

Toxicity Test of Flavonoid Compounds from Ethyl Acetate Extract of Malacca Leaves with Brine Shrimp Lethality Test

Muhammad Zakwan¹, Amalia Sutriana², Nurliana^{3*}, Nuzul Asmilia⁴,
Muhammad Ammar⁵, Arindita Niatazya Novianti⁶

¹Veterinary Public Health Study Program, Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia, ²Laboratory of Pharmacology, Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia, ³Laboratory of Veterinary Public Health, Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia, ⁴Laboratory of Clinic, Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia, ⁵Study Program of Veterinary Medicine, Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia, ⁶Division of Basic Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

*Corresponding author: nurliana@unsyiah.ac.id

Abstract

Flavonoids are a group of polyphenolic compounds produced in plants as secondary metabolites. Flavonoids have favorable biochemical effects on multiple diseases and other bioactivities. This study aimed to isolate the active compounds contained in malacca (*Phyllanthus emblica*) leaves and analyze toxic properties using the Brine Shrimp Lethality Test (BSLT) method in *Artemia salina* leach shrimp larvae. This study used malacca leaf powder with ethyl acetate solvent then macerated. Ethyl acetate extract was concentrated and fractionated, then toxicity tests were carried out. Extracts from the evaporation results were further fractionated using solvent petroleum ether, diethyl ether, and ethyl acetate. Then the ethyl acetate fraction was hydrolyzed using reflagged with 7% sulfuric acid for two hours, then the filtrate was extracted with ethanol solvent. The obtained fraction washed with aquades and dried using a vacuum desiccator. The toxicity test results showed that the flavonoid compound of malacca leaves was not toxic with a value of $LC_{50} > 1000$ ppm, an increase in the concentration of the extract was followed by an increase in the average mortality of larvae.

Keywords: *Artemia salina*, Brine Shrimp Lethality Test, flavonoids, *Phyllanthus emblica*

Received: 10 June 2023

Revised: 27 October 2023

Accepted: 27 November 2023

INTRODUCTION

The medicinal plant has been used as a folk treatment for various human diseases for thousands of years in many parts of the world. Currently, there is a renewed interest in the study of safer biologically active compounds isolated from natural products with an acceptable therapeutic index for the development of new drugs (Bajuber *et al.*, 2020). Malacca (*Phyllanthus emblica*) is commonly known as Amla or Indian gooseberry. In Indonesia, the malacca plant is widely known by various names including kimlaka (Malay), balaka (Minangkabau), malacca (Sunda and Betawi), kemloko (Java), malacca (Madura) (Fauzi *et al.*, 2018), bak rem (Aceh) balakka (North Sumatra), matengo (Ternate) (Asmilia *et al.*, 2020). Malacca belongs to the Euphorbiaceae family and has been often used in traditional medicine to treat

a wide variety of diseases including antibacterial (Elangovan *et al.*, 2015) then extracts from malacca leaves can also be used as a substitute for inflammation (Asmilia *et al.*, 2020), chemoprotective (Singh *et al.*, 2011), antidiabetic, anti-dioxide, anticancer, and anti-inflammatory (Kaur *et al.*, 2013).

Malacca has several chemical contents that have been successfully isolated, i.e. polyphenols, tannins, flavonoids, essential oils, alkaloids, lignins, glyceroids, carbohydrates, phenols, saponins, and terpenoids are widely contained in the leaves, fruits, and roots of the malacca plant (Fauzi *et al.*, 2018). Flavonoids are phenolic glycoside compounds that are very much found in plants (Musman *et al.*, 2017) and function to give aroma, and color to flowers, and taste in seeds and fruits (Mierziac *et al.*, 2014; Qonitatillah *et al.*, 2020), protect plants from environmental influences, as antimicrobials, and protection from

exposure to ultraviolet rays (Alfaridz and Amalia, 2018). Flavonoid compounds contain C15 which consists of two phenolic nuclei and are then connected with three units of carbon (Nugraha *et al.*, 2017; Puspitasari *et al.*, 2021).

The flavonoid group has a carbon skeleton consisting of two substituted benzene rings connected by a three-carbon aliphatic chain. The classification of flavonoids is based on the heterocyclic ring of supplemental oxygen and the dispersed hydroxyl group. The largest group of flavonoids has a Piran ring that connects a three-carbon chain with one of the benzene rings (Wahyulianingsih *et al.*, 2016). This study aimed to determine the potential for acute toxicity of flavonoid compounds from ethyl acetate extract of malacca leaves against *Artemia salina* larvae using the Brine Shrimp Lethality Test (BSLT) method.

MATERIALS AND METHODS

Ethical Approval

This study did not require ethical approval because it did not use laboratory animals.

Study Period and Location

The process of isolating flavonoid compounds was performed at the Pharmacology Laboratory, Faculty of Veterinary Medicine, Syiah Kuala University. The sample used in this study was malacca leaves taken from Bha Ulee Tutu village, Simpang Tiga District, Aceh Besar Regency in 2021.

Sample Preparation

The collected malacca leaves were thoroughly washed and then dried for 5 days, then separated from the leaf bones. Simplisia distorted dry, further mashed into powder.

Extract Preparation

The extraction process of malacca leaves was performed using the maceration method in a ratio of 1:10 between malacca leaf powder and solvent. A total of 250 g of malacca leaf simplisia powder was put into the maceration vessel and then maceration was carried out using N-hexane

solvent as much as 2.5 L for 3 days. The maceration process obtained filtrate and its residue. Then re-macerated using a solvent of ethyl acetate as much as 2.5 L for 3 days, after which it was filtered until a Caltrate of ethyl acetate of malacca leaves was obtained. Furthermore, the filtrate was collected into the evaporator flask, then evaporation was carried out using a rotary evaporator at a temperature of 40–60°C until an ethyl acetate extract of malacca leaves was obtained. Then the isolation of flavonoid compounds was carried out (Asmilia *et al.*, 2020).

Isolation of Flavonoid Compounds

Ethyl acetate extract from evaporation results was further fractionated using solvent petroleum ether, diethyl ether, and ethyl acetate. Then the ethyl acetate fraction was hydrolyzed by being reflagged with 7% sulfuric acid (H₂SO₄, 10 ml/g residue) for 2 hours, then the filtrate was extracted using ethyl acetate solvent. The obtained fraction was washed with aquades and dried using a vacuum desiccator. The flavonoid compounds obtained were continued with acute toxicity tests using the BSLT method (Saputra *et al.*, 2023).

Toxicity Test Procedure using BSLT Method

This study was an experimental study with a post-test-only control group design to evaluate the toxicity of malacca leaf flavonoid compounds against *A. salina* larvae using the BSLT method. The concentration of flavonoid compounds used was 1000 ppm, 750 ppm, 500 ppm, 250 ppm, 100 ppm, 75 ppm, 50 ppm, 25 ppm, and 10 ppm.

The test media was prepared by pouring 5 mL of seawater into each test tube. Transferred some of the 48-hour-old larvae of *A. salina* into a petri dish to facilitate the retrieval of the larvae. On the respective test tube, 10 shrimp larvae were poured using a drip pipette. To facilitate observation, lup was used. After that, 1 mL of the compound dripped into the test tube using a micropipette. Then, seawater was dripped at a 10 mL volume in the respective test tube.

Each test tube was covered and topped using aluminum foil slightly perforated for airflow.

These methods were replicated 3 times for the respective tubes. Stirring was carried out to homogenate the solution and solvent. Prepared a negative control, which was a test tube containing seawater and 10 shrimp larvae without the addition of an extract solution. After that, the solution was allowed for 24 hours, then the number of surviving larvae in each test tube was calculated.

The standard criterion for measuring shrimp larvae mortality was if the shrimp larvae did not show movement for several seconds of observation. Manual calculation of the larvae was done by observing the larvae in the test tube using a lup. The number of dead larvae was calculated by subtracting the total number of larvae in

respective concentrations from the number of surviving larvae.

Data Analysis

Data was represented in tables and analyzed descriptively.

RESULTS AND DISCUSSION

The died rate of larvae using flavonoid compounds at concentrations of 10 ppm, 25 ppm, 50 ppm, 75 ppm, 100 ppm, 250 ppm, 500 ppm, and 750 ppm has not been able to eradicate the larvae. The concentration that affects larvae mortality seen in concentrate 1000 ppm is only able to eradicate 4 larvae out of 30 larvae tested.

Table 1. BSLT results in the respective groups

Concentration (ppm)	ppm logs	Probit	% Mortality	Mortality	Total
10	1.000	0.000	0.00	0	30
25	1.398	0.000	0.00	0	30
50	1.699	0.000	0.00	0	30
75	1.875	0.000	0.00	0	30
100	2.000	0.000	0.00	0	30
250	2.398	0.000	0.00	0	30
500	2.699	0.000	0.00	0	30
750	2.875	0.000	0.00	0	30
1000	3.000	3.887	13.33	4	30

The toxic properties of the malacca leaf flavonoid compound are not seen at any concentration, this can be seen from the number of larvae that died in the BSLT test at only a concentration of 1000 ppm, and from 30 only 4 larvae died (Table 1).

The number of dead larvae can be seen in the percent mortality of test animals. The mortality percentage of the flavonoid compound of malacca leaves is found at a concentration of 1000 ppm, which is 13.33%. This is because flavonoid compounds are not toxic to *A. salina* larvae. According to Nurhayati and Setiawan (2006), stated that the presence of test larvae in the control that die is due to natural death. This can be seen from the behavior of *A. salina* just before died. *A. salina* who died on the controls experienced a decrease in activity. *A. salina* in the control is getting lower and continues to be at the base of the tube. Meanwhile, *A. salina* who died in the

experimental tube due to treatment, exposed disorientation of movements.

Table 2. Toxicity categories based on LC₅₀

Category	LC ₅₀ (µg/mL)
Highly toxic	< 30
Toxic	30–1000
Non-toxic	> 1000

(Meyer *et al.*, 1982).

Determination of the toxicity of compounds or extracts acutely using shrimp larvae *Artemia salina* is a simple preliminary/prescriptive test of biological activity. This BSLT method can be used as a prescriptive test in the study of compounds that lead to cytotoxic activity tests. If a natural material compound provides a toxic effect on LC₅₀ with a concentration of more than 1000 ppm, it is represented in the category of non-

toxic compounds. Meanwhile, if LC_{50} with a concentration of less than 1000 ppm, it is represented in the category of toxic compounds (Table 2).

A compound is declared to have potential toxicity if has LC_{50} less than 1000 ppm. Sundari *et al.*, (1996) stated that LC_{50} is a concentration of substances that cause death in 50% of experimental animals. Based on the calculation results using Microsoft Excel showed the LC_{50} value of malacca leaf flavonoid compounds which was 150999017,865 ppm.

CONCLUSION

Malacca leaf flavonoid compounds were not toxic to *A. salina* larvae using the Brine Shrimp Lethality Test (BSLT) method because the LC_{50} value was greater than 1000 ppm. LC_{50} malacca leaf flavonoid compound is 150999017,865 ppm.

ACKNOWLEDGEMENTS

The authors express their gratitude to the Faculty of Veterinary Medicine, Syiah Kuala University for supporting this study.

AUTHORS' CONTRIBUTIONS

N and AS: Conceptualization and drafted the manuscript. MZ, NA, and MA: Performed extraction. ANN: Validation, supervision, and formal analysis. N and AS: Performed the data analysis and the preparation of table. NA and MA: Performed BSLT evaluation. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

- Alfaridz, F., & Amalia, R. (2018). Review jurnal: klasifikasi dan aktivitas farmakologi dari senyawa aktif flavonoid. *Farmaka*, 16(3), 1–9.
- Amalia, A., Sari, I., & Nursanty, R. (2017). Aktivitas antibakteri ekstrak etil asetat daun sembung (*Blumea balsamifera* (L.) DC.) terhadap pertumbuhan bakteri *Methicilin Resistent Stapylococcus aureus* (MRSA). Prosiding Seminar Nasional Biotik, 387–391.
- Asmilia, N., Fahrimal, Y., Abrar, M., & Rinidar, R. (2020). Chemical compounds of Malacca leaf (*Phyllanthus emblica*) after triple extraction with n-hexane, ethyl acetate, and ethanol. *The Scientific World Journal*, 2, 1–5.
- Bajuber, Q., Indiasuti, D. N., & Kusuma, E. (2020). The Analgesic Effect of *Zingiber cassumunar Roxb.* Ethanol Extract on Mice using Writhing Test Method. *Jurnal Medik Veteriner*, 3(1), 45–50.
- Elangovan, N. M., Dhanarajan, M. S., & Elangovan, I. (2015). Evaluation of antibacterial and antifungal activity of *Phyllanthus emblica* leaf extract. *International Research Journal Pharmacy Bioscience*, 2(2), 59–66.
- Fauzi, N. I., Ardiansyah, S. A., & Hidayat, S. (2018). Skrining mekanisme kerja daun malaka (*Phyllanthus emblica* L.) sebagai antibiabetes. *Tropical Medicine Conference Series*, 1(1), 106–110.
- Kaur, J., Kaur, D., Singh, H., & Khan, M. U. (2013). Emblica officinalis: a meritocratic drug for treating various disorders. *Indo American Journal of Pharmaceutical Research*, 4447–4496.
- Meyer, B. N., Ferrighni, N. R., Put-nam, J. E., Jacobson, L. B., Nichols, D. E., & McLaughlin, J. L. (1982). Brine shrimp: a convenient general bioassay for active plant constituent. *Planta Medica*, 45(5), 31–34.

- Mierziak, J., Kostyn, K., & Kulma, A. (2014). Flavonoids as important molecules of plant interactions with the environment. *Molecular Diversity Preservation International*, 19, 16240–16265.
- Musman, M., Audina, E. Ratu, F. I. R., Erlidawati, E., & Safarida, S. (2017). Assessment of type 2 anti-diabetes on bound flavonoid of *Barringtonia racemosa* (L.) Spreng. kernel in glucose induced diabetic rats. *American Journal of Pharmacology and Toxicology*, 12(3), 48–61.
- Nurhayati, P. E., & Setiawan, N. C. E. (2018). Aktivitas antibakteri ekstrak etanol daun bandotan (*Ageratum Conizoides* L.) terhadap bakteri *Staphylococcus aureus* dengan metode difusi sumuran (Doctoral dissertation). Akademi Farmasi Putera Indonesia, Malang.
- Puspitasari, V., Wahjuni, R. S., Saputro, A. L., Hamid, I. S., & Wibawati, P. A. (2021). Effectiveness of Clove Flower Extract (*Syzygium Aromaticum* L.) as Analgesic on Licking Time Reaction in Male Mice with Formalin Induction. *Jurnal Medik Veteriner*, 4(2), 226–230.
- Qonitatillah, A., Wigati, K. W., & Irawan, R. (2020). Physical Exercise Does Not Improve Colon Inflammation in Mice Induced Lambda Carrageenan. *Jurnal Medik Veteriner*, 3(1), 57–64.
- Saputra, M. G. A. S., Sudira, I. W., Merdana, I. M., Dharmayudha, A. A. G. O., & Sudisma, I. G. N. (2023). Evaluation of Acute Dermal Toxicity of Hibiscus Leaves as Simplicial Ointment on Albino Rats. *Jurnal Medik Veterinar*, 6(2), 216–229.
- Singh, E., Sharma, S., Pareek, A., Dwivedi, J., Yadav, S., & Sharma, S. (2011). Phytochemistry, traditional uses and cancer chemopreventive activity of amla (*Phyllanthus emblica*): the sustainer. *Journal of Applied Pharmaceutical Science*, 2(1), 176–183.
- Sundari, D., Kosasih, P., & Ruslan, K. (1996). Analisis Fitokimia Ekstrak Etanol Daging Buah Pare (*Momordica charantia* L.). Tesis. Jurusan Farmasi. Institut Teknologi Bandung. Bandung.
- Wahyulianingsih, Handayani, S., & Malik, A. (2016). Penetapan kadar flavonoid total ekstrak daun cengkeh (*Syzygium aromaticum* (L.) Merr & Perry). *Jurnal Fitofarmaka Indonesia*, 3(2), 188–193.
