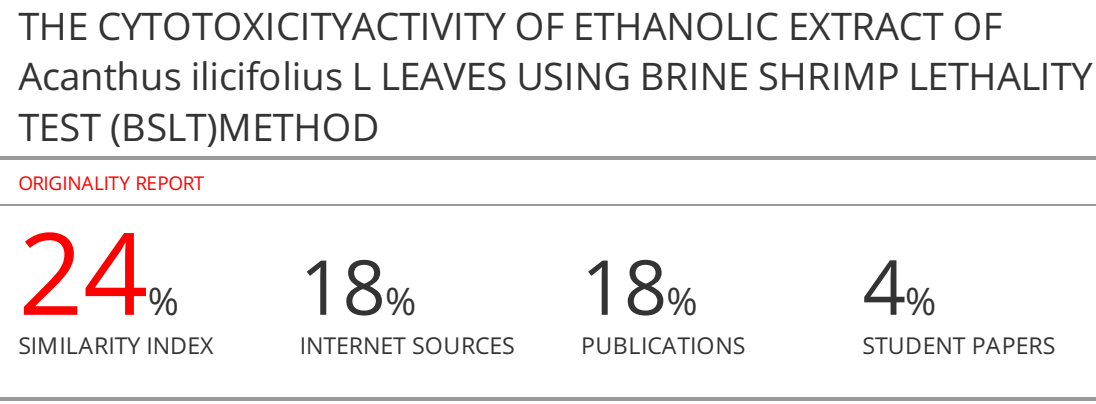
**Reviewer’s Comments**

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**THE CYTOTOXICITY ACTIVITY OF ETHANOLIC EXTRACT OF *Acanthus ilicifolius*** *L* **LEAVES USING BRINE SHRIMP LETHALITY TEST (BSLT)METHOD**

**ABSTRACT**

**Aim and objective :** Acanthus ilicifolius also known as jeruju is a plant that has a lot of bioactivity that can be used as a potential medicinal plant development. This study aimed to determine the cytotoxicity activity of ethanol extract of A. ilicifolius leaves.

**Methods :** This study used Brine Shrimp Lethality Test (BSLT) method using *Artemia salina* larvae with test solution series concentration of 10, 50, 100, 500, 1000 ppm and control without extract.*A. salina* were added to each test tube. After 24 hours, the larvae mortality was observed. LC50assessment was analyzedwith probit analysisby Microsoft excel.

**Result :**The largest mortality percentage was shown at a concentration of 1000 ppm ethanol extract of *A. ilicifolius* leaves with an average mortality value of 80%, while the concentrations of 10 ppm, 50 ppm, 100 ppm, and 500 ppm had an average mortality value of 20%, 40%, 57% and 63%.The ethanol extract of *A. ilicifolius* leaves categorized as toxic with a LC50 value of 103,6 ppm.

**Conclusion :**It was concluded that ethanol extract of *A. ilicifolius* leaves has cytotoxicity activity potential

**Keywords:** *Acanthus ilicifolius*,*Artemia salina*, Cytotoxicity, BSLT

**INTRODUCTION**

Nowadays, the use of medicinal plants is very popular almost all over the world. They have been used traditionally as medicine for thousands of years in countries such as China, India, Thailand, Japan and Indonesia. Indonesia is a country that has abundant biodiversity. Indonesia has tropical forests with 3000 species of plants and 1,845 of them are medicinal plants**1**.Medicinal plants are very popular plants that can be used as raw materials for traditional medicine and herbal medicine.Medicinal plants are plants that contain active substances in part or all of plant parts that can be used to treat or prevent disease**2**.

Some medicinal plants that are widely used are *Artocarpus altilis, Centella asiatica, Piper betle Hibiscus rosa sinensis, Blumea balsamifera, Alium sativum, Curcuma longa, Mimosa pudica, Carica papaya,* andAcanthus ilicifolius. The various kinds of pharmacological effects of these plants are as antioxidants, antibacterial, antihypertension, antidiabetic, anti-inflammatory, analgesic and many other effects**3,4,5**. This pharmacological activity is caused by the presence of secondary metabolites contained in the plants.Secondary metabolites are various chemical compounds produced by plant cells with various biological effects**6**. Different types of secondary metabolites found in the medicinal plants which play an importantrole in many kinds of diseases.

Acanthus ilicifolius also known asJeruju is one of the plants that is used as a medicinal plant. *A. ilicifolius* is a member of the Acanthaceae family and is a mangrove shrub grows in tropical and subtropical intertidal habitats**7**. *A. ilicifolius* has bioactive compounds such as alkaloids, flavonoids, tannins, saponins, terpenoid, and steroids**8**. Andriani *et al.,* reported that methanolic extractof *A. ilicifolius* leaves contained alkaloids, flavonoids, polyphenols,tannins, steroids, and glycosides**9**. Phenylethanoid glycosides mostly found in *A. ilicifolius* leaf ethanolic extract**10**. The main phenylethanoid glycosides in *A. ilicifolius* are isoacteoside and acteoside**11**. Pharmacology studies showed that *A. ilicifolius* leaves extract has antifungal, antioxidant, analgesic, antimicrobial, and hepatoprotective activities**11,12,13**. *A. ilicifolius* leaves infusion at a concentration of 40% has potential effect as analgesic in mice**13**. Previous studies have reported that methanolic extract of *A. ilicifolius* leaves have strong antioxidant (IC50 : 17.51 µg/ml) andantifungal activity against *Candida albicans***9**. Zhang *et al*. reported the potential hepotoprotective activity of phenylethanoid glycosides from *A. ilicifolius* against carbon tetrachloride (CCl4)-induced liver injury in vivo and in vitro**11**

The pharmacological effects of plants are due to the presence of secondary metabolites contained in them. The effectiveness of these active components as herbal medicines can be determinedthrough a preliminary analysis in the form of a cytotoxicity analysis.The method that is often used in the analysis of cytotoxicity is the Brine Shrimp Lethality Test (BSLT).This test describes the level of toxicity of the extract against *Artemia salina* larvae. The results of this test can be used to identify a wider range of plant bioactivity. Therefore, the present study was carried out to determinecytotoxicity assayof the ethanol extract of *A. ilicifolius* leavesagainst larvae of *A. salina.*

**MATERIALS AND METHOD**

**Solvents**

96% ethanol, aquadest, Tween 80, sea water, H2SO4, FeCl3, HCL,chloroform, ammonia

**Sample Collection and Preparation**

Fresh leaves of *A. ilicifolius*were collected from Gitgit Village, Sukasada District, Buleleng Regency, Bali and were botanically identified by Balai Konservasi Tumbuhan Kebun Raya “Eka Karya” Bali (LIPI).

**Extraction**

The fresh leaves of *A. ilicifolius* were washed with water and dried at room temperature. The dried leaves were powdered by blender. Powder extracted with 96% ethanol for five days with stirring. The filtrate was collected and evaporated using rotary evaporator.

**Phytochemical Screening**

* **Test for Alkaloids**

A quantity of 0.1 g of the extract added with 10 ml of chloroform and a few drops of ammonia. The chloroform fraction was separated and acidified with a few drops of concentrated sulfuric acid. The acid fraction was taken and divided into 2 tubes, then Dragendorf and Meyer reagents were added. The presence of alkaloids was indicated by the formation of a white precipitate in the Meyer reagent, and a red precipitate in the Dragendorf reagent.

* **Test for Flavonoids**

2 ml of *A. ilicifolius* leaf extract was heated, then ethanol was added. Magnesium powder was added to the solution and then HCl was added. Red or orange coloration indicates the presence of flavonoid.

* **Test for Phenolic Compound**

0.1 g of *A. ilicifolius* leaves extract was dissolved in methanol, then 2-3 drops of 5% FeCl3 solution were added. Dark green color indicates the presence of phenolic compounds

* **Test for Saponins**

*A. ilicifolius* leaves extract was added 5 mL of water, shaken in a test tube, a stable foam was formed (1 cm high foam and stable for 30 minutes).

* **Test for Steroids**

Identification of steroids was carried out using Libermann-Burchard reagent. 5 grams of sample was extracted with ± 10 mL n-hexane, then filtered. The extract was dried on a test stain board, added three drops of acetic anhydride and then one drop of concentrated sulfuric acid (H2SO4), the presence of a steroid group compound was indicated by the formation of a brownish or violet ring at the solution boundary.

* **Test for Tannins**

0.1 g of *A. ilicifolius* leaves extract was dissolved in methanol, then 2-3 drops of 1% FeCl3 solution were added and observed forDark green or bluish black color.

**The Cytotoxicity Assay**

The cytotoxicity assay was carried out with the Brine Shrimp Lethality Test (BSLT) by using *Artemia salina*. Preparation of *A. salina* larvae is carried out by incubating the eggs for 48 hours in an aquarium filled with seawater. The test solution of *A. ilicifolius* leaves ethanol extract was made in a series of concentrations of 10, 50, 100, 500, 1000 ppm. After the solvent evaporated, 50 L of tween, 1 mL of seawater, and 10 larvae of *A. salina* were added to each test tube. Then the sea water is added again up to 5 ml. Normal control was also made without the addition of extract.After 24 hours, the larvae mortality was observed.

LC50assessment was analyzedwith Probit analysisby Microsoft excel. Determination of the toxicity category is carried out based on theTable 1.

**Table 1 : LC50 Categories14**

|  |  |
| --- | --- |
| **Categories** | **LC50 (ppm)** |
| Non Toxic | >1000 |
| Toxic | 30-1000 |
| Very Toxic | <30 |

**RESULTS AND DISCUSSION**

**Phytochemical Screening**

The phytochemical group test were performed and the result are presented in Table 2. Result indicated that alkaloids, flavonoids, phenols, saponins, steroids, and tannins were detected in the ethanol extract of *A. ilicifolius* leaves.

**Table 2 : Phytochemical Screening Result**

|  |  |  |
| --- | --- | --- |
| **Chemical Group Test** | **Observation** | **Inference** |
| Alkaloids | (+) | Presence of alkaloid |
| Flavonoids | (+) | Presence of flavonoids |
| Phenols | (+) | Presence of phenols |
| Saponins | (+) | Presence of saponins |
| Steroids | (+) | Presence of steroids |
| Tannins | (+) | Presence of tannins |

**Cytotoxicity Activity**

Cytotoxic activity assay was carried out by Brine Shrimp Lethality Test (BSLT). This method is a simple method that is often used for cytotoxic assay. Based on the Table 3, it showed thatin the control group there was no larvae mortality.The largest mortality percentage was shown at a concentration of 1000 ppm ethanol extract of *A. ilicifolius* leaves with an average mortality value of 80%, while the concentrations of 10 ppm, 50 ppm, 100 ppm, and 500 ppm had an average mortality value of 20%, 40%, 57% and 63%. It shows that larvae mortality is not affected by seawater but by *A. ilicifolius* leaf extract.The higher the concentration of the extract, the higher the mortality of *Artemia salina* larvae.Based on the result, it showed that the ethanol extract of *A. ilicifolius* leaves categorized as toxic with a LC50 value of 103,6 ppm. So that it has cytotoxicity potential,this is related to secondary metabolite compounds such as phenolics, flavonoids and tannins, which are contained in the extract which at certain levels has the potential for cytotoxicity and caused larvae mortality.The mechanism of larvae mortality is related to the function of phenolic compounds, flavonoids and tannins in *A. ilicifolius* leaves which can inhibit larval feeding power (antifedant). The way these compounds work is by acting as stomach poisoning.Therefore, when these compounds enter the larva's body, the digestive system will be disturbed.This causes the larvae to fail to get a taste stimulus so they are unable to recognize the food so that the larvae starve to death**15**.

**Table 3 : Percentage of Larvae Mortality and LC50 of Ethanol Extract of *A. ilicifolius* Leaves**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Concentration**  **(ppm)** | **Log 10** | **Replication** | **Total larvae** | **Mortality**  **(%)** | **~~% Mortality~~** | **Probit** | **LC 50**  **(ppm)** |
| Control | - | 1 | 10 | 0 (0) | ~~0%~~ | 0 | 0 |
| 2 | 10 | 0 | ~~0%~~ | 0 |
| 3 | 10 | 0 | ~~0 %~~ | 0 |
| 10 | 5.00 | 1 | 10 | 2 | ~~20%~~ | 4,16 | 103,6 |
| 2 | 10 | 2 |
| 3 | 10 | 2 |
| 50 | 5.70 | 1 | 10 | 3 | ~~40%~~ | 4,75 |
| 2 | 10 | 5 |
| 3 | 10 | 4 |
| 100 | 6.00 | 1 | 10 | 6 | ~~57%~~ | 5,18 |
| 2 | 10 | 4 |
| 3 | 10 | 7 |
| 500 | 6.70 | 1 | 10 | 7 | ~~63%~~ | 5,33 |
| 2 | 10 | 4 |
| 3 | 10 | 8 |
| 1000 | 7.00 | 1 | 10 | 8 | ~~80%~~ | 5,84 |
| 2 | 10 | 6 |
| 3 | 10 | 10 |

**CONCLUSION**

Ethanol extract of *A. ilicifolius* leavesis toxic based on Brine Shrimp Lethality Testand hascytotoxicity activity potential with a LC50 value of 103,6 ppm.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHOR`S CONTRIBUTION**

This research was designed and conducted in collaboration of all authors

**REFERENCES**

1. Zuraida Z. Analisis Toksisitas Beberapa Tumbuhan Hutan Dengan Metode Brine Shrimp Lethality Test (Bslt). J Penelit Has Hutan. 2018;36(3):239–46.

2. Siregar RS, Tanjung AF, Siregar AF, Bangun IH, Mulya MO. Studi Literatur Tentang Pemanfaatan Tanaman Obat Tradisional. Semin Soc Sci Eng Hum. 2020;(e-ISSN 2775-4049):385–91.

3. Sari DRAP, Ahmad FF, Djabir YY, Yulianty R. Breadfruit leaves extract (Artocarpus altilis) effect on pancreatic damage in diabetic type II animal model induced by alloxan–nicotinamide. Med Clin Pract [Internet]. 2020;3:100099. Available from: https://doi.org/10.1016/j.mcpsp.2020.100099

4. Yunita E, Sari DRAP. Potensi Antibakteri Pegagan (Centella asiatica) Terhadap Bakteri Gram Positif dan Gram Negatif. J Edukasi Mat dan Sains. 2020;IX(2):236–40.

5. Bamola N, Verma P, Negi C. A Review on Some Traditional Medicinal Plants. Int J Life-Sciences Sci Res. 2018;4(1):1550–6.

6. Hussein RA, El-Anssary A. Plants Secondary Metabolites: The Key Drivers of the Pharmacological Actions of Medicinal Plants. Herb Med [Internet]. 2019;11–30. Available from: http://www.intechopen.com/books/trends-in-telecommunications-technologies/gps-total-electron-content-tec-prediction-at-ionosphere-layer-over-theequatorial-region%0AInTec

7. Singh D, Aeri V. Phytochemical and pharmacological potential of Acanthus ilicifolius. J Pharm Bioallied Sci. 2013 Jan;5(1):17–20.

8. Ernianingsih WS, Mukarlina, Rizalinda. Etnofarmakologi Tumbuhan Mangrove Achantus ilicifolius L ., Acrostichum speciosum L . dan Xylocarpus rumphii Mabb . Di Desa Sungai Tekong Kecamatan Sungai Kakap Kabupaten Kubu Raya. J Protobiont. 2014;3 (2)(2):252–8.

9. Andriani D, Revianti S, Prananingrum W. Identification of compounds isolated from a methanolic extract of acanthus ilicifolius leaves and evaluation of their antifungal and antioxidant activity. Biodiversitas. 2020;21(6):2521–5.

10. Zhang M, Ren X, Yue S, Zhao Q, Shao C, Wang C. Simultaneous Quantification of Four Phenylethanoid Glycosides in Rat Plasma by UPLC-MS/MS and Its Application to a Pharmacokinetic Study of Acanthus Ilicifolius Herb. Molecules. 2019 Aug;24(17).

11. Zhang M-Q, Ren X, Zhao Q, Yue S-J, Fu X-M, Li X, et al. Hepatoprotective effects of total phenylethanoid glycosides from Acanthus ilicifolius L. against carbon tetrachloride-induced hepatotoxicity. J Ethnopharmacol. 2020 Jun;256:112795.

12. Avijit D, Md RS, Md SIH, Md H, A.S.M. M –Al-H. Phytochemical Screening and the Evaluation of the Antioxidant, Cytotoxic and Antimicrobial Properties of Acanthus Ilicifolius (Family: Acanthaceae). Int Res J Pharm. 2012;3(8):153–6.

13. Lara AD, K FS, Lara AD. Uji Aktivitas Analgesik Infusa Daun *A. ilicifolius* ( Acanthus ilicifolius L .) Pada Mencit Putih Jantan ( Mus musculus ) Test The Analgesic Activity Of *A. ilicifolius* Leaf Infusion ( Acanthus ilicifolius L .) On Male White Mice ( Mus musculus ). 2021;3(2):71–80.

14. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 1982 May;45(5):31–4.

15. Muaja AD, Koleangan HSJ, Runtuwene MRJ. Uji Toksisitas dengan Metode BSLT dan Analisis Kandungan Fitokimia Ekstrak Daun Soyogik (Saurauia bracteosa DC) dengan Metode Soxhletasi. J MIPA. 2013;2(2):115.