

Antioxidant Activity and Toxicity Tests of Leaf Extract (*Ayapana triplinervis* (Vahl) RM) against Shrimp Larvae (*Artemia salina* Leach)

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Abstract. Research on the antioxidant activity test and toxicity test of leaf extract (*Ayapana triplinervis* (ValH) RM) has been conducted against the larval shrimp (*Artemia salina* Leach). The dried leaves weighing 500 grams were macerated using methanol, filtered, and concentrated using a rotary evaporator. Methanol crude extract was fractionated with n-hexane, ethyl acetate, and methanol water. Based on Test Phytochemicals screening on secondary metabolites showed that the crude extract of methanol, n-hexane fraction, and methanol-water containing secondary metabolites alkaloids, flavonoids, and phenolic in ethyl acetate fraction of the content of secondary metabolites, alkaloids, and flavonoids, and the fraction of n-hexane and ethyl acetate contain steroids. In contrast, the crude extract of methanol and n-hexane fraction contains phenolic compounds. Test mortality of shrimp larvae (Brine Shrimp Lethality Test) showed mortality of shrimp larvae (*Artemia salina* Leach) using Probit analysis (SAS) to determine the value of 50% Lethal Concentration (LC50). In this test, the most active is the methanol crude extract of archery leaves, with a value of 18.8608 ppm, in a test of antioxidant activity using DPPH free radical reduction in the spectrophotometer and IC50 values obtained. In methanol crude extract was 430.73 ppm; n-hexane fraction was 168.5 ppm; ethyl acetate fraction was 114.87 ppm; and methanol-water fraction was 37.23 ppm.

Keywords: *Ayapana triplinervis*, *Artemia salina*, antioxidants, and DPPH.

INTRODUCTION

Traditional medicine has been widely accepted in almost all countries in the world. According to the *World Health Organization* (WHO), countries in Africa, Asia, and Latin America use traditional medicine to complement the primary treatment they receive. Even in Africa, as much as 80% of the population uses herbal medicine for primary treatment. The driving factor for the increase in the use of traditional medicine in developed countries is more comprehensive access to information about traditional medicine throughout the world. Using plants as medicine in Indonesia has been going on for a

long time, and people have used them from generation to generation based on experience. They are still limited to traditional, and few compounds and other benefits are known. Only a few species have known their contents out of the 1260 species of medicinal plants in Indonesia.^[1]

One of the plants used by Indonesian people to treat diseases is leaves (*Ayapana triplinervis* (Vahl) RM). *Ayapana* plant, in particular, the leaves are often used by people as a medicine for dengue fever wounds, to improve urine flow, and as a medicine for high blood pressure. Usually, the part of the plant used is the leaves. Regarding the chemical content of *Ayapana leaves*, from the literature, it is known that *Ayapana leaves* contain phenolic compounds, tannins, and saponins.^[2]

Potential compounds as antioxidants are generally flavonoids, phenolics, and alkaloids. Flavonoid and polyphenolic compounds have properties such as antioxidant, antidiabetic, anticancer, antiseptic, and anti-inflammatory. Meanwhile, alkaloids compounds have antineoplastic properties, which are also effective in inhibiting the growth of cancer cells.^[3]

This medicinal plant has been well known for its usefulness from generation to generation and passed down from one generation to the next. However, only a small portion has been thoroughly researched regarding their active compound content. Therefore, the author wants to prove the truth scientifically by conducting further research and testing through phytochemical tests, antioxidant activity using the DPPH method, and toxicity testing against *Artemia salina* Leach using the Brine Shrimp Lethality Test (BSLT) method. It is often used as an initial screening for active compounds contained in plant extracts.

METHODOLOGY

Chemicals and Equipment

The tools used in this research are Erlenmeyer, beaker, measuring cup, analytical balance, rotary evaporator, vacuum pump, volume pipette, drop pipette, tube rack, test tube, micropipette, hot plate, stirrer rod, magnetic stirrer, freezer, separating funnel, funnel, scissors, blender, digital scale, water bath, spatula, digital camera, stationery, shaker, UV-Visible spectrophotometer, reagent bottle, desiccator, incubator, TL lamp, measuring cylinder flask.

Material

The materials used are Archery leaves (*A. triplinervis* (Vahl) RM), methanol, *n*-hexane, ethyl acetate, distilled water, acetone, FeCl₃, concentrated H₂SO₄, butanol, Mg powder, Dragendorff reagent, chloroform-ammonia, H₂SO₄ 2 M, glacial Acetic Acid, Bi(NO₃)₂ · 5H₂O, HgCl₂, concentrated HNO₃, KI, FeCl₃ 1%, concentrated HCl, Mg powder, diethyl ether, CHCl₃, aluminum foil, filter paper, adhesive plastic, label paper, tissue, DPPH reagent, vitamin C, DMSO.

Research procedure

Extraction and Fractionation

Samples of leaves (*A. triplinervis* (Valh) RM) The ground mixture is weighed and then extracted by maceration, soaking the sample in methanol solvent at room temperature. The filtrate obtained was filtered using Whatman filter paper and a glass funnel. Then, the solvent was evaporated using a rotary evaporator to obtain a crude methanol extract.

Next, the crude methanol extract is fractionated based on the differences in polarity of the organic solvents. The method is as follows: the methanol-free crude extract is added to a mixture of methanol and *n*-hexane in a ratio of 2:1 (V/V). Fractionation was carried out using a separating funnel so that two fractions were obtained, namely the methanol fraction and the *n*-hexane fraction. The *n*-hexane fraction is concentrated using a rotary evaporator and is referred to as the *n*-hexane fraction extract.

Next, the methanol fraction was fractionated with ethyl acetate and was repeated until an apparent ethyl acetate fraction was obtained. The ethyl acetate fraction is concentrated using a rotary evaporator and is referred to as the ethyl acetate fraction extract.

In crude extracts, methanol and the three fractions (*n*-hexane fraction, ethyl acetate fraction, and methanol-water fraction) will be subjected to phytochemical and shrimp larvae mortality tests (BSLT).

Phytochemical Test

Samples of each extract were weighed at 10 mg, dissolved in 20 mL of ethanol, then divided into 6 test tubes. Alkaloid phytochemical tests were carried out using Dragendorff's reagent, steroid/triterpenoid tests with Liebermann-Burchard reagent, flavonoid tests with concentrated Mg and HCl bands, phenolic tests by adding FeCl₃ 1% solution and saponin tests by adding hot water and then shaking vigorously.

Toxicity Test (Brine Shrimp Lethality Test)

Samples of each extract were weighed at 1 mg dissolved in 100 μ L DMSO while stirring. Dilute with 150 μ L seawater so that the total volume is 250 μ L. Next, 200 μ L is taken and then diluted with 600 μ L. The total volume becomes 800 μ L, so the concentration becomes ^[3,4]:

$$\frac{200 \mu\text{L}/250 \mu\text{L} \times 1 \text{ mg}}{800 \mu\text{L}} = \frac{0.8 \text{ mg}}{800 \mu\text{L}} = 1000 \text{ ppm}$$

The control solution was prepared in the same way as the procedure above without using a sample.

Shrimp seeds (± 1000 seeds) were placed in 100 mL of filtered seawater using a small aquarium for 48 hours with lighting. After that, the shrimp seeds are ready for toxicity testing.

Prepare two standard microplates each for the test plate and control plate. Into rows I and II, each of the three columns, put 100 μ L of sample on the test plate and 100 μ L of control solution on the control plate.

Row II solution was diluted with 100 μ L seawater and stirred. Then 100 μ L was pipetted again and put into row III. The row III solution was diluted again with 100 μ L of seawater while stirring and put into row and done the same way until the last row. So the concentration of the solution for each row is as follows: row I = 1000 ppm, row II = 500 ppm, row III = 250 ppm, row IV = 125 ppm, row V = 62.5 ppm, row VI = 31.25 ppm, line VII = 15.625 ppm, and line VIII = 7.8 ppm.

Next, 100 μ L of seawater containing 8-15 shrimp larvae was added to the sample solution on the test plate and the control solution on the control plate, then left for 24 hours. After that, the average number of dead and live shrimp larvae was calculated for each row on the test plate. The LC₅₀ value is determined by a probit test using SAS (Statistical Analysis System)

RESULTS AND DISCUSSION

In this study, the sample used was leaves (*A. triplinervis* (Valh) RM). Then, the sample to be used is washed clean, air-dried, and ground. The dry weight of ground archery leaf powder (*A. triplinervis* (Valh) RM) were 500 gr. The archery leaf sample (*A. triplinervis* (Valh) RM) was then macerated using methanol solvent, filtered with the help of a vacuum pump, and concentrated using a rotary evaporator, and a concentrated methanol extract was obtained.

The crude methanol extract was then fractionated using solvents based on their polarity, including *n*-hexane, ethyl acetate, and methanol water.

Table 1. Weight of Crude Extract and each Fraction

No	Extract Type	Heavy (Grams)
1.	Methanol Crude Extract	3,884
2.	<i>n</i> -hexane fraction	2,436
3.	Ethyl Acetate Fraction	1,132
4.	Methanol-water fraction	0.573

The resulting fraction was reconcentrated using a rotary evaporator. From this fractionation process, *n*-hexane fraction extract, ethyl acetate fraction, and methanol-water fraction are obtained, which will be used for phytochemical tests, toxicity tests, and antioxidant tests.

The concentrated extract obtained was used for phytochemical tests, toxicity tests, and antioxidant activity tests using the DPPH method.

Phytochemical Test

Based on the results of phytochemical tests on crude methanol extract, *n*-hexane fraction, ethyl acetate fraction, and methanol-water fraction from Panahan (*A. triplinervis* (Vahl) RM) leaves, the content of secondary metabolite compounds is known, which are presented in the following table 2.

Table 2. Phytochemical test results of crude methanol extract and each fraction on (*Ayapana triplinervis* (Vahl) RM)

leaves

Secondary Metabolites	Extract Type			
	Extract Rough Methanol	Fraction <i>n</i> - hexane	Fraction Ethyl Acetate	Fraction Methanol-water
Alkaloids	+	+	+	+
Flavonoids	+	+	+	+
Phenolic	+	+	-	+
Saponins	-	-	-	-
Steroids	-	+	+	-
Terpenoids	-	+	+	-

Information :

(+) : Contains Secondary Metabolite Compounds.

(-) : Does not contain secondary metabolite compounds.

Toxicity test (Brine Shrimp Lethality Test)

The toxicity test used the BSLT method. The *A. salina* Leach shrimp are used, which are dropped in seawater and assisted by a TL lamp. After the shrimp larvae have hatched and are approximately 24 to 48 hours old, samples are inserted using seawater solvent. Then, the number of live and dead shrimp larvae was counted to determine the level of toxicity (LC₅₀).

Based on calculations using SAS Probit Analysis of crude methanol extract, *n*-hexane fraction, ethyl acetate fraction, and the methanol-water fraction of arrowroot leaves (*A. triplinervis* (Vahl) RM), LC₅₀ (Lethal Concentration 50%) was shown in Table 3. Meanwhile, the shrimp larvae mortality results (Brine Shrimp Lethality Test) from crude methanol extract and each fraction were obtained after 24 hours and calculated as LC₅₀ (Lethal Concentration value of 50% populations).

Lethal Concentration value of 50% (LC₅₀), which is a value that indicates the concentration of toxic substances that can cause the death of organisms up to 50%. The level of toxicity of an extract includes:

$$\begin{aligned} \text{LC}_{50} \leq 30 \text{ ppm} &= \text{Very Toxic} \\ 31 \text{ ppm} \leq \text{LC}_{50} \leq 100 \text{ ppm} &= \text{Toxic} \\ \text{LC}_{50} > 1000 \text{ ppm} &= \text{Not Toxic} \end{aligned}$$

Table 3. LC₅₀ value of shrimp larvae mortality test for crude methanol extract and each fraction.

No.	Extract Type	LC ₅₀ (ppm)
1.	Archery Leaf Methanol Crude Extract	18.8608
2.	Panahan Leaf <i>n</i> -Hexane Fraction	19.8369
3.	Ethyl Acetate Fraction	24.5851
4.	Methanol-water fraction	2751.6108

According to Meyer, this value shows that the extract is included in the toxic level ranging from 31 ppm $\leq$ LC₅₀ $\leq$ 1000 ppm. Based on the calculation results, it was found that the extract from crude ethanol extract had the lowest potential for toxicity compared to the *n*-hexane fraction and the ethyl acetate fraction. This result is related to the secondary metabolite compounds contained in each extract, which at certain levels have a higher toxic level so that they can cause more significant death in shrimp larvae.

Antioxidant Activity Test

In testing the antioxidants of arrowroot (panahan or prasman) leaves (*A. triplinervis* (Vahl) RM) using the DPPH method. This DPPH method can measure free radical inhibitor activity in a sample, which is based on the absorbance value obtained from the maximum wavelength.

The test data resulting from the antioxidant activity test using the DPPH radical reduction method for each extract and vitamin C can be seen in the following Tables.

Table 4. Percent reduction of DPPH radicals (%AA) in crude methanol extract of (*A. triplinervis* (Vahl) RM) leaves.

Concentration				
12.5 ppm	25 ppm	50 ppm	75 ppm	100 ppm
8.99%	9.37 %	9.93 %	54.48%	12.18%

Table 5. Percent silencing of DPPH radicals (%AA) in the *n*-hexane fraction of (*Ayapana triplinervis* (Vahl) RM) leaves.

Concentration				
12.5 ppm	25 ppm	50 ppm	75 ppm	100 ppm
11.43 %	11.99%	19.99%	28.66%	31.48%

Table 6. Percent silencing of DPPH radicals (%AA) in the Ethyl Acetate fraction Archery leaf (*A. triplinervis* (Vahl) RM).

Concentration				
12.5 ppm	25 ppm	50 ppm	75 ppm	100 ppm
6.91%	11.05%	13.11%	28.4 %	35.4%

Table 7. Percent reduction of DPPH radicals (%AA) in the methanol-water fraction of Panahan (*A. triplinervis* (Vahl) RM) leaves.

Concentration				
12.5 ppm	25 ppm	50 ppm	75 ppm	100 ppm
8.99%	10.11%	47.00%	84.82%	97.00%

Table 8. Percent silencing of DPPH radicals (%AA) in vitamin C at various concentrations of Panahan (*A. triplinervis* (Vahl) RM) leaves.

Concentration				
12.5 ppm	25 ppm	50 ppm	75 ppm	100 ppm
25.8%	32.44%	41.02%	61.98%	83.33%

Based on the results of the data above, a graph of the relationship between the concentration of each extract and the reduction of DPPH radicals (%AA) can be seen in the following Figures:

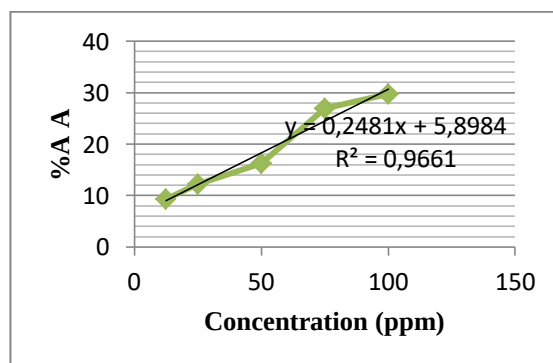


Figure 1. Correlation Curve %AA concentration of crude methanol extract of Arrowroot Leaves.

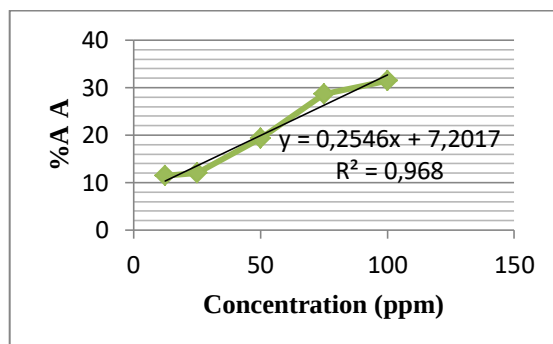


Figure 2. Relationship Curve of %AA concentration of n-hexane fraction of Panahan leaves

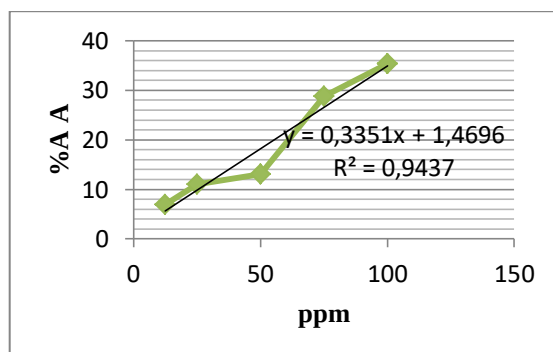


Figure 3 . Correlation Curve of %AA concentration of Ethyl Acetate fraction of Arrowroot Leaves.

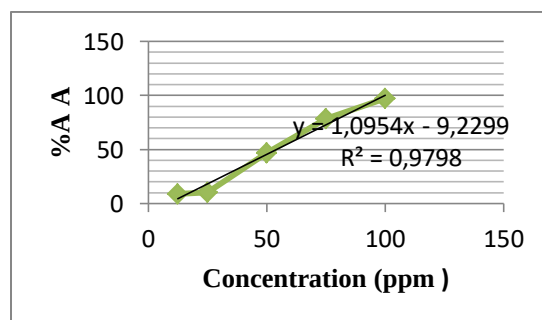


Figure 4. Relationship Curve of %AA Concentration of methanol-water fraction of Arrowroot Leaves.

The IC₅₀ value for the crude methanol extract was 430.73 ppm, the *n*-hexane fraction was 168.5 ppm, the ethyl acetate fraction was 144.87 ppm, and the methanol-water fraction was 37.23 ppm. Meanwhile, the antioxidant activity of vitamin C using the DPPH radical reduction test obtained an IC₅₀ value of 54.23 ppm.

The IC₅₀ values for the crude methanol extract, *n*-hexane fraction, ethyl acetate fraction, and vitamin C as a comparison can be seen in the following graphic image (Figure 5):

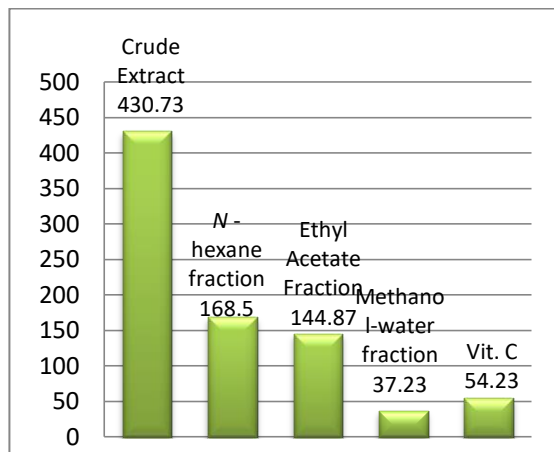


Figure 5. IC Value₅₀ Archery Leaves (*A. triplinervis* (Valh) RM).

The parameter used for the DPPH radical capture test is the IC₅₀ value, namely the extract concentration required to capture DPPH radicals of 50%. The IC₅₀ value is obtained from a linear regression equation, which states the relationship between the concentration of the test extract and the percentage of radical capture. The lower the IC₅₀ value, the more active the extract is as a DPPH radical scavenger.

Vitamin C in this study is a control because vitamin C is commonly used as an antioxidant. Free radicals readily oxidize vitamin C because it has double bonds. Free radicals will capture hydrogen atoms and cause a negative charge on oxygen atoms, which will then be decollated through resonance, resulting in stable and harmless free radicals. In addition, because vitamin C has two hydrogen interaction sites that are connected internally, there are further interactions after the first hydrogen interaction by DPPH, which means two DPPH molecules are captured or reduced by one vitamin C molecule. Apart from that, vitamin C was chosen because vitamin C can neutralize free radicals throughout the body^[8-10].

One of the compounds that can act as an antioxidant is flavonoid because it can transfer an electron to a free radical compound, where R^{*} is a free radical compound, FI-OH is a flavonoid compound, and FI-OH^{*} is a flavonoid radical. Flavonoid compounds are also good reducers, inhibiting many oxidation reactions, both enzyme and non-enzyme. The antioxidant activity of specific flavonoids as active components is used to inhibit blood circulation. Their antioxidant activity may explain why certain flavonoids are active components of plants traditionally used to treat liver dysfunction. The following is an example of the reaction mechanism for reducing free radicals by flavonoids.^[16-19]

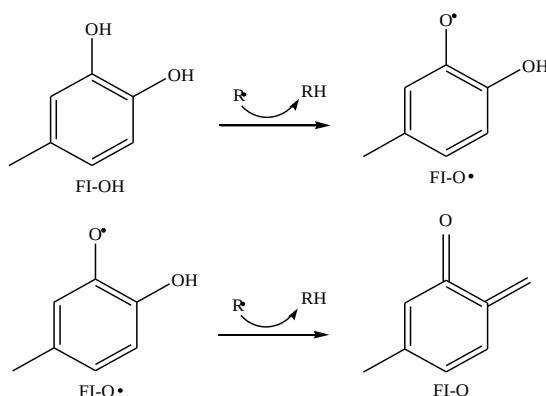


Figure 6: reaction for recuding radical by flavoniids

Apart from that, alkaloid compounds also can act as antioxidants. For example, indole alkaloids such as stricin and brusine, when viewed from their structure, can inhibit O₂, and caffeine can act as a hydroxyl radical reducer. Nitrogen-based compounds from plants have the potential to inhibit various oxidative processes. Radical compounds derived from amine compounds have a very long termination stage, thus stopping radical chain reactions efficiently. The following is an example of the reaction mechanism for reducing free radicals by alkaloids^[22,23]

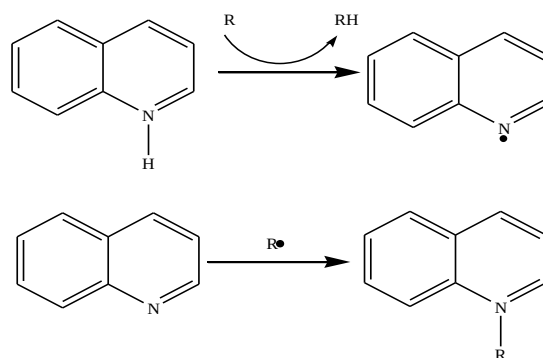


Figure 7: Reaction for reducing radicals by alkaloids

CONCLUSION

Based on phytochemical tests, there are several secondary metabolites in the crude methanol extract, *n*-hexane fraction, and water-ethanol containing alkaloid, flavonoid, and phenolic secondary metabolites. In the ethyl acetate fraction, there are alkaloid and flavonoid secondary metabolites. The *n*-hexane and ethyl acetate fractions contain steroids, while in the crude methanol extract and *n*-hexane fractions contain phenolics.

The amount of antioxidant activity using the DPPH free radical reduction test method on the crude methanol extract and each fraction of arrowroot leaves (*A. triplinervis* (Vahl) RM) were obtained from the IC₅₀ (Inhibition Concentration) values, namely for the crude methanol extract of 430.73 ppm; *n*-hexane fraction of 168.5 ppm; ethyl acetate fraction of 114.87 ppm; and the water-methanol fraction was 37.23 ppm.

Based on the LC₅₀ (Lethal Concentration) values, arrowroot leaves (*A. triplinervis* (Vahl) RM) in crude methanol extract have the highest activity of each fraction, namely 18.8608 ppm, for the *n*-hexane fraction it is 19.8369 ppm; and in the ethyl acetate fraction it was 24.5851 ppm; which is potentially active because it has the highest effectiveness against toxicity and the resulting LC₅₀ value is less than 1,000 ppm, while in the methanol-water fraction, the LC₅₀ value has the lowest effectiveness (2751.6 ppm).

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