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Brine Shrimp (*Artemia salina* Leach.) Lethality Test of Ethanolic Extract from Green Betel (*Piper betle* Linn.) and Red Betel (*Piper crocatum* Ruiz and Pav.) through the Soxhletation Method for Cytotoxicity Test

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Abstract

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BACKGROUND: Traditional medicine is currently being explored and developed widely by various countries because its cost effectiveness and reduced side effects. The green betel (*Piper betle* Linn.) and the red betel (*Piper crocatum* Ruiz and Pav.) are plants from the same *Piper* genus that are widely cultivated in Indonesia and have various phytochemical properties and pharmacological effects.

AIM: The objective of this research is to determine the cytotoxicity of green leaves and red betel leaves.

METHODS: This research was conducted in two main stages: First, the extraction and preparation through the soxhletation method; and second, a cytotoxicity test through the brine shrimp (*Artemia salina* Leach) lethality test.

RESULTS: The results showed that there was lethal concentration 50% (LC₅₀) value in both green betel leaves ethanolic extract and red betel leaves ethanolic extract, with 44.975 µg/mL and 31.556 µg/mL, respectively. The red betel leaves ethanolic extract has a higher cytotoxicity than green betel leaves ethanolic extract which can be seen from the lower LC₅₀ value.

CONCLUSIONS: The difference in cytotoxicity is due to differences in plant varieties that cause variations in phytochemical content which affects the pharmacological effects. The green betel leaves ethanolic extract and the red betel leaves ethanolic extract are cytotoxic and have potential anticancer properties.

Introduction

Traditional medicine is defined as medicine that comes from herbal plants and has been known since ancient times. Traditional medicine has been relied on for generations because it is inexpensive and often has fewer side effects compared to modern day medicine [1], [2]. Additional research on plants used as traditional medicines is still needed through preclinical testing and clinical testing so that they can safely be used in health care facilities [3]. Many herbal plants still have unknown cytotoxicity levels. Traditional medicines that have cytotoxicity have the potential to be developed as anticancer drugs [4].

The number of cancer patients has increased from 2010 to 2020 and is the leading cause of death worldwide. The mortality versus success rates are

very much influenced by the type of cancer, the stage of the cancer, the patient's general health level, and the passion of the patient to recover [5]. There are many ways to treat cancer and one of them is through medicinal plants. In fact, the advancement of modern science and technology has not shifted the role of traditional medicine, but rather enhanced and complemented its practices [6].

The betel plant of the *Piper* genus with the subcategories of the green betel (*Piper betle* Linn.) and red betel (*Piper crocatum* Ruiz and Pav.) is widely cultivated in Indonesia. The leaves have phytochemicals that are pharmacologically active and are often used for several treatments. This is because the green betel leaves and the red betel leaves contain antioxidant [7], antidiabetic [8], antifungal [9], and antibacterial [10] properties.

The brine shrimp (*Artemia salina* Leach) lethality test (BSLT) method is often used to detect the

presence of compounds that have cytotoxicity in the process of active compounds isolation from natural materials by determination of the lethal concentration 50% (LC₅₀) value [11]. Based on the preliminary studies, the researchers are interested in using the brine shrimp lethality to measure the cytotoxicity of green betel leaves ethanolic extract and red betel leaves ethanolic extract obtained through the soxhletation method.

Methods

Research description

This study is an experimental study to test the cytotoxicity of green betel leaves ethanolic extract and red betel leaves ethanolic extract. The extraction process is carried out through the soxhletation method using a Soxhlet extractor, then evaporated with a rotary evaporator, and concentrated with a water bath. A cytotoxic effects test was carried out through the BSLT method at various extract concentrations.

Samples

This study used samples of green betel leaves and red betel leaves each obtained from Research Plantations in Mutiara Mukti Farma Pharmaceutical Industry. The plant determination and taxonom⁷ were carried out at the Herbarium Medanese by Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara.

Animal test

This study involved brine shrimp larvae which were obtained from the Research Laboratory in Mutiara Mukti Farma Pharmaceutical Industry. The animal determination was⁷ carried out at the Animal Systematic Laboratory by Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara.

Ethical clearance

¹³ The research protocol was reviewed by the Biology Department, Faculty of Mathematics and Natural Sciences, University of North Sumatra's Ethics Commission to obtain ethical clearances.

Tools

This study used the following tools: A cutter (Tekiro), drying cabinet (Alumex), blender (Philips), analytical balance (Sartorius), hotplate stirrer (Thermo), Soxhlet extractor (Iwaki), filter paper (Whatman), rotary

evaporator (Buchi), water bath (Memmert), vortex (Ika), desiccator (Brand), aerator (Amara), lamp (Hannoch), brine incubator (Vivaria), and loupe (Joyart).

Materials

This study relied on ethanol (Merck), larvae of *A. salina* Leach (Fishowner), and sodium chloride (Merck).

Extract preparation

To extract the green and red²⁴ tel leaves, the soxletation method from Dianursanti *et al.* (2020) was used with minor modifications [12]. The green betel leaves and red betel leaves were picked in the morning, washed under running water, drained to dry, weighed, dried in a drying cabinet for 3 days until dry, blended in a blender to powder form, and weighed again. The extraction was carried out through the soxhletation method by weighing 50 g of dried sample, which was then wrapped in filter paper. The sample was inserted into the Soxhlet extractor, combined with 500 mL of ethanol in a round bottom flask, coupled with a soxlet extractor with a condenser, extracted until the liquid soaking the sample is colorless, evaporated the dilute extract using an evaporator, then evaporated using a water bath until a concentrate extract was obtained.

BSLT

The cytotoxicity test used was the BSLT from Supomo *et al.* (2019) with minor modification [13]. The brine shrimp larvae were prepared by weighing 5 g of brine shrimp eggs, inserting them into a brine incubator, soaking the eggs in 250 mL of artificial sea water (40 g salt in 2 L of water), lighting them with a 50 watt lamp, and aerating them for 48 h. ¹¹ The cytotoxicity test was carried out by weighing 50 mg of the extract, which was dissolved in 5 ml of ethanol (obtained from a stock solution with an extract concentrati⁸ of 10000 µg/mL), pipetted in concentrations of 1000 µL, 500 µL, 250 µL, 100 µL, 50 µL, 25 µL, and 10 µL, inserted in²⁶ vials, and left for 24 h to evaporate the solvent. Next 5 mL of artificial sea water was added and homogenized with a ³ortex (obtained test solution with extract concentration 1000 µg/mL, 500 µg/mL, 250 µg/mL, ²⁵ 0 µg/mL, 50 µg/mL, 25 µg/mL, and 10 µg/mL). Ten brine shrimp larvae were added to each vial, along with 50 µL of yeast suspension as food. The vials were placed under⁶ the heat lamp and left for 24 h with observation at 0 h, ¹ 1 h, ² 2 h, ³ 3 h, ⁴ 4 h, ⁵ 5 h, ⁶ 6 h, 12 h, 18 h, and 24 h. At each interval the dead larvae were counted using a loupe (larvae that did not show movement for 10 s were declared dead) and the death percentage was calculated. This was repeated 3 times for each extract concentration ¹⁶ and determined the LC₅₀ value with probit analysis using the Statistical Package for the Social Sciences (SPSS)

application with 26th Version. An extract is declared active if it has a LC₅₀ value <1000 µg/mL. There was a total of 420 brine shrimp larvae used throughout the experiment. The larvae used are 48 h old, because at this age, the larvae have complete limbs.

Results and Discussion

The green betel leaves' ethanolic extract appeared to have a viscous texture, specific aroma, and brownish-green color. The red betel leaves ethanolic extract appeared to have a viscous texture, specific aroma, and brownish red color. The yield percentage of green betel leaves ethanolic extract and red betel leaves ethanolic extract each was calculated based on fresh sample and dry samples. Table 1 showed the yield percentage of green leaves ethanolic extract and red betel leaves ethanolic extract based on fresh and dry samples.

Table 1: The yield percentage of green betel (*P. betle* Linn.) leaves ethanolic extract and red betel (*P. crocatum* Ruiz and Pav.) leaves ethanolic extract based on fresh and dry samples

Sample	Weight			Yield percentage of extract	
	Fresh	Dry	Extract	Based on Fresh Sample	Based on Dry Sample
Green betel leaves (<i>P. betle</i> Linn.)	1 kg	497 g	4.72 g	4.69	9.44
Red betel leaves (<i>P. crocatum</i> Ruiz and Pav.)	1 kg	519 g	5.12 g	5.31	10.24

P. betle: Piper betle, P. crocatum: Piper crocatum.

The yield percentage of red betel leaves ethanolic extract was higher than the yield percentage of green betel leaves ethanolic extract. Macroscopic examination results show that green betel leaf is a single leaf, brownish green leaf color. Macroscopic examination results show that red betel leaf is a single leaf, reddish green leaf color. Both green betel leaves and red betel leaves have oval to oval blades; a pointed tip; heart-shaped, rounded, or curved base; and have a distinctive smell. The results of microscopic examination of green betel leaves and red betel leaves have found fragments in the form of anomocytic-type stomata, essential oil in the parenchyma cells, trichomes, and oil cells in the hypodermis and xylem [14].

The test results show that various concentrations of extract could kill brine shrimp larvae in series concentrations, including, 1000 µg/mL, 500 µg/mL, 250 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL, and 0 µg/mL. Table 2 displays the data for the brine shrimp larvae death rate at various observation intervals and concentration levels of green betel leaves ethanolic extract. Table 3 displays the data for the brine shrimp larvae death rate at various observation intervals and concentration levels of red betel leaves ethanolic extract. Figure 1 charts the brine shrimp larvae deaths at various observation intervals in various concentrations of green betel leaves ethanolic extract. Figure 2 charts the brine shrimp larvae deaths at various observation

Table 2: The deaths of brine shrimp (*A. salina* Leach.) larvae data at various observation intervals in various concentrations of green betel (*P. betle* Linn.) leaves ethanolic extract

Sample	Concentration	Average deaths of brine shrimp (<i>A. salina</i> Leach.) Larvae											
		0 h	1 h	2 h	3 h	4 h	5 h	6 h	12 h	18 h	24 h		
Green betel leaves (<i>P. betle</i> Linn.)	10	0.00	0.00	0.00	0.00	0.00	0.33	1.00	1.67	2.00	3.00		
	25	0.00	0.00	0.00	0.00	0.67	1.33	1.67	2.67	3.67	4.33		
	50	0.00	0.00	0.00	0.67	1.00	1.67	2.00	3.00	4.00	5.00		
	100	0.00	0.67	1.00	1.33	2.00	2.33	3.00	4.00	5.00	6.00		
	250	0.00	1.33	2.33	2.67	3.33	3.67	4.33	5.33	6.33	7.33		
	500	0.00	1.67	2.33	3.00	3.67	4.67	5.33	6.33	7.33	8.33		
	1000	0.00	2.33	3.00	3.67	4.67	5.33	6.00	7.00	8.00	9.00		

P. betle: Piper betle, A. salina: Artemia salina.

intervals in various concentrations of red betel leaves ethanolic extract.

Table 3: The deaths of brine shrimp (*A. salina* Leach.) larvae data at various observation intervals in various concentrations of red betel (*P. crocatum* Ruiz and Pav.) leaves ethanolic extract

Sample	Concentration	Average deaths of brine shrimp (<i>A. salina</i> Leach.) Larvae											
		0 h	1 h	2 h	3 h	4 h	5 h	6 h	12 h	18 h	24 h		
Red betel leaves (<i>P. crocatum</i> Ruiz and Pav.)	10	0.00	0.00	0.00	0.00	0.00	1.33	1.67	2.00	2.67	3.33		
	25	0.00	0.00	0.00	0.33	1.33	2.00	2.67	3.33	4.00	4.67		
	50	0.00	0.00	0.67	1.00	1.67	2.00	2.67	3.67	4.67	5.67		
	100	0.33	0.67	1.33	1.67	2.33	3.00	3.67	4.67	5.67	6.67		
	250	0.00	1.67	2.67	3.33	3.67	4.33	5.00	6.00	7.00	8.00		
	500	0.00	2.33	3.00	3.33	4.33	5.33	6.00	7.00	8.00	9.00		
	1000	0.00	3.67	4.33	5.33	5.67	6.33	7.00	8.00	9.00	10.00		

P. crocatum: Piper crocatum, A. salina: Artemia salina.

total mortality rate was obtained by counting the larvae that died at each concentration. The response to death at a concentration of 1000 µg/mL was the fastest mortality rate and the greatest mortality value. The results of the total deaths after 24 h were analyzed with probit analysis using the 26th version of the SPSS application to obtain a LC₅₀ value. The analysis results showed that correlation coefficient of the logarithm of the concentration to the percentage of mortality from brine shrimp larvae was 0.998 for green betel leaves ethanolic extract and 0.999 for red betel leaves ethanolic extract. This correlation coefficient meets the requirements of >0.99 to indicate the almost perfect correlation and almost perfect relationship between axis and ordinate [15]. Therefore, increasing the green betel leaves ethanolic extract concentration and the red betel leaves ethanolic extract concentration further increases the mortality rate and the mortality number of brine shrimp larvae. The medicinal plants extract generally provides cytotoxicity to brine shrimp larvae with a concentration dependent activity profile [16].

The LC₅₀ value of green betel leaves ethanolic extract and red betel leaves ethanolic extract was, respectively, 44.975 µg/mL and 31.556 µg/mL. The extract showed cytotoxicity in a BSLT if the extract could cause the death of 50% of the tested animals at a concentration <1000 µg/mL [17], [18]. Based on this previous research green betel leaves ethanolic extract and red betel leaves ethanolic extract have a cytotoxic effect and may have promising potential for further development in anticancer drugs [19].

Variation in the concentration of green betel leaves ethanolic extract and red betel leaves ethanolic extract in this experiment results in different mortality rates for the brine shrimp larvae. This indicates that cytotoxicity

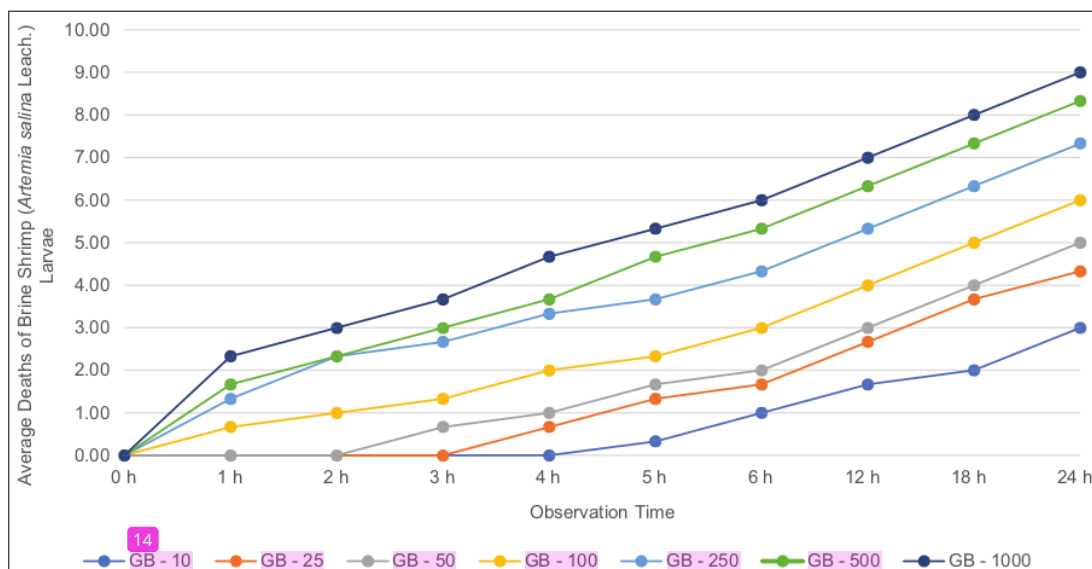


Figure 1: The chart of deaths of brine shrimp (*Artemia salina* Leach.) larvae at various observation intervals in various concentrations of green betel (*Piper betle* Linn.) leaves ethanolic extract. GB: Green betel (*Piper betle* Linn.) leaves ethanolic extract

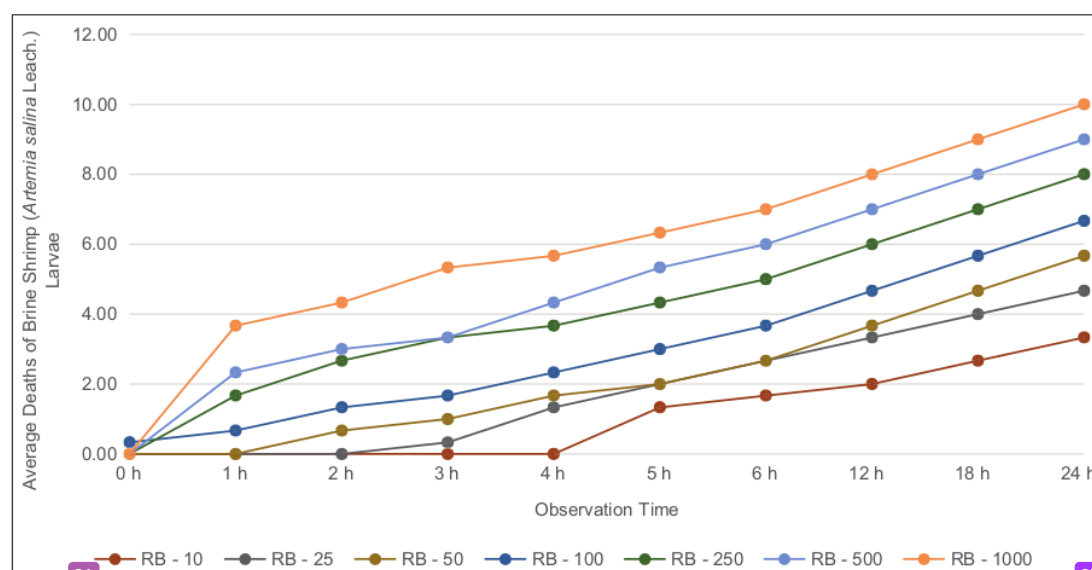


Figure 2: The chart of deaths of brine shrimp (*Artemia salina* Leach.) larvae at various observation intervals in various concentrations of red betel (*Piper crocatum* Ruiz and Pav.) leaves ethanolic extract. RB: Red betel (*Piper crocatum* Ruiz and Pav.) leaves ethanolic extract

of the green leaves ethanolic extract and red betel leaves ethanolic extract were concentration dependent. This result is in accordance with several previous studies that show cytotoxicity is concentration dependent in traditionally medicinal plants' extracts [20], [21], [22].

The cytotoxicity test using the BSLT method is very important as a preliminary test for anticancer properties. If the test results show cytotoxicity, the

cytotoxicity test can be continued on human cancer cells [23]. The brine shrimp (*A. salina* Leach.) larvae used are 48 h old, because at this age the larvae have complete limbs, were in the I-III instar phase, and were the most sensitive to the test material [24].

Plant compounds are an important source of therapeutic compounds for cancer treatment. Phytochemical content contained in green betel leaves

ethanolic extract and red betel leaves ethanolic extract has potentially anticancer properties. The phytochemical content contained in green betel leaves ethanolic extract and red betel leaves ethanolic extract is alkaloids, flavonoids, steroids, monoterpenes, triterpenes, sesquiterpenes, tannins, and glycosides [25], [26].

The death mechanism of brine shrimp larvae is related to the function of alkaloid compounds in green betel leaves ethanolic extract and red betel leaves ethanolic extract which can diffuse through the cell membrane of brine shrimp larvae, causing damage or modification of the membrane's permeability and disrupting the substance transfer system which can interfere with biochemical processes and physiological processes [27]. The entry of toxic substances into the brine shrimp larvae's digestive tracts as non-selective filter so that anything that can enter the mouth of brine shrimp seems to be food. The compounds that enter can interact with the target (e.g. enzymes, fats, cell membranes, and nucleic acids) so the compounds affect the body's mechanisms which eventually lead to death [28], [29].

Flavonoids in green betel leaves ethanolic extract and red betel leaves ethanolic extract contribute to brine shrimp larvae death through the mechanism of causing stomach poisoning which will irritate the brine shrimp larvae's digestive systems. Flavonoids also work by inhibiting the taste receptors of the brine shrimp larvae which cause the larvae to fail in the food intake so the brine shrimp larvae experience hunger and die [30]. Flavonoid compounds play a role in providing cytotoxic effects on brine shrimp larvae both in extract and pure isolate form [31]. Higher alkaloid levels and higher flavonoid levels result in an extract becoming more cytotoxic and result in a decrease LC_{50} values so that there is higher anticancer potential [32].

Terpenoids have been proposed as a relevant class found in several plant species, which have pharmacological properties [33]. Terpenoids are believed to facilitate medicinal effects, as several studies have shown that terpenoids compounds show chemopreventive and chemotherapeutic properties against cancer in humans [34]. Terpenoids, the monoterpene class, triterpene class, and sesquiterpene class are useful as a beneficial agent for use as an anticancer drug for the treatment of tumors that are resistant to chemotherapy and for minimizing the side effects of current treatments [35], [36], [37].

From the results, it can be seen that red betel leaves ethanolic extract has a lower LC_{50} value than green betel leaves ethanolic extract. These results indicate that red betel leaves ethanolic extract has a higher cytotoxicity than green betel leaves ethanolic extract. The lower LC_{50} value indicates a higher cytotoxic effect [38]. This difference in cytotoxicity because of differences in plant species and variety that cause qualitatively differences and quantitatively differences in the phytochemical content of primary and secondary metabolites contained in the extract [39].

Conclusions

The cytotoxicity test using the BSLT method of green betel (*P. betle* Linn.) leaves ethanolic extract and red betel (*P. crocatum* Ruiz and Pav.) leaves ethanolic extract obtained by the soxhletation method have LC_{50} value, respectively, 44.975 μ g/mL and 31.556 μ g/mL. These results show that green betel leaves ethanolic extract and red betel leaves ethanolic extract are cytotoxic and have the potential anticancer properties.

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