



Brine Shrimp (*Artemia salina*) Bioassay of the medicinal plant *Pseudelephantopus spicatus* from Iligan City, Philippines

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Abstract

Medicinal plants are commonly distributed in different regions of the Philippines. Based on ethnomedicinal significance, *P. spicatus* used in traditional medicine was collected and evaluated for biological activity using the Brine Shrimp Bioassay. Plant extracts were obtained through decoction, ethanolic extraction and extraction with ethanol-water. Four concentrations (10, 100, 500, 1000 ppm) of the *P. spicatus* extract were used. Mortality of the brine shrimp was observed after 6 hours and 24 hours. The results showed that the decoction and ethanol-water extract were inactive against brine shrimp. However, the ethanol extract showed a toxicity effect after 6h and 24h exposures with LC₅₀ values at 944.07 and 266.07 ppm, respectively. Results indicate that the ethanol extract may have substances that are cytotoxic and that active components of the plant are better extracted with absolute ethanol than with hot water or mixture of ethanol and water. The active components present may have medicinal importance with no adverse effects, and may support the therapeutic use of *P. spicatus*.

Keywords: Cytotoxicity, decoction, ethanol extract, LC₅₀, traditional medicine.

Introduction

In many developing countries, many people depend on traditional and alternative medicine¹. About four billion people (80%) of the world's population are estimated to use botanical medicine². Even though modern medicine may be accessible in these countries, herbal medicines represent a considerable proportion of the global drug market and have often maintained popularity given that these countries, including the Philippines possess rich floristic wealth. Herbal drugs are safe than the synthetic drugs and are biofriendly and eco-friendly³. Many plants used in traditional medicine contain chemical substances called phytochemicals⁴ that produce a physiological action on the human body⁵. *Pseudelephantopus spicatus* is one of the most important plants found in arid regions of the world. *P. spicatus* is used for treating poisonous snake bites in India⁶. In Jamaica the plant is used for fever, sprains, and eye problems⁷. In Taiwan, the plant which was originally useful to treat dampness, nephritis, edema, scabies, and pneumonia, was shown to induce acute hepatic damages in rats and exhibits antifungal activity against *Candida albicans*, *A. niger*⁸ and performs antileishmanial activity⁹. In the Philippines, leaves are considered specific for eczema and are used as a topical agent and as vulnerary-a medicine used in the healing of wounds. However, data on the pharmacological properties and toxicity of this plant are lacking and no study has been conducted that shows the extensive diversity of its metabolites.¹⁰ Documentation of antimicrobial properties and toxicity of medicinal plants is essential to build a comprehensive database from which it may be potential to search new leads in development of drugs^{11,12}.

The study of bioactive components from plant extracts in the laboratory is frequently hindered by the lack of simple, suitable, and fast screening procedure^{13, 14}. Many bioassay methods are applied using whole animals, biochemical system or isolated tissues but these procedures are somewhat expensive and complicated. Therefore, brine shrimp bioassay is a more convenient procedure for general toxicity screening. The lethality assay using *Artemia salina* was used in this study because it has been proven to effectively biomonitor the isolation of insecticidal, cytotoxic, antineoplastic, antimalarial, and antifeedant compounds from plant extracts¹⁵. The method is attractive because it is very simple, inexpensive and little toxin amounts are enough to perform the test in the micro-well scale^{16, 17}. Since its introduction, this *in vivo* lethality bioassay has been successively used for providing a frontline screen that can be backed up by more specific and more sophisticated bioassays once the active compounds are isolated^{18, 19}. With this, the *P. spicatus* safety or toxicity is assessed, since the findings are important considering the usage of the plant by human beings. Thus, the findings of this present work may provide baseline information on the promising plant species that could be used as a basis for the development of new tools of great therapeutic importance.

Material and Methods

Collection of Plant Material: For the purpose of botanical identification, small branches or twigs with reproductive structures, healthy leaves, stipules, bark and wood samples from each plant were collected in duplicate following accurate documentation. Mature *P. spicatus* leaves were collected from

Dalipuga, Iligan City, Philippines and were identified by Professor Aranico from the Department of Biological Sciences, Mindanao State University-Iligan Institute of Technology, Philippines.

Preparation of Plant Extract: For the preparation of the crude extract, about 2-3kg of plant or plant parts were cleaned by washing with tap water followed by distilled water. The sample was air-dried for about 2-3 weeks, and the dried samples were pulverized using a sterile electric blender. The half portion of the pulverized leaves was soaked in pure absolute ethanol, and the other was soaked in 50:50 ethanol-water mixtures for three days. Each solution was filtered with Whatman No. 1 filter paper and concentrated at 40°-50°C under reduced pressure using the rotary evaporator²¹.

For the preparation of the plant decoction, about 1 kg fresh and clean samples of the plant were cut into pieces and boiled in sufficient amount of distilled water (1:2 ratio) for 5 minutes. The mixture was filtered, cooled and stored in glass containers and freeze-dried until all the water was removed to give concentrated decoction. It was then kept until required.

Stock solutions were prepared. Thirty milligrams of dried samples from decoction, crude ethanol extract and crude ethanol-water extract, were dissolved with 3000 ppm ethanol and distilled water respectively and then sonicated to dissolve the dried samples. From the stock solution, 10, 100, 500, and 1000 ppm concentrations were prepared by the addition of 5 ppm, 50 ppm, 250 ppm and 500 ppm of solution, respectively in a 20 mL test tube. Addition of a minimal amount of dimethyl sulfoxide (DMSO) was done to completely dissolve the solution in each test tube.

Brine Shrimp Lethality Bioassay: Hatching of Brine Shrimp: Brine shrimp (*Artemia salina*) lethality bioassay was carried out to investigate the cytotoxicity of extracts of medicinal plants. Artificial seawater was prepared by dissolving 40 grams of natural table salt in every liter of distilled water. Sea water was kept in a small tank, and *A. salina* eggs were added to the divided tank²¹. Constant temperature (around 37° C) was maintained and constant supply of oxygen was carried out. Brine shrimps were allowed to mature and hatch as nauplii for two days. The newly hatched shrimp was collected using a dropper.

Assay Proper: Ten brine shrimp larvae were introduced into each sample vials containing different concentrations of the extracts. Seawater was added to make a total volume of 5 ml. The vials were maintained under illumination. Survivors were counted after 6, and 24 hours and the deaths at control and each dose level were determined.

Lethal concentration Determination: After 6h and 24h, the lethal concentrations of the *P. spicatus* extract resulting to 50% mortality of the brine shrimp (LC₅₀) were determined. Then, by

means of a trendline fit linear regression analysis (MS Excel version 7) the dose-response data were transformed into a straight line. From the best-fit line obtained the LC₅₀ was derived.

Statistical Analysis: Reed-Muench statistical method was used to determine the relative toxicity of the *P. spicatus* extracts to living organisms. It was done by testing the response of *A. salina* under various concentrations of the extract. LC₅₀ represents the dose lethal to the half members of the *A. salina*. This was determined by plotting the mortality (y-axis) versus log of concentration (x-axis). The concentration that rendered 50% mortality was the LC₅₀.

Results and Discussion

Table 1 shows the toxicity of the *P. spicatus* extracts on the brine shrimp after 6 and 24-hour exposure. The extracts obtained from decoction and ethanol-water extract exhibited no lethality on the brine shrimps at any of the concentrations at 6h and 24h. The brine shrimps were still actively moving, and no signs of behavioral changes were observed. Crude plant extract with LC₅₀ value of less than 1000 ppm is toxic while non-toxic (inactive) if it is higher than 1000 ppm²⁰. Since the LC₅₀ in the both of this extract taken from decoction and ethanol-water mixture was higher than 1000 ppm, it was considered inactive. It may be because the active components present in the *P. spicatus* were not extracted through the two methods mentioned above. Even though decoction process is economical due to its low cost in terms of instrumentation and reagents²¹ it may be an inefficient process given that ingredients may be damaged during the prolonged heating of substances, and other ingredients may be oxidized and lose activity²². During the decoction process, many aromatic herbs with high levels of volatile oils are easily lost through evaporation²³. Also, ethanol-water mixture extraction process was still ineffective and it is in accordance with the previous study in which the alcohol/water mixture (typically 20–40% alcohol) is actually a poor medium for extraction. It is because it causes the desired components to condense out of the liquid therefore none is left in the finished product²⁴. The ethanol extract of *P. spicatus* showed a toxicity effect at 6h and 24h, with LC₅₀ value at 944.07 and 266.07 ppm, respectively. This suggests that the extract could have compounds that are cytotoxic as the LC₅₀ value was lower than 1000 ppm (table 1). The brine shrimp mortality rate at different concentrations in the ethanol extract was found to increase with increasing concentration of the sample, and it clearly shows that the extraction with ethanol was a better way of obtaining *P. spicatus* extract bioactive components. The previous studies show that ethanolic extract of *P. spicatus* demonstrated strong biological activity toward *Leishmania amazonensis*⁸. Ursolic acid and the two hirsutinolides (the 8-acetyl-13-O-ethyl-piptocarphol and 8, 13-diacetyl-piptocarphol 8-acetyl-13-O-ethyl-piptocarphol) isolated through phytochemical screening might be responsible for its pharmacological activities thus giving support to its use in Peru⁹.

Table- 1
Effects of the *P. spicatus* Extracts on the Brine Shrimp *A. salina* after 6- and 24-hr Exposure

Extracts	Concentration of the Extract (ppm)	Brine Shrimp Mortality (%)		Acute LC ₅₀ (after 6 hrs)	Chronic LC ₅₀ (after 24 hrs)
		After 6 hrs.	After 24 hrs.		
Decoction	10	0	0	>1,000ppm	>1,000ppm
	100	0	0		
	500	0	0		
	1000	0	0		
Ethanol	10	0	0	944.07ppm	266.07ppm
	100	6.67	3.33		
	500	26.67	16.67		
	1000	36.67	76.67		
Ethanol-water	10	0	0	>1,000ppm	>1,000ppm
	100	0	0		
	500	0	0		
	1000	0	0		

Herbal medicines have received high interest as a substitute to clinical treatment, and the demand for herbal remedies has currently increased rapidly. The increase in the number of herbal users as opposed to the insufficiency of scientific evidences on its safety has raised concerns regarding its detrimental effects²⁵ and related concerns apply to the *P. spicatus* in this study. Although there was no mortality in the control group, in the extract obtained by decoction and mixture of ethanol and water, it is evident that the ethanol extract possessed a mild toxicity effect with acute and chronic values of LC₅₀ 944.07 ppm and 266.07 ppm, respectively with the percent mortality increasing with concentration. This present study shows that *P. spicatus* plant extract has some evident toxicity and may be used as herbal medicine in known dosages, especially in rural areas, where conventional medicine is too expensive. In addition, the *P. spicatus* extract can be further tested for acute toxicity on the animal model to compare the two methods of toxicity evaluation.

Conclusion

This study presents the toxicity of the *P. spicatus* ethanol extract, which should be very useful for any future *in vivo* or clinical study of this plant extract. Results indicated that bioactive compounds present might account for the plant's pharmacological or toxicological effects. The results somehow support the use of this plant species in traditional medicine. In addition, the present study confirms the utilization of the brine shrimp (*Artemia salina*) bioassay as a reliable, simple, and convenient method in monitoring bioactivity of medicinal plants.

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