

Short Communication: Morphological diversity and the addition of golden snail protein: Its effect on flavonoid content on *Echinacea purpurea*

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Abstract. *Ferdiana WC, Widiyastuti Y, Pujiasmanto B, Sakya AT, Yunus A. 2021. Short Communication: Morphological diversity and the addition of golden snail protein: Its effect on flavonoid content on Echinacea purpurea. Biodiversitas 23: 62-66. Echinacea purpurea* is a subtropical medicinal plant that contains flavonoids. Flavonoids function as antioxidants and immunomodulators. However, information on the method to increase flavonoid content in *E. purpurea* is still limited. The golden snail protein increases flavonoid content. The purpose of this study was to determine the best accession and optimal concentration of golden snail protein on biomass production and flavonoid content in *E. purpurea*. The study used a Factorial Completely Randomized Design (CRD). The first factor was three accessions of *E. purpurea* (A1, A2, A3); the second factor was 5 levels of the addition of golden snail protein (0 mL/L/week, 10 mL/L/week, 20 mL/L/week, 30 mL/L/week, and 40 mL /L/week). There were differences in morphological characters of three *E. purpurea* accessions, i.e., stem and the shape of the flower crown. Accession 2 had the highest flavonoid content except for the 10 mL/L/week treatment. The highest increase in flavonoid content in three accessions was obtained in the treatment of 10 mL/L/week gold snail protein by 19% flavonoid content compared to control treatment (0 mL/L/week). The concentration of 0 mL/l/week or control had the lowest flavonoid content.

Keywords: Accession, *Echinacea purpurea*, flavonoid, golden snail protein

INTRODUCTION

Echinacea purpurea L. is a subtropical plant originating from North America (Bodiment et al. 2002) and was first cultivated in Indonesia in 1998 (Rahardjo 2000). *E. purpurea* produces various secondary metabolites, namely caffeic acid derivatives, phenols, flavonoids, etc. *E. purpurea* is used as one of the raw materials for herbal medicines (Lee et al. 2010). Flavonoids are secondary metabolites produced by *E. purpurea* that have the immunomodulatory or antioxidant activity, and function to maintain human body resistance (Aarland et al. 2017).

In Indonesia, *E. purpurea* has not been widely cultivated. The Center for Research and Development of Medicinal Plants and Traditional Medicines is also still studying the cultivation of *E. purpurea*. Many drugs, pharmaceutical, and herbal medicine manufacturers in Indonesia also import *E. purpurea* simplicia as raw material for their processed products (Rahardjo 2005). Information on standardization in cultivation and increasing secondary metabolites content of *E. purpurea* is also still very limited in Indonesia.

The addition of golden snail protein has been proven to improve cultivation and total flavonoid content. The golden snail was one of the pests that cause damage to rice crops, causing losses to farmers, however, the golden snail contains a fairly high crude protein, ranging from 18.33% (Hertrampf and Piedad-Pascual 2000) to 86.36% (Jintasataporn et al. 2014). The golden snail protein could be extracted and used as liquid organic fertilizer. The protein of golden snails undergoes a nitrogen fixation process, followed by a degradation process into amino acids. Plants absorb NO_3^- reduced to NO_2^- by Nitrate Reductase. Then some NO_2^- will be reacted enzymatically with glutathione and NH_4^+ , and some NO_2^- will be transported to chloroplasts to form NH_4^+ . The NH_4^+ is then assimilated into amino acids in a series of reactions facilitated by a series of enzymes (Fringilla et al. 2014). The use of liquid organic fertilizer based on golden snail protein can increase the yield of *E. purpurea* quantitatively and qualitatively (Hajagha et al. 2019). The purpose of this study was to determine the best accession and optimal concentration of golden snail protein on biomass production and flavonoid content in *E. purpurea*.

MATERIALS AND METHODS

Plant materials

Three accessions of *E. purpurea* (A1, A2, and A3) used in this study were from the collection of the Center for Research and Development of Traditional Medicinal and Medicinal Plants (B2P2TOOT) Tawangmangu, Karanganyar, Central Java, Indonesia which had different morphological characteristics.

Experimental design

The study was conducted at the Jumantono screen house, Karanganyar, Central Java, Indonesia from April to August 2020. The experimental design used was a Completely Randomized Design (CRD) with a factorial pattern consisting of 2 factors. The first factor was 3 accessions of *E. purpurea* (A1, A2, and A3). The second factor was 5 levels of the addition of golden snail protein, i.e., (P0: 0 mL/L/week; P1: 10 mL/L/week; P2: 20 mL/L/week; P3: 30 mL/L/week; P4: 40 mL/L/week). Each treatment had 4 replications, and in total there were 60 experimental units.

Field experimental procedure

Preparation of golden snail protein

The golden snail protein-based liquid organic fertilizer was made based on the method from Andriani (2019) as follows, the golden snail was boiled for approximately 15-20 minutes, then the shell was separated from the golden snail meat. After that, the golden snail meat was mashed using a blender. Meanwhile, 2 jerry cans (5 liters) were prepared and each jerry can be filled with 4 liters of coconut water, 500 mL of molasses, 160 mL of EM4, and 1 kg of mashed golden snail meat. Stir until homogenous then cover tightly with an insulating adhesive. The jerry can cap is perforated to attach a small hose with a diameter of 1 cm hose that is connected to a mineral water bottle. The bottle was half-filled with water to reduce gas in the jerry can. The fertilizer mixture was fermented for approximately 15 days. Protein analysis was carried out using the Kjeldahl method. The protein yield is 22%.

Treatment of golden snail protein

One and half-month-old seedlings of three accessions of *E. purpurea* were obtained from the Center for Research and Development of Medicinal Plants and Traditional Medicines. The transplanted seedlings should have at least 3 true leaves, and be treated with golden snail protein once a week.

Harvest

Echinacea purpurea was harvested at 120 DAP (days after planting). The plants were weighed to obtain fresh weight. After weighing *E. purpurea* plants were dried at room temperature for 7 days followed by drying in an oven at 50°C to constant dry weight.

Analysis of herb extract

Dried *E. purpurea* were ground into powder. Five g of plant powder was weighed as the initial sample weight and

then macerated with 50 mL of 70% ethanol for 3 days. After the maceration process, it was filtered and the filtrate was put into a cup that had been weighed previously as the empty weight of the cup and dried in the oven at 50°C. The weight of the extract was calculated by subtracting the final weight of the cup from the empty weight of the cup (Research and Development Center of Medicinal Plants and Traditional Medicines 2018). The percentage of *E. purpurea* extract was calculated using the formula:

$$r(\%) = \frac{x}{y} \times 100$$

Where:

r : Percentage of extracts (%)

x : Weight of herbal extract (g)

y : Initial sample weight (g)

Analysis of total flavonoid content (TFC)

Analysis of total flavonoid content was carried out using the Aluminum chloride colorimetric method from Chang et al. (2002) with a slight modification. One hundred mg of concentrated extract was dissolved in 70% ethanol to a volume of 10 mL, then sonicated for 15 minutes and precipitated overnight. Four mL extract was put in the oven at 50°C, then diluted with 8 mL of methanol, then sonicated for 15 minutes and precipitated overnight. Flavonoid testing was carried out as follows: Solution 1: 0.2 mL of extract added with 4.8 mL of aquabidest. Solution 2: 0.2 mL of extract added with 1 mL of AlCl₃ and 3.8 mL of aquabidest. Solution and 2 were incubated for 15 minutes. The first sample solution was read at the wavelength of 415 nm using a UV-Vis Spectrophotometer. A standard curve using quercetin solution was made by pipetting 2 mL of the second sample solution. After that, the absorbance value was read at the maximum wavelength and operating time of the first sample solution. The quercetin concentration was calculated by the curve equation $y = 0.0078x + 0.0427$, the Y value was the standard absorbance curve and the X value was the quercetin concentration (mg/L). The accumulation of flavonoids in the extract was calculated using the formula:

$$F = \frac{C \times V \times FP \times 10^{-3}}{M} \times 100$$

Where:

F : Total flavonoid content (%)

C : Quercetin concentration (mg/L)

V : Extract volume (L)

FP : Dilution factor

M : Sample weight (mg)

Data analysis

Qualitative data as morphological diversity were analyzed descriptively. Quantitative data was obtained from the average of each observation based on the available graphs.

RESULTS AND DISCUSSION

Morphological diversity

Morphological characteristics were observed to determine certain characteristics of the accessions of *E. purpurea* after being treated with golden snail protein. The differences in morphological characters of three *E. purpurea* accessions were in the color of the stem (Figure 1), the shape of the flower crown (Figure 2), and the leaf shape (Figure 3).

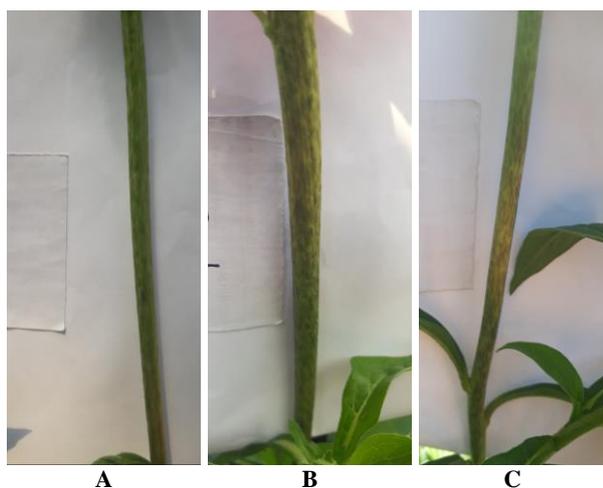


Figure 1. Stems morphology of three *Echinacea purpurea* accession. A: Accession 1. B: Accession 2. C: Accession 3

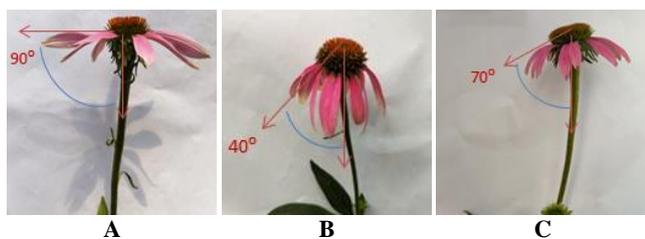


Figure 2. Flowers morphology of three *Echinacea purpurea* accession. A: Accession 1. B: Accession 2. C: Accession 3

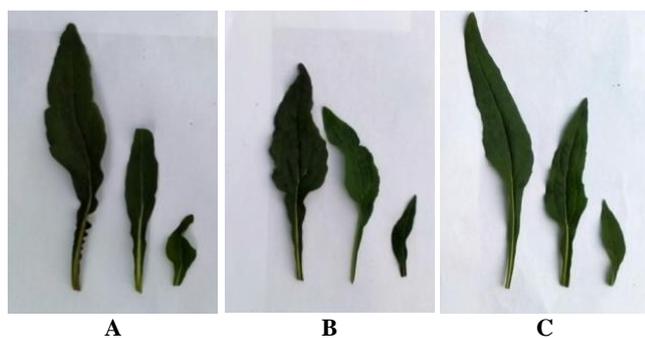


Figure 3. Leaf morphology of three *Echinacea purpurea* accession. A: Accession 1. B: Accession 2. C: Accession 3

Morphological stem of three accessions of *E. purpurea*

The stem morphology of three *E. purpurea* accessions was different. Accession 1 has light green stems with green spots, while Accession 2 has green stems with dark green spots. Accession 3 has a different stem color and is more conspicuous than Accession 1 and Accession 2, which has a green stem color with dark purple spots. The differences in stem color can be influenced by the surrounding environment, the nutrients or fertilizers applied, and genetic factors. According to Allen (2010), plant morphology such as the color or shape of plant stems can be influenced by metabolic processes in plants and external factors such as microclimate which includes temperature, humidity, stress, environmental changes.

Morphological flower of three accessions of *E. purpurea*

The floral morphological characters of the three *E. purpurea* accessions also varied. Accession 1 has a flower crown that is almost parallel or forms a right angle (90°) from the flower stalk, while Accession 2 has a striking flower crown shape, namely the crown is bent downwards or forms an angle of approximately 40° from the flower stalk. Accession 3 has a crown shape that is not too bent down or forms an angle of approximately 70° from the flower stalk. In line with Shidiq et al. (2020) that the agronomic and morphological diversity of *E. purpurea* is also influenced by cross-pollination and produces *E. purpurea* seeds with different morphological characters from the parent. Previous studies by Choirunnisa et al. (2021), showed that propagation using seeds can produce morphological variations of stem color, flower shape, and new leaves that are different from the morphological characters of the parent.

Morphological leaf of three accessions of *E. purpurea*

The three *E. purpurea* accessions studied had dark green leaves. Accession 1 has an elongated leaf shape with wavy leaf edges and blunt leaf tips. Accessions 2 and 3 had the same leaf shape, which was elongated with pointed leaf edges and pointed leaf tips. In line with the research of Shidiq et al. (2020), accessions 2 and 3 of *E. purpurea* had dark green leaf color with curved, serrated, and pointed leaf edges. Indentation on the edges of young leaves is not visible but is visible on mature leaves. The morphological character of the leaf shape in the *E. purpurea* accession used this time was the same as the parent, but the leaf color for accession 1 was different from the parent, which was light green. Differences in leaf color from the parent can be caused by environmental influences and fertilizer application. According to Pratiwi et al. (2019), changes in leaf color of *Mentha spicata* were caused by lack of sunlight and absorption of nutrients and water.

Extraction

Extraction of *E. purpurea* was carried out by maceration. The maceration method is a simple extraction method with the principle of immersing and stirring the sample in an appropriate solvent in extracting flavonoid compounds, and maceration extraction have the advantage

of using more solvents than other extraction methods (Sapiun et al. 2020).

The ethanol extract of *E. purpurea* had a blackish green color that contains active ingredients. According to Mahajan et al. (2020), secondary metabolites were produced as one of the adaptive mechanisms of plants under stress conditions. Figure 4 shows the graph of extract from three accessions of *E. purpurea* treated with golden snail protein. The extract percentage of A1, A2, and A3 was 7.53%, 7.98%, and 8.42%, respectively. Application of golden snail protein produces various extract percentages ranging from 7.78% to 8.49%. The addition of golden snail protein at high concentrations has a low extract yield. The might be due to the primary metabolic processes in the plant running well and not experiencing stress, therefore the plant produces only a small amount of secondary metabolites such as flavonoids. According to Tuteja (2007), the production of secondary metabolites in plants directly depends on the physiological conditions and development of these plants.

The extract yield is related to the chemical compounds contained in the plant. The high yield of extract does not correlate with the amount of specific active ingredients such as flavonoids because the herb extract is an accumulation of all the active ingredients contained in plants. The solvent used is ethanol which is polar which is used to extract polar components such as flavonoids. Further testing needs to be done to obtain more specific active ingredients or secondary metabolites. According to Mumtazah et al. (2020) stated that a high yield of extract does not necessarily produce high active ingredients.

Total flavonoid content

Flavonoids are one of the secondary metabolites that have been known to be contained in *E. purpurea*. According to Heldt (2011), flavonoids are a combination of organic pigments that form the color of leaves, flowers, stems, and

fruit in plants and are also useful for humans, specifically as antioxidants. The biosynthesis of flavonoids is carried out through the phenylpropanoid pathway and involves PAL (*Phenylalanine Ammonia-Lyase*) enzyme (Taiz and Zeiger 2002).

Accession 2 in each treatment of golden snail protein showed the highest yield except in the treatment of 10 mL/l/week. The highest flavonoid content in 3 accessions was found in the golden snail protein treatment at the concentration of 10 mL/l/week. The concentration of 10 mL/L/week increased the flavonoid content by 19% compared to the control treatment (0 mL/L/week). The concentration of 0 mL/L/week or control had the lowest flavonoid content (Figure 5). The application of golden snail protein more than 10% reduced the total flavonoid content compared to the 10% treatment. This is due to the higher the concentration of golden snail protein, the higher the nitrogen content. Nitrogen has an important role in plant growth. An adequate supply of nitrogen could result in good plant growth and a stable primary metabolic process so that primary metabolic products can be properly translocated throughout the plant body and the plant does not experience stress. Plants that grow well do not trigger *Phenylalanine Ammonia Lyase* (PAL) enzyme activity which plays an important role in the formation of flavonoids in the shikimate or phenylpropanoid pathway so that low PAL enzyme activity results in low flavonoid accumulation.

The low concentration of golden snail protein resulted in high total flavonoid content. The lower the concentration of golden snail protein, the lower the nitrogen content. According to the research of Ibrahim et al. (2011) nitrogen deficiency increases the accumulation and synthesis of phenolic compounds and flavonoids through the phenylpropanoid pathway. A study by Liu et al. (2010) showed that flavonoid content in *C. morifolium* leaves was high at low N supply.

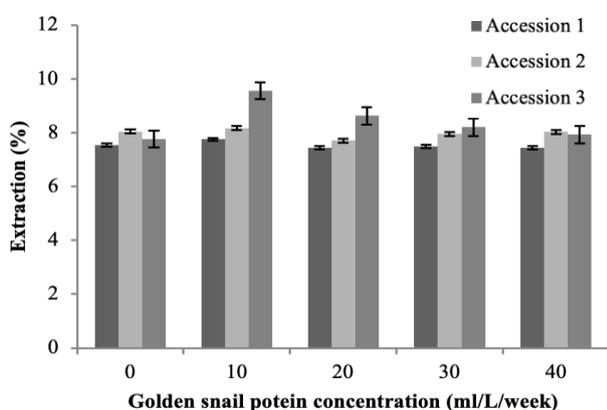


Figure 4. Percentage of extraction of three *Echinacea purpurea* accessions treated with different concentrations of golden snail protein

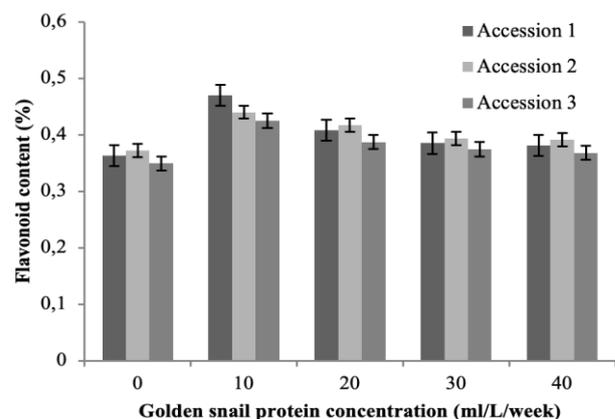


Figure 5. Total flavonoid content of three *Echinacea purpurea* accessions treated with different concentrations of golden snail protein

The formation of flavonoids compounds in *E. purpurea* plants was caused by the plant experiencing nutritional stress, namely the lack of availability of nitrogen nutrients. Stress caused by plants will increase the activity of the enzyme PAL which plays a role in the formation of secondary metabolites through the phenylpropanoid pathway that produces phenols and their derivatives, namely flavonoids. According to Liu et al. (2010), the increase in PAL enzyme activity is an important mechanism that contributes to the increased accumulation of flavonoids. The control treatment had the lowest flavonoid content because the plants only utilized nutrients from the soil without the addition of a continuous supply of golden snail protein. The nitrogen available in the soil is limited and is used by plants for the growth process so that over time the availability of nitrogen runs out and plants cannot survive even though they have produced secondary metabolites. Low nitrogen availability causes inhibition of flavonoid biosynthesis.

In conclusion, there were differences in the morphological characteristics of the three accessions of *E. purpurea* in stem color, flower crown shape, and leaf shape. The highest total flavonoid content was found in Accession 2 and the highest extract yield was found in Accession 3. The concentration of golden snail protein 10 mL/L/week could increase the flavonoid content by 19% compared to the control treatment (0 mL/L/week).

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