu, 19. Tawau

Table 1. Sampling areas, sample size (N), and geographic coordinates of 19 *Euthynnus affinis* populations were determined through COI gene analysis

Sampling locations	Coordinates	Marine region	N
Batu Maung, Penang (BM)	5° 16' 60.00" N, 100° 16' 60.00" E	M	24
Lumut, Perak (LUM)	4° 13' 56.28" N, 100° 37' 47.28" E	M	24
Sungai Besar, Selangor (SB)	3°39'50.4"N, 100° 59' 16.6"E	M	14
Kuala Selangor, Selangor (KS)	3° 21' 0.00" N, 101° 15' 0.00" E	M	24
Kukup, Johor (KUK)	1° 18' 60.00" N, 103° 26' 59.99" E	M	12
Pasir Puteh, Kelantan (PP)	5° 49' 58.28"N, 102° 24' 19.02"E	SCS	18
Tok Bali, Kelantan (TB)	5°52'35.5"N, 102° 27'29.9"E	SCS	14
Pulau Tenggol, Terengganu (PT)	4° 47' 59.99" N, 103° 40' 59.99" E	SCS	21
Pantai Kijal, Terengganu (PK)	4°18'20.2"N, 103°28'57.2"E	SCS	6
Kuantan, Pahang (KUA)	3° 48' 27.72" N, 103° 19' 33.60" E	SCS	24
Endau, Johor (END)	2° 38' 59.99" N, 103° 36' 59.99" E	SCS	24
Bintawa, Sarawak (BIN)	1°33'50.96"N, 110°23'15.6"E	SCS	13
Pulau Bruit, Sarawak (PB)	2° 30' 59.99" N, 111° 25' 59.99" E	SCS	14
Mukah, Sarawak (MUK)	2° 53' 45.5784" N, 112° 6' 13.4820" E	SCS	24
Kota Kinabalu, Sabah (KK)	5° 58' 29.64" N, 116° 04' 20.64" E	SCS	24
Kudat, Sabah (KU)	6° 53' 14.35" N, 116° 49' 25.10" E	SS	24
Sandakan, Sabah (SAN)	5° 50' 24.72" N, 118° 07' 4.44" E	SS	20
Lahad Datu, Sabah (LD)	5° 01' 36.48" N, 118° 19' 37.20" E	CS	24
Tawau, Sabah (TA)	4° 14' 41.35" N, 117° 53' 28.14" E	CS	24
Total			372

Note: M: Straits of Malacca, SCS: South China Sea, SS: Sulu Sea, CS: Celebes Sea

Isolation of DNA extraction and PCR amplification

The DNA samples obtained from the clipped fins were isolated using the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method, as described by Bakar et al. (2018), with minor changes to the proteinase K concentration to enhance the quality of the extracted DNA. Subsequently, the pelleted DNA underwent purification and elution using ddH₂O, then visualization through electrophoresis in an agarose gel (1.7%) stained with ethidium bromide (EtBr). Utilizing a spectrophotometer Q3000 (Quawell, Korea), the DNA purity was evaluated and fixed at -20°C until further use. Polymerase Chain Reaction (PCR) amplifications were used to amplify the cytochrome oxidase I (COI) gene. The expected length of the COI gene was 632 bp, and the primer sequences used for DNA amplification were FishF1 (5'TCAACCAACCACAAAGACATTGGCAC3') and FishR1 (5'TAGACTTCTGGGTGGCCAAAGAATCA3') (Ward et al. 2005). The PCR amplification was conducted in 25 µL volume including 16.25 µL molecular grade water, 2.5 µL 10X PCR buffer, 2.0 µL MgCl₂, 1.0 µL dNTPs, 0.5 µL of each primer (10 mM), 0.25 µL i- Taq (Intron, South Korea), and 2.0 µL DNA template. Gradients PCR were initially utilized to obtain the best annealing temperature using a T100TM Thermal-cycler (BIO-RAD, USA). The PCR reaction was performed in a Major Cycler, CyCLER-25 (Major Science, USA). The temperature profile was: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, annealing temperature 47.9-48.5°C for 50 sec, extension step 72°C for 1 min, followed by a final extension at 72°C for 7 min, and final hold at 4°C. PCR products were visualized in a 1.7% agarose gel (Vivantis Sdn.Bhd.) and stained with ethidium bromide to check for the presence of DNA. The PCR materials were cleaned using the Intron Purification Kit (Intron, South Korea) to

confirm that no impurities or extra suppressors were present. The purified products of PCR were later delivered to NHK Bioscience (Korea) for the sequencing process.

Data analysis

The sequences were processed using MEGA ver. 11 software (Tamura et al. 2021) for trimming and alignment, utilizing the ClustalW algorithm. The nucleotide composition was determined using MEGA ver. 11 (Tamura et al. 2021). The DNA sequences acquired were effectively identified through the utilization of the Basic Local Alignment Search Tool (BLAST) within the National Centre for Biotechnology Information (NCBI) database, which can be accessed at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. The *E. affinis* samples were assigned using homogeneity/similarity threshold values exceeding 99%. The pairwise genetic disparities within and between populations were computed using the Kimura 2-parameter (K2P) model in MEGA version 11 software. The variance of genes, haplotype variation (Hd), and nucleotide composition (π) was measured using DnaSP ver. 6.12 (Rozas et al. 2017). The reconstruction of the phylogenetic tree was carried out using the Neighbour Joining (NJ) tree method. The investigation involved the analysis of evolutionary relationships and sequence variation using the Kimura 2-parameter method, which was implemented in MEGA ver. 11 (Tamura et al. 2021). The estimation of the values of all phylogenetic nodes was conducted using 1000 replicates, and only bootstrap values exceeding 50% are presented. The phylogenetic analysis incorporated the sequence of *Lutjanus erythropterus* Bloch, 1790 (Accession no: GU673202.1) retrieved from GenBank (<https://blast.ncbi.nlm.nih.gov>) as an outgroup. In addition, a sequence of *E. affinis* obtained from GenBank (Accession

no: KY371510.1) was included in the analysis to verify the identification of the specimens as *E. affinis*.

A minimum spanning network was created using the median-joining method performed in PopART ver.1.7 (Leigh and Bryant 2015) to infer the relationships among haplotypes. Next, to estimate the population genetic structure of 19 *E. affinis* populations, an analysis of molecular variance (AMOVA) was conducted in Arlequin ver.3.5 (Excoffier and Lischer 2010). The isolation by distance (IBD) or Mantel test was performed in Arlequin ver.3.5 to verify the association between geographical and genetic distances. Population pairwise genetic distance values represented the gene distances, while geographical intervals between populations were estimated using the ruler tool in Google Earth. The neutrality test was conducted utilizing DnaSP version 6.12 (Rozas et al. 2017), which incorporates Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) analyses. Tajima's D statistic can identify departures from neutrality that arise due to factors such as population bottleneck, population expansion, directional selection, or introgression. This is achieved by analyzing data on the frequency of mutations or segregation sites. Tajima's D is a statistical measure that provides insights into various evolutionary processes; a positive value of Tajima's D indicates balancing selection, population substructuring, or a recent population bottleneck. Conversely, negative values of Tajima's D suggest recent directional selection or population growth beyond the presence of rare alleles (Tajima 1989). On the other hand, Fu's (1997) Fs analyses employ haplotype distribution to detect historical variations in population size. Additionally, the mismatch distribution was computed using DnaSP ver. 6.12 (Rozas et al. 2017). Analyzing the pattern can provide insights into the population's past demographic changes (Chen et al. 2015). An unimodal distribution pattern indicates a population recently experienced extension, whereas a multimodal dispersal model suggests a stable population (Rogers and Harpending 1992). The accession numbers OP595397-OP595523 were utilized to deposit all sequences into the GenBank database.

RESULTS AND DISCUSSION

Sampling data

Moreover, 372 specimens of *E. affinis* were sequenced for the COI gene. The specimens were obtained from a total of 19 distinct locations situated within four primary geographic regions: Straits of Malacca, South China Sea, Sulu Sea, and Celebes Sea (Figure 1 and Table 1).

Genetic distance and genetic diversity

The DNA sample sequence successfully produced fragments measuring 632 base pairs (bp) for the COI gene. The findings of this study demonstrate a 100% success rate in amplification for the COI gene. The intra and inter-population genetic distances of all *E. affinis* populations are displayed in Table 2. The intrapopulation genetic distance exhibited a range of values from 0.000 to 0.029. The population of TA demonstrated the highest level of

intrapopulation genetic distance (0.029), whereas the populations of TB, PK, PT, SB, KS, KUK, and SAN exhibited the lowest intrapopulation genetic distance (0.000).

Conversely, the interpopulation genetic distance ranged from 0.000 to 0.082. The interpopulation genetic distance between KUA and MUK populations was the highest at 0.082. On the other hand, the populations of PK and TB, PT and TB, SB and TB, SB and PK, SB and PT, KUK and TB, KUK and PK, KUK and PT, KUK and SB, SAN and TB, SAN and PK, SAN and PT, SAN and SB, SAN and KUK exhibited the lowest interpopulation genetic distance at 0.000 (Table 2). Overall, the genetic divergence among 19 populations of *E. affinis* varied from low to high.

The nucleotide sequences of COI in *E. affinis* exhibited a high A+T content, with percentages of 51.7%. The observed values exceeded the G+C content, consistent with the range observed in vertebrates (Nei and Kumar 2000). The haplotype diversity (Hd) values varied between 0.0000 and 0.8961, with an average value of 0.5401. Similarly, the nucleotide diversity (π) values spanned between 0.0000 to 0.0137, with a mean value of 0.0045 (Table 3). The findings suggest that each of the 19 populations of *E. affinis* exhibited significant values of haplotype variations (Hd >0.5) and comparatively low values of nucleotide diversity (π <0.5).

Phylogenetic analysis and Minimum Spanning Network (MSN)

The Neighbor Joining (NJ) tree analysis demonstrated the formation of three distinct clades among the *E. affinis* samples collected from Malaysian waters (Figure 2). The first clade consisted of individuals from the following locations: END, LUM, KUA, and KU. The second clade comprised samples from PK, PT, KUK, SAN, LUM, SB, PP, TB, KS, BM, TA, and KK, whereas the third clade encompassed individuals from LD, TA, BIN, PB, TB, PK, PT, KUK, SAN, and MUK. The NJ tree analysis also demonstrated that the GenBank sequence of *E.affinis* exhibited a closer relationship with the *E.affinis* specimens studied, as opposed to the outgroup species (*L. erythropterus*). This finding confirms that the collected individuals belong to the *E. affinis* species. The analysis of the Minimum Spanning Network (MSN) revealed that Hap 2 had the most frequent occurrence among the haplotypes. The remaining haplotypes exhibited limited distribution across a few populations or exclusive to a specific population. The presence of three distinct clades (1, 2, and 3) was further supported by the MSN analysis, revealing a nucleotide divergence of over 5 differences between them (Figure 3).

Haplotype distribution

The analysis of haplotype distribution reveals that 22 distinct haplotypes were identified for the COI gene across 19 populations. Haplotype 2 (N=277) emerged as the dominant haplotype, exhibiting authority across all populations except for KS and BM. Haplotype 1 was present in two distinct populations, namely PP and KS. Conversely, Haplotype 5 was identified in three separate populations: TA, KUA, and LUM. The remaining haplotypes were private or specific to a particular population (Figure 3).

Table 2. Pairwise genetic distance within and between *Euthynnus affinis* populations from Malaysian waters for COI gene based on Kimura 2 parameter model.

	PP	TB	PK	PT	SB	KS	KUK	SAN	LD	TA	KU	KK	BIN	PB	MUK	KUA	LUM	END	BM
PP	0.001																		
TB	0.001	0.000																	
PK	0.001	0.000	0.000																
PT	0.001	0.000	0.000	0.000															
SB	0.001	0.000	0.000	0.000	0.000														
KS	0.002	0.003	0.003	0.003	0.003	0.000													
KUK	0.001	0.000	0.000	0.000	0.000	0.003	0.000												
SAN	0.001	0.000	0.000	0.000	0.000	0.003	0.000	0.000											
LD	0.005	0.004	0.004	0.004	0.004	0.007	0.004	0.004	0.008										
TA	0.022	0.021	0.021	0.021	0.021	0.025	0.021	0.021	0.024	0.029									
KU	0.008	0.007	0.007	0.007	0.008	0.011	0.007	0.007	0.011	0.018	0.005								
KK	0.021	0.020	0.020	0.020	0.020	0.024	0.020	0.020	0.023	0.030	0.017	0.009							
BIN	0.028	0.027	0.027	0.027	0.027	0.031	0.027	0.027	0.033	0.044	0.032	0.051	0.014						
PB	0.040	0.039	0.039	0.039	0.039	0.044	0.039	0.039	0.044	0.051	0.040	0.057	0.064	0.016					
MUK	0.060	0.058	0.058	0.058	0.058	0.064	0.058	0.058	0.064	0.071	0.060	0.079	0.081	0.015	0.004				
KUA	0.023	0.022	0.022	0.022	0.022	0.026	0.022	0.022	0.026	0.040	0.030	0.038	0.052	0.063	0.082	0.020			
LUM	0.019	0.018	0.018	0.018	0.018	0.022	0.018	0.018	0.023	0.036	0.026	0.036	0.049	0.060	0.079	0.022	0.015		
END	0.018	0.017	0.017	0.017	0.017	0.021	0.017	0.017	0.021	0.036	0.025	0.038	0.053	0.061	0.080	0.028	0.019	0.008	
BM	0.014	0.016	0.016	0.016	0.016	0.013	0.016	0.016	0.020	0.048	0.025	0.038	0.048	0.059	0.079	0.046	0.039	0.041	0.013

Note: Bold indicates intrapopulation values

Population genetic structure

The genetic compositions of 19 populations of *E. affinis* originating from Malaysia were examined by analyzing the COI gene. In comparison, the Analysis of Molecular variance (AMOVA) results indicate a significant genetic variation level observed among populations, accounting for 53.54% of this variation. Conversely, genetic variation within populations is relatively low, accounting for 46.46%. The results revealed significant genetic structure among all populations of *E. affinis* examined. This was indicated by the values of F_{ST} and P , showing an F_{ST} value of 0.5354 and a P value of less than 0.05 (Table 4). The Mantel test results reveal a significant correlation between genetic differentiation (measured by the F_{ST} value) and geographical distance ($r = 0.6815$, $P = 0.0000$) among the examined populations.

Neutrality test and mismatch distribution

The neutrality assessment of the COI gene revealed a statistically significant negative Tajima's D statistic (-0.6416, $P > 0.05$) across all populations. However, the F_s statistic calculated by Fu (1997) yielded a positive value, although it did not reach statistical significance (0.4203, $P > 0.05$) (Table 3); this mismatch distribution analysis exhibited an unimodal pattern (Figure 4).

Table 3. Haplotype diversity, nucleotide diversity, Tajima's D, and Fu's F_s statistics for 19 *Euthynnus affinis* populations from Malaysian waters based on COI gene

Population	N	h	Hd	π	Tajima's D	Fu's F_s
PP	18	2	0.7994	0.0009	1.5476	1.4280
TB	14	1	0.0000	0.0000	—	—
PK	6	1	0.0000	0.0000	—	—
PT	21	1	0.0000	0.0000	—	—
SB	14	1	0.0000	0.0000	—	—
KS	24	1	0.0000	0.0000	—	—
KUK	12	1	0.0000	0.0000	—	—
SAN	20	1	0.0000	0.0000	—	—
LD	24	3	0.8961	0.0047	-2.3034*	-4.3770
TA	24	3	0.8833	0.0110	0.0275	4.6530*
KU	24	2	0.8419	0.0036	-1.7842	0.2360
KK	24	1	0.8806	0.0070	-1.2180	-0.7560
BIN	13	2	0.8508	0.0105	-2.2971*	0.7870
PB	14	1	0.8891	0.0132	-0.9982	0.0140
MUK	24	6	0.8452	0.0033	-2.1814*	-3.2320
KUA	24	2	0.8788	0.0137	1.5295	6.2490*
LUM	24	2	0.7849	0.0089	-0.9948	3.5950
END	24	8	0.8503	0.0054	-2.3601*	-2.5950
BM	24	2	0.8614	0.0033	-1.1581	1.9840
Total	372	22	0.5401	0.0045	-0.6416*	0.4203

Notes: N: Number of samples, h: Number of haplotypes, Hd: Haplotype diversity, π : Nucleotide diversity. *Significant at $P > 0.05$

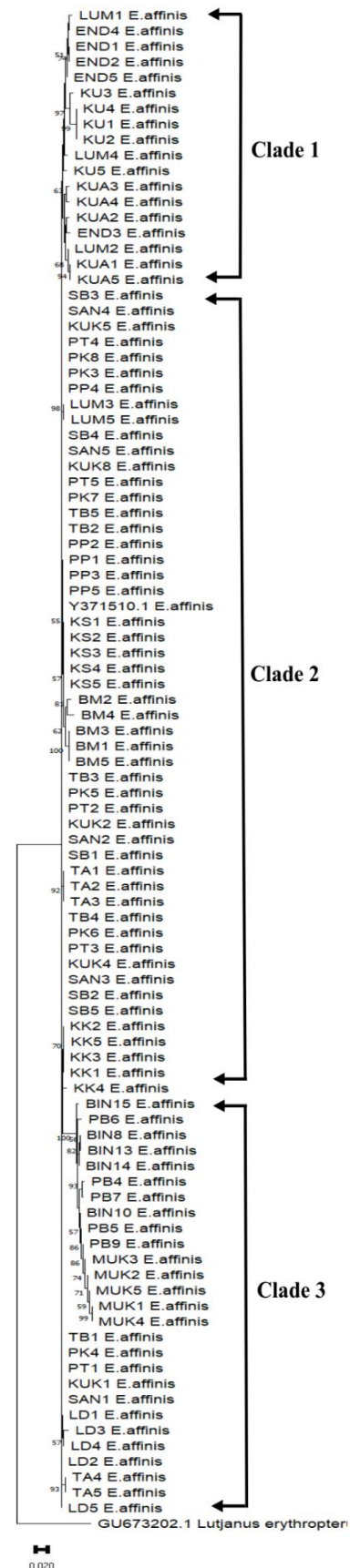


Figure 2. Neighbor Joining (NJ) tree illustrating the correlation among COI haplotypes of *Euthynnus affinis* specimens obtained from Malaysian waters. Only bootstrap values greater than 50% are displayed

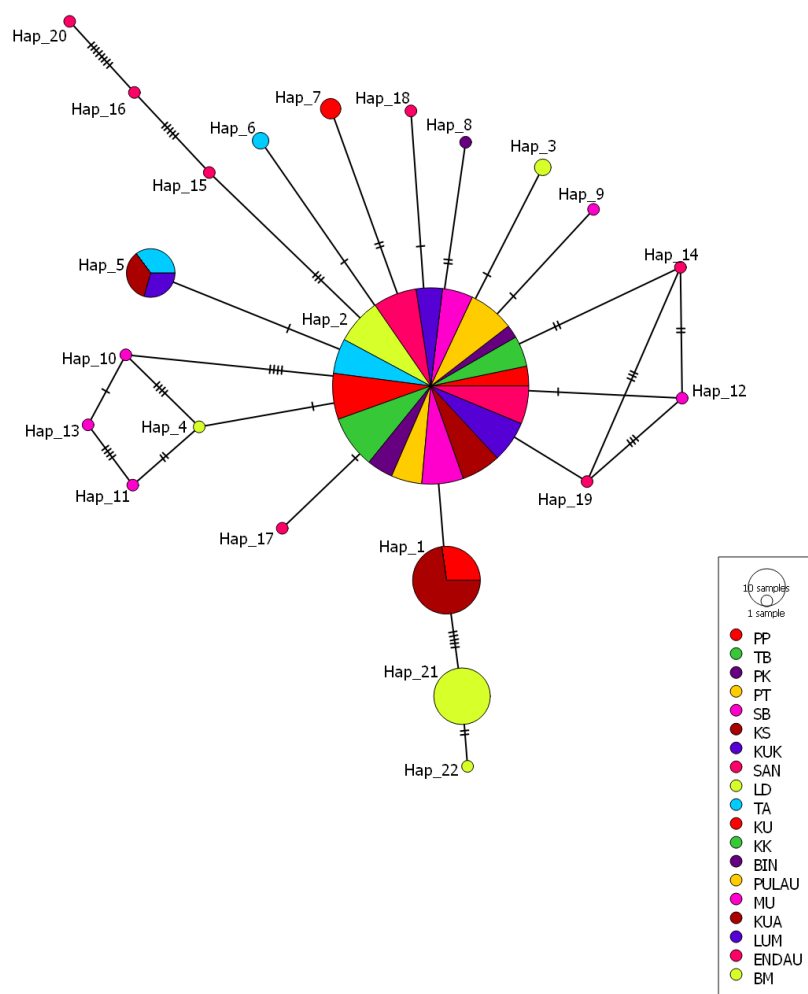


Figure 3. Minimum spanning network (MSN) using 22 COI haplotypes of *E. affinis* obtained from 19 distinct populations from Malaysian waters

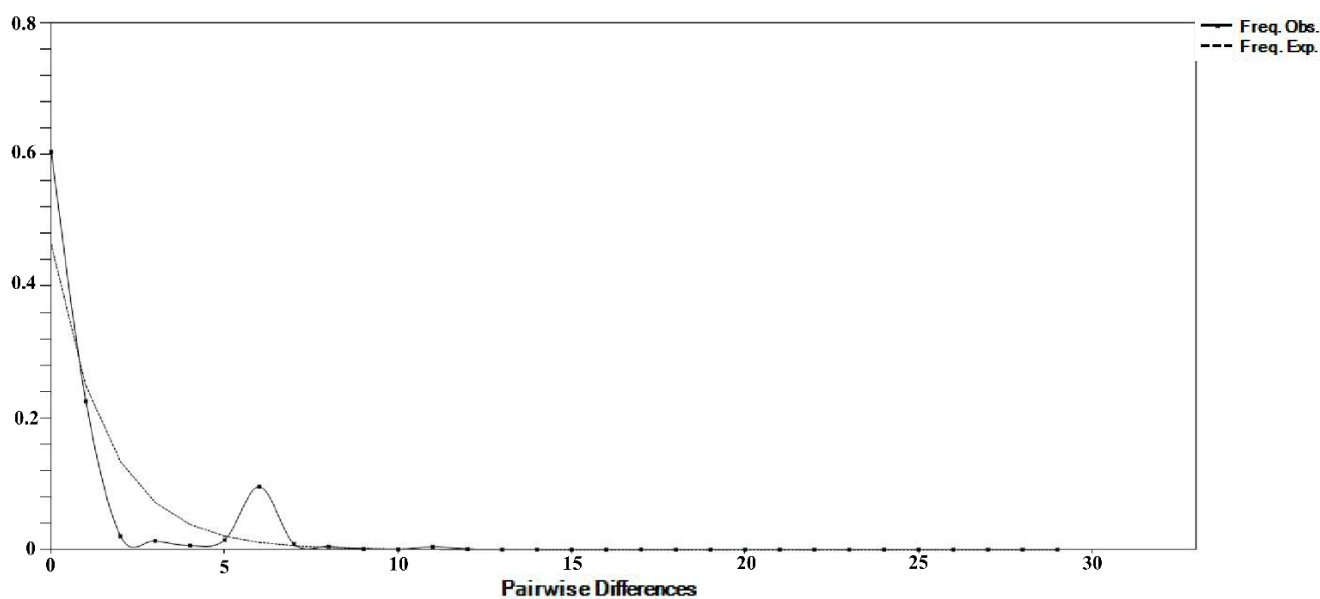


Figure 4. Mismatch distributions (pairwise number of differences) for the COI gene of *E. affinis* showing the expected and observed pairwise differences between sequences with the respective frequencies

Table 4. Analysis of molecular variance (AMOVA) of 19 populations of *Euthynnus affinis* collected from Malaysian waters based on the COI gene

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	F_{ST}	P
Among populations	18	2633.509	7.18584 Va	53.54	0.5354	0.0000
Within populations	353	2201.244	6.23582 Vb	46.46		
Total	371	4834.753	13.42166	100		

Discussion

The presence of skilled taxonomists is crucial in ensuring the accurate identification of species using morphological identification techniques or traditional methods, as there is a significant risk of misidentification resulting from phenotypic plasticity. Therefore, DNA barcoding has demonstrated significant utility in the identification of species, particularly for specimens that are imperfect, damaged, or at distinct morphological stages (Wang et al. 2018). The COI barcoding enables the precise identification of nearly 98% of documented marine fish species. This approach has been widely adopted across diverse geographic regions to classify and record fish populations (Becker et al. 2015). The current investigation effectively identified over 99% of *E. affinis* samples obtained using the Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) database utilizing the COI gene. This study demonstrates the efficacy of the COI gene in distinguishing *E. affinis* specimens and assessing the genetic diversity and population pattern of *E. affinis* collected from four important oceanic areas in Malaysia, encompassing a total of 19 landing sites.

The genetic distance within and between 19 populations of *E. affinis* exhibited a range from low to high values (0.000–0.082) (Table 2). The findings indicate that there exists a genetic differentiation among a total of 19 populations of *E. affinis*. The assessment of genetic distance is frequently employed to assess the taxonomic classification of closely related species and for evolutionary investigation. According to Hebert et al. (2003), a higher genetic distance indicates a substantial decrease in relatedness between the samples, accompanied by extensive variations in their nucleotide bases. Marine species commonly exhibit a substantial population size, the capacity to disperse extensively during their pelagic larval stages, and a wide geographical distribution (Palumbi 1992). For example, the limited genetic diversity observed among tuna populations across different oceans can be attributed to a consistent and extensive pelagic habitat and abundant spawning areas (Durand et al. 2005). In contrast, the physical obstacles in the marine environment, such as ocean currents, reefs, and other land formations, are thought to be why marine fish display genetic diversity and population structure. The presence of these barriers has the potential to impede the movement of genes between populations, resulting in genetic differentiation (Ward et al. 1994).

Furthermore, it is important to note that various fish species exhibit distinct environmental preferences, including but not limited to water temperature, salinity, and

depth. When populations of a given species inhabit distinct habitats, they have the potential to undergo distinct genetic adaptations in response to their respective environments. It is important to acknowledge that genetic differentiation is an inherent and customary phenomenon in all species. However, human activities such as overfishing, pollution, and habitat degradation may also lead to genetic differentiation and population fragmentation among marine fish species (Conover et al. 2006).

These present findings are consistent with prior studies that have examined the genetic structure of *E. affinis* in various marine regions, including the North Indian Ocean (Kumar et al. 2012), the Straits of Malacca (Masazurah et al. 2012), and the coastal waters of Tanzania (Johnson et al. 2016). These studies have identified discrete clades of *E. affinis*. Similarly, the findings of the current investigation align with the findings presented by Kasim et al. (2020), wherein a minimal genetic differentiation was observed among 11 distinct populations of *Thunnus tonggol* (Bleeker, 1851) (Longtail Tuna) in Malaysia. In their study, Akbar et al. (2018) provided findings on the genetic distance observed in *Thunnus obesus* (Lowe, 1839) (Bigeye Tuna). Their research focused on two distinct populations inhabiting Indonesia's North and South Moluccas Seas. The reported genetic distance values ranged from 0.023 to 0.027, indicating the level of genetic differentiation within and between these populations. Furthermore, evaluating the GC content in commercial fish helps understand nucleotide diversity and assess evolutionary lineages, leading to better interpretations of mutation pressures on taxa by selecting substitution models (Figueroa et al. 2014). For instance, higher GC content corresponds to more nucleotide diversity and a greater likelihood of mutations in a population (Imtiaz 2018).

An examination of haplotype diversity (H_d) and nucleotide diversity (π) within populations can offer valuable insights into the demographic history of a population (Grant and Bowen 1998). Low nucleotide diversity and high haplotype diversity indicate that the population is immense, has recently expanded, and contains unique genetic variations. Nevertheless, this also implies that there has been an insufficient duration for the accumulation of supplementary nucleotide substitutions across the various haplotypes (Grant and Bowen 1998; Kasim et al. 2020). As stated by Kumar et al. (2012), it is widely accepted that the substantial genetic diversity observed in marine fishes can be attributed to their large population sizes. The extensive haplotype diversity observed in this study can be attributed to the wide geographic range and substantial population sizes of *E. affinis*. The present findings are consistent with previous

studies conducted on various species of tuna, including *Kawakawa* (*E. affinis*) ($H_d = 0.67$, $\pi = 0.0124$) (Santos et al. 2010), Longtail Tuna (*T. tonggol*) ($H_d = 0.990$, $\pi = 0.0195$) (Kasim et al. 2020), bigeye tuna (*T. obesus*) ($H_d = 0.981$, $\pi = 0.028$) (Pertiwi et al. 2017). According to Vandewoestijne et al. (2004), the substantial diversity of haplotypes can be attributed, at least in part, to the elevated mutation rate observed in mitochondrial DNA. This assertion is corroborated by the analysis of MSN (Figure 3), which indicates that a minimum of three mutations occurred before a novel haplotype emerged.

The phylogenetic tree and MSN analysis demonstrate that the 19 populations of *E. affinis* obtained from Malaysian waters are classified into three distinct clades (Figures 2 and 3). The first clade comprises END, LUM, KUA, and KU samples. The second clade, comprising 12 populations (PK, PT, KUK, SAN, LUM, SB, PP, TB, KS, BM, TA, and KK), is notably dominated by haplotype Hap 2, which is the most prevalent. In comparison, the third clade encompasses individuals from LD, TA, BIN, PB, TB, PK, PT, KUK, SAN, and MUK. The remaining haplotypes are encompassed by the first and third clades (Figures 2 and 3).

Moreover, various genetic groups inside a species may be linked to secondary interaction and continuous interbreeding amongst populations that have been geographically apart for many generations (Durand et al. 2005). The results of MSN analysis indicate that a significant proportion of the populations exhibited shared genetic characteristics with Hap 2. This finding suggests that there has been a recent exchange of genetic material between these populations, as reported by Koopman et al. (2007). Furthermore, Horne et al. (2008) demonstrated that over a long period, significant genetic exchange and movement of individuals between geographically distant populations resulted in the sharing of haplotypes. In addition, LD, TA, BIN, MUK, END, and BM populations exhibited unique COI haplotypes. The findings of this study indicate a potential decrease in the exchange of genetic material between the studied locations, most probably caused by oceanic currents (Halim et al. 2022). Oceanic currents can affect gene flow among regions by creating barriers that isolate populations and increase differentiation levels or promoting long-distance dispersal (Gouvêa et al. 2023). The direction and strength of ocean currents can influence the movement of marine species and their larvae, which affects the distribution of genetic variation among populations (Snead et al. 2023). Therefore, oceanic currents are crucial in shaping marine populations' genetic structure by promoting or hindering gene flow among different regions. These currents are a key factor in the evolution and adaptation of marine species and have significant ecological and conservation implications (White et al. 2010).

The AMOVA analysis corroborated the findings derived from the analyses of the phylogenetic tree and MSN, thus emphasizing the presence of a genetic structure among the populations of *E. affinis*. Using F_{ST} values is a common practice in estimating gene flow, whereby a greater F_{ST} value signifies a substantial level of genetic

differentiation. In comparison, a lesser F_{ST} value indicates a minimal level of genetic differentiation (Jose et al. 2023). AMOVA analysis indicated a substantial level of F_{ST} values (0.5354) with a statistically significant difference ($P < 0.05$, 0.0000) (Table 4). Furthermore, the Mantel test or isolation by distance (IBD) demonstrates a substantial relationship between genetic difference and geographical distance. The findings of the present investigation confirm the emergence of a structural framework within *E. affinis* populations. Moreover, earlier studies have reported the existence of a genetic framework within tuna species (e.g., Kunal et al. 2013; Li et al. 2015).

The analysis of demographic history examines fluctuations in the size of a population over time, using nucleotide sequences to infer historical variations in mutation patterns (Tajima 1989). The present study employed an independent demographic history test to ascertain that populations of *E. affinis* obtained from Malaysia experienced a period of population expansion. The neutrality test yielded a statistically significant negative Tajima's D statistic for all populations (-0.6416 , $P > 0.05$). Additionally, Fu's F_s statistic was positive, although it did not reach statistical significance (0.4203 , $P > 0.05$) (Table 3). The findings show that Fu's F_s ' effectiveness in detecting significant population changes surpasses Tajima's D, thereby addressing the discrepancy between the two measures (Fu 1997). The results of this study indicate the occurrence of a population change in the past, suggesting a population expansion based on Tajima's D (Tajima 1989). Tajima's D is a widely employed method for assessing whether the nucleotide sequence is in a state of mutation-drift equilibrium. According to Korneliussen et al. (2013), a negative value of Tajima's D suggests the possibility of population growth, expansion, or the occurrence of purifying selection. A positive value of Tajima's D indicates balancing selection, population sub-structuring, or a recent population bottleneck. As stated by Fu (1997), the F_s test is susceptible to detecting evolutionary forces resulting from an abundance of new mutations within a population. Moreover, it is considered a potent tool for identifying instances of population expansion. Furthermore, the appearance of a unimodal mismatch distribution pattern, as shown in Figure 4, supports the concept of a dramatic population increase in the taxa's current state. These findings align with prior studies conducted on pelagic species, including *Thunnus albacares* (Bonnaterre, 1788) (Kunal et al. 2013; Li et al. 2015), *T. tonggol* (Kasim et al. 2020), and *Scomberomorus commerson* (Lacepède, 1800) (Johnson et al. 2021). In general, the findings of this study indicate that the *E. affinis* specimens collected from different geographical locations underwent a period of population expansion.

In conclusion, this study represents the inaugural documentation encompassing multiple locations in Malaysian waters, examining the genetic diversity and population structure of *E. affinis* through the utilization of the COI gene. The mitochondrial DNA gene, specifically COI, has been effectively utilized to discern 19 distinct populations of *E. affinis* in the waters of Malaysia. This analysis has unveiled the presence of three distinct lineages

within these populations, which are distributed across four marine regions: Straits of Malacca, South China Sea, Sulu Sea, and Celebes Sea. Moreover, the study findings will build a solid basis for successfully administering and protecting neritic tuna stocks in Malaysia. Further studies on *E. affinis* may necessitate the application of various DNA markers and an increased amount of sampling locations and specimens.

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Morphometric variations of *Kawakawa* (*Euthynnus affinis* Cantor, 1849) in Malaysian and Yemeni waters

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Abstract. Binashikhbubkr K, Setyawan AD, Naim DM. 2023. Morphometric variations of *Kawakawa* (*Euthynnus affinis* Cantor, 1849) in Malaysian and Yemeni waters. *Nusantara Bioscience* 15: 269-278. *Euthynnus affinis* Cantor, 1849 (*Kawakawa*), a small epipelagic tuna that migrates, is one of the most commercially significant tunas in Indo-Pacific subtropical and tropical waters. Unfortunately, there is a lack of knowledge on the region's stock structure and management of the particular migratory species. The present investigation aimed to distinguish body shape differences among 20 populations of *E. affinis* across Malaysian and Yemen coastal waters. A total of 416 specimens of *E. affinis* were sampled from five central geographic regions: the South China Sea, Straits of Malacca, Sulu Sea, Celebes Sea, and the Arabian Sea. Multivariate analyses, such as Principal Component Analysis (PCA), Discriminant Function Analysis (DFA), and Cluster Analysis (CA) of 12 morphometric variables, were conducted to differentiate 20 populations of *E. affinis*. The findings revealed significant differences in the body form among 20 populations. Morphological homogeneity was observed among populations from Peninsular Malaysia and Malaysian Borneo (Sabah and Sarawak). As expected, Yemen's (Mukalla City) populations were clearly segregated and formed a separate cluster. The highest percentage of correctly classified specimens (100%) came from populations in Pulau Tenggol (Terengganu) and Kukup (Johor). In contrast, the highest percentage of misclassified specimens (100%) came from populations in Kota Kinabalu (Sabah), Bintawa (Sarawak), Lumut (Perak), and Endau (Johor). Based on the Mahalanobis distances, hierarchical cluster analyses revealed that 20 populations of *E. affinis* could be clustered into three distinct groups. This is the first report on *E. affinis* from Peninsular Malaysia, Malaysian Borneo (Sabah and Sarawak), and the waters of Yemen using morphometric techniques.

Keywords: DFA, *Euthynnus affinis*, morphometric, PCA, population discrimination

INTRODUCTION

The marine fisheries sectors in Malaysia and Yemen are poised for a significant growth phase, propelling them towards becoming prominent fishing hubs in Asia. Their strategic geographical locations strongly bolster this promising outlook, as both countries are surrounded by vital oceans, endowing them with abundant marine resources. In the case of Malaysia, the marine fisheries sector has made substantial contributions to the nation's economy. In 2021 alone, this sector yielded approximately 1.7 million metric tons of seafood, estimated at around RM 15 billion (Annual Fisheries Statistics 2021). Meanwhile, Yemen boasts a wealth of fisheries resources, as evidenced by a total production of 228,655 metric tons recorded in 2012 (Ministry of Fish Wealth 2015). This industry also provides several job possibilities and exports and protein supplies worldwide to Malaysians and other regional countries (Bakar et al. 2018).

The major Malaysian landing places exhibit a diverse range of marine fish species, estimated to be approximately 200-300. Daily, the fish landing site at these locations typically offers an average of 50-100 distinct species. During monsoon season, various species exhibit regular emergence patterns, with certain species potentially

dominating the landing sites. In contrast, permanent resident species of estuaries, bays, and reefs can be found throughout the year (Bakar et al. 2018). Fish possess significant economic value and serve as a valuable source of animal protein for human consumption while also being crucial in maintaining biodiversity (Marchetti et al. 2020). The Thunnini tribe comprises the genera *Thunnus*, *Auxis*, *Katsuwonus*, and *Euthynnus*, which hold significant economic value (Pertiwi et al. 2017; Sato et al. 2020). *Euthynnus affinis*, *Euthynnus alletteratus*, and *Euthynnus lineatus* species belong to the *Euthynnus* Genus. *Kawakawa*, scientifically known as *E. affinis* (Cantor 1849), holds significant ecological importance as one species in the Scombridae family. This species is distributed in the Indo-Pacific region across tropical and sub-tropical waters (Yazawa et al. 2019; Khoa et al. 2021). Due to its migratory neritic epipelagic nature, this species has received significant attention as Malaysia's most significant tuna species with commercial value (Yazawa et al. 2015). In contrast, *E. alletteratus* and *E. lineatus* are found exclusively in the Atlantic and Pacific Oceans, respectively (Kumar and Kunal 2013; Sulistyaningsih et al. 2014).

Morphometric analysis of fish is very important in fisheries management, as it allows the calculation of

evolutionary characteristics and the identification of variations in form, ontogeny, function, and evolutionary relationships between individual animals. In this regard, identifying changes among populations of the same fish species across different geographical areas is the main function of morphometric characteristics (Meshram et al. 2021). Multiple multivariate techniques are commonly used to distinguish between populations of different fish species. These techniques include Principal Component Analysis (PCA), Discriminant Function Analysis (DFA), and Cluster Analysis (CA). Several researches have been conducted using DFA, PCA, and CA to evaluate morphometric variation between different fish species (e.g., Jalili et al. 2015; Pérez-Quinón et al. 2017; Mounir et al. 2019; Aminan et al. 2020; Binashikhbubkr et al. 2022).

Previous research on *E. affinis* has mostly focused on various factors, including reproductive behavior, biological characteristics, stock assessment, genetic structure, and population dynamics (Santos et al. 2010; Rohit et al. 2012; Johnson and Tamatamah 2013; Sulistyaningsih et al. 2014; Nissar et al. 2015; Kumar et al. 2019; Binashikhbubkr et al. 2023). On the other hand, an investigation managed by Griffiths et al. (2017) focused on the morphological relationships of 4 fish species in the Scombridae family found in Australian waters. This study specifically determined the relationship between fork length and total length, as well as the relationship between length and weight for several species such as *E. affinis*, *Thunnus tonggol*, *Cybiosarda elegans*, and *Rastrelliger kanagurta*. However, there have been no morphometric investigations concerning *E. affinis* populations in many geographical areas, including Peninsular Malaysia, Malaysian Borneo (Sabah and Sarawak), and the Arabian Sea (specifically Mukalla City, Yemen). Therefore, to fill the gap in this field of study, this current study attempts to examine and differentiate morphological differences across 20 different populations of *E. affinis* found in Peninsular Malaysia, Borneo Malaysia, and Mukalla City, Yemen.

MATERIALS AND METHODS

Study area and sampling activities

Approximately 416 individuals of *Kawakawa* hailing from diverse regions, including Peninsular Malaysia, Malaysian Borneo, and Mukalla City (Yemen), were meticulously gathered directly by local fishermen at 20 distinct commercial fish landing sites between November 2019 and September 2021. These landing sites comprised 19 in Malaysia and one in Mukalla City (Figure 1). The specimens were obtained from 5 prominent geographical areas: South China Sea (SCS), Straits of Malacca (M), Sulu Sea (SS), Celebes Sea (CS), and Arabian Sea (AS). Specifically, within the Straits of Malacca, data were collected from 5 landing sites: Sungai Besar and Kuala Selangor in Selangor, Kukup in Johor, Lumut in Perak, and

Batu Maung in Penang. In the South China Sea region, 10 landing sites were chosen: Pasir Puteh and Tok Bali in Kelantan, Pantai Kijal and Pulau Tenggol in Terengganu, Kota Kinabalu in Sabah, Bintawa, Pulau Bruit, and Mukah in Sarawak, Kuantan in Pahang, and Endau in Johor. Additionally, the Sulu Sea was represented by 2 landing sites, Sandakan and Kudat in Sabah, while the Celebes Sea featured 2 sites: Lahad Datu and Tawau in Sabah. Finally, 1 landing site was selected from the Arabian Sea, specifically Mukalla City in Yemen (Figure 1).

Subsequently, samples from Malaysian waters were transported in an icebox to the School of Biological Science at Universiti Sains Malaysia for investigation. Following the taxonomic guidelines provided by Collette and Nauen (1983), the collected specimens were morphologically identified and validated. Upon their arrival at the School of Biological Science, Universiti Sains Malaysia, the Malaysian samples underwent a thorough cleaning process with running water. Subsequently, they were gently tapped and systematically arranged on an even top with contrasting dark surrounding to ensure optimal clarity and visibility for further examinations. Conversely, the specimens originating from Mukalla City were subjected to measurements and photographic documentation within the confines of the Laboratory of Biology, Faculty of Science, Hadhramout University, Yemen. Each sample was meticulously labeled and snapshotted with a Nikon D90 digital camera. To assure precision, a digital caliper was employed to measure the morphometric variables, with measurements recorded to the nearest 0.1 cm (Figure 2).

Morphometric analyses

Moreover, the morphometric analysis of *E. affinis* involved the measurement of 12 characters using a digital caliper : Standard Length (SL), Fork Length (FL), Total Length (TL), Head Length (HL), Body Depth (BD), Snout length (Snl), Eye Diameter (ED), pectoral fin distance (DP), first dorsal fin distance (DD1), second dorsal fin distance (DD2), anal fin distance (DA), and ventral fin distance (DV) (Figure 2). Therefore, to mitigate the influence of size, all morphometric variables were normalized using the equation proposed by Elliott et al. (1995):

$$M_{adj} = M(L_s/L_0)^b$$

Where:

M : Original measurement

M_{adj}: Size-adjusted measurement

L_s : Overall mean of the standard length for all samples

L₀ : Standard length of each sample

b : Each character from the observed data as the slope of the log M on log L₀ regression, encompassing all fish from all populations.

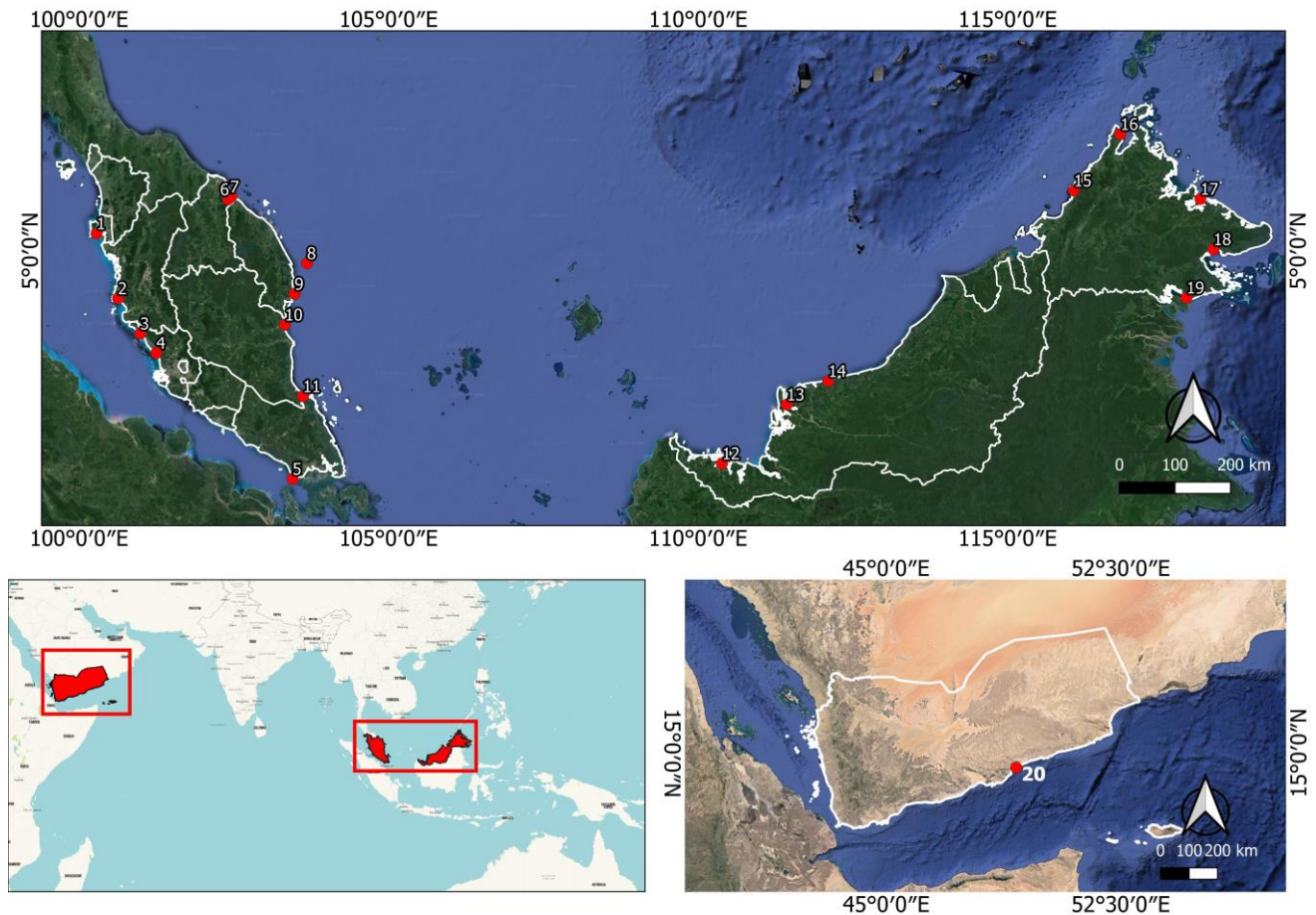


Figure 1. Sampling locations of *Euthynnus affinis* specimens collected from the Straits of Malacca, South China Sea, Sulu Sea, Celebes Sea, and Arabian Sea regions are as follows: 1. Batu Maung, Penang; 2. Lumut, Perak; 3. Sungai Besar, Selangor; 4. Kuala Selangor, Selangor; 5. Kukup, Johor; 6. Pasir Puteh, Kelantan; 7. Tok Bali, Kelantan; 8. Pulau Tenggol, Terengganu; 9. Pantai Kijal, Terengganu; 10. Kuantan, Pahang; 11. Endau, Johor; 12. Bintawa, Serawak; 13. Pulau Bruit, Serawak; 14. Mukah, Serawak; 15. Kota Kinabalu, Sabah; 16. Kudat, Sabah; 17. Sandakan, Sabah; 18. Lahad Datu, Sabah; 19. Tawau, Sabah; 20. Mukalla, Yemen

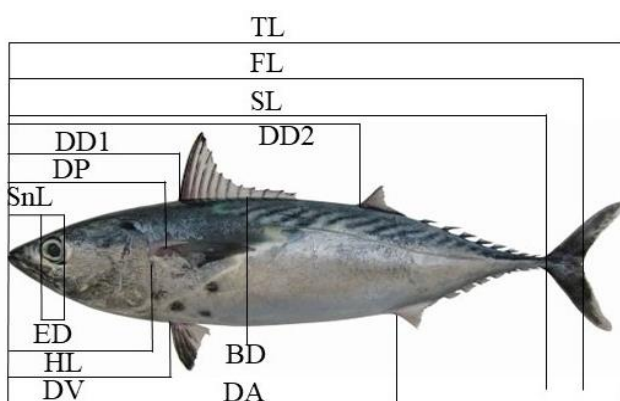


Figure 2. Morphometric measurements of *Euthynnus affinis* (Kawakawa). TL: Total Length, FL: Fork Length, SL: Standard Length, HL: Head Length, BD: Body Depth, ED: Eye Diameter, SnL: Snout length, DP: Distance of pectoral fin, DD1: Distance of the first dorsal fin, DD2: Distance of the second dorsal fin, DV: Distance of ventral fin, DA: Distance of anal fin.

A multivariate Discriminant Function Analysis (DFA) was employed to distinguish among 20 *E. affinis*

populations by identifying a combination of features that maximized population divergence. The discriminant analysis used the results to determine correctly differentiated characters' proportions. Wilks' lambda analysis was utilized to distinguish variations among the populations. In this study, Discriminant Function Analysis (DFA) and Principal Component Analysis (PCA) were conducted using SPSS software version 25 to pinpoint the most influential differences in body shape for distinguishing between populations. To assess population relationships among 20 *E. affinis* populations, hierarchical cluster analysis was carried out based on Mahalanobis distances, employing the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Hammer et al. 2001).

RESULTS AND DISCUSSION

Sampling data

Moreover, 416 samples of *E. affinis* were collected from 20 distinct locations within 5 primary geographic regions: Straits of Malacca, South China Sea, Sulu Sea, Celebes Sea, and Arabian Sea (AS).

Table 1. Eigenvalue and Wilk's lambda of each function of DFA of *Euthynnus affinis*

Function	Eigenvalue	% of variance	Cumulative %	Canonical correlation	Wilks' Lambda	Sig.
1	2.897 ^a	47.9	47.9	0.862	0.026	0.000
2	1.500 ^a	24.8	72.7	0.775	0.102	0.000
3	0.667 ^a	11.0	83.7	0.632	0.254	0.000
4	0.501 ^a	8.3	92.0	0.578	0.423	0.000
5	0.188 ^a	3.1	95.1	0.398	0.635	0.000
6	0.176 ^a	2.9	98.0	0.387	0.755	0.001
7	0.076 ^a	1.3	99.2	0.267	0.888	0.649
8	0.028 ^a	0.5	99.7	0.165	0.956	0.994
9	0.010 ^a	0.2	99.9	0.099	0.982	0.999
10	0.008 ^a	0.1	100.0	0.089	0.992	0.977

Note: ^a) indicates the first 10 discriminant functions used in the analysis

Table 2. The structure matrix represents the distributed correlations among measurement characteristics and functions for morphometric characteristics.

Variables	Structure Matrix									
	Function									
	1	2	3	4	5	6	7	8	9	10
DP	.969*	.004	.026	.039	.014	-.043	-.051	-.224	-.058	-.005
DD1	.912*	-.038	.010	.150	.115	-.031	.020	-.299	-.035	-.002
DV	.876*	-.024	.005	-.080	.119	-.049	.159	-.386	.134	.132
DA	.798*	.128	-.099	.400	.222	-.040	.006	-.273	-.080	.213
HL	.711*	.401	-.178	.387	-.281	.147	.136	.033	.070	.166
DD2	.686*	.144	-.031	.564	.168	.150	.061	-.362	-.044	-.046
TL	.683*	.598	-.050	.290	-.006	.078	-.079	-.152	-.115	.144
FL	.672*	.577	-.105	.298	.013	.093	-.006	-.218	-.197	.146
SL	.660*	.610	-.116	.293	.051	.083	-.079	-.236	-.103	.110
BD	.541	.620*	.322	.409	-.045	-.189	-.006	.066	-.018	.095
ED	.526	.572*	-.256	.262	.286	.280	.211	.184	-.096	-.121
SnL	.422	.508*	.461	.210	-.017	.247	-.291	.131	.196	.326

Note: *Largest absolute correlation between each variable and any discriminant function

Table 3. Correct and misclassifications for 20 populations of *Euthynnus affinis* collected from Peninsular Malaysia, Malaysian Borneo, and Mukalla City (Yemen)

Sampling Locations	N	% of Correctly Classified	% of Misclassified	Total (%)
Pasir puteh, Kelantan	18	38.9	61.1	100
Tok Bali, Kelantan	14	71.4	28.6	100
Pantai Kijal, Terengganu	8	62.5	37.5	100
Pulau Tenggol, Terengganu	21	100	0	100
Sungai Besar, Selangor	14	92.9	7.1	100
Kuala Selangor, Selangor	24	37.5	62.5	100
Kukup, Johor	15	100	0	100
Mukalla, Yemen	18	94.4	5.6	100
Sandakan, Sabah	20	25	75	100
Lahad Datu, Sabah	24	4.2	95.8	100
Tawau, Sabah	24	4.2	95.8	100
Kudat, Sabah	24	50	50	100
Kota Kinabalu, Sabah	24	0	100	100
Bintawa, Sarawak	24	0	100	100
Pulau Bruit, Sarawak	24	16.7	83.3	100
Mukah, Sarawak	24	8.3	91.7	100
Kuantan, Pahang	24	4.2	95.8	100
Lumut, Perak	24	0	100	100
Endau, Johor	24	0	100	100
Batu Maung, Penang	24	25	75	100
Total	416			

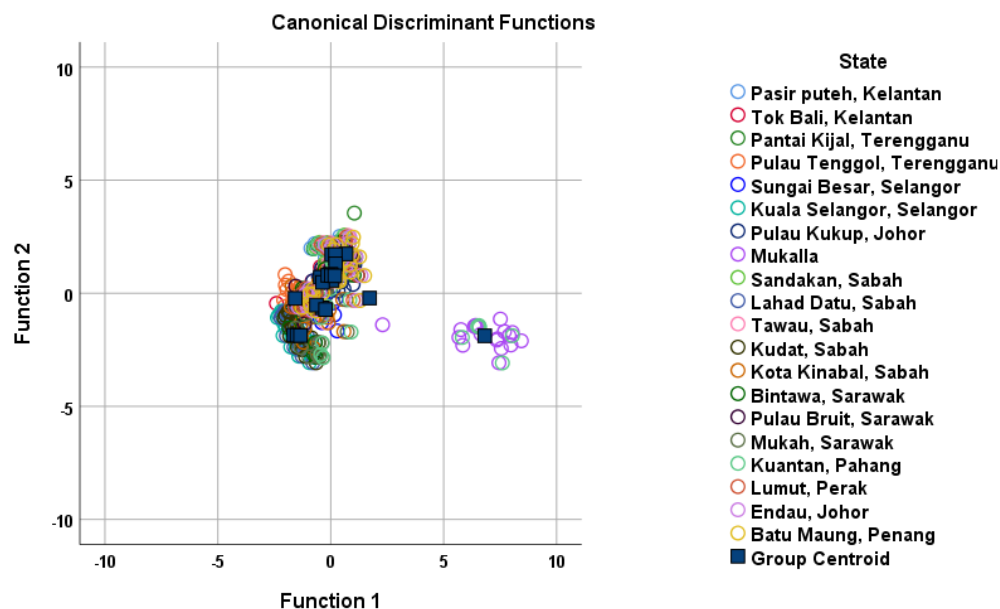
Table 4. Principal component analysis scores of variations within the population of *Euthynnus affinis*

PC	Eigenvalue	% Variance	Cumulative %
1	9.948	82.902	82.902
2	1.141	9.511	92.414
3	0.420	3.499	95.912
4	0.149	1.245	97.157
5	0.109	0.909	98.066
6	0.093	0.773	98.839
7	0.076	0.634	99.474
8	0.027	0.228	99.702
9	0.021	0.175	99.877
10	0.007	0.057	99.935
11	0.005	0.041	99.976
12	0.003	0.024	100.000

Discriminant Function Analysis (DFA)

The discriminant function analysis has properly recognized ten functions, namely functions 1 to 10, with the variances being 47.9%, 24.8%, 11.0%, 8.3%, 3.1%, 2.9%, 1.3%, 0.5%, 0.2%, and 0.1%, respectively. Functions 1 to 4 revealed a notable association for population differentiation with values of 0.86, 0.78, 0.63, and 0.58, respectively (Table 1). Wilk's Lambda values were derived from analyzing 10 functions (designated as functions 1 through 10). The corresponding significance values for the first six functions are as follows: 0.026, 0.102, 0.254,

0.423, 0.635, and 0.755, yielded probabilities of $p = 0.000$ and 0.001 (Table 1). We discover that the distance of the pectoral fin (DP) had the highest correlation score at 0.969 among the measurement variables, followed closely by the length of the first dorsal fin (DD1) at 0.912, ventral fin's length (DV) at 0.876, anal fin's length (DA) at 0.798, Head Length (HL) at 0.711, second dorsal fin's length (DD2) at 0.686, Total Length (TL) at 0.683, Fork Length (FL) at 0.672, and Standard Length (SL) at 0.660 when setting up the DFA based on the variables' scores in function 1 (Table 2). Therefore, based on DFA, the highest percentage of correct identifications (100%) among 20 populations of *E. affinis* into their original groups was observed in Pulau Tenggol and Kukup. In contrast, the lowest percentage of correct identifications (0%) was found in the populations from Kota Kinabalu, Bintawa, Lumut, and Endau (Table 3). In this current study, the assessment of DFA demonstrated that the highest percentage of accurately classified was from Pulau Tenggol (Terengganu) and Kukup (Johor), both with 100%. In contrast, the highest percentage of misclassification was observed in the populations from Kota Kinabalu (Sabah), Bintawa (Sarawak), Lumut (Perak), and Endau (Johor), all with 100%, respectively. The canonical discriminant function analysis indicated that the mean body patterns of specimens from Malaysian Borneo and Peninsular Malaysia were morphologically identical. In contrast, the mean body patterns for populations from Mukalla City (Yemen) were segregated (Figure 3).

**Figure 3.** Canonical discriminant analysis based on morphometric measurements of 20 populations of *Euthynnus affinis*

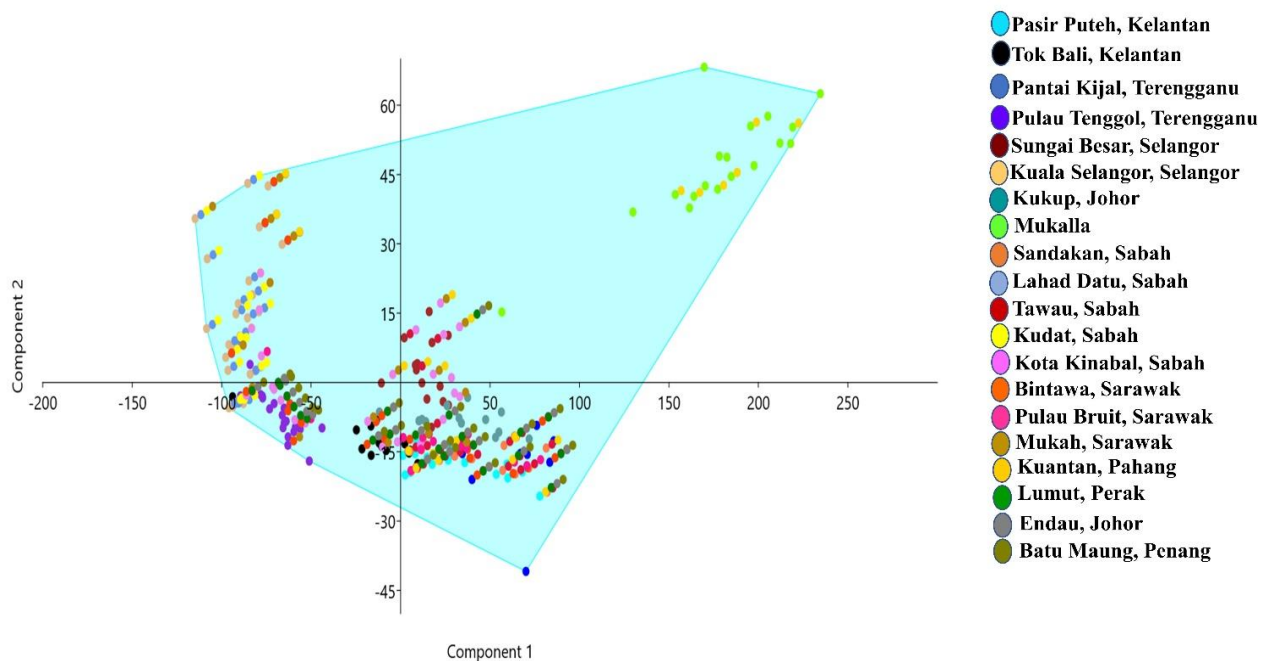


Figure 4. The principal components analysis of *Euthynnus affinis* shows PC1= 82.902% and PC2= 9.511%, accounting for 92.414% of the total variation in 416 samples

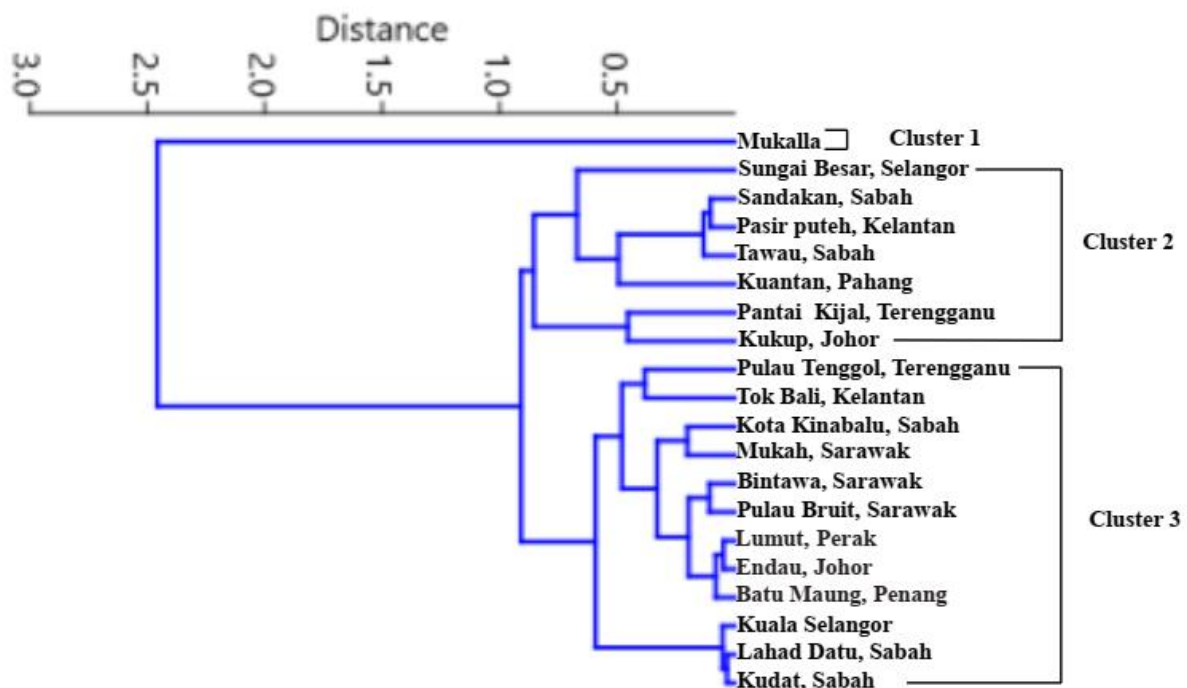


Figure 5. A dendrogram was generated from cluster analysis based on Mahalanobis distances among 20 populations of *Euthynnus affinis* using morphometric measurements

Principal Component Analysis (PCA)

The analysis of PCA performed on 416 individuals revealed 12 components that were instrumental in identifying variations in body shape. The first component exhibited the highest variance, accounting for 82.902% and possessing an eigenvalue of 9.948, which attests to its significant influence (an eigenvalue more than 0.3 is

regarded as significant). However, when considering the first four Principal Components (PCs), the breakdown of variance was as follows: 82.902% for PC1, 9.511% for PC2, 3.499% for PC3, and 1.245% for PC4, summing up to an overall variance of 97.157% that elucidates the variations in body shape (Table 4). Our findings indicate that the subtle morphological deviation among *E. affinis*

individuals was primarily influenced by the overlaying structure observed in the graph of PC1 vs. PC2, where a remarkably notable eigenvalue was significant in discerning *E. affinis* based on shared body shape characteristics (Figure 4). In summary, Figure 4 illustrates that the populations exhibited significant overlap, with no clear difference, signifying a high degree of morphological homogeneity across Peninsular Malaysia and Malaysian Borneo populations. Notably, the population from Mukalla City (Yemen) stood out as a distinct and separate group or cluster. In general, the results obtained from PCA confirmed the DFA results. Hierarchical cluster analysis based on the Mahalanobis distances demonstrated that 20 populations of *E. affinis* were grouped into three separate clusters (Figure 5). The first group comprises populations exclusively from Mukalla City. The second group encompasses populations from Sungai Besar (Selangor), Sandakan (Sabah), Pasir Puteh (Kelantan), Tawau (Sabah), Kuantan (Pahang), Pantai Kijal (Terengganu), and Kukup (Johor). The third and final group includes populations from Pulau Tenggol (Terengganu), Tok Bali (Kelantan), Kota Kinabalu (Sabah), Mukah (Sarawak), Bintawa (Sarawak), Pulau Bruit (Sarawak), Lumut (Perak), Endau (Johor), Batu Maung (Penang), Kuala Selangor, Lahad Datu (Sabah), and Kudat (Sabah).

Discussion

Discriminant Function Analysis (DFA)

In identifying *E. affinis*, function 1 in DFA is the best attribute with a variance value of 47.9% (Table 1), and Wilk's lambda score is 0.026. When loading into the analysis, distance of pectoral fin (DP) is revealed to have a crucial role in recognizing *E. affinis* with the greatest contribution values/score, followed by distance of the first dorsal fin (DD1), ventral fin's distance (DV), anal fin's distance (DA), head length (HL), second dorsal fin's distance (DD2), Total Length (TL), Fork Length (FL), and Standard Length (SL) in function 1 (Table 2). Our results confirm the study by Aminan et al. (2020) and Gain et al. (2017), in which they highlighted that function 1 in DFA was able to distinguish intrapopulation in samples of *Rasbora* spp. and *Cirrhinus cirrhosus*. Furthermore, a similar study in Tunisian waters revealed that fork length, standard length, and pectoral fin are important characteristics in differentiating between bluefin tuna populations *Auxis rochei* (Allaya et al. 2016). Likewise, a study conducted by Karakulak et al. (2016) demonstrated that there is a clear relationship between the length of the head and the length of the pectoral fin, the first predorsal, and the height of the second dorsal fin between *Thunnus thynnus* and *E. alletteratus*. Furthermore, Griffiths et al. (2017) divulged a highly significant relationship between fork length-total length and length-weight among four Scombridae fish species (*E. affinis*, *T. tonggol*, *R. kanagurta*, and *C. elegans*). Body characteristic variations were influenced by exterior factors such as food and water quality availability and dependent and independent elements (Kumar et al. 2019; Sawalman and Madduppa 2020). Differentiation in the shape of head pattern is also

attributable to using different environmental niches, food, and prey species (Cronin-Fine et al. 2013).

The assessment of DFA carried out in this study demonstrated that the group member of *E. affinis* was accurately recognized, with a maximum percentage (100%) of classifications for the populations from Pulau Tenggol (Terengganu) and Kukup (Johor), followed by populations from Mukalla City (94.4%), Sungai Besar (Selangor) (92.9%), Tok Bali (Kelantan) (71.4%), and Pantai Kijal (Terengganu) (62.5%). On the other hand, the high percentage of misclassified (100%) was in the populations from Kota Kinabalu (Sabah), Bintawa (Sarawak), Lumut (Perak), and Endau (Johor), followed by populations (95.5%) in the populations from Lahad Datu and Tawau (Sabah), Kuantan (Pahang), and Mukah (Sarawak) (91.7%), Pulau Bruit (Sarawak) (83.3%), Kuala Selangor (Selangor) (62.5%), Pasir Puteh (Kelantan) (61.1%), Sandakan (Sabah), and Batu Maung (Penang) (75%), and Kudat (Sabah) (50%) (Table 3). The results from our study were comparable to Ganjali et al. (2021), who reported that the overall correctly classified topmouth gudgeon (*Pseudorasbora parva*) from Iranian waters was 74.5%. The data on predicted group membership was derived from the study of components that exhibited a significant level of similarity (Kočišová and Mišanková 2014; Aminan et al. 2020). Furthermore, canonical discriminant assessment asserted that morphological similarities prevailed between Peninsular Malaysia and Malaysian Borneo populations. The population from Mukalla City is clearly separate from the population caught in Malaysian waters (Figure 3). Generally, DFA has exhibited significant variabilities between 20 *E. affinis* populations sampled. Our findings were analogous to those of Hajjej et al. (2013) and Pérez-Quinonez et al. (2017), who reported the presence of morphologically segregated groups of *E. alletteratus* and *Opisthonema*, respectively using DFA. According to some theories, environmental and genetic variations could have caused the gap across populations (see Verma et al. 2014; Madduppa et al. 2017). Generally, various causes influence morphological variances; environmental influences, genetic factors, and habitat diversity are only a few examples (Tzeng 2004). Because of the genetic effects on the ontogenies of individuals, selection, and environment, geographic separation possibly occurs in the evolution of varied physical features between fish populations (Sajina et al. 2011). The primary factor contributing to the divergence of fish populations in distinct aquatic habitats is geographical segregation, which frequently results in reduced gene flow between these populations. Following Konan et al. (2010), geographical obstacles foster genetic and morphological variations across populations, facilitating differentiation. As Mahfuj et al. (2022) specified, the variation in phenotypic features observed among stocks can be attributed to genetic variations arising from population migration, natural selection, mutation, and environmental factors. These factors, in turn, are influenced by the geographical location. Based on the characteristics of the Arabian Sea, it exhibits various features that can lead to differences in the fish body shape, including high productivity, food availability, salinity, biodiversity, a wide

range of temperatures (Jawad 2021), geographical barriers, and discrete spawning time (Barth et al. 2017). Interestingly, prior research has indicated notable dissimilarities between Yellowfin tuna (*Thunnus albacares*) specimens procured from the Arabian Sea and those sourced from the Atlantic, Indo-Pacific regions, and the Bay of Bengal (Kunal et al. 2013; Barth et al. 2017).

Principal Component Analysis (PCA)

The first four principal components (PCs) addressed 97.157% of the variation in body shape across all variables, demonstrating high significance with eigenvalues exceeding 0.3. An eigenvalue greater than 0.3 is considered significant (Lombarte et al. 2012). The elevated scores of the first four PCs indicate substantial variations in the overall body shape among all examined populations of *E. affinis*. (Figure 4 and Table 4). This finding aligns with the outcomes achieved by Claverie and Wainwright (2014), who found that the first four PCs consequences in 75.3% of morphological variations in the reef fishes. Similar findings were also recorded in studies by Imtiaz and Naim (2018) (80% of body shape variations in the Genus *Nemipterus*) and Mounir et al. (2019) (88.96% of body shape variations in *Sardina pilchardus*). In the same way, Addis et al. (2014) revealed that the first two PCs accounted for 56.97% of the shape variations in *T. thynnus*. Particularly, we outlined that the differences in the morphological patterns of *E. affinis* prevailed in the overall body pattern. As a result, our findings are comparable to those of Jalili et al. (2015), which determined that phenotypic differences between *Alburnus filippii* depend on snout tip and body depth variations. Similarly, Mounir et al. (2019) highlighted head length, dorsal fins, and body depth as critical variables characterizing differences in *S. pilchardus* populations along the Moroccan Atlantic coast.

The PCA results generally corroborated the DFA findings, indicating that morphological homogeneity existed among Peninsular Malaysia and Malaysian Borneo populations. However, specimens in Mukalla City were clearly separated and established their cluster. The samples' overlapping distribution could be attributed to significant seasonal migration in these waters, showing that these populations have a temporal and spatial residence in these areas. Interestingly, our findings proved that the previous genetic studies on the genetic makeup of *E. affinis* from various oceanic provinces such as Philippines and Peninsular Malaysia (Pangkor Island, Penang) (Santos et al. 2010), North Indian Ocean (Kumar et al. 2012), Straits of Malacca (Masazurah et al. 2012), and Tanzania coastal waters (Johnson et al. 2016), which reported that the genetic structure of *E. affinis* was a single stock. Based on the outcomes derived from the hierarchical cluster analysis, the populations of *E. affinis* were clustered into three clusters, as illustrated in Figure 5. These differences between population differences can be ascribed to each population's distinct geographical locations and environmental constraints (Siddik et al. 2016). According to the cluster analysis, our findings are comparable to the results obtained by Siddik et al. (2016) and El Mghazli et al. (2021), who found that the populations of Gangetic

whiting (*Sillaginopsis panijus*) from the Bangladesh and Atlantic horse mackerel (*Trachurus trachurus*) from the Moroccan Atlantic coast were each clustered into two groups. Fish exhibit notable morphological diversity within and across populations, making them particularly sensitive to environmental adaptation, eventually influencing their phenotypes (Verma et al. 2014). Furthermore, these variations have been linked to marine ecology, encompassing light penetration, food availability, and water currents (Imtiaz and Naim 2018).

In conclusion, this current research is the first of its kind to employ morphometric techniques for determining and discriminating Malaysian populations of *E. affinis* [Peninsular Malaysia, Borneo (Sabah and Sarawak)], and Mukalla City, Yemen. Discriminant function analysis (DFA), principal component analysis (PCA), and cluster analysis (CA) have revealed significant differences in body form among 20 *E. affinis* populations investigated. The findings will deliver precious information for the proper governance and preservation of *E. affinis* in these regions. Further research combining morphometric and molecular techniques is needed to interpret population delimitation accurately.

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Optimization of L-asparaginase production from endophytic bacteria isolated from the mangrove *Rhizophora mucronata*

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Abstract. Nafisaturrahmah A, Susilowati A, Pangastuti A. 2023. Optimization of L-asparaginase production from endophytic bacteria isolated from the mangrove *Rhizophora mucronata*. Nusantara Bioscience 15: 279-287. L-asparaginase is an enzyme that hydrolyses L-asparagine to L-aspartate and ammonia. L-asparaginase has the potential to treat acute lymphoblastic leukemia and other malignant cancers. So far, purified L-asparaginase from *Escherichia coli* and *Erwinea chrysanthemi* has been available and applied clinically in humans. However, this treatment has side effects such as allergy, cross-interaction, immune system stimulation, drug resistance, and nonspecific L-glutaminase activity. These side effects can be overcome by discovering new sources of L-asparaginase, which are serologically different but have similar therapeutic effects. This study aims to determine the optimal conditions of endophytic bacterial culture in producing L-asparaginase. Endophytic bacteria were screened using an M9 medium with asparagine as a substrate; the L-asparaginase-producing isolates showed pink zones around the colonies. Optimization of L-asparaginase production by endophytic bacteria is carried out by One Factor at A Time (OFAT). Optimization of enzyme production includes incubation time, temperature, pH, ammonium sulfate levels, and glucose concentration in the bacterial growth medium; determination of enzyme production by Nesslerization method. The results showed that 8 isolates could produce high L-asparaginase, 14 isolates had medium ability, 30 isolates had low ability, and 2 bacterial isolates did not produce L-asparaginase. Endophytic isolates were able to produce the highest L-asparaginase under different optimal conditions. The optimal incubation time for endophytic isolates in this study was 60-84 hours, the optimal temperature was 37°C, the optimal pH was 7, the nitrogen content was 0.25 mg/L, and the optimal glucose level was 3%.

Keywords: Endophytic bacteria, enzyme production, L-asparaginase, mangrove *Rhizophora mucronata*, optimization

INTRODUCTION

L-asparaginase is a catalytic enzyme in the hydrolysis of L-asparagine to L-aspartate and ammonia. L-asparagine is a nonessential amino acid used for protein synthesis and cell growth. L-asparaginase has the potential to treat acute lymphoblastic leukemia and other malignant cancers. Asparagine synthetase can work normally in normal cells, producing sufficient amounts of asparagine (Micu et al. 2020). Meanwhile, in patients with acute lymphoblastic leukemia, the amount of asparagine synthetase in cells is limited, so additional asparagine is needed from nutrients outside the cells (Konecna et al. 2004; Lomelino et al. 2017). L-asparaginase degrades L-asparagine, which leukemia cells need, so the cancer cell's need for L-asparagine is not met sufficiently. Consequently, cell growth will be inhibited, and death will occur in leukemia cells (Narta et al. 2007; Radadiya et al. 2020). L-asparaginase activity cannot interfere with normal cells since the normal cells can produce asparagine synthetase in the proper amount so that the cells' needs for asparagine are sufficiently fulfilled by the normal cells. This L-asparaginase potential will be developed as an alternative treatment for cancer cells.

The mangrove forest in Kulon Progo, Indonesia is a mangrove tourist area directly adjacent to residential areas. This area has a high risk of anthropogenic threats in the

form of final waste storage, such as agricultural waste, household waste, and oil spills, affecting water quality (Saru 2013). Mangrove ecosystems are coastal ecosystems composed of many vegetation and living organisms with biological and physiological adaptations in synthesizing metabolites or enzymes specific to environmental stress (Prihanto et al. 2019). In this habitat, *Rhizophora mucronata* Lam. adapts to grow resistant to stress conditions by producing different metabolites compared to a serene environment, allowing specific types of endophytes to be found.

L-asparaginase can be produced by animals, plants, and microorganisms such as fungi, yeast, and bacteria (Talluri et al. 2014). Bacteria are a better source of L-asparaginase; it is easier to culture, and optimizing enzyme production and extraction is easier to do (Savitri et al. 2003; Cachumba et al. 2016). Bacteria need a substrate in the form of asparagine as the main source to be processed and broken down into L-aspartate and ammonia. Asparagine is abundant in plants, about 60-80% in the roots, leaves, and fruit, 40-45% in the xylem and 20-30% in the phloem. One of the plants that contain asparagine and can be used as a source of L-asparaginase-producing microorganisms is the mangrove *R. mucronata* (Nur et al. 2013). However, L-asparaginase purified from *Escherichia coli* and *Erwinea chrysanthemi* has been available and has been clinically applied to humans, but this treatment has side effects such

as allergies, cross interactions, stimulation of the immune system, drug resistance, and nonspecific L-glutaminase activity (Kotzia and Labrou 2007). These side effects can be reduced by discovering new sources of L-asparaginase, which are serologically different but have similar therapeutic effects. This requires screening samples from various sources to obtain isolates with L-asparaginase non-glutaminase, which have the same or greater therapeutic effect and different serology characteristics (Joshi and Kulkarni 2018).

Endophytic microbes can produce compounds or metabolites similar to bioactive compounds in host plants because of their close symbiotic relationship with the host plant (Chow et al. 2018). This was proven by Harper et al. (2003), who stated that taxol, an anticancer agent, was originally only found in the *Taxus brevifolia* tree, but many researchers found that the endophytic *Taxomyces andreanae* in this plant also contained taxol. Salini et al. (2016) research stated that the stems of the mangrove *R. mucronata*, originating from the mangrove forest on the south coast of Kerala, India, contain many endophytic bacteria that produce L-asparaginase, capable of treating cancer.

Further research and promising applications in L-asparaginase is that the L-asparaginase gene (GkASN) from the thermophilic bacterium, *Geobacillus kaustophilus*, has been cloned and expressed in *E. coli* Rosetta™2 (DE3) cells using the pET-22b(+) vector. GkASN-induced asparagine deficiency effectively reduced metastatic synergy in SNU387 HCC cells on the xCELLigence system with differentiated epithelial Hep3B and poorly differentiated metastatic mesenchymal HCC SNU387 cells (Özdemir et al. 2022). Another study (Aktar et al. 2023) performing a structural comparison of ten L-ASNases with very different kinetic properties showed that residues with a catalytic role are conserved and some differences at position 59 close to the substrate may influence the kinetic parameters. L-ASNase type I from the yeast *Lachancea thermotolerans* (LtASNase) showed the highest specific activity (313.82 U/mg) and catalytic efficiency for L-Asn. Therefore, LtASNase is a promising oncological therapeutic candidate to be further investigated in pharmaceutical applications. Furthermore, research that can successfully modify L-ASNase such as reducing glutaminase activity, increasing the in vivo stability, and enhancing thermostability, will increase its commercialization in the pharmaceutical field (Wang et al. 2021) and also improve the knowledge about its production current biotechnological developments in L-ASNase production and biochemical characterization is also being carried out (Castro et al. 2021).

This research was conducted to determine whether the roots, stems, and leaves of the mangrove *R. mucronata* in the Wana Tirta, Yogyakarta mangrove area contain endophytic microorganisms that can produce L-asparaginase.

MATERIALS AND METHODS

Study area

The Wana Tirta mangrove forest is located in Kulon Progo District; one of five districts/cities in the Special Region of Yogyakarta Province, Indonesia; located in the western part, with coordinates -7.89267, 110.01983. The climate in Kulon Progo can be characterized by two main seasons - the rainy season and the dry season. The rainy season generally falls between November and April. Most of the rain falls in a short time and is heavy, but sometimes there is prolonged rain that can last all day. January is usually the wettest month. During the rainy season, the average daily high temperature ranges from 28-30°C. The dry season occurs between May and October. Although there may be occasional rainfall at the beginning and end of this period. During these months, the average daily high temperature is around 32°C and the nighttime low is 25°C. During 2021 in Kulon Progo District the average monthly rainfall was 213.56 mm and 12.10 rainy days per month, the lowest humidity was 78.6% (August), and the highest was 85.9% (January). Pemerintah Kabupaten Kulon Progo <https://kulonprogokab.go.id/v31/detil/7672/kondisi-umum>. 23 Juni 2020

The mangrove *R. mucronata* was taken from the Wana Tirta ecotourism area, Kulon Progo, Yogyakarta. The sampling technique was carried out based on the cluster sampling method. Approximately 10% of the mangrove area of Wana Tirta Kulon Progo is divided into 3 regions, namely region 1 (beach), region 2 (coastal), and region 3 (land). Each area has 3 sampling points with a distance of 200 m in each area. At each sampling point, samples were taken from 3 plants. The samples used in this study were the roots, stems, and leaves. Fresh samples were taken, put in a newsprint, and taken to the laboratory. Next, samples were stored in the refrigerator (Prihanto et al. 2019). The following is a map of the sampling locations for the mangrove *R. mucronata* (Figure 1).

Isolation of endophytic bacteria

Endophytic bacteria were isolated from samples of roots, stems, and leaves of mangrove *R. mucronata*. The surface sterilization of the sample was done by washing it with running water and immersing it in 70% alcohol for 30 seconds and 2% sodium hypochlorite for 2 minutes. Samples were rinsed with 70% alcohol for 30 seconds and ddH₂O 3-4 times (Lashgarian et al. 2021). Sterile samples were cut and grown on Nutrient Agar (NA) growth media. The bacterial colonies from the plant tissue were purified as pure cultures using the streak-plate method, then observed for morphology and screened for their ability to produce L-asparaginase.

Screening of L-asparaginase-producing endophytes

Screening of L-asparaginase-producing endophytic is based on increased pH due to enzymatic release of ammonia on asparagine, glutamine, urea, or NaNO₃ substrates by L-asparaginase-producing bacteria. Rapid L-asparaginase activity detection is demonstrated by staining techniques using a pH indicator mixed in culture media

containing asparagine as an N source (Gulati et al. 1997). The commonly used pH indicator is phenol red, which is yellow under acidic conditions and changes color to pink under alkaline conditions due to increased pH. Endophytic bacteria producing *L*-asparaginase were screened qualitatively based on the method in Gulati et al. (1997). Pure cultures of endophytic isolates were inoculated using the single dot-plate method on a modified M9 medium; this M9 medium is minimal, and the only source of N is added *L*-asparaginase. The composition of M9 medium is D-Glucose 2 g/L, Na_2HPO_4 6g/L, KH_2PO_4 3g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02g/L, NaCl 0.5g/L, CaCl_2 0.002g/L, Agar 3g/L, *L*-asparagin 5g/L, and phenol red 0.009%). Cultures were incubated at 37°C for 24-48 hours. The pink zone around the colony indicates that the isolate produces *L*-asparaginase; the pink zone diameter formed around the colony was measured. Screening is done with two repetitions; next, the 10 isolates with the highest ability were tested for optimization of *L*-asparaginase production.

Preparation of ammonium chloride standard solution

A standard solution was prepared by diluting a standard ammonium chloride solution with a concentration of 100 ppm (Karimullah et al. 2018). The standard solution is prepared by dissolving 0.01g of NH_4Cl in 100 mL of distilled water. Standard solutions were made with concentrations of 1 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm, and 25 ppm.

Preparation of the standard curve

The standard curve between ammonium chloride's absorbance and ammonium chloride concentration is obtained by adding 100 mL of standard solution with 15

drops of Nessler's reagent. The solution was allowed to stand at room temperature for 10 minutes, and the absorbance was measured at a wavelength of 450 nm.

Determination of optimum incubation time

The optimum incubation time is the optimal time for *L*-asparaginase production. *L*-asparaginase production is indicated by the enzyme activity shown by breaking asparagine into ammonium sulfate. The isolate that produced *L*-asparaginase was grown in liquid M9 medium and incubated on a 200 rpm incubator shaker at 26-29°C for 96 hours. Every 12 hours, the samples were centrifuged at 3,000 rpm for 10 minutes at 4°C to separate the supernatant and cells. The supernatant was taken to estimate enzyme production (Abhini and Zuhara 2018).

Measurement of *L*-asparaginase production

Enzyme production is expressed as enzyme activity U/mL. Enzyme production was measured using the Nesslerization method (Abhini and Zuhara 2018). The reaction will be seen by adding 0.5 mL of the supernatant containing the enzyme into 1.7 mL of 0.04 M *L*-Asparagine and 1 mL of 0.05M Tris-HCl pH 8.6. The supernatant containing enzymes and substrates was incubated at 37°C for 30 minutes. The reaction was stopped by adding 0.5 mL of 1.5 M Trichloroacetic Acid (TCA). 0.2 mL of Nessler's reagent was added to a tube containing 0.1 mL of the reaction mixture and 3.75 mL of ddH₂O. The tube was incubated at room temperature for 10 minutes. The absorbance of the sample was measured at a wavelength of 450 nm with a spectrophotometer. Next, 1 unit of *L*-asparaginase enzyme is defined as the amount of enzyme that breaks down 1 μmol of ammonia for 1 minute at 37°C.

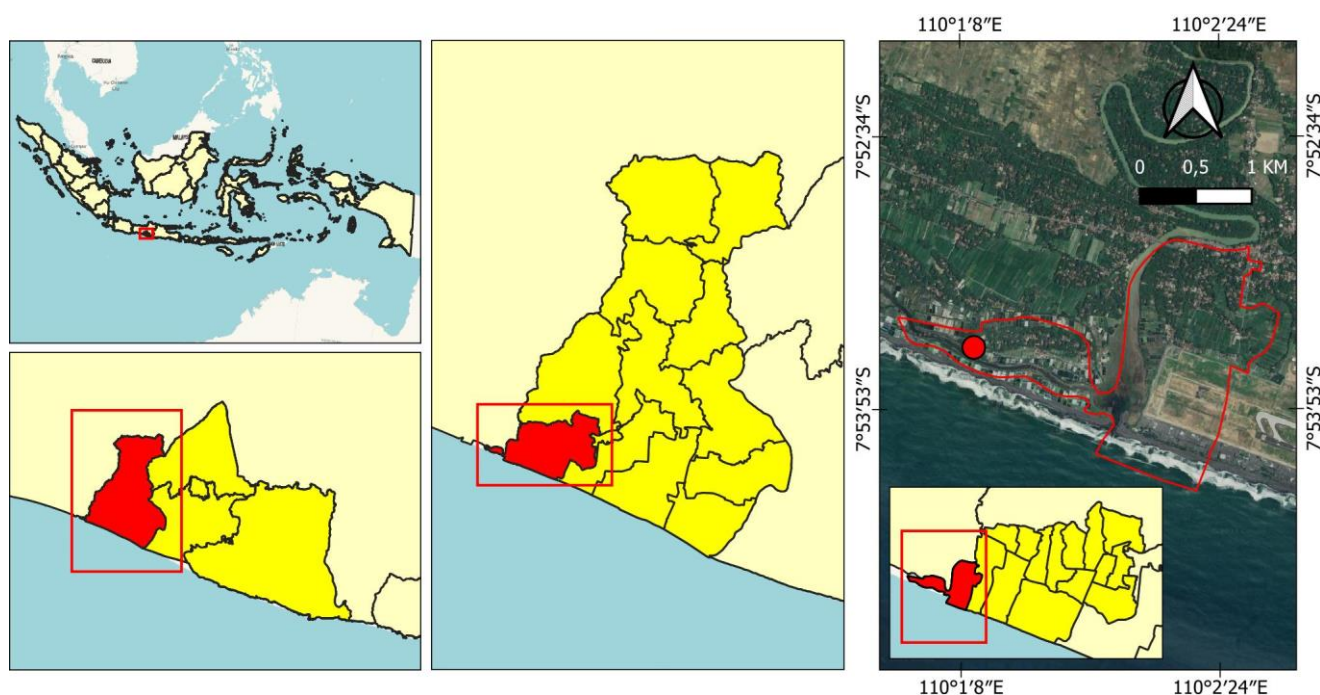


Figure 1. Locations of mangrove sampling: the Wana Tirta ecotourism area, Jangkarán, Temon, Kulon Progo, Yogyakarta, Indonesia

Optimization of L-asparaginase enzyme production by endophytic bacteria

Optimization of media and endophytic growth conditions for enzyme production follows Lashgarian et al. (2021). Optimization of L-asparaginase production by bacteria is carried out with One Factor at A Time (OFAT) method. Optimization factors performed include incubation time, incubation temperature (27, 37, and 50°C); pH media (6,7,8); and ammonium sulfate 0.25 mg/L, 0.5 mg/L, 1 mg/L; glucose level 0.5, 1, 3%. Bacterial isolates producing L-Asparaginase were cultured in 100 mL of liquid M9 medium. In the temperature optimization treatment, the bacterial culture was incubated in an incubator shaker with a speed of 200 rpm at 27°C, 37°C, and 50°C, respectively.

In the pH variation treatment, the growth medium was adjusted until it reached a pH of 6, 7, and 8. In the glucose variation, the growth medium was adjusted with D-glucose levels to 0.5%, 1%, and 3%, respectively. In the ammonium sulfate variation, the growth medium was added as much as 0.25 mg/L, 0.5 mg/L, and 1 mg/L. Endophytic cultures were incubated on an incubator shaker at 200 rpm at 37°C. Endophytic cultures were incubated during the optimal incubation time that had been obtained. After reaching the optimum incubation time, the culture was taken and centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant was taken for measurement of enzyme activity.

The absorbance value obtained is substituted into the ammonium-chloride standard curve equation with the regression equation $y = ax+b$

Ammonia level:

$$y = ax+b \quad (y-b)$$

$$x = ((y-b)/a)$$

Where: x: ammonia content (μmol/L); y: absorbance; a: slope; b: intercepts

The value of L-Asparaginase production is obtained by using the equation:

$$\text{Enzyme activity (Unit/mL)} = (y-b)/a \times (V.\text{Total})/(V.\text{Analysis}) \times 1/(V.\text{Enzyme}) \times 1/(t.\text{Incubation})$$

Where: V.Total = Enzyme Volume + Substrate + Buffer +TCA; V. Analysis = Total Volume analyzed; V. Enzymes = Volume of enzymes analyzed; t. Incubation time = 30 minutes

Data analysis

The data obtained in this study were the isolation and screening of endophytic bacteria from the mangrove *R. mucronata*, which produce L-asparaginase. Optimization of L-asparaginase production was done using a One-Factor-at-The-Time method by calculating the concentration of ammonia and enzyme activity. The data obtained was then analyzed descriptively by displaying the data in the form of pictures and tables.

RESULTS AND DISCUSSION

Endophytic bacteria in the mangrove *Rhizopora mucronata*

A total of 54 isolates of endophytic bacteria have been isolated from the mangrove plant *R. mucronata* from 3 sampling areas, namely the beach, the coastal, and the land; the beach area had the highest number of isolates 35 and the most isolates obtained from leaf samples as many as 20. The number of endophytic isolates from all samples is presented in Table 1.

In these beach areas, water circulation is higher when compared to other areas, so the amount of nutrients dissolved in the water is also relatively high. Plants submerged in water absorb nutrients dissolved and are used for bacterial growth. The coastal area has the fewest bacterial isolates; in this area, the plants are not always submerged in water and sometimes experience dry conditions. This area will be submerged by brackish water during high tide, whereas during low tide, this area will not be submerged. A total of 15 bacterial isolates obtained were from roots, 19 isolates were derived from stems, and 20 isolates were derived from leaves. Most of the isolates obtained in this study came from leaves, and the least isolates came from roots.

Screening of L-asparaginase-producing endophytic bacteria from the mangrove *R. mucronata*

The screening process determines whether the production of the L-Asparaginase by endophyte isolates is large, moderate, or low. Enzyme production is estimated from an increase in the pH of the medium, which forms a pink zone due to the breakdown of the substrate by the growing bacteria. M9 medium is a minimal medium, so it will not affect changes in pH when bacterial growth occurs. The M9 medium negates the influence of other carbon and nitrogen sources, which may influence L-asparaginase production (Chow and Ting 2017). Determination of the ability to produce enzymes based on the pink zone formed on the M9 medium. The larger the pink zone formed, the greater the production of L-asparaginase in the bacterial isolates (Figure 2.B, C, D). Bacterial isolates that did not produce L-Asparaginase did not form pink zones around the colonies (Figure 2.A).

Table 1. Number of endophytic bacteria isolated from mangroves *Rhizopora mucronata*

	Number of Endophytic Isolates		
	Root	Stem	Leaf
Beach	12	12	11
Coastal	2	3	2
Land	1	4	7
Total	15	19	20

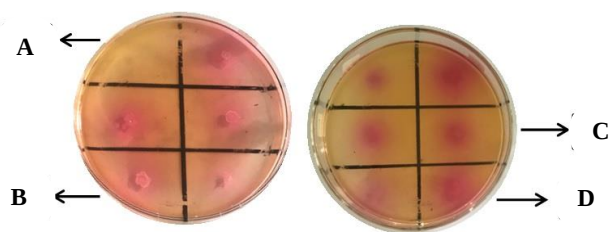


Figure 2. Pink zone showing L-asparaginase production of an endophytic isolate from *Rhizopora mucronata*. Note: a. Endophyte bacterial colony does not produce L-asparaginase (-), b. Endophyte bacterial colony that produces low L-Asparaginase (pink zone diameter <1.5 cm), c. Endophyte bacterial colony that produces moderate L-asparaginase (diameter of the pink zone 1.5-2.5 cm), d. Endophyte bacterial colony that produces large L-asparaginase (pink zone diameter >2.5 cm)

The diameter of the pink zone is formed based on an increase in the media's pH due to the substrate's breakdown by growing bacteria. The bacteria convert L-asparagine to L-aspartate and ammonia in the media, resulting in a change in pH as an indication of the bacteria producing L-asparaginase. The ammonia that is formed cause the pH of the media to become more alkaline, bringing about a color change in the media from yellow to pink (Mahajan et al. 2014). Endophytic bacteria in solid media break down L-asparagine into L-aspartate and ammonia in the media, indicating that bacteria can produce L-asparaginase. The ammonia that is formed cause the pH content of the media to become more alkaline; it will change the media's color from yellow to pink (Mahajan et al. 2014). A total of 10 bacterial isolates with high and moderate enzyme activity were selected for further optimization of enzyme production.

Based on screening on the M9 medium, endophytic bacteria produced L-asparaginase; of all endophytic bacterial isolates, 52 endophytic isolates produced L-asparaginase, and 2 endophytic isolates did not produce L-asparaginase. Endophytic isolates have different L-asparaginase production abilities. A total of 8 isolates produced high L-asparaginase, 14 isolates produced moderate L-asparaginase, 30 isolates produced low L-asparaginase, and 2 bacterial isolates could not produce L-asparaginase (Table 2).

The grouping of low, medium, and high enzyme production is based on Abhini and Zuhara (2018). Endophytic isolates were categorized as having low enzyme production if the diameter of the pink zone formed around the colony was less than 1.5 cm and classified as moderate enzyme production if they had a pink zone diameter of 1.5 cm to 2.5 cm. Endophytic isolates were categorized as having large L-asparaginase enzyme production if the diameter of the pink zone formed around the colony was more than 2.5 cm.

Optimization of L-asparaginase enzyme production

A total of 10 endophytic isolates with high and medium L-asparaginase production were optimized for enzyme production. Optimization of enzyme production was carried out by the Nesslerization method. The production of the L-asparaginase enzyme is very important for evaluating the efficiency of enzyme production so that it can be increased several times by manipulating environmental conditions and growth factors (Pola et al. 2018). Bacterial L-asparaginase activity is distinguished based on different levels of stability, and production levels are influenced by concentrations of C and N sources, temperature, aeration rate, pH, incubation time, and inoculum (Ali et al. 2016).

Table 2. Qualitative production of L-asparaginase endophytic bacterial isolate from *Rhizopora mucronata* on M9 medium

Endophytic Isolate	Enzyme Production* (cm)	Endophytic Isolate	Enzyme Production* (cm)	Endophytic Isolate	Enzyme Production* (cm)
1	3.6	21	1.3	41	1.1
2	1.7	22	1.3	42	0.9
3	2.7	23	1.9	43	0.6
4	2	24	1.7	44	0.4
5	3	25	1.3	45	1.6
6	1.8	26	0	46	1.5
7	1.3	27	1.4	47	0.6
8	1	28	1	48	0.9
9	1.5	29	0.8	49	1.6
10	3.2	30	0.9	50	0
11	1.2	31	0.3	51	0.6
12	2.6	32	0.5	52	1.7
13	1.5	33	0.4	53	0.2
14	1	34	0.3	54	0.4
15	2.4	35	0.3		
16	3.3	36	0.4		
17	3.2	37	0.3		
18	3.7	38	0.2		
19	1.9	39	1.5		
20	1	40	0.3		

Note: *Diameter of the pink zone around the colony

Optimal incubation time

Each bacterial isolate grown on culture media has its own incubation time to produce L-asparaginase. Each isolate will experience an increase in enzyme activity along with the longer the incubation period. The increase in enzyme activity will continue until it reaches the optimum point, and then the enzyme activity decreases. The optimal incubation time for the bacterial isolates in this study was 60-84 hours. The relationship curve for L-asparaginase production and incubation time for each endophytic bacterial isolate can be followed as shown in Figure 3.

The relationship curve of L-asparaginase production and incubation time for each bacterial isolate has almost the same pattern (Figure 3). In the first 12 hours, the amount of L-asparaginase produced by endophytic bacterial isolates was low. The bacterial isolates still adapt to the culture media, so L-asparaginase production is low. Bacterial isolates experienced increased growth and enzyme production as the incubation continued until it reached the optimal point. The optimal point is the optimal incubation time of an endophytic bacteria in producing the highest enzyme, and each bacterial isolate is different. The optimal incubation time for the bacterial isolates in this study was 60-84 hours. A total of 3 bacterial isolates produced L-Aparaginase optimally at the 60th hour of incubation, namely isolate 3, isolate 45, and isolate 49. Moreover, 4 bacterial isolates produced enzymes optimally at the 72th incubation time, namely isolate 1, isolate 5, isolate 39, and isolate 46. A total of 3 isolates of endophytic bacteria obtained optimally produced L-Aparaginase at the 84th hour incubation period, namely isolate 12, isolate 18, and isolate 23. Once at the optimal point, the enzyme activity will experience a slight decrease but is still relatively stable. Optimal incubation time, ammonia levels as a result of substrate breakdown by the presence of L-asparaginase, and enzyme production expressed as measured enzyme activity are presented in Table 3.

The levels of ammonia formed from the breakdown of asparagine by L-asparaginase endophytic isolates at the

optimal incubation time varied (Table 3). Ammonia levels obtained from endophytic bacterial isolates ranged from 188.1 $\mu\text{mol/L}$ to 333.9 $\mu\text{mol/L}$. Bacterial isolate 39 had the lowest L-asparaginase activity compared to other bacterial isolates, 188.1 $\mu\text{mol/L}$ ammonia, and 57.3 IU/mL enzyme activity. Isolate 18 had the highest L-asparaginase activity at 333.9 $\mu\text{mol/L}$ ammonia and 101.7 IU/mL enzyme activity. The research of Joshi and Kulkarni (2018) stated the L-asparaginase activity of the bacteria was relatively the same as the enzyme activity values from this study; namely, *B. pseudomycoides* of 70.4 IU/mL, *B. licheniformis* of 80 IU/mL, and *P. denitriformis* of 77.56 IU/mL enzyme activity has a linear relationship with the level of ammonia. The greater the level of ammonia formed, the greater the enzyme activity value.

Optimum temperature

Enzyme biosynthesis is influenced by environmental factors, one of which is temperature. Each endophytic bacterial isolate has a different relationship curve between incubation temperature and L-asparaginase production. Apart from isolates 39 and 45, endophytic bacterial isolates had a low ability to produce enzymes at 27°C and continued to increase until they reached the optimum temperature, namely 37°C. At 50°C, enzyme production will decrease (Figure 4).

Generally, the optimal temperature of enzyme production of most endophytic isolates is 37°C. However, isolate 39 had optimal temperature of production of L-asparaginase at 27°C, and the enzyme production continued to decrease at higher temperatures at 37°C and 50°C. This result was inversely proportional to the results of isolate 45. Isolate 45 had low enzyme production activity at 27°C and increased to 50°C. Isolate 45 can produce L-asparaginase optimally at 50°C. Optimal incubation temperature, ammonia levels as a result of substrate breakdown by the presence of L-asparaginase, and enzyme production expressed as measured enzyme activity are presented in Table 4.

Table 3. Optimal incubation time for L-asparaginase production by endophytic bacterial isolates from *Rhizopora mucronata*

Endophytic Isolates	Optimum Incubation Time (hours)	Absorbance	Ammonia Level ($\mu\text{mol/L}$)	Enzyme Activity (U/mL)
Isolate 1	72	2.234	301.2	91.7
Isolate 3	60	2.176	293.4	89.3
Isolate 5	72	2.393	322.8	98.3
Isolate 12	84	2.132	287.4	87.5
Isolate 18	84	2.476	333.9	101.7
Isolate 23	84	2.506	202.9	61.8
Isolate 39	72	1.397	188.1	57.3
Isolate 45	60	1.45	195.3	59.5
Isolate 46	72	1.503	202.5	61.7
Isolate 49	60	1.416	190.7	59.1

Table 4. Optimal incubation temperature for L-asparaginase production by endophytic bacterial isolates from *Rhizopora mucronata*

Endophytic Isolates	Optimum Incubation Temperature (°C)	Ammonia Level ($\mu\text{mol/L}$)	Enzyme Activity (U/mL)
Isolate 1	37	300.9	91.6
Isolate 3	37	293.1	89.2
Isolate 5	37	322.2	98.2
Isolate 12	37	287	87.4
Isolate 18	37	333.6	101.6
Isolate 23	37	337.7	102.8
Isolate 39	27	243.4	74.1
Isolate 45	50	201	61.2
Isolate 46	37	202.1	62.6
Isolate 49	37	190.4	58

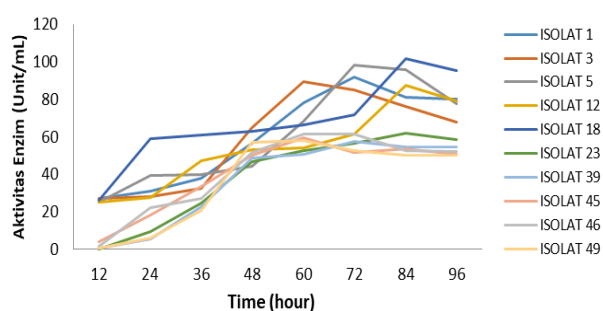


Figure 3. The relationship curve between incubation time and enzyme production by endophytic isolates

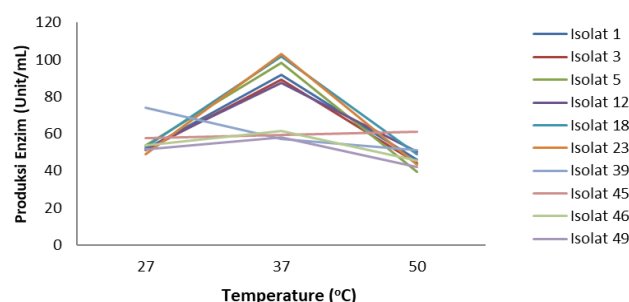


Figure 4. Relationship curve between incubation temperature and enzyme production of endophytic isolates

Isolate 23 had the highest production of L-asparaginase when compared to other endophytic isolates, namely 337.7 μmol ammonia content and an enzyme activity value of 102.8 U/mL at 37°C. Isolate 49 had the lowest ability to produce L-asparaginase, which was 190.4 μmol of ammonia content and 58U /mL of enzyme activity at 37°C. Endophytic bacterial isolates could produce L-asparaginase optimally at 37°C, except for isolates 39 and 45 (Table 4). This is following Mohtashami et al. (2019), which stated that a temperature of 37°C is the most suitable for L-asparaginase production using *B. licheniformis* and will experience a decrease in enzyme activity at 42°C by 5.19% and at 47°C by 32.19%. Isolate 39 was produced L-asparaginase optimally at 27°C with an ammonia level of 243.4 $\mu\text{mol/L}$ and an enzyme activity of 74.1 U/mL. Isolate 45 produced L-asparaginase optimally at 50°C with an ammonia level of 201 $\mu\text{mol/L}$ and an enzyme activity of 61.2 U/mL. This follows Joshi and Kulkarni (2018), which showed that *E. carotovora* showed optimum activity at 50°C.

pH media optimal

Enzyme production is very sensitive to pH media fluctuations, and enzyme production will be inhibited at a certain pH range. Endophytic isolates have different optimum pH media in L-asparaginase production. A total of 5 endophytic isolates produced the highest L-asparaginase in media with a pH of 7, namely isolate 1, isolate 5, isolate 12, isolate 18, isolate 23, and isolate 45. Isolate 3 and isolate 39 produced optimal L-asparaginase in media with low pH at 6. Isolate 46 has optimal L-asparaginase production at a pH of 8 (Figure 5).

Isolate 39 had the highest enzyme production, with 382.4 $\mu\text{mol/L}$ ammonia content and 116.5 U/mL enzyme activity at pH 6. Isolate 49 had the lowest enzyme production, with ammonia levels of 250.3 $\mu\text{mol/L}$ and 76.2 U/mL of enzyme activity at pH 6 (Table 5).

Enzyme production can increase or decrease depending on pH changes because it affects bacteria growth. pH

media plays an important role in changes in bacterial physiology and enzyme production (Purnawan et al. 2016). The pH of the media affects the absorption of nutrients and physiological activity, so it can affect the growth of biomass and the formation of its products (Mohtashami et al. 2019). The optimum concentration of ammonium sulfate in the media.

Certain nitrogen sources are preferred in increasing the production of L-asparaginase, both organic and inorganic forms. Each endophytic isolate has a relationship curve for L-asparaginase production with different optimal ammonium sulfate concentrations in the media. A total of 5 endophytic isolates produced the highest L-asparaginase in the medium with the lowest ammonium sulfate concentration (0.25 mg/L), namely isolate 3, isolate 12, isolate 18, isolate 39, and isolate 45. Isolate 1 and isolate 23 produced L-asparaginase optimal at the highest ammonium sulfate concentration, i.e., 1 mg/L. Isolate 5 and isolate 46 produced the highest L-asparaginase at a concentration of 0.5 mg/L (Figure 6).

Table 5. Optimal pH media for L-asparaginase production by endophytic bacterial isolates from *Rhizopora mucronata*

Endophytic Isolates	Optimum pH Media	Ammonia Level ($\mu\text{mol/L}$)	Enzyme Activity (U/mL)
Isolate 1	7	300.9	91.6
Isolate 3	6	367.9	112
Isolate 5	7	322.4	98.2
Isolate 12	7	287.1	87.4
Isolate 18	7	333.6	101.6
Isolate 23	7	337.6	102.8
Isolate 39	6	382.4	116.5
Isolate 45	7	274	83.4
Isolate 46	8	251.8	76.7
Isolate 49	6	250.3	76.2

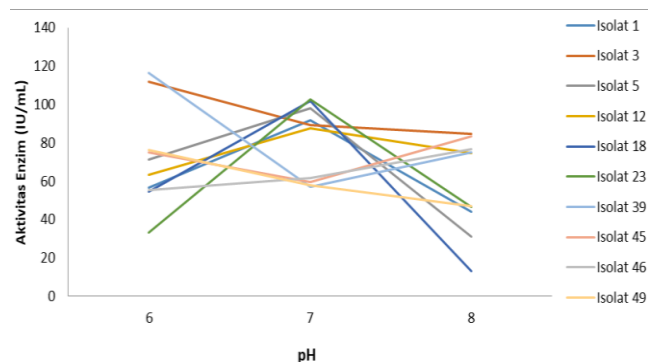


Figure 5. Relationship curve between pH media and enzyme production of endophytic isolates

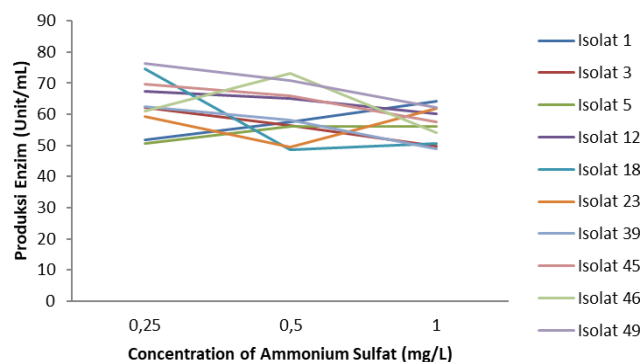


Figure 6. Relationship curve between concentration of ammonium sulfate in the media and enzyme production of endophytic isolates

Isolate 49 had the highest L-asparaginase enzyme production, with an ammonia level of 250.7 $\mu\text{mol/L}$ and an enzyme activity of 76.3 Units/mL at an ammonium sulfate concentration of 0.25 mg/L. Isolate 5 had the lowest enzyme production when compared to other isolates. In comparison, isolate 5 produced an 184.4 $\mu\text{mol/mL}$ ammonia level and an enzyme activity of 56.2 Units/mL at an ammonium sulfate concentration of 0.5 mg/L (Table 6). Ammonium is the best nitrogen source for L-asparaginase production. This follows Alrumman (2019), which states that ammonium sulfate is the optimal nitrogen source for *B. licheniformis*, which can increase L-asparaginase production by 35.56%.

The optimum concentration of glucose in the media

Glucose as a carbon source increases bacterial cell growth and synthesizes primary metabolites such as enzymes. The concentration of carbon sources positively affects the production of the enzyme L-asparaginase and high yields can be obtained from media rich in carbon sources (Chow and Ting 2017). Each endophytic isolate had almost the same relationship curve between glucose concentration in the media and L-asparaginase production. All endophytic isolates produced the highest L-

asparaginase at a glucose concentration of 3% (Figure 7). Isolate 5 had the highest enzyme production compared to other isolates, namely 343.8 $\mu\text{mol/L}$ ammonia content and 104.7 U/mL enzyme activity. Isolate 45 had the lowest enzyme activity at 141.3 $\mu\text{mol/L}$ ammonia content and 43 U/mL enzyme activity (Table 7).

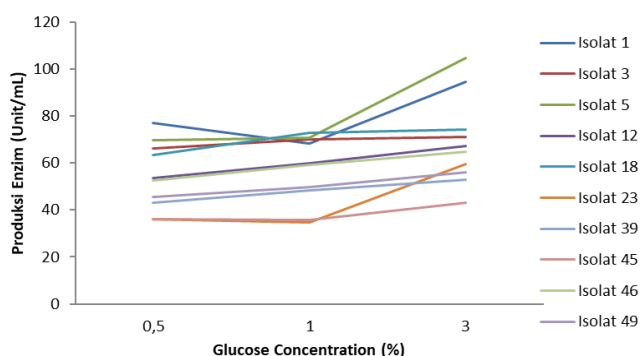


Figure 7. Relationship curve between concentration of glucose in the media and enzyme production of endophytic isolates

Table 6. The optimum concentration of ammonium sulfate in the media for L-asparaginase production by endophytic bacterial isolates from *Rhizopora mucronata*

Endophytic Isolates	Concentration of Ammonium Sulfate (mg/L)	Ammonia Level ($\mu\text{mol/L}$)	Enzyme Activity (U/mL)
Isolate 1	1	211	64.2
Isolate 3	0.25	204.3	62.2
Isolate 5	0.5	184.4	56.2
Isolate 12	0.25	221.5	67.5
Isolate 18	0.25	245	74.6
Isolate 23	1	203.7	62
Isolate 39	0.25	205.4	62.5
Isolate 45	0.25	229.3	69.8
Isolate 46	0.5	240.3	73.2
Isolate 49	0.25	250.7	76.3

Table 7. The optimum concentration of glucose in the media for L-asparaginase production by endophytic bacterial isolates from *Rhizopora mucronata*

Endophytic Isolates	Concentration of Glucose (%)	Ammonia Level ($\mu\text{mol/L}$)	Enzyme Activity (U/mL)
Isolate 1	3	310.4	94.5
Isolate 3	3	233.5	71.1
Isolate 5	3	343.8	104.7
Isolate 12	3	221.1	67.3
Isolate 18	3	243.7	74.2
Isolate 23	3	195.8	59.6
Isolate 39	3	173.4	52.8
Isolate 45	3	141.3	43
Isolate 46	3	213	64.9
Isolate 49	3	184.3	56.1

According to Chow and Ting (2017), incorporating glucose as a carbon source and L-asparagine as a nitrogen source affects the induction of L-asparaginase production because glucose can increase L-asparaginase production in various bacterial isolates. Alrumman et al. (2019) researched the best carbon source for *B. licheniformis* in producing L-asparaginase; the carbon source of glucose had a significant effect on enzyme activity with an increase of 11.2%, and the lowest yield was using a carbon source of starch and glycerol which resulted in a decrease in enzyme activity of 40.36% and 53.1%. Several studies on optimizing L-asparaginase activity showed several optimum conditions for each isolate used. The optimization of L-asparaginase from endophytic *Fusarium proliferatum* (isolate CCH) revealed that glucose concentration, nitrogen source, L-asparagine concentration and temperature influenced the L-asparaginase production. As such, the recommended conditions were 0.20% of glucose, 0.99% of L-asparagine and 5.34 days incubation at 30.50 °C. The L-asparaginase production of CCH increased from 16.75 ± 0.76 IU/mL to 22.42 ± 0.20 IU/mL after optimization (Yap et al. 2021). Effects of eight parameters including temperature, pH, incubation time, inoculum size, agitation speed, the concentration of starch, L-asparagine, and yeast extract were studied on L-asparaginase production by the Arctic isolate *S. koyangensis* SK4. Maximum enzyme activity of 136 IU/ml was obtained at 20°C on the seventh day of incubation in the asparagine dextrose broth maintained at pH 7.5, agitation speed 125 rpm, and L-asparagine concentration of 7.5 g/L. The statistical optimization method described in this study proved effective for increasing the L-asparaginase production by Arctic actinomycetes (Saleena et al. 2023).

In this study there were 54 isolates of endophytic bacteria were found in mangroves *R. mucronata*. Bacterial isolates produce different L-asparaginases. A total of 8 endophytic isolates could produce high L-asparaginase; 14 isolates were moderate, 30 isolates were low, and 2 bacterial isolates did not produce L-asparaginase. Endophytic isolates were able to produce the highest L-asparaginase under different optimal conditions. The optimal incubation time for endophytic isolates in this study was 60-84 hours, the optimal temperature was 37°C, the optimal pH media was 7, the nitrogen content was 0.25 mg/L, and the optimal glucose level was 3%. L-asparaginase from endophytic isolates from mangrove *R. mucronata* shows high production and enzyme activity; in the future, it can be produced according to optimal conditions and used to treat leukemia cancer.

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Potential effects of fermented moringa (*Moringa oleifera*) leaf extract to increase the color brightness of the Oranda goldfish (*Carassius auratus*)

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Abstract. Umaiya S, Putra AN, Herjayanto M, Syamsunarno MB. 2023. Potential effects of fermented moringa (*Moringa oleifera*) leaf extract to increase the color brightness of the Oranda goldfish (*Carassius auratus*). Nusantara Bioscience 15: 289-297. Moringa (*Moringa oleifera* Lam.) leaf extract serves as a natural reservoir of carotenoids, prominently β -carotene, effectively employed to augment the color quality of ornamental fish. This study aimed to evaluate the potential effects of adding fermented moringa leaf extract to feed to enhance the color vibrancy of Oranda goldfish (*Carassius auratus* (Linnaeus, 1758)). Moringa leaves were also fermented using *Aspergillus niger* Tiegh. at a dosage of 0.5 g/100 g for 48 hours, followed by extraction using 70% ethanol. Four doses of fermented moringa leaf extract (0, 40, 50, 60 mL/kg) with three replications were added to a commercial feed using the coating method. The results showed that the carotene in fermented moringa leaf extract is 94.22 ppm/50 mL. Adding moringa leaf extract to the feed enhanced the Oranda goldfish's brightness and color diversity. The addition of fermented moringa leaf extract at a dose of 60 mL/kg led to the highest color diversity values on the body (57.81%), caudal (71.71%), and head (71.13%) compared to other treatments. Moringa leaf extract did not affect growth rate, survival, blood profile, and water quality parameters for maintaining Oranda goldfish. The extract from *A. niger* had the potential to be used in the cultivation of Oranda goldfish, and the addition of 60 mL/kg achieved the best color brightness and diversity.

Keywords: *Aspergillus niger*, feed, fermented, ornamental fish

INTRODUCTION

The Oranda goldfish (*Carassius auratus* (Linnaeus, 1758)) is one of the ornamental fish species with high economic value, resulting in significant demand and cultivation potential (Hedayati et al. 2017). The fish has various colors, such as black, white, red, orange, brown, and yellow (Gurung et al. 2018) and belongs to the Cyprinidae family (Hedayati et al. 2017). Oranda is a goldfish widely cultivated in Indonesia due to its beautiful colors and unique body shape (Yan et al. 2022). Oranda goldfish has long fins, including the dorsal fin and long double caudal fin, the goldfish most widely cultivated in Indonesia (Prakoso et al. 2023). Improving fish color quality is an effort that can lead to higher selling value of ornamental fish (Sawant et al. 2020). The better and unique the color and the more perfect the physical form of the ornamental fish, the more expensive the selling price will be on the market (Hoseinifar et al. 2023). The color quality and appearance of the Oranda goldfish are directly proportional to the market price (Kautsar et al. 2022). However, the cultivation faces many challenges, such as the degradation of color quality, both in brightness and diversity (Andriani et al. 2021). Enhancing fish color can be achieved by providing feed containing color pigments or carotenoids (Dananjaya et al. 2017), and carotenoids in the diet naturally enhance the color brightness (Sahin et al. 2022).

Carotenoids are natural pigments in animals, plants, and microorganisms (Astari et al. 2016; Wongphonprateep and

Pichitkul 2021). Carotenoids are the primary source of coloration on fish skin, and based on their chemical structure, carotenoids are divided into 2 groups: carotenes and xanthophyll (Sathyaruban et al. 2021). Fish cannot synthesize carotenoids, which must be added to their diet (Hekimoglu et al. 2017; Koncara et al. 2019). The improvement in fish color begins with the intake of carotenoid-containing feed, which enters the fish body and is synthesized into pigments. Furthermore, these pigments were carried through the bloodstream and deposited in chromatophores in the dermis/skin (Das and Biswas 2016). Fish pigmentation depends on the additional feed containing carotenoids. Fish only modify pigments from their dietary sources (Meilisza et al. 2019). However, the high cost of synthetic carotenoids limits their use in feed formulations due to increased expenses (Besen et al. 2019).

Moringa leaves (*Moringa oleifera* Lam.) can serve as an alternative source of carotenoids due to their beta-carotene content, one of the provitamin A compounds (Angelica et al. 2020). Jebaraja et al. (2013) found that Moringa leaf contains a significant amount of β -carotene, specifically lutein, at 520 mg/kg. Beta-carotene belongs to the carotenoid group and is crucial in skin color regulation by providing orange to red colors (Chapman and Miles 2018). The findings of Takdir et al. (2022) explained that applying Moringa leaf extract in feed enhances growth and color vibrancy in betta fish. However, the leaves also contain anti-nutritional compounds such as saponins, tannins, phytates, and polyphenols (Putra et al. 2020a). These anti-nutritional substances limit the use of Moringa

leaves due to their potential to inhibit growth and physiological processes within the fish body (Abdel-Latif et al. 2022). Fermentation process using *Aspergillus niger* Tiegh. can reduce the crude fiber and anti-nutritional content in fish feed ingredients (Putra et al. 2022a). Several studies have reported natural potential sources of carotenoid to improve the color quality of goldfish such as astaxanthin (Weeratunge and Perera 2016), red paprika (Kumar et al. 2017), bixin (Dananjaya et al. 2017), Lutein (Besen et al. 2019), annatto seeds extract (Dananjaya et al. 2020), red yam flour (Putra and Romdhonah 2019), carrot flour (Tiewsoh et al. 2019), lobster meal (Bell et al. 2019), *Spirulina platensis* (Gomont) Geitler (Kargun and Dikbas 2020), sweet potato and yellow pumpkin flour (Kautsar et al. 2022). However, no data reports the utilization of fermented moringa leaf extract for enhancing pigmentation of goldfish. Therefore, the study is needed to explore the potential of the Moringa leaves as a source of carotenoids in fish feed. This study aimed to evaluate the potential effects of adding the fermented extract of Moringa leaf to feed to enhance the color brightness of Oranda goldfish.

MATERIALS AND METHODS

Fermented moringa leaf extract preparation

The fermentation of moringa leaf powder started with steaming for 30 min at a temperature of 100°C. *A. niger* inoculum with a dosage of 0.5 g/100 g was mixed with moringa leaf powder and incubated for 48 hours, following the study findings of Ikhwanuddin et al. (2018). Moringa leaf powder was dried in an oven at 60°C for 10 min. The powder was extracted using 70% ethanol in a 1:1 (w/v) ratio and stirred using a magnetic stirrer for 15 hours at room temperature, following the method described by Putra et al. (2015). The powder was filtered using filter paper, and the obtained filtrate was evaporated with a vacuum evaporator at 60 °C for 45 min to remove ethanol residue. Subsequently, the remaining solution was diluted with distilled water until the total dissolved solids reached 5%.

Preparation of test feed and experimental design

Fermented Moringa leaf extract was weighed according to the treatment doses and homogenized at 250 mL/kg of water and 2% egg yolk, following Putra et al. (2020b). The extract was then coated by evenly spraying onto the commercial feed and air-drying at room temperature for 40 minutes. The feed coating process followed the method described by Putra and Romdhonah (2019). The commercial feed used as the test diet was ornamental (PF500, Indonesia), with a particle size of 0.5-0.7 mm, minimum protein content of 39.5%, fat 5.3%, crude fiber 4.0%, ash content 11.6%, moisture content 10.4%, nitrogen-free extracts 39.6%, dry matter 89.6%, organic matter 78.0%, and energy 433.78 kcal/g (protein: 5.6 kcal/g, fat: 9.4 kcal/g, BETN: 4.11 kcal/g). Feeding was conducted three times a day ad libitum at 08:00, 12:00, and 16:00 local time. This study employed an experimental method with a Completely Randomized Design (CRD)

consisting of four groups of fermented Moringa leaf extract treatments. The treatments included A (without the addition of fermented Moringa leaf extract, 0 mL/kg), B (fermented Moringa leaf extract 40 mL/kg), C (fermented Moringa leaf extract 50 mL/kg), and D (fermented Moringa leaf extract 60 mL/kg).

The test fish used were Oranda goldfish sourced from an ornamental fish breeder in Pandeglang, Banten, Indonesia, with an average length of 4.5±0.20 cm and weight of 3.2±0.27 g. The fish were acclimated and fasting for 24 hours to eliminate the influence of residue in their bodies. A total of 120 were randomly distributed into 30x30x30 cm aquariums with a water volume of 20 L/aquarium and a density of 10 fish per aquarium. The aquariums used are colorless (colored glass), and all treatments are given the same light, namely bright during the day and dark at night, to minimize the effect of light and tank color on Oranda goldfish. Fish maintenance was carried out for 40 days using a recirculation system, with one aerator as an oxygen source and one water heater (Amara HT-50) in each aquarium. Water quality management is carried out by daily siphoning of waste and feed residue was performed, and 30% of the total aquarium water volume was replaced daily. Water quality measurements consist of temperature with a digital thermometer (TPM 10), pH with a pH meter (Backlight PH-2011), and oxygen content with a DO meter (Lutron DO-551).

Test parameters

Fish color quality

Color quality measurements were taken every 10 days (10, 20, 30, and 40 days) using the measurement method proposed by Kautsar et al. (2022). The measurements were conducted visually using the Toca Color Finder (TCF) standard values by 8 healthy and non-colorblind panelists. The assessment ranged from a score of 1 (lowest) to 7 (highest), with color gradations from yellow to orange and red. Three test fish were assessed per replication at each measurement interval, and the scores and colors are presented in Table 1.

Table 1. Toca Color Finder (TCF) score in ornamental fish

Color Score	Color	TCF Code
1		TCF code 0305
2		TCF code 0405
3		TCF code 0505
4		TCF code 0604
5		TCF code 0625
6		TCF code 0605
7		TCF code 0616

Fish color diversity

Observations were made at the end of the rearing period, covering three sample points: head, body, and caudal peduncle. Color diversity observations followed the method proposed by Andriani et al. (2021). Test fish were photographed using a 13-megapixel camera, and the obtained photos were analyzed using color gradient conversion methods on a scale and percentage basis using Adobe Photoshop CS4. Color diversity observations were conducted on 3 fish from each treatment.

Chromatophore cell count

Four randomly selected fish were used to prepare histological slides at the end of the rearing period. The preparation was conducted at the Laboratory of Histopathology, Fish and Environmental Disease Examination Station, Serang, Banten. Following the method described by Putra et al. (2021a). Histological preparation started with the fixation step, where the sampled fish organ was immersed in 10% phosphate-buffered formalin and 5% ethylenediaminetetraacetic acid solution for 24 hours. Dehydration followed, involving immersion of the fish organ samples in alcohol with gradually increasing concentrations of 70%, 80%, 96%, and absolute alcohol for 3 hours; the next step involved immersing the samples in xylene solution for 1 hour before embedding them in paraffin. The staining phase was then conducted using hematoxylin and eosin (H and E), and the prepared slides were observed under a microscope (Leica DM500) at 10x magnification. Meanwhile, chromatophore cell counts were performed at three randomly selected points on each slide. The number of cells in each treatment group of Oranda goldfish was counted and statistically analyzed based on the obtained data.

Fish blood profile

The measurement of fish blood profiles, including leukocyte, erythrocyte, hematocrit, and hemoglobin counts, was conducted at the end of the rearing period. Blood samples were taken from four fish in each treatment using a 1 mL syringe containing 0.1 mL of anticoagulant to prevent blood coagulation. Blood samples from each treatment were mixed with Hayem (for erythrocyte measurement) and Turk solutions (for leukocyte measurement) until homogenous. Subsequently, 1-2 drops of blood were placed into a hemocytometer and counted under a microscope at 400x magnification. Erythrocyte and leukocyte measurements followed the method described by Kumar et al. (2015), and hemoglobin levels were measured using the Sahli method (Pawar and Bhilave 2019). Blood was drawn into a Sahli pipette up to the 2 mL mark and was transferred to a Hb-meter tube containing 0.1 N HCl. The solution was allowed to stand for 3-5 minutes for hemoglobin to react with HCl, forming acid hematin, and distilled water was added until the color matched the standard in the Hb-meter tube. Hemoglobin values were also determined based on the height scale of the formed surface of the solution, indicating the amount in grams per 100 mL of blood. The hematocrit level was calculated according to the method proposed by Yaji et al. (2018).

Hematocrit tubes containing fish blood samples were centrifuged for 5 minutes at 5,000 rpm, and the levels were expressed as a percentage of the volume of packed blood cells.

Growth parameters

Growth parameters, including feed consumption, specific growth rate, and survival rate, were measured following the formulas proposed by Rifai et al. (2022). The feed conversion ratio was calculated using the formula proposed by Hermawan et al. (2021). These parameters included (i) Feed consumption (g) = initial feed amount (g) - final feed amount (g), (ii) Absolute weight (g) = mean final fish weight (g) - mean initial fish weight (g), (iii) Absolute length (cm) = mean final fish length (cm) - mean initial fish length (cm), (iv) Weight/length growth rate = $((\ln \text{ final weight/length}) - (\ln \text{ initial weight/length})) / 40 \text{ days}$, (v) Feed conversion ratio = feed consumption / (final weight - initial weight), (vi) Survival rate (%) = (number of fish at the end/ beginning of the study) x 100.

Chemical analysis

Proximate analysis was conducted to determine the protein, fat, crude fiber, ash, and moisture content of the test feed at the Fish Nutrition Laboratory, Institut Pertanian Bogor, Bogor, Indonesia, following the procedures outlined by AOAC (2005). Carotenoid content analysis in the fermented Moringa leaf extract was conducted at the Postharvest Agricultural Research and Development Center, Bogor, using the method proposed by Meiliszka et al. (2019) with a spectrophotometer at 480 nm.

Statistical analysis

Color variation data, the number of chromatophore cells, and growth were analyzed using Analysis of Variance (ANOVA) through SPSS software version 17.0. Significant differences in the results were tested using Duncan's Multiple Range Test with a confidence interval of 95%. The obtained data were presented in tables and graphs, and the color brightness and fish blood profile data were analyzed descriptively.

RESULTS AND DISCUSSION

Fish color brightness

The values for Oranda goldfish with the addition of fermented Moringa leaf extract are presented in Figure 1. The results indicated that adding fermented Moringa leaf extract to the feed enhances the intensity of color brightness in Oranda goldfish. The color brightness scores of Oranda goldfish on the 10th day and 20th day ranged from 3.72 to 3.75 and 3.73 to 3.80, respectively. The improvement in color enhancement was noticed on the 20th day, with the highest score recorded in treatment D at 4.75, followed by C, B, and A at 4.53, 4.34, and 3.75, respectively. The same trend was observed on the 40th day, where the highest score was found in treatment D at 5.04, followed by C, B, and A at 4.79, 4.44, and 3.70.

Color variation in Oranda goldfish

Color variation calculations were observed at the head, body, and caudal peduncle. The color variation analysis in Oranda goldfish with different doses of fermented Moringa leaf extract is presented in Table 2, while the images of Oranda goldfish at the end of the rearing period are shown in Figure 2. The color range values on the body, caudal, and head ranged from 47.28% to 57.81%, 48.37% to 71.71%, and 54.59% to 71.13%, respectively.

Table 2. Color variation analysis in *C. auratus* Oranda goldfish using Adobe Photoshop CS4*

Feed Test**	Color Variation (%)		
	Body	Caudal	Head
A	47.28 ± 5.58 ^a	48.37 ± 6.93 ^a	54.59 ± 3.71 ^a
B	50.65 ± 2.40 ^{ab}	54.14 ± 8.88 ^a	62.96 ± 0.45 ^b
C	52.22 ± 4.64 ^{ab}	55.86 ± 1.98 ^a	65.93 ± 0.87 ^{bc}
D	57.81 ± 6.31 ^b	71.71 ± 2.70 ^b	71.13 ± 4.45 ^c

Note: *Different superscript letters behind the standard deviation (±) values indicate significant differences ($P < 0.05$). ** A (control), B (40 mL/kg fermented Moringa leaf extract), C (50 mL/kg fermented Moringa leaf extract), and D (60 mL/kg fermented Moringa leaf extract)

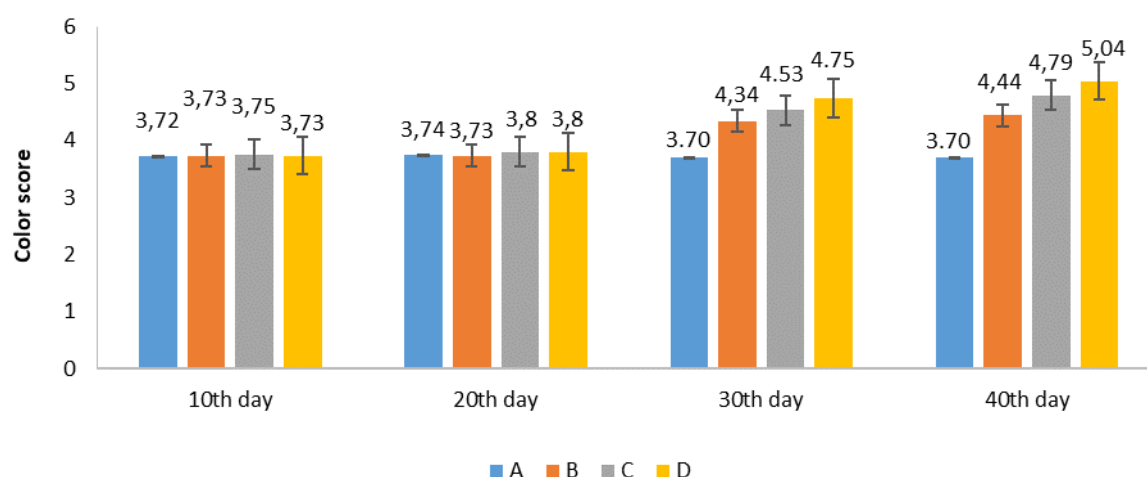


Figure 1. Scores of color brightness in Oranda goldfish with different fermented Moringa leaf extract additions. A (control), B (40 mL/kg fermented Moringa leaf extract), C (50 mL/kg fermented Moringa leaf extract), and D (60 mL/kg fermented Moringa leaf extract)

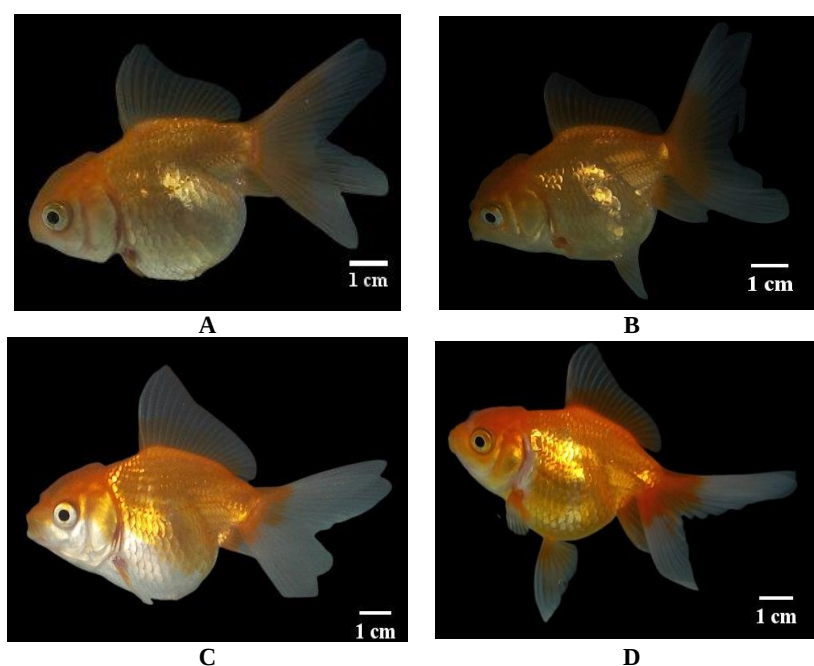


Figure 2. Visual appearance of color quality in Oranda goldfish at the end of the rearing period. A (control), B (40 mL/kg fermented Moringa leaf extract), C (50 mL/kg fermented Moringa leaf extract), and D (60 mL/kg fermented Moringa leaf extract)

Number of chromatophore cells

The number of cells in Oranda goldfish with different doses of the extract in the feed is presented in Figure 3, while the histological results are shown in Figure 4. The result showed that the highest ($P<0.05$) number of chromatophore cells was found in treatment D of 136 cells, followed by treatment C of 106 cells, treatment B of 100 cells and treatment A of 83 cells. Chromatophore cells appear as dark purple spherical cells under a 10x microscope magnification.

Growth and survival rate of Oranda goldfish

The growth and survival rate of Oranda goldfish with different doses of fermented Moringa leaf extract in the feed are presented in Table 3. The average final, absolute, and specific growth rate length ranged from 5.90 to 6.24 cm, 1.57 to 1.64 cm, and 0.74 to 0.78 % cm/day. The final weight ranged from 7.14 to 7.25 g, while the absolute weight and specific growth rate in weight ranged from 3.41 to 3.52 g and 1.62 to 1.66 % g/day, respectively. Feed consumption values, feed conversion ratio, and survival rate did not differ significantly ($P>0.05$) among treatments. The feed consumption and conversion ratio ranged from

12.17 to 12.88 g and 3.54 to 3.76, and the treatments showed the same survival rate of 100%.

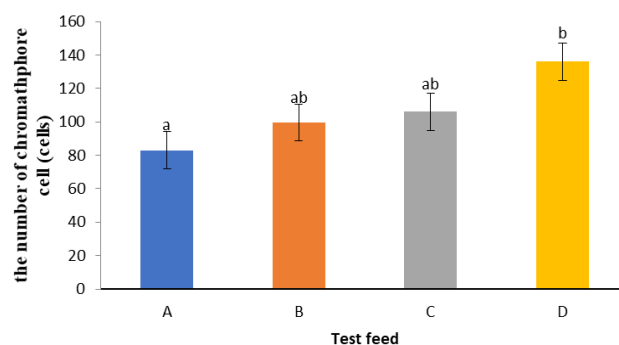


Figure 3. Number of chromatophore cells in Oranda goldfish with fermented Moringa leaf extract in the feed. Different superscript letters (a, ab, b) above the bar graph indicate significant differences ($P<0.05$). A (control), B (40 mL/kg fermented Moringa leaf extract), C (50 mL/kg fermented Moringa leaf extract), and D (60 mL/kg fermented Moringa leaf extract)

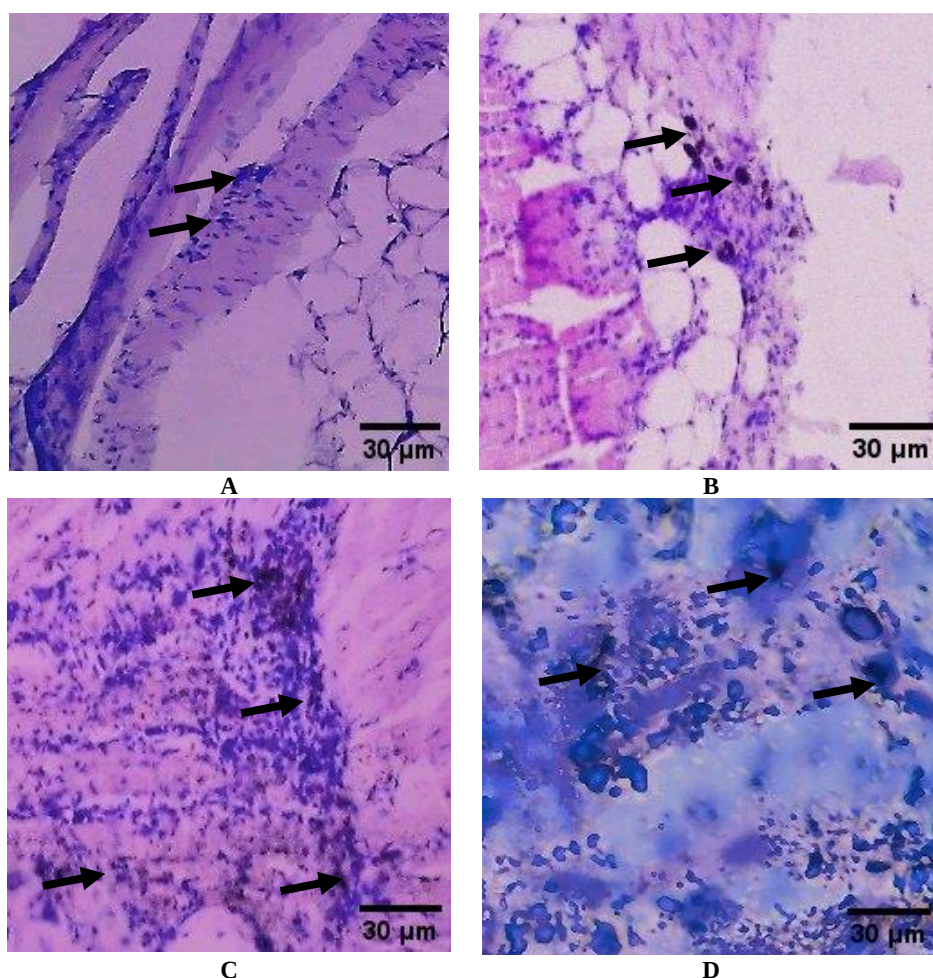


Figure 4. Histological observation results of chromatophore cells (yellow circle) in the skin tissue of *C. auratus* Oranda goldfish. A (control), B (40 mL/kg fermented Moringa leaf extract), C (50 mL/kg fermented Moringa leaf extract), and D (60 mL/kg fermented Moringa leaf extract)

Blood profile of Oranda goldfish

The blood profile values of Oranda goldfish, including hemoglobin, hematocrit, erythrocytes, and leukocytes, are presented in Table 4. The study indicated that hemoglobin and hematocrit values ranged from 5.0 to 5.8 g/dL and 10.81 to 22.58%. The erythrocyte values ranged from 0.55 to 2.11×10^6 cells/mm³. Leukocyte values also ranged from 4.75 to 7.17×10^4 cells/mm³.

Water quality

Water quality is crucial as the primary medium for fish survival, and the range of values in the environment for Oranda goldfish with the addition of fermented Moringa leaf extract is presented in Table 5.

Discussion

Color brightness is a primary factor in determining the quality of ornamental fish and is influenced by genetics, nutrition, color pigment content in the feed, and environment (Koncara et al. 2019). The result showed that adding fermented Moringa leaf extract produced higher scores of color brightness in Oranda goldfish on the 30th

and 40th day. The higher scores in the treatments with the addition of fermented Moringa leaf extract were attributed to the presence of carotenoid pigments efficiently absorbed and utilized by the fish to enhance and maintain their color quality. Sathyaruban et al. (2021) stated that goldfish cannot improve and maintain their body color without carotenoid content in their diet. One of the functions of carotenoids is to maintain and enhance color brightness (Chapman and Miles 2018). These compounds are absorbed into pigment cells through various processes, and the distribution of pigment cells affects the increase and decrease of fish color. Similar findings have been reported by Takdir et al. (2022) that Moringa leaf extract supplementation significantly influenced the coloring of betta fish. This was supported by Angelica et al. (2020), who said that one of the antioxidant compounds in Moringa leaves was beta-carotene, which is responsible for orange, red-orange, and yellow pigments. According to Dananjaya et al. (2020), increasing pigmentation on the skin and fins of goldfish occurs at different times depending on the environment and carotenoid concentration in the diet.

Table 3. Average survival rate, absolute weight, absolute length, and feed consumption of Oranda goldfish (*C. auratus*)*

Growth Parameters	Feed Test**			
	A	B	C	D
Initial length (cm)	4.65±0.10 ^a	4.51±0.28 ^a	4.33±0.15 ^a	4.56±0.10 ^a
Final length (cm)	6.24±0.03 ^a	6.15±0.37 ^a	5.90±0.08 ^a	6.15±0.13 ^a
Absolute length (cm)	1.59±0.13 ^a	1.64±0.32 ^a	1.57±0.21 ^a	1.59±0.28 ^a
Specific growth rate of length (%.cm/hari)	0.74±0.07 ^a	0.78±0.14 ^a	0.77±0.11 ^a	0.75±0.13 ^a
Initial weight (g)	3.73±0.06 ^a	3.68±0.09 ^a	3.73±0.06 ^a	3.73±0.06 ^a
Final weight (g)	7.25±0.69 ^a	7.16±0.66 ^a	7.14±0.40 ^a	7.24±0.34 ^a
Absolute weight (g)	3.52±0.66 ^a	3.49±0.60 ^a	3.41±0.43 ^a	3.50±0.30 ^a
Specific growth rate of weight (%.g/hari)	1.65±0.23 ^a	1.66±0.20 ^a	1.62±0.16 ^a	1.65±0.09 ^a
Feed consumption (g)	12.88±0.14 ^a	12.17±0.30 ^a	12.60±0.41 ^a	12.31±0.48 ^a
Feed conversion ratio	3.76±0.81 ^a	3.56±0.63 ^a	3.74±0.56 ^a	3.54±0.43 ^a
Survival rate (%)	100.00±0.00 ^a	100.00 ± 0.00 ^a	100.00 ± 0.00 ^a	100.00 ± 0.00 ^a

Note: *Different superscript letters behind the standard deviation (±) values indicate significant differences ($P < 0.05$). ** A (control), B (40 mL/kg fermented Moringa leaf extract), C (50 mL/kg fermented Moringa leaf extract), and D (60 mL/kg fermented Moringa leaf extract)

Table 4. Blood profile of Oranda goldfish with the addition of fermented Moringa leaf extract in the feed

Feed Test*	Blood Profiles			
	Hemoglobin (g.%)	Hematocrit (%)	Erythrocyte (10^6 cells/mm ³)	Leukocyte (10^4 cells/mm ³)
A	5.7±1.6	13.33±1.2	1.38±15.6	4.75±28.6
B	5.4±2.1	10.81±1.7	0.82±16.4	5.70±31.6
C	5.0±1.8	20.68±1.4	0.55±18.2	6.36±15.7
D	5.8±1.1	22.58±1.6	2.11±21.4	7.17±14.0

Note: *A (control), B (40 mL/kg fermented Moringa leaf extract), C (50 mL/kg fermented Moringa leaf extract), and D (60 mL/kg fermented Moringa leaf extract)

Table 5. Water quality parameters in the environment for Oranda goldfish rearing

Parameters	Feed Test*			
	A	B	C	D
Temperature (°C)	26-29	26-29	26-29	26-29
pH	7.89-8.63	7.77-8.70	7.53-8.50	7.81-8.55
DO (mg/L)	5.80-6.7	5.31-7.3	5.85-7.50	5.21-7.7
Ammonia (mg/L)	0-0.025	0-0.25	0-0.25	0-0.25

Note: *A (control), B (40 mL/kg fermented Moringa leaf extract), C (50 mL/kg fermented Moringa leaf extract), and D (60 mL/kg fermented Moringa leaf extract)

In the present study, adding the highest dose of fermented Moringa leaf extract (60 mL/kg) resulted in the highest color brightness score compared to other treatments. This suggests that a higher dose has a higher carotene content, resulting in better color quality of the Oranda goldfish. The same results have been reported by Andriani et al. (2021). The addition of butterfly pea leaf meal as a carotenoid source in the feed with the highest dose of 12% produced the best quality score (87.33) compared to treatment with additional doses of 6% (73.83), 1% (32.17) and 0% (24.67). Nutritional strategy is an effort widely used to improve the color quality of ornamental fish. Using fish feed rich in carotenoids has been proven to increase the brightness of ornamental fish (Sawant et al. 2020). Visual color variation in the present study was also observed at the end of the rearing period at the head, body, and caudal. The result demonstrated that the percentage of visual color variation increased with the fermented Moringa leaf extract dose. Treatment D significantly ($P < 0.05$) resulted in the highest color variation level in the body (57.81%), caudal (71.71%), and head (71.13%) compared to the others. This finding suggested that higher doses of the extract lead to higher carotenoid content in the feed, resulting in better color variation than other treatments. Moringa leaves are a source of carotenoids in the feed, enhancing the color quality of ornamental fish (Angelica et al. 2020). The results showed that the leaf extract's carotene content is 94.22 ppm/50 mL. Similar findings have been reported by other studies using natural ingredients as a source of carotenoids.

Dananjaya et al. (2017) reported that increasing the dietary carotenoid level enhances pigmentation on the skin and fin of goldfish. Kurnia et al. (2019) stated that the addition of red dragon fruit peel meal extract as a potential carotenoid source with the highest dose (15%) resulted in the best color quality in koi carp compared to other treatments (0%, 5% and 10%). Chromatophores are cells responsible for increasing or decreasing the brightness of fish scales (Astari et al. 2016). When the number of chromatophore cells increases, the color of the fish body becomes brighter and more attractive when the number of cells increases. In the present study, we found the highest number of chromatophore cells in treatment D, while the smallest number was found in treatment A. These results align with the brightness level and color variation of goldfish in this study. The number and combination of chromatophore cells in each fish species vary depending on the feed and environment (Sabrina et al. 2023).

Carotenoids have a positive effect on increasing color pigmentation and goldfish growth (Besen et al. 2019). However, the present study showed no significant differences in the growth parameters of Oranda goldfish among treatments. The addition of fermented Moringa leaf extract to the feed is thought not to affect macro and micronutrient content in the feed so that the growth performance of the Oranda goldfish produced in each treatment is the same. The same results have been reported by Hekimoglu et al. (2017), who found that supplementation of algae carotenoids in the diet did not affect the growth and feed conversion ratio of tomato

clownfish. Gurung et al. (2018) also reported that adding natural carotenoid sources (cassava leaves, potato leaves, and colocasia leaves) in the feed does not affect the Oranda goldfish's specific growth rate and feed conversion ratio. In a similar study, Bell et al. (2019) revealed that including lobster meal (0-15%) in the diet did not affect the growth performance of goldfish. Similarly, there is no significant effect on the diet's absolute weight, absolute length specific growth rate and survival rate of goldfish with different concentrations of red yam flour (0-12%) as carotenoid sources (Putra and Romdhonah 2019). These results are also in line with the report by Kautsar et al. (2022) that the addition of natural ingredients (sweet potato, carrot, and pumpkin meal) as a source of β -carotene has no impact on absolute weight growth and absolute length growth of Oranda goldfish. There were no significant differences ($P > 0.05$) in the amount of feed consumption, where the addition of fermented extract to the feed did not affect the palatability of Oranda goldfish. Feed palatability is determined by taste, smell, and color, which affect feed consumption, digestibility, and fish growth levels. Putra et al. (2020a) stated that using Moringa leaves as feed ingredients did not affect the feed consumption of catfish. This study found no difference in the survival rate of Oranda goldfish; the same results were found by Dananjaya et al. (2017). Using natural bixin as a potential carotenoid with different levels in feed did not affect the survival rate of goldfish. A similar result was found by Besen et al. (2019), who found that dietary lutein as a carotenoid in the diet (50 mg/kg) did not influence goldfish juvenile survival rate, growth, and feed efficiency.

The blood profile values of Oranda goldfish in each treatment fall within the normal range. Hemoglobin in the blood functions as an oxygen transport tool found within erythrocytes, while hematocrit represents the percentage of the volume serving as an indicator of fish stress (Madyowati and Muhajir 2018). The normal hemoglobin value of fish is 4.70-16.6 g/dL (Fazio et al. 2019), while the hematocrit range for teleosts fish is 9.4-33.53% (Witeska et al. 2022). According to Esmaeili et al. (2021), the normal range of red and white blood cells in fish was 0.4 to 5.2×10^6 cells/mm³ and 2.17 to 116.5×10^4 cells/mm³. Based on these results, adding fermented Moringa leaf extract to the feed is assumed not to affect the physiological processes within the body of Oranda goldfish. The analysis can evaluate the physiological responses within the fish's body, indicating stress. Sources of stress, such as environmental factors, including temperature, pH, fish handling, and light, as well as biotic factors, including pathogenic infections, can negatively impact the physiological state (Royan et al. 2014). The anti-nutritional substances present in Moringa leaves can interfere with the physiological processes within the body of fish (Putra et al. 2022b). The fermentation process of *A. niger* is believed to have reduced the crude fiber value and anti-nutritional content in the leaves. This aligns with the statement by Putra et al. (2022a) that the fermentation of feed ingredients using *A. niger* can decrease the crude fiber value and anti-nutritional substances. However, in the present study, the authors did

not measure the anti-nutritional substances in Moringa leaf extract before and after fermentation.

Water quality plays an important role for ornamental fish because good water quality produces bright colors and fish growth (Indriani et al. 2023). Changes in rearing water temperature will affect the metabolism, growth, and feed consumption of the fish's body (Charlot et al. 2017). In this study, the obtained temperature values from 26 to 29°C indicated the normal range of fish rearing temperature. According to Chen et al. (2019), the optimal temperature value for goldfish is 28°C. Catalano et al. (2019) added that goldfish still grow to a temperature of 18°C. Roslan et al. (2021) stated that the normal range for Dissolved Oxygen (DO) and pH values in freshwater fish are >4 mg/L and 6.5-8.5. In this study, DO and pH values ranged from 5.21 to 7.50 mg/L and 7.53 to 8.70; these results indicate that the DO and pH values in this study are in the normal range. Ammonia is toxic and the most abundant product of nitrogen metabolism produced by intensive aquaculture systems (Putra et al. (2021b)). The result showed that the value of ammonia produced was 0 to 0.25 mg/L; the results are within the normal range of freshwater fish rearing. Mustaqim et al. (2022) stated that the ammonia value for fish rearing should not exceed 1.5 mg/L. The addition of fermented Moringa leaf extract in the feed did not affect the water quality values in the environment for Oranda goldfish.

In conclusion, the carotene in fermented Moringa leaf extract showed a content of 94.22 ppm/50 mL. Adding Moringa leaf extract to the feed enhanced and maintained the color of the Oranda goldfish. The addition of fermented Moringa leaf extract at a dosage of 60 mL/kg resulted in the highest color diversity values on the body (57.81%), caudal peduncle (71.71%), and head (71.13%) compared to others. The addition of fermented Moringa leaf extract did not affect growth, survival rate, blood profile, and water quality. Fermented Moringa leaf extract using *A. niger* at a dosage of 60 mL/kg could be used in Oranda goldfish cultivation. Further study should be conducted to explore the addition of carotenoid content in Oranda goldfish after being fed with fermented Moringa leaf extract to optimize the dosage.

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