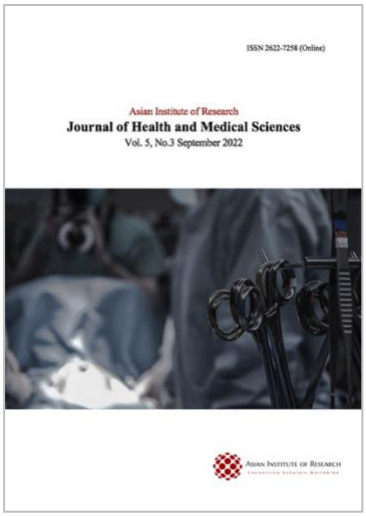

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Evaluation of Toxicity of the Bioactive Components from the Stem of Buyo (*Piper betle L.*) Extracts

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Abstract
Extracts of Buyo (Piper betle L) were subjected to a bioscreening study to detect cytotoxicity activity by the brine shrimp lethality bioassay. Specifically, the researchers opted to use the stem part of the plant since there has been no study about its cytotoxic activity. The result obtained for the ethanol extract was promising. The researchers concluded that ethanol extract is the most active in cytotoxicity activity, with a value of 205.3525, against the other extracts. This extract can be regarded as a promising candidate for a plant-derived antitumor or anticancer compound. Also, it is suggested that the extracts should be subjected to other tests and further experimentations to elaborate on its essential biological properties.

Keywords: Brine Shrimp Lethality Test, Cytotoxicity, *Piper betle L.*, Medicinal Properties, Green Heart

1. Introduction

The Philippines has a diverse species of plants (Sakilan JM et al., 2019). These plants have unique components that give the plants a unique chemical composition. Buyo, for example, is one of the most valuable plants that inhabits not only in the Philippines but also in other nearby countries. This plant was named as the Golden Heart of Nature since it has many important biological activities and significant phytoconstituents that bring about outstanding contributions to people using the plant. Buyo has written its history to the world of herbal medicine with its promising power to alleviate some of the people's illnesses. The leaves of this plant contributed a lot of biological activities such as antibacterial activity, gastroprotective activity, antioxidant activity, antidiabetic activity, radioprotective activity, the effect on the cardiovascular system and platelet inhibition activity, antifertility activity, immunomodulatory activity, cholinomimetic effect, hepatoprotective activity, as an oral care agent, and neuropharmacological profile.

Medicinal plants are natural resources yielding valuable phytochemical products, which are often used in the treatment of various diseases. A substantial part of the population in developing countries, uses folk medicines for their daily healthcare. In the Philippines, most of the people use folk medicines as part of their everyday healthcare.

Some of the traditional medicine involves the use of crude plant extracts, which may contain an extensive diversity of molecules, often with indefinite biological effects (Olowa and Nuñeza, 2013).

Anthropologists have found traces of betel in the spirit caves in Northwest Thailand dating back as to 5500-7000BC, which is even before systematic and organized agriculture came to be practiced. There have been similar findings in Timor in Indonesia going back to 3000 BC and in the blacked teeth of a human skeleton in Palawan in (Pradhan et al., 2013) the Philippines, going back to 2600 BC. It had found a place in the most ancient Sri Lanka Historical Book "Mahawamsa" written in *palli*. Even today, some hardened betel chewers in Thailand, Myanmar, and Indonesia with black teeth as (Pradhan et al., 2013) a result of long years of chewing (Chaveerach et al., 2006). There is archaeological evidence that the betel leaves have been chewed along with the Arica nut since very ancient times it is not known when these two different (Pradhan at al., 2013) stimulant substances were first put together. It may be difficult to ascertain the period when the tradition of chewing was started. However, it was mentioned in the Vatsyayana's Kamsutra, and Kalidas' Raghuvamsa reflects the antiquity of this practice. The social status of betel can also be appreciated from the fact that it was a great honor to receive betel from kings and nobles. Such was the status of betel in ancient India. During this period (Circa 600 AD), words like Tambuladhikara, Tambuladyaka, Tambuladayini, and Tambulika were used in different texts. Some of the common usages are mentioned in Kadamberi. Betel has been referred to in Sakta-tantra as one of the means of achieving siddhi. It was believed that without betel chewing and offering betel to Guru, no siddhi could be gained. Tambool has also been referred to as facilitating the sadhak in chewing dharma, yasha aisvarya, Srivairagya, and mukti. Tambool finds frequent mention in writings from the fifth century onwards especially, Reetikaaleen Hindi poetry (Wenke et al., 1983; Sharma, 2003; Prokopczyk et al., 1991).

Piper betle Linn. (Local name 'Buyo') Piperaceae, a dioecious, annual creeper, climbing by many small adventitious rootless, grows to a height of about one (10) meter, generally grown in hotter and damper parts of the country (Wenke et al., 1983; Sharma, 2003). It is extensively found in damp forests and is propagated in the Philippines and other countries in South-East Asia, such as India, Vietnam, and China. In India, it is found in Uttar Pradesh, Bihar, Bengal, Orissa, Tamilnadu, Andhra Pradesh, and Karnataka. In Tamilnadu, three varieties of *Piper betle* leaves, Sirugamani, Karpoori, and Vellaikodi, are accessible mostly (Prokopczyk et al., 1991).

The study of the bioactive compounds from plant sources and extracts in the chemical laboratory is often hampered by the lack of a suitable, simple, and rapid screening procedure. There are many procedures for bioassay that are employed using whole animals, isolated tissues, or biochemical systems. A practical method for general toxicity screening is, therefore, essential as a preliminary stage in the study of bioactive plants. A model animal that has been used for this purpose is the brine shrimp, *Artemia salina* (Kumar, 1993; Wenke et al., 1983; Sharma, 2003; Prokopczyk et al., 1991; Imran and Amin, 2011).

Availability of the eggs, the ease of hatching them into larvae, the rapid growth of the nauplii, and the relative ease of maintaining a population under laboratory conditions (Svoboda and Hampson, 1999) have made the brine shrimp a simple and effective animal test in biological sciences and toxicology. Combined with a reference standard, the brine shrimp test offers a bioassay that can be rapid, simple, bench-top, and, more importantly, inexpensive, and reproducible (Imran and Amin, 2011).

The physiological or biological effect to be observed in the screening is critical. One of the most superficial biological responses is to monitor the lethality since there is only one criterion: either dead or alive. In that case, the statistical analysis is relatively easy. The lethal concentration for 50% mortality after 24 hours of exposure (the chronic LC_{50}) is determined as the measure of the toxicity of the extract or compound. The choice of time, governed by the solubility of the extractor substance, is mostly one of convenience since the test is to be rapid and kept simple (Wenke et al., 1983; Sharma, 2003; Prokopczyk et al., 1991; Imran and Amin, 2011).

The brine shrimp (*Artemia salina*) is a simple zoologic organism (an arthropod). The use of brine shrimp test (BST) as a tool to measure general bioactivity in plant extracts was initiated in 1982 and then modified in 1991 as a simple, rapid, in-house, bench-top, and low-cost prescreen for cytotoxicity and 9KB (human epidermoid carcinoma of the nasopharynx) cytotoxicity was observed in an initial study. The usefulness of brine shrimp as a prescreen for antitumor activity was confirmed in a blind comparison with *in vitro* cytotoxicity and 3PS activity.

The brine shrimp bioassay has been implemented as a test for the last 20 years and has led to the discovery of the cytotoxic effects of a wide range of plants and bioactive compounds so diverse in their chemical structure. This method is now widely used all over the world with great success (Kumar, 1993; Wenke et al., 1983; Sharma, 2003; Prokopczyk et al., 1991; Imran and Amin, 2011).

The primary purpose of the study is to evaluate the toxicity of the bioactive components of Buyo (*Piper betle*) in the different stem extracts. The brine shrimp lethality test was used for the evaluation of cytotoxicity of the plant extracts.

2. Materials and Methods

2.1. Plant Materials

The plant samples (Figure 1) were collected from Barangay Hinaplanon, Iligan City, Lanao del Norte, Philippines. The Taxonomic identity of the plant was confirmed by Prof. Edgardo C. Aranico, plant taxonomist of the Department of Biological Sciences of the College of Science and Mathematics, Mindanao State University-Iligan Institute of Technology.

Figure 1: Piper betle

The researchers opted to use the stem part of the plant because there has been no study about its biological activities (e.g., cytotoxicity). The stems were washed with running tap water to remove any dirt before the drying process. The stems were cut into small pieces and dried at 40°C for two weeks.

2.2. Extract Preparation

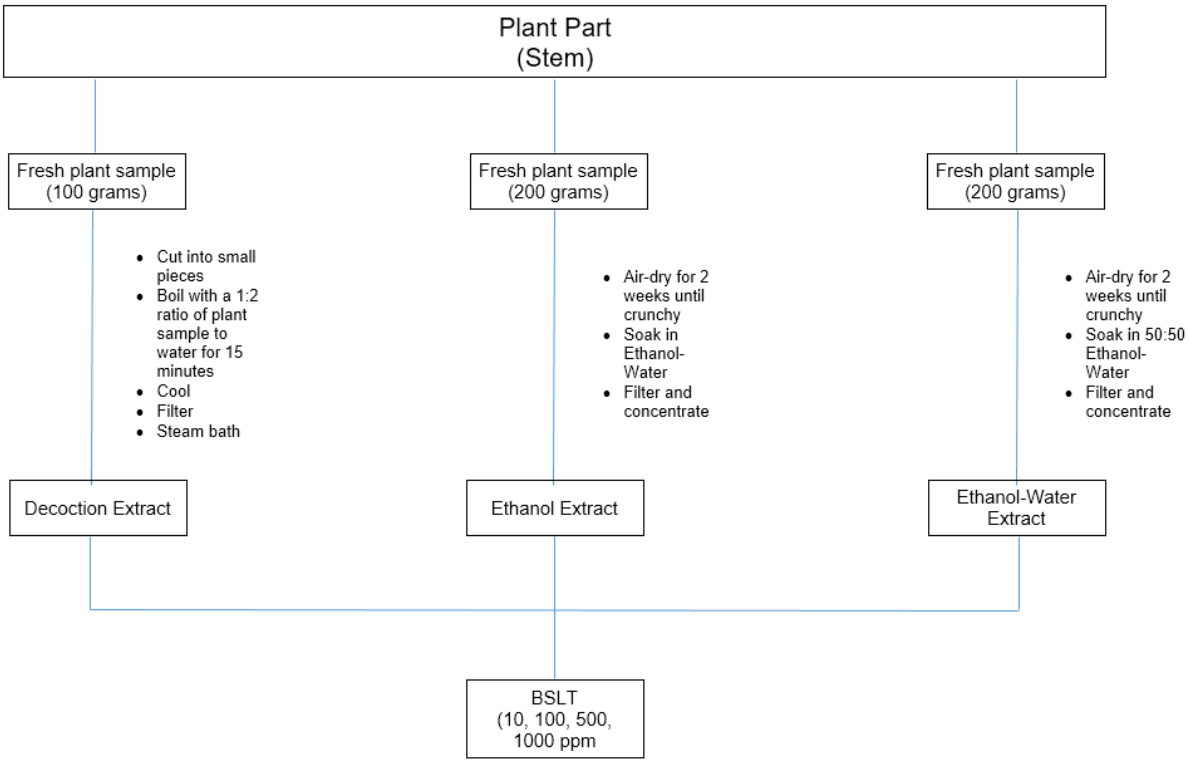

Figure 2: Schematic Diagram of the Procedures and Protocol of the Experiment

2.3. Brine Shrimp Lethality Test

Test samples of the plant extracts, in triplicate, were prepared as follows: One hundred grams (100 g) each of the crude extract was initially dissolved in 10 mL of dimethyl sulfoxide (DMSO) and further diluted with seawater to produce the required concentrations. Appropriate amounts (5000-, 500-, 50- and 5- µL for 10,000-, 1,000-, 100- and 10-ppm, respectively) were transferred to vials containing small filter paper discs, air-dried overnight to evaporate the solvent, and further dried under nitrogen gas. Ten brine shrimps were transferred to each sample vial, and boiled, filtered seawater was added to make 5mL. Tests for each concentration were done in triplicate. Control experiments using DMSO was also performed for the four concentrations in triplicates. All the vials were maintained under illumination. The number of dead and alive nauplii was counted after 6 and 24 hours. The results were evaluated, and the acute and chronic LC_{50} values were determined using the Reed-Muench method.

3. Results and Discussion

The extracts studied in this work showed significant lethality against brine shrimp, which has been successfully used as a simple biological test to guide the experiment.

Table 1: Mortality Rate (%)

Extracts	6 hours of Exposure				24 hours of Exposure			
	1000ppm	500ppm	100ppm	10ppm	1000ppm	500ppm	100ppm	10ppm
PbE	100%	21.43%	0%	0%	100%	93.75%	21.88%	8.33%
PbEW	11.76%	6.35%	3.30%	1.69%	73.68%	54.72%	14.93%	6.02%
PbD	15.15%	5.00%	2.25%	1.71%	43.59%	18.64%	11.25%	4.04%

The mortality rate of the brine shrimp after 6 hours and 24 hours of exposure to various doses of plant stem extracts of *Piper betle* is shown in Table 1. For 6 hours of exposure, at 100ppm and 10ppm, almost the PbEW (*Piper betle*

ethanol: water, 50:50) and PbD (*Piper betle* decoction extract) showed no toxicity against the brine shrimps. Also, for 24 hours of exposure, at 10ppm, almost the three extracts showed no toxicity against the brine shrimps. Meanwhile, for the 6 hours of exposure, at 100ppm and 10ppm, the PbE (*Piper betle* ethanol extract) showed no toxicity against brine shrimps. Thus, the plant stem extracts of *P. betle* are not toxic or harmful when prepared at lower concentrations (100ppm and 10ppm). The results suggested that a higher concentration of plant stem extracts of *P. betle* is needed to kill 50% of the brine shrimps.

Table 2: LC50 Results of the Various Extracts

Extracts	Acute LC_{50}	Chronic LC_{50}
PbE	668.3439 ppm	205.3525 ppm
PbEW	>1000 ppm	434.010 ppm
PbD	>1000 ppm	>1000 ppm

The interpretation of results was based on the concept of the rate of LC_{50} and its corresponding range of concentration. If the value of LC_{50} is lower than 50 ppm, it signifies high mortality rates; if the value of LC_{50} is up to 500 ppm, it signifies promising results; and if the value of LC_{50} is up to 800 ppm, it signifies moderate toxicities.

The LC_{50} results of the three extracts evaluated in this screening are listed in Table 2. From the assay, it was found that acute LC_{50} in PbE (*Piper betle* ethanol extract) showed moderate toxicity with 668.3439. The chronic LC_{50} of PbE showed promising results having the value of 205.3525. The acute LC_{50} of PbEW (*Piper betle* ethanol: water, 50:50) showed >1000ppm. The chronic LC_{50} of PbEW showed promising results with a value of 434.010. The acute LC_{50} and chronic LC_{50} of PbD (*Piper betle* decoction extract) showed >1000ppm. Based on the results, the brine shrimp lethality of *Piper betle* extracts was found concentration dependent. The crude extract is toxic if it has an LC_{50} value of less than 1000 µg/mL, while non-toxic if it has greater than 1000 µg/mL (Meyer et al., as cited by Cabrido, 2015).

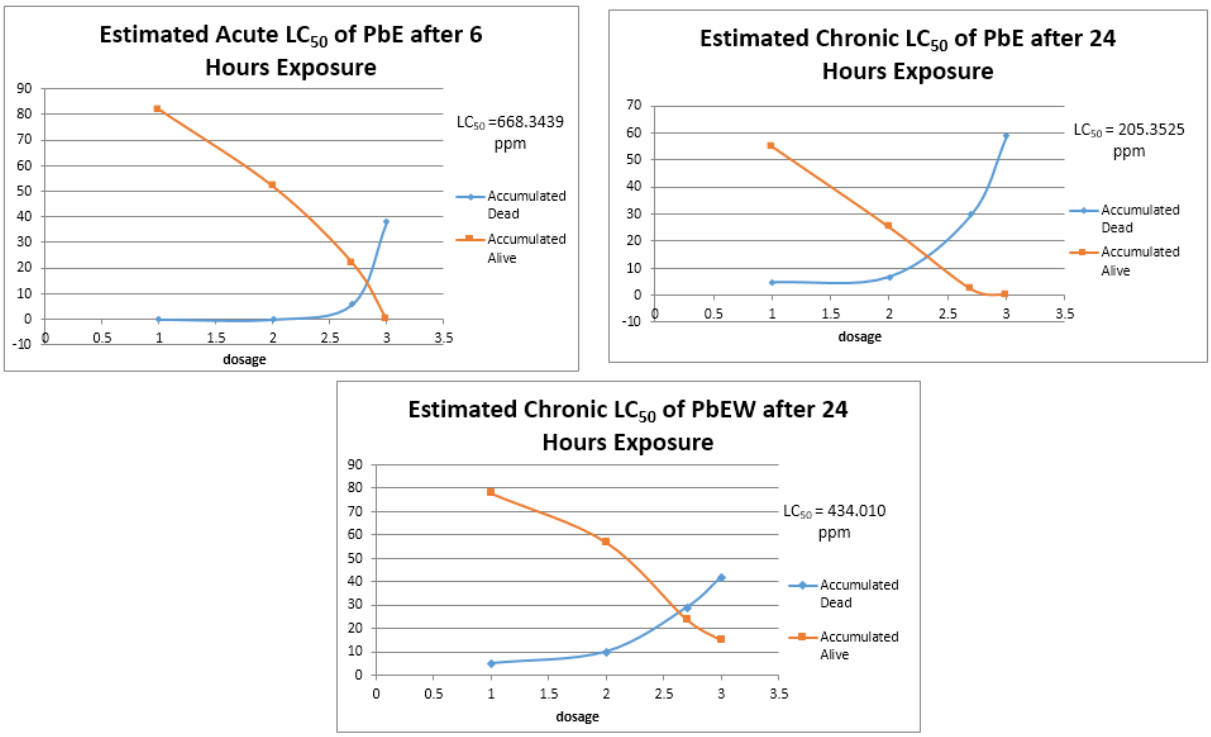

Figure 3: Estimated LC50 of the Active Extracts with Different Times of Exposure

4. Conclusions

Crude stem extracts of *Piper betle* does not exhibits toxicity. Among the three extracts, only PbE and PbEW showed moderate toxicity against *Piper betle* with a value of 205.3525 and 434.010, respectively. These extracts

can be regarded as a promising candidate for a plant-derived antitumor or anticancer compound. PbD showed no significant cytotoxicity activity since the value of LC_{50} is greater than 1000 µg/mL. The assay is limited only to the evaluation of the cytotoxicity activity of the plant stem extracts; hence this study insufficiently determines the properties of the potential and essential bioactive compounds. It is suggested that the extracts should be subjected to other tests and further experimentations to elaborate on its essential biological properties.

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