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Toxicity Test of Ethanol Extract *Stelechocarpus Burahol* (Kepel) Fruits through Brine Shrimp Toxicity Assay

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ABSTRAK

Pendahuluan: *Stelechocarpus burahol* (Kepel) merupakan tanaman yang tumbuh alami di Indonesia. Kepel termasuk dalam famili *Annonaceae*. Evaluasi terhadap toksisitas ekstrak tanaman ini sangat penting untuk mendukung untuk penelitian selanjutnya. *Stelechocarpus burahol* mengandung senyawa flavonoid, sebagai antimikroba dan kontrasepsi, selain itu flavonoid mengaktifkan jalur antioksidan yang memberikan efek antiinflamasi. Kontrasepsi yang cocok untuk pria dan wanita harus mampu mencegah pembuahan, murah, tidak memiliki efek samping, sehingga diperlukan tanaman obat alami sebagai agen kontrasepsi (KA) karena aman, murah, memiliki antioksidan, dan antiinflamasi. *Brine Shrimp Toxicity Assay* terhadap ekstrak etanol tanaman ini telah diuji.
Metode: Desain true eksperimen, sampel terdiri 6 kelompok, dan tiap kelompok terdiri dari 10 nauplii hidup. Konsentrasi ekstrak : 250 ppm, 500 ppm, 750 ppm, dan 1000 ppm. Sepuluh nauplii hidup ditambahkan ke setiap konsentrasi, dan volume akhir disesuaikan menjadi 1 ml menggunakan air laut buatan (35 ppt). Botol kontrol negatif berisi 1 ml air laut buatan, sedangkan botol kontrol positif berisi 50% etanol dalam air laut. Setelah 24 jam, semua botol diperiksa dengan senter/kaca pembesar, dan jumlah nauplii yang bertahan hidup dihitung. Percobaan diulang tiga kali, dan data direpresentasikan sebagai Mean±SD
Hasil: Ekstrak etanol buah *Stelechocarpus burahol* (EESB) yang diuji toksisitasnya terhadap udang air asin memiliki nilai konsentrasi mematikan 50% (LC50) lebih dari 1000 ppm (2787,225 ppm).
Kesimpulan: Ekstrak etanol buah kepel tidak menimbulkan toksisitas terhadap udang air asin. Oleh karena itu, ekstrak etanol buah kepel dapat dipertimbangkan untuk penelitian lebih lanjut dengan uji akut, subakut, dan antifertilitas.

Kata Kunci: *stelechocarpus burahol*; ekstrak etanol; *brine shrimp toxicity assay*

ABSTRACT

Introduction: *stelechocarpus burahol (Kepel) is a plant that grows naturally in Indonesia and belongs to the Annonaceae family. Evaluation of the toxicity of this plant extract is very important to support further research. Stelechocarpus burahol contains flavonoid compounds, which act as antimicrobials and provide protection. Additionally, flavonoids activate antioxidant pathways that provide anti-inflammatory effects. Contraceptives suitable for men and women must be able to prevent fertilization, be inexpensive, and have no side effects. Therefore, natural medicinal plants are needed as contraceptive agents (CA) because they are safe, inexpensive, have antioxidants, and are anti-inflammatory. The Brine Shrimp Toxicity Assay of the ethanol extract of this plant has been tested.*
Methods: *A true experimental design was used, with samples consisting of 6 groups, and each group consisting of 10 live nauplii. The extract concentrations were 250 ppm, 500 ppm, 750 ppm, and 1000 ppm. Ten live nauplii*

were added to each concentration, and the final volume was adjusted to 1 ml using artificial seawater (35 ppt). The negative control bottle contained 1 ml of artificial seawater, while the positive control bottle contained 50% ethanol in seawater. After 24 hours, all bottles were examined with a flashlight/magnifying glass, and the number of surviving nauplii was counted. The experiment was repeated three times, and data were represented as Mean±SD.

Results: *The ethanol extract of Stelechocarpus burahol fruit (EESB) tested for its toxicity to brine shrimp had a lethal concentration of 50% (LC50) value of more than 1000 ppm (2787.225 ppm).*

Conclusion: *The ethanol extract of kepel fruit did not cause toxicity to brine shrimp. Therefore, the ethanol extract of kepel fruit can be considered for further research with acute, subacute, and antifertility tests.*

Keywords: stelechocarpus burahol; ethanol extract; shrimp brine toxicity test

INTRODUCTION

Stelechocarpus burahol is believed to be a type of fruit plant native to Indonesia. Kepel plants can grow in secondary forests in the lowlands up to an altitude of 600 m above sea level with sufficient sunlight conditions. The flowering season occurs in September–October and fruiting in March–April (Angio & Firdiana, 2021). *Stelechocarpus burahol* is a plant containing flavonoid compounds (Habibah et al., 2017). Numerous synthetic and naturally occurring substances, such as flavonoids, tannins, and hydrangenols; curcumins from the spice cumin; gossypol; sodium aurothiomalate; fenoprofen; glycerrhizic acid; fatty acids; plant-derived substances; heparin; and O-sulfated hyaluronic acid, have been shown to have antihyaluronidase activity (sHA) (Srivastav et al., 2010). Hyaluronidase inhibitors have long since been researched as antimicrobials and contraceptive (Garg et al., 2005). *Stelechocarpus burahol* has been shown to have antibacterial properties, inhibiting the growth of bacteria in the S. epidermis bacterial strain. Epidermis and leaf extracts from S. burahol have also been used to overcome body odor, as a treatment for halitosis, and as an antimicrobial against oral bacteria. Testing with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method showed that high antiradical activity was exhibited, and microorganisms were inhibited, killed, and reduced in number. Additionally, the potential for pharmacological activity as an oral deodorant through adsorbent function and probrote activation has been demonstrated (Shadrina et al., 2022).

Besides that, flavonoids have antioxidant properties (Wu et al., 2021). Flavonoids can scavenge reactive oxygen species (ROS) under biotic and abiotic stress. Flavonoids limit the activity of metabolic enzymes in the ROS formation pathway, thereby stimulating the antioxidant defense system (Shomali et al., 2022). Consumption of plants that contain antioxidants can increase SOD levels, which leads to a decrease in oxidative stress (Panjaitan, 2022). Oxidative stress in rats is characterized by lipid peroxidation. End result of lipid peroxidation is malondialdehyde (MDA), which has toxic properties for cells (Wirastuti et al., 2022).

Flavonoids activate antioxidant pathways that render an anti-inflammatory effect. They inhibit the secretions of enzymes such as lysozymes and -glucuronidase and inhibit the secretion of arachidonic acid, which reduces inflammatory reactions (Al-Khayri et al., 2022). Flavonoids widely distributed in natural plants have been reported to have anti-inflammatory properties (Wu et al., 2021). IL-6 and TNF α, are cytokines involved in the activation of the immune response (Gurnida, 2011). Flavonoids decreased the expression of pro-inflammatory cytokines such as IL-6 and TNF-α (Al-Khayri et al., 2022).

Stelechocarpus burahol has many ingredients that are very beneficial for health, Therefore, before further research, it is necessary to carry out further studies on the toxicity activity of Stelechocarpus burahol fruit. The Stelechocarpus burahol toxicity test was carried out using the Brine Shrimp Lethality Test (BSLT) because it is a simple, rapid, and inexpensive bioassay. BSLT are

utilised in the first investigation of natural product toxicity, screening marine natural products, dental materials, heavy metals, metal ions, algae, and cyanobacteria (Konan et al., 2022).

METHOD

This research utilized a true experimental design, employing the Brine Shrimp Lethality Test (BSLT) method for the toxicity test procedure. The chemicals and ingredients used included distilled water, ammonia (merck), sulfuric acid (merck), hydrochloric acid (merck), fruit peel from B. macrocarpa, glacial acetic acid (merck), DMSO (merck), ethyl acetate (technical), Mg powder (Merck), TLC plate G60 F245 (merck), FeCl3 (merck), n-hexane (technical), Mayer, Dragendorff, Wagner reagents, sea water, and methanol (technical). The tools used comprised glassware, TLC plates (Merck), TLC vessels, 254 nm and 366 nm UV lamps, analytical balances, evaporators (Heidolph), thermometers, jars, plastic boxes, corks, aerators (Amara), and aquarium neon lights. Shrimp eggs were hatched in dark and light vessels. In the dark zone, eggs and aerators were placed, while in the bright zone, a lamp was provided for lighting to facilitate hatching and to separate the cysts. The vessel was filled with approximately 50-100 mg of shrimp eggs to be hatched. The vessel was divided into two parts, a dark zone and a light zone, with a light that was turned on for 48 hours. Ten larvae were then pipetted into 2500 µL of seawater. To dissolve the sample, 2 drops of DMSO were added. The extract to be tested was prepared in concentrations of 250, 500, 750, and 1000 ppm. The sample solution to be tested was pipetted, each from 2.5 ml or 2500 µL to 5 ml or 5000 µL, to obtain concentrations of 250, 500, 750, and 1000 ppm. For each concentration, 3 repetitions were carried out. For the control, no samples were added; only 2 drops of DMSO were added as a negative control and 50% ethanol as a positive control. The solution was left for 24 hours, after which the number of dead and alive larvae in each vial was counted. Probit analysis was then used to determine LC50 (Meyer et al., 1982). The extract was prepared by dissolving 50 mg of extract in 5 ml of methanol. The division of concentration differences was done using Mayer's concentration modification (Meyer et al., 1982). Different concentrations (250 ppm, 500 ppm, 750 ppm, and 1000 ppm) of plant etanol extracts are prepared in vials . Ten alive nauplii are added and then the final volume is raised up to 1 ml by adding artificial sea water (35 ppt). Artificial sea water (1 ml) vial is used as negative control and 50% ethanol in sea water is used as positive control (Audah et al., 2022). After 24 hours all vials are inspected with torch/ magnifying glass and surviving nauplii number is counted. Toxicity of plant aqueous extract is checked by mean percentage of alive and dead brine shrimp nauplii. Experiment is repeated three times and the data represented as Mean±SD. The death and alive rate of brine shrimp nauplii is calculated by using following formulae (Saddar et al., 2022). The number of dead and alive larvae from each vial was subsequently counted and analyzed using probit analysis to determine the LC50 (Meyer et al., 1982).

$$\text{Death \%} = \frac{\text{No. of dead brine shrimps}}{\text{Total no. of brine shrimps}} \times 100$$

$$\text{Alive \%} = \frac{\text{No. of alive brine shrimps}}{\text{Total no. of brine shrimps}} \times 100$$

RESULT

Table 1. The Brine Shrimp Lethality Assay results

Number	Concentration	R1		R2		R3		R4	
		Mortality	Total BS	Mortality	Total BS	Mortality	Total BS	Mortality	Total BS
	(ppm)	(%)		(%)		(%)		(%)	
1	250	1	10	0	10	1	10	0	10
2	500	1	10	0	10	2	10	0	10
3	750	1	10	2	10	1	10	1	10
4	1000	2	10	1	10	2	10	2	10

Based on Table 1, this study demonstrated when the concentration of a certain substance increased, the mortality rate of brine shrimp rose, with the LC50 value was 2787.23 ppm. In addition, its value was higher than the maximum concentrate of 1000 ppm, indicating that the extracted sample used in a present work was non-toxic.

DISCUSSION

Toxicity is the harmful effect of a chemical or drug on target organs. Generally, the potential to cause disorders or death is possessed by chemical compounds if given to living organisms in sufficient quantities. In this study, the ethanol extract of Stelechocarpus burahol fruit (EESB) concentrations were set at 250 ppm, 500 ppm, 750 ppm, and 1000 ppm, and no toxicity was demonstrated by EESB in the brine shrimp toxicity assay, as evidenced by an LC50 value of 2787.225 ppm. A value significantly higher than 1000 ppm was confirmed, indicating that the extract is non-toxic.

The brine shrimp bioassay is a rapid preliminary screening method used to detect biochemical activity and determine the toxicity of crude extracts. This assay is widely recognized for its efficiency and simplicity, providing an initial indication of the presence of bioactive compounds. By exposing brine shrimp larvae to various concentrations of a test substance, researchers can quickly assess the potential toxicity, making it a valuable tool in early-stage drug discovery and environmental monitoring (Syahmi et al., 2010).

The Toxicity Test Method used is the Brine Shrimp Lethality bioassay, or BSLB/BSLT, which aims to determine the concentration needed for tea extract to kill half of the initial population of test animals. The method is obtained faster (24 hours), is inexpensive, and is easier to process than other tests because special equipment and training are not required, and relatively few samples are used. The death of the larvae due to the influence of the test substance allows toxic effects to be known or measured. By carrying out the BSLT test, the correct LC50 value of the EESB, an acute toxicity parameter, can be determined based on probit analysis. LC50 is defined as the concentration of a chemical compound in air or in water that can cause 50% death in a population of test animals or certain living things (Jelita et al., 2020).

The level of toxicity of plant extracts can be determined by looking at their LC50 values. If the LC50 (ppm) value is <30 ppm, it is said to be Highly toxic; if the LC50 (ppm) is less than 1000 ppm, it is said to be toxic; conversely, if the LC50 value is greater than 1000 ppm, it is said to be non-toxic (Meyer et al., 1982). In this study, LC50 = 2787.225 ppm, which means this can kill half of the total 10 shrimp, namely 5 shrimp. And the LC50 value of the microplate method has non-toxic potential because the value is greater than 1000 ppm (Meyer et al., 1982).

According to comparative references from several journals, research on early screening for anticancer activity has been carried out using the Brine Shrimp Lethality Test (BSLT) method for toxicity tests. The sample used had been extracted from Lutjanus sp. bone. Collagen had been extracted using the hydroextraction method and identified by FTIR. The results showed that the yield of collagen was 4.535% with a protein concentration of 8.815 mg/mL. Collagen had been identified from the spectra of amides A, B, I, II, and III at 3421.72, 2926.01, 1651.07, 1541.12, and 1240.23 cm-1. The toxicity test showed an LC50 value of 8.760 µg/mL. Collagen from Lutjanus sp. bone can be used as a natural anticancer agent (Ramli et al., 2019).

Furthermore, research on the level of cytotoxicity of collagen-chitosan wound dressings used the Brine Shrimp Lethality Test method; the samples used were collagen and chitosan from snakehead fish. Cytotoxicity testing used the Brine Shrimp Level Test (BSLT) method with concentrations for each sample group of 10, 50, 100, 250, 500, 750, and 1000 ppm. The results showed that each group of wound dressings, such as K0, K1, K2, and K3, had LC50 > 1000 ppm, which indicated non-toxic wound dressings (Andini et al., 2020).

In addition, research on the Lethality Test of Brine Shrimp (Artemia salina Leach) and ethanolic extracts of Green Betel (Piper betle Linn.) and Red Betel (Piper crocatum Ruiz and Pav.) through the Soxhletation Method for Cytotoxicity Test with samples of green leaves and red betel leaves showed that a lethal concentration of 50% (LC50) was found in the ethanol extract of green betel leaves and the ethanol extract of red betel leaves, respectively at 44.975 µg/mL and 31.556 µg/mL. Higher cytotoxicity was observed in the ethanol extract of red betel leaves compared to the ethanol extract of green betel leaves, as indicated by the lower LC50 value (Nerdy et al., 2021).

In further research on the Cytotoxicity and Lethality of Brine Shrimp from Rotenoid and Sarcolobus globosus Extract, the samples used were Rotenoid and its extracts, with concentrations of each sample group of 1–1000 µg/mL. High toxicity in the brine shrimp test was shown by three rotenoid isolates from S. globosus, tephrosin, sarcolobin, and 12a-hydroxyrotenone, with LC50 values of 2.2, 2.8, and 1.9 µg/mL (Wangensteena et al., 2007).

Then research on Phytochemical Screening and Toxicity Study of Imperata cylindrica (L.) P. Beauv. (Poaceae) Crude Extract with Brine Shrimp (Artemia salina) Lethality Assay yielded moderately toxic root extract (LC50: 168.47 µg/mL) and weakly toxic leaf extract (LC50: 527.25 µg/mL) (Konan et al., 2022). Extract and Fractions, whole plant Heliotropium europaeum The extract and fractions of the whole plant of *Heliotropium europaeum* were used with concentrations of 10, 100, and 1000 µg/ml. Etoposide was used as the standard drug in this research study with a concentration of 7.4625 µg/ml. The Finney computer program was used for the determination of LD50 (Achakzai et al., 2020). The brine shrimp lethality test (BSLT) technique was used to determine the LC50 values of clove flower extract (*Syzygium aromaticum*), and values of 227.1 µg/ml were found, with concentrations of 50 ppm, 250 ppm, 500 ppm, 750 ppm, 1000 ppm, and 0 ppm (seawater) as the control (Aksono et al., 2022).

The cytotoxicity of Coleus amboinicus (CA) was conducted using the Brine Shrimp Lethality Assay, where the solution of the extract was added to achieve concentrations of 1000, 500, 250, 125, 50, and 10 µg/ml in the wells. A negative control was prepared using 2 ml of artificial seawater without the extract, while potassium chromate (K2CrO4) (Sigma Aldrich, St. Louis, MO, USA) served as the positive control with concentrations of 5.0, 10.0, 15.0, 20.0, and 25.0 µg/ml in artificial seawater. The lethality concentration (LC50) of the extract, determined using the Probit test, was 34.545 µg/ml with a 95% confidence interval of 0.731 to 1.432. This result was higher than the positive control potassium chromate, which had an LC50 of 5.520 µg/ml. Based on these results, the LC50 value of the methanol extract of CA falls within its criteria, indicating the presence of potent cytotoxic compounds and potentially antitumor agents in the methanol extract of CA (Laila et al., 2020).

The Brine Shrimp Lethality Assay was employed to assess the toxicity of young barley grass extracts using six graded doses (1600 µg/mL, 800 µg/mL, 400 µg/mL, 200 µg/mL, 100 µg/mL, and 50 µg/mL). Evaluation of plant extracts via this bioassay indicates that LD50 values below 1000 µg/mL are indicative of bioactivity, suggesting the potential of these extracts as promising candidates for plant-derived anti-tumor compounds (Panthi et al., 2020).

In evaluating the cytotoxicity of three different plant samples (*Gaultheria odorata*, J *Justicia spicigera*, and *Leucaena collinsii*), concentrations ranging from 1000 µg/mL down to 10 µg/mL were utilized, with sea water, DMSO (1%), and K2Cr2O7 serving as controls. Based on the LD50 values obtained, G. odorata, J. spicigera, and L. collinsii exhibited LD50 values of 780.9 ± 11 µg/mL, 841.2 ± 27 µg/mL, and 820.5 ± 40 µg/mL, respectively, indicating they are slightly toxic. Conversely, C. dodecandra (310.5 ± 15 µg/mL) and H. angiospermum (430.2 ± 20 µg/mL) demonstrated moderate toxicity levels. Notably, T. nelsonii exhibited a highly toxic profile with an LD50 of 14 µg/mL, which was lower than the positive control K2Cr2O7 (LD50 of 18 µg/mL). These results suggest varying degrees of cytotoxic potential among the tested plant samples, with T. nelsonii showing particularly potent cytotoxic activity compared to the positive control (De La Cruz-Jiménez et al., 2022).

The ethanolic extracts of M. piperita demonstrated a concentration-dependent increase in brine shrimp mortality rates, indicating the presence of potent bioactive compounds in the crude extracts. Specifically, the ethanolic extract exhibited the highest efficacy, achieving 50% mortality of brine shrimp nauplii. In contrast, the water extract showed lower cytotoxicity. The standard treatment resulted in complete mortality of the brine shrimp nauplii, underscoring the significant inhibitory effect of the extracts' deadly chemicals (Iqbal et al., 2022).

The brine shrimp lethality bioassay is a widely accepted method for initial cytotoxicity assessment of plant extracts and compounds. It categorizes LC50 values below 1 mg/mL as indicative of significant activity, thus labeling the aqueous extract of Streblus asper with an LC50 of 3235.9 µg/mL as non-toxic. In contrast, the methanol extract of bark (MEB) and methanol extract of leaf (MEL) extracts of S. asper exhibit higher cytotoxicity with LC50 values of 32.36 µg/mL and 173.78 µg/mL, respectively, indicating moderate to high toxicity levels. Herbal toxicity can vary due to factors such as differing concentrations of active compounds in different plant parts, variations in harvesting times, developmental stages, and specific geographical and climatic conditions during plant growth. These factors underscore the complexity in evaluating the safety and efficacy of herbal extracts, necessitating careful consideration of multiple variables in pharmacological studies (Rahman et al., 2021).

The brine shrimp lethality assay exhibited a notable correlation with the in vitro cytotoxicity assay, highlighting the ADAE extract as highly active. This extract induced a mortality rate of 70% among the brine shrimp nauplii, coupled with an LC50 value of 300.1 µg/mL. These results underscore the potency of the ADAE extract in inducing cytotoxic effects, suggesting its potential as a candidate for further investigation in developing bioactive compounds (Maqsood et al., 2023).

The brine shrimp lethality assay revealed varying degrees of toxicity with different concentrations of Sargassum polycystum methanol extracts. At the highest concentration tested (10 mg/mL), the extract exhibited significant toxicity, resulting in 90% mortality of the brine shrimp nauplii, whereas at the lowest concentration (0.63 mg/mL), no mortality was observed. The calculated LC50 value for the methanol extract was 15.60 mg/mL, indicating low toxicity as LC50 values above 1 mg/mL are generally considered non-toxic. These findings suggest that while Sargassum polycystum methanol extract shows dose-dependent toxicity against brine shrimp, it falls within safe limits for potential further exploration as a bioactive compound source (Wong, 2022).

Brine shrimp were used to calculate the overall toxicity, with the maximum fatality occurring at a concentration of 1000 µg/mL and the least fatality occurring at a concentration of 10 µg/mL. These were discovered to be nontoxic between LC50 and 1000 µg/mL, weak between 500 and 1000

µg/mL (LC50), moderate between 100 and 500 µg/mL (LC50), and strong between 0 and 100 µg/mL (LC50) [58]. With an LC50 value of 115.11 µg/mL, the MELHS plant extract was shown to be moderately toxic to cells, and this was related to the study's death rate. The presence of flavonoids and anti-tumor components in the MELHS plant extract may account for its cytotoxic bioactivity (Fahad et al., 2021).

Stelechocarpus burahol is a plant containing flavonoid compounds (Habibah et al., 2017). Numerous synthetic and naturally occurring substances, such as flavonoids, tannins, and hydrangenols; curcumins from the spice cumin; gossypol; sodium aurothiomalate; fenoprofen; glycerrhizic acid; fatty acids; plant-derived substances; heparin; and O-sulfated hyaluronic acid, have been shown to have antihyaluronidase activity (sHA) (Srivastav et al., 2010). Hyaluronidase inhibitors have long since been researched as antimicrobials and contraceptive (Garg et al., 2005).

Based on the study's findings, the following implications can be drawn: Evaluating the toxicity of the plant extract EESB is essential to support further research aimed at determining its toxic effects using both acute and sub-acute toxicity assays in rat models. Such evaluations are critical to understanding the safety profile of EESB, which is necessary for advancing research on its potential antifertility effects.

Subsequent research should investigate the antifertility effects of EESB in rat models to elucidate the underlying mechanisms of its action. Particular focus should be placed on the hormone-regulating properties of EESB, as well as its antioxidant and anti-inflammatory activities. Understanding these mechanisms is vital for the potential development of contraceptives that are effective, affordable, and devoid of significant side effects.

Natural medicinal plants, including EESB, are promising candidates for contraceptive agents (CA) due to their safety, affordability, antioxidant properties, and anti-inflammatory effects. These attributes are critical in the search for contraceptives that are suitable for both men and women, capable of preventing fertilization without adverse effects. The study highlights the importance of continuing research in this area, as the results indicate no significant limitations to the current research conducted on EESB, thereby supporting its potential for future contraceptive development.

CONCLUSION

The ethanol extract Stelechocarpus burahol Fruit (EESB) is nontoxic.

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