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Identification of Phytochemicals, Antioxidant Activity Using DPPH Method (*1.1=Diphenyl-2-Picrylhydrazyl*), Toxicity Using Brine Shrimp Lethality Test of *Diplazium esculentum* Tea

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ABSTRACT

Vegetable fern/pakis/paku *(Diplazium esculentum)* contains active compounds such as total phenols, steroids, triterpenoids, flavone amino acids, and flavonoids, which are rich sources of medically necessary phytochemicals. This study aimed to determine the potential antioxidant content of pakis (*Diplazium esculentum*) tea. The research methods used included the antioxidant test procedure (IC_{50}), DPPH method, phytochemical procedure, and BSLT procedure. The results of the phytochemical test showed a positive (+) value on the alkaloid, flavonoid, saponin, and phenolic test. The DPPH test showed 130.38468, 131.1340, 131.5681 ppm with an average value of 131.0289 ppm; meanwhile, moderate antioxidant activity and BSLT testing showed LC_{50} of 9817.479 ppm, where the tea was found not toxic since the LC_{50} value <1000 ppm. Based on the findings, the brewed vegetable fern/pakis *(Diplazium esculentum)* contains alkaloids, flavonoids, saponins, and phenolics based on phytochemical testing, potential as a non-toxic antioxidant source according to DPPH and BSLT tests. *Diplazium esculentum* is a vegetable that contains antioxidants and is safe for consumption.

Keywords: Antioxidant activity, BSLT*Diplazium esculentum*, DPPH, Phytochemicals

INTRODUCTION

Indonesia is rich in biodiversity in vegetables and fruits, which are believed to contain antioxidant compounds and have been consumed for generations. According to the Culture Importance Index (CI), the most important vegetable species is *Diplazium esculentum* (Bhatia et al., 2018). Vegetable fern/paku/pakis *(Diplazium esculentum)* is a plant with high economic value, is used as medicine, consumption, and has many benefits (Koniyo et al., 2019).

Diplazium esculentum (Family: Athyriaceae) is one of the most popular yields of biodiversity, considered a pharmacologically diverse ethnomedicinal plant (Chaudhuri & Roy, 2020). *Diplazium* is a genus of the cliff fern family with an estimated 400 known species (Lim et al., 2020), ferns that can be consumed are useful as certain drugs contain sources of natural bioactive compounds that can be used as natural medicines and have the potential to be developed as new drugs (Zannah et al., 2017). This plant grows well in areas with moist soil (Kunkeaw et al., 2021), such as in river areas and even agricultural land (Apriyanti et al., 2017). All over the world, many species of ferns are used as traditional medicine (such as diabetes, smallpox, asthma, diarrhea, rheumatism, dysentery, headache, fever, and others) (Semwal et al., 2021) and consumed as a food source or made into vegetables (Awang et al., 2020).

LITERATURE REVIEW

Green vegetables such as ferns *(Diplazium esculentum)* are rich in Fe, a source of protein (Yusuf et al., 2020), and effectively inhibit the activity of the secretase enzyme (Sirichai et al., 2022). Fern/pakis/paku *(Diplazium esculentum)* is often used by the community as delicious food, raw vegetable, or salad, some believe this plant has the properties to cure various diseases, and the leaves have activity against Leishmaniasis parasites (anti-leishmanial) (Jyoti et al., 2019).

Diplazium esculentum contains major non-nutritive components such as flavonoids and polyphenols (Alfonita, 2018). Flavonoids are a group of phenolic compounds with two prominent roles, namely antioxidants and antibacterials. Flavonoids as antioxidants can prevent the emergence of diseases caused by free radicals (Alfonita, 2018). Polyphenol compounds from plants have many benefits, including antioxidants, and can be applied to develop new drugs (Halimatussakdiah et al., 2020).
Natural phenolic compounds that are antioxidants can be classified into lipophilic and hydrophilic groups (including phenolic compounds). The antioxidant activity of phenolic compounds is formed due to the ability of phenolic compounds to form phenoxide ions which can donate one electron to free radicals to form compounds that are not radical (Dhianawaty & Ruslin, 2014). Antioxidants neutralize free radicals by donating electrons and help prevent cell and tissue damage (Zihad et al., 2019).

RESEARCH METHOD

Tools and materials

Yellow light, hatching media, micropipette, spectrophotometer, filter dropper pipette, test tube, analytical balance, and container.
Aquades, Arthemia Salina, seawater, DMSO, DPPH, fresh Folium *(Diplazium Esculentum)*, dried Folium *(Diplazium Esculentum)*, FeCl3, concentrated HCL,

Methanol, Mg powder, 2% NaOCl, Pb (CH3COO)2, Liberman-Burchad reagent, Dragendorff's reagent, and Mayer's reagent.

Phytochemicals determination

1. *Alkaloid*

   The sample was pipetted as much as 1 mL into two different tubes. In the first tube, a few drops of Dragendorff's reagent (the appearance of an orange precipitate indicated a positive test result). The second tube was added a few drops of Mayer's reagent (a positive test result was indicated by the appearance of a yellowish cream-colored precipitate).
2. *Flavanoid*

   The sample was pipetted as much as 1 mL into two different tubes. In the first tube was added a few drops of Pb $(CH_3COO)_2$ (the appearance of a yellow precipitate indicated a positive test result). The second tube II was added a little Mg powder, shaken, and added a few drops of concentrated HCl (the appearance of a red solution indicated positive test results).
3. *Terpenoid*

   A sample of 1 mL was added with 1 mL of Lieberman-Burchard reagent (a positive test result was indicated by the appearance of a green/blue solution for steroids and purple/red for triterpenoids).
4. *Phenolic*

   A sample of 1 mL was added with 1 mL of distilled water, and then a few drops of FeCl3 were added (a positive test result was indicated by the appearance of a green/black precipitate).
5. *Saponin*

   A 1 mL sample was added with a few drops of distilled water and then shaken vigorously (a positive test result is indicated by the appearance of foam/foam that lasts more than 30 seconds).

Determination of Antioxidants (IC_{50}) by DPPH Method

1. *Preparation of stock solution 5000 ppm*

   The sample was weighed as much as 0.05 g, then dissolved in 10 mL of methanol to obtain a sample of 5000 ppm.
2. *Antioxidant testing (IC_{50})*

   Samples of 5000 ppm were pipetted: 0.05, 0.1, 0.2, 0.4, and 0.8 mL into different test tubes for the concentration of 50, 100, 200, 400, and 800 ppm. Then, 1 mL of 0.4 mM DPPH was added, the solution volume was made up to 5 mL with methanol, then homogenized. Allowed them to stand in a dark place for 30 minutes, then the absorbance was measured with a spectrophotometer at the maximum wavelength (515 nm).

   Antioxidant activity was calculated using the following equation (Junejo et al., 2018):

$$\text{Antioxidant Activity (\%)} = \frac{\text{A control - A sample}}{\text{A control}} \times 100$$

   The IC_{50} value was calculated based on a linear equation obtained from the graph between concentration and antioxidant activity, so the equation for calculating IC_{50} was as follows:

$$Y = ax + b$$

(y: antioxidant activity (y = 50); x: concentration (IC_{50}))

IC_{50} calculation:

$x = (y-b)/a$

$x = (50-b)/a$

BSLT Procedure

1. *Artemia salina preparation*

   Seawater was filtered about 500 mL, put into a hatching container, and then aerated for 2 hours, referred to as hatching media. ± 0.5 g of *A. salina* eggs were added with 2% NaOCl until the eggs were submerged and then allowed to stand for 5 minutes, then filtered and rinsed with seawater until the filtrate was clear. Then the *A. salina* eggs were put into the hatching medium, then given yellow light radiation, and allowed to continue the aeration for 48 hours until the eggs hatched. Then, 10 larvae were put into a test tube and ready to be tested with samples.

2. *Sample Preparation and Testing*

   The sample was made into 10 mL of 500 ppm of the stock solution by weighing 0.005 g, then dissolved in 10 mL of DMSO + seawater (1:1). Samples were put into tubes containing 10 *A. salina* with variations in sample concentrations of 1, 10, and 100 ppm, and each was replicated three times. The volume of the reaction mixture is shown in table 1.

Table 1. Variation of Sample Volume and *Artemia salina*

[Sample] (ppm)	V. Sample (mL)	V. *A. salina* (mL)	V. Additional seawater (mL)	V. Total (mL)
0.1	0.001	1	3.99	5
1	0.01	1	3.9	5
10	0.1	1	3	5

After filling the volume of the mixture about 5 mL, allowed to stand for 24 hours, then observed and counted the number of dead larvae.

RESULTS

Table 2. Phytochemicals of brewed *D. esculentum* tea

No.	Phytochemicals	Test Results
1	Alkaloid	+
	a. Dragendorff	+
	b. Mayer	-
2	Flavonoid	+
	a. Lead acetate	+
	b. Mg powder	-
3	Triterpenoids	-
4	Saponins	+
5	Phenolic	+

Table 3. Antioxidant (IC_{50}) of brewed *D. esculentum tea* using DPPH Method

Samples	IC 50 (ppm)				Antioxidant Category
	Test 1	Test 2	Test 3	Average	

Brewed *D. Esculentum* tea	130.3846	131.1340	131.5681	131.0289	Medium

Figure 1. Regression curve of log [sample] VS value of probit of brewed *D. esculentum tea*

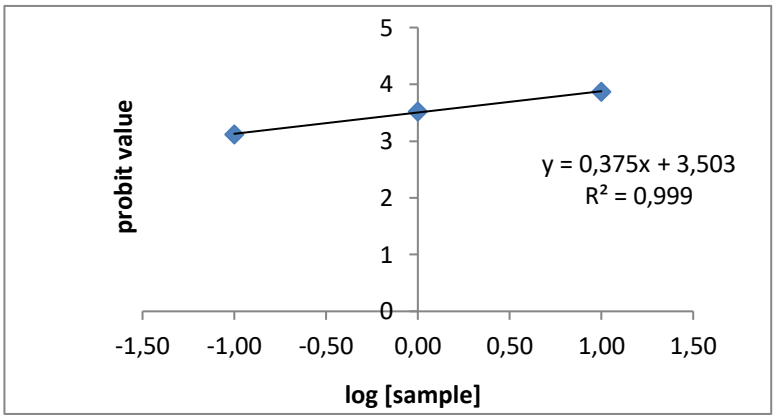

Table 4. LC_{50} of brewed *D. esculentum* tea

Log [sample]	Probit value
-1.00	3.12
0.00	3.52
1.00	3.87

For LC 50(x), the probit value is 5(y), enter to the regression equation y = 0.375x + 3.503
y - 3.503/0.375 = x
5 - 3.503/0.375 = 3.992
Log x = 3.992
x = antilog 3.992
x = 9817.479 ppm
y - 3.503/0.375 = x

DISCUSSION

Antioxidants can inhibit and prevent oxidation (Prasetyo et al., 2021). This compound has a small molecular weight but can inactivate oxidation reactions by preventing the formation of radicals (Syafitri et al., 2017). Antioxidants act as electron donors (electron donors) or reductants. It works to stop free radical reactions from metabolism in the body or the environment (Prasetyo et al., 2021).

Phytochemical screening is a step to qualitatively determine the chemical compounds' content in the brewing of Diplazium esculentum tea (Syafitri et al., 2017). The simplicia phytochemical screening test results can be seen in Table 2. Where the value of the alkaloid test gave a positive (+) result using Dragendroff's reagent and Mayer's reagent; the flavonoid test gave a positive (+) result on lead (II) acetate and negative (-) on Mg powder; triterpenoids gave negative results (-); saponins and phenolics gave positive (+)

results. The results obtained from the brewed vegetable fern/pakis/paku tea have antioxidant activity seen from the flavonoid test's positive (+) results. Flavonoids are promising reducing compounds, inhibiting many reducing reactions, both enzymatically and non-enzymatically. Flavonoids act as reservoirs for hydroxy radicals and superoxide and thus protect membrane lipids against damaging reactions (Hermawan et al., 2017).

Testing antioxidant activity in brewed Diplazium esculentum tea was done using the DPPH reduction method. DPPH compounds will react with antioxidant compounds by taking hydrogen atoms from antioxidant compounds to get electron pairs. In this case, the parameter used was IC_{50}, which determines the amount of sample concentration that can inhibit DPPH free radical activity by 50%; the smaller the IC_{50} value, the higher the antioxidant activity in the sample (Syafitri et al., 2017).

The DPPH test begins making a 5000-ppm stock solution, where the sample was weighed as much as 0.05 grams which would be dissolved in 10 mL of methanol so that a sample of 5000 ppm was obtained. It was continued with antioxidant testing (IC_{50}) where samples of 5000 ppm were pipetted about 0.05 each; 0.1; 0.2; 0.4; and 0.8 mL into different test tubes with variations of 50, 100, 200, 400, and 800 ppm, then 1 mL of 0.4 mM DPPH was added. The volume was made up to 5 mL with methanol and homogenized. Allowed them to stand in the dark for 30 minutes, then the absorbance was measured with a UV-VIS spectrometer at the maximum wavelength (515 nm).

The antioxidant activity of brewed D. esculentum tea is shown in table 3. It was carried out with three replications and found IC_{50} values of 130.3846, 131.1340, and 131.5681 ppm with an average of 131.0289. From the data, the brewed D. esculentum tea has moderate antioxidant activity with an IC_{50} value of < 101-150 ppm. This category is divided into five levels: very strong <50 ppm, strong 50-100 ppm, moderate 101-150 ppm, weak 250-500 ppm, and inactive >500 ppm (Syafitri et al., 2017).

The third test of LC_{50} used the BSLT procedure, where the initial step was the preparation of Artemia salina by taking 500 mL of filtered seawater and then placing it in a hatching container for 24 hours. A. salina L. is the number of shrimp larvae mortality due to the effect of giving the compound a predetermined dose (Kurniawan & Ropiqa, 2021).

Commercial availability in dry cysts ±0.5 g of A. salina eggs were added with 2% NaOCl until the eggs were submerged and then allowed to stand for 5 minutes, then filtered and rinsed with seawater until the filtrate was clear. Then the eggs were put into the hatching medium, given yellow light radiation, and allowed to continue the aeration for 48 hours until the eggs hatched. Ten larvae were put in a test tube and ready to be tested with samples. Sample testing was carried out by making 10 mL of the sample into 500 ppm of the stock solution by weighing the sample as much as 0.005 g and then dissolving it in 10 mL of DMSO + seawater (1:1). The sample was put into a tube containing 10 A. salina with variations in 1, 10, and 100 ppm variations in sample concentrations. It was repeated three times, as shown in table 1. After the volume of the mixture was filled with 5 mL, it was allowed to stand for 24 hours, then observed and counted the number of dead larvae.

The BSLT test using shrimp eggs (Artemia salina Leach) is not only used to study the toxicity of samples in general but can also be used to determine the potential of compounds as anticancer, antibacterial, and antifungal. The results of the BSLT test are based on the relationship between Log C (concentration) and the probit value, which represents the percent mortality of the test animals. Probit analysis has a 95%

confidence level; the probit value is obtained from table 4 (Izzaty et al., 1967). LC_{50} (Median Lethal Concentration) is the concentration that causes the death of 50% of the test organisms that graphs and calculations can estimate at a particular time of observation (Tanbiyaskur et al., 2019). The results of the measurement of the value of the toxicity test based on the results of the linear equation of 9817.479 ppm showed non-toxic where the LC50 value <1,000 ppm (Kurniawan & Ropiqa, 2021).

CONCLUSION

Based on the results obtained in this study, it can be concluded that the phytochemical testing of brewed vegetable fern/pakis/paku *(Diplazium esculentum)* tea showed positive (+) results on the testing of alkaloids, flavonoids, saponins, and phenolics. The antioxidant test (IC_{50}) of the DPPH method on brewed *D. esculentum* tea showed 130.38468, 131.1340, and 131.5681 ppm with an average value of 131. 0289 ppm, categorized moderate antioxidant activity. The BSLT test was based on a linear equation of 9817.479 ppm, which showed non-toxic results with an LC_{50} value of <1.000 ppm.

LIMITATION (OPTIONAL)

As for problems that may arise when diplazium esculentum samples are kept in the refrigerator for a long time before being processed, leading the DPPH test findings to be less than optimal.

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DECLARATION OF CONFLICTING INTERESTS

The authors state that there are no potential conflicts of interest in this work, authorship, or publication.

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