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Brine Shrimp Lethality Assay of the Ethanolic Extracts of Three Selected Species of Medicinal Plants from Iligan City, Philippines

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Abstract

The present study was conducted to test for in vivo Brine Shrimp Lethality Assay (BSLA) of the ethanolic extracts of Lantana camara, Chromolaena odorata, and Euphorbia hirta and correlate cytotoxicity results with known pharmacological activities of the plants. Novel cytotoxic, antitumor, and pesticidal compounds can be isolated from potential plant sources through the assessment of cytotoxic activity against brine shrimps. Cytotoxicity was evaluated in terms of LC_{50} (lethality concentration). Ten nauplii were added into three replicates of each concentration of the plant extract. After 24 hours the surviving brine shrimp larvae were counted and LC_{50} was assessed. Results showed that the extracts of L. camara, C. odorata, and E. hirta were potent against the brine shrimp with LC_{50} values of 55, 10, and 100 ppm (µg/mL), respectively. It indicated that bioactive components are present in these plants that could be accounted for its pharmacological effects. Thus, the results support the uses of these plant species in traditional medicine.

Keywords: Brine shrimp lethality assay, *Lantana camara*, *Chromolaena odorata*, *Euphorbia hirta*, LC_{50}, potent, cytotoxicity.

Introduction

Many of today's drugs have been derived from plant resources. Historically, medicinal plants have provided a source of inspiration for novel therapeutic drugs, as plant derived medicines have made large contributions to the health and well being of humans. According to the World Health Organization (WHO), nowadays, 80% of the world's population rely on plants for their primary health care[1]. The medicinal value of plants is due to the substances that it contains which produce a physiological action on the human body. Some examples of these substances are alkaloids, essential oils, tannins, resins, and many others. Moreover, some negative effects obtained in the use of local plants as source of medicine are basically due to over-dosage and lack of adequate knowledge of other detrimental by-products contained in some plants[2].

For centuries, *Lantana camara* L. have been used by folk healers in Asia and South America to treat various dermatological and gastrointestinal diseases, tetanus, malaria, and tumors[3]. Leaf extracts of *L. camara* have been found to have an antimicrobial, fungicidal, insecticidal, and nematicidal potential. *Lantana* oil is used for treating skin itches, as an antiseptic for wounds, and sometimes used externally for leprosy and scabies[4]. *Chromolaena odorata* is mainly used by some tribal groups for the treatment of cuts and wounds where young leaves are crushed. *C. odorata* has an antihelminthic, antimalarial, anti-inflammatory, antimicrobial, fungicidal, insecticidal, and wound healing properties[5]. Further, *Euphorbia hirta* is considered to have an antiasthmatic, antibacterial, antifungal, antimalarial, antihelminthic, antitumor, diuretic, and hemostatic activities. In the Philippines, the leaves of *E. hirta* are mixed with *Datura metel* leaves and flowers in the preparation of "asthma-cigarettes"[6].

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[7]. However, most of the available information regarding the medicinal potential of these plants is not provided with credible scientific data. For this reason, several researches have been conducted to determine the toxicity of medicinal plants[3]. A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in plant crude extracts is the Brine Shrimp (*Artemia* sp.) Lethality Assay (BSLA)[8]. BSLA is used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticidal compounds[9]. The low cost and ease of performing the assay and the commercial availability of inexpensive brine shrimp eggs makes BSLA a very useful bench top method[10]. This assay has been noted as a useful tool for the isolation of bioactive compounds from plant extracts[11].

In this present study, ethanolic extracts of the three selected medicinal plants collected in Iligan City were tested *in vivo* for their cytotoxic effect against the brine shrimp nauplii and relate toxicity results with their known ethno-pharmacological activities. In vivo lethality test has been successfully used as a preliminary study of cytotoxic and antitumor agents[12]. Thus, the findings of this present work would give baseline information on the most promising plant species that could be use as a basis for the development of new tools of great therapeutic importance.

Material and Methods

Plant Materials: Fresh leaves of *Lantana camara* and *Chromolaena odorata* and whole plant of *Euphorbia hirta* were collected randomly from different areas in Iligan City. These plants were selected because of their availability (common in wasteland and road side) in the area aside from their medicinal potentials. The plants were identified by comparing it with the herbarium specimens at the Botany Museum of the College of Science and Mathematics of the Mindanao State University-Iligan Institute of Technology, Iligan City. The plants were washed with water and air dried in shade for about one to two weeks. Then air-dried leaves were pounded and kept (at 20°C) in closed plastic containers.

Preparation of Plant Extracts: Twenty grams of the fine powder of each plant samples were weighed and added into an Erlenmeyer flask containing 250 mL of 95% ethanol. The solution was covered and shaken every 30 min for about six (6) hours and allowed to stand for about 48 hours in room temperature. Then, it was shaken and filtered using Whatman filter paper (No.1). After filtration, the solvent was removed by evaporation using a rotary evaporator under reduced pressure at temperature below 55°C. An alternative dilution procedure developed by McLaughlin et al.[13] were adopted in the preparation of the different dilutions of the plant extracts for BSLA where 20 mg of each extract was dissolved in 2 mL of the solvent. The final concentrations were 1000, 100, 10, and 1 ppm (µg/mL). There were three (3) replicates in each concentration. A control test was also prepared.

Brine Shrimp Lethality Assay (BSLA): Brine shrimp eggs were obtained from the New Aqua Laboratory in Naawan, Misamis Oriental, as a gift sample for the research work. Filtered, artificial seawater was prepared by dissolving 38 g of sea salt in 1 liter of distilled water for hatching the shrimp eggs. The seawater was put in a small plastic container (hatching chamber) with a partition for dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber while the lamp above the other side (light) will attract the hatched shrimp. Two days were allowed for the shrimp to hatch and mature as nauplii (larva). After two days, when the shrimp larvae are ready, 4 mL of the artificial seawater was added to each test tube and 10 brine shrimps were introduced into each tube. Thus, there were a total of 30 shrimps per dilution. Then the volume was adjusted with artificial seawater up to 5 mL per test tube. The test tubes were left uncovered under the lamp. The number of surviving shrimps were counted and recorded after 24 hours. Using probit analysis, the lethality concentration (LC_{50}) was assessed at 95% confidence intervals. LC_{50} of less than 100 ppm was considered as potent (active)[14]. As mentioned by Meyer and others[9], LC_{50} value of less than 1000 µg/mL is toxic while LC_{50} value of greater than 1000 µg/mL is non-toxic. The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.

Results and Discussion

The ethanolic extracts of the three plants tested showed good brine shrimp larvicidal activity. The lethality concentration (LC_{50}) of *Lantana camara, Chromolaena odorata,* and *Euphorbia hirta* extracts were 55 ppm (µg/mL), 10 ppm, and 100 ppm respectively (table 1). The degree of lethality was directly proportional to the concentration of the extract. Maximum mortalities (100%) were observed at a concentration of 1000 ppm in both *Lantana camara* and *Euphorbia hirta* extracts while that of *Chromolaena odorata* was at 100 and 1000 ppm. Based on the results, the brine shrimp lethality of the three plant extracts were found to be concentration-dependent. The observed lethality of the three plant extracts to brine shrimps indicated the presence of potent cytotoxic and probably antitumor components of these plants. According to Meyer et al.[9], crude plant extract is toxic (active) if it has an LC_{50} value of less than 1000 µg/mL while non-toxic (inactive) if it is greater than 1000 µg/mL.

Table-1
The number of shrimp nauplii that survived after treating with the three plant extracts and the percentage mortality

Plant Extracts	Concentration (ppm or µg/mL)	Number of Surviving Nauplii After 24 h			Total Number of Survivors	% Mortality
		T1*	T2	T3		
Lantana camara	1	7	8	8	23	23%
	10	5	6	5	16	47%
	100	4	5	5	14	53%
	1000	0	0	0	0	100%
Chromolaena odorata	1	7	7	8	22	27%
	10	4	6	5	15	50%
	100	0	0	0	0	100%
	1000	0	0	0	0	100%
Euphorbia hirta	1	10	9	8	27	10%
	10	7	8	6	21	30%
	100	6	5	4	15	50%
	1000	0	0	0	0	100%

*T=Trial

The results showed that the ethanolic extracts of the three selected medicinal plants were potent or active against brine shrimps where *Chromolaena odorata* was the most active at 10 ppm. In the study conducted by Vital and Rivera[15], ethanolic extract of *C. odorata* leaves was observed as having an antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Salmonella typhimurium* and more effective than ampicillin even at small amount. According to them, the possible antimicrobial potential of *C. odorata* could be due to its ability to bind to the cell wall of the bacteria, thereby inhibiting its synthesis probably because of the flavonoids and tannins present in the plant. The flavonoids, triterpenes, chalcones, and steroids isolated from *C. odorata* through phytochemical screening might be responsible for its pharmacological activities[5].

Lantana camara ranks as the second potent or active plant species against brine shrimps at 55 ppm in this present study. Fatope et al.[16] screened for the activity of the leaves, stems, and roots extracts of *L. camara* on brine shrimp larvae and was able to isolate oleanonic acid, lantadene A, and oleanolic acid which exhibited significant toxicity on the larvae. Zani and others[17] also found out that *L. camara* (root extracts) were active in the BSLA. Moreover, they also noted that *L. camara* inhibit the development of the phytopathogenic fungus *Cladosporium sphaerospermum*. This could be attributed to the presence of camaraside and lantanoside cytotoxic substances which were already isolated from *L. camara*[18]. Previous related studies showed that the essential oil from this plant is active against fungi[18] and bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*[19]. The findings of Medeiros and et al[20] indicated that the major compound of *L. camara* essential oils from leaves and stems are sesquiterpenes which were cytotoxic to V79 mammalian cells and also to *Artemia salina*, showing 50% lethal concentration (LC50) values from 0.23 µg/mL. Their *in vitro* data suggested that essential oil of this plant may also be effective in treating yeast infection.

Further, Dibua et al.[21] exhibited that *L. camara* (and *Allium sativum*) has an antitubercular activity on multiple-drug-resistant *Mycobacterium* species which could be associated to the presence of cardiac glycosides enhancing myocardiac contraction, and exerting hypotensive effect by inhibiting Na+ and K^+ ions[21]. Also the alkaloids, flavonoids, saponins, and oils isolated from this plant might be the cause of its observed activity against the isolates. Alkaloids interferes with the cell division process while flavonoids exert an antioxidant activity against the superoxide radical, thereby inhibiting low density lipid oxidation[22,23].

The result on the lethality of *Euphorbia hirta* on brine shrimps is in agreement with other studies where its LC_{50} values are 71.15 µg/mL[24] and 118.88 µg/mL[25]. In contrast, the study of Amutha et al.[26] have recorded an LC_{50} values of 0.71, 0.66, 0.41 and 0.03 mg/mL for the stems, leaves, roots, and flowers of *E. hirta*, respectively against brine shrimps. They also observed that *E. hirta* extracts showed antibacterial (against *Staphylococcus aureus* and *Micrococcus* sp.) and anticandidal (against *Candida albicans*) activities. The presence of alkaloids, tannins, and flavonoids could be accounted for its antibacterial properties. In the other hand, some studies have shown that *E. hirta* extracts exhibited selective cytotoxicity against several cancer cell lines. In one study, the extract of this plant species was noted as having an anti-proliferative activity against the normal mouse fibroblast cells. Furthermore, the methanolic extract of *E. hirta* leaves showed an anti-proliferative activity against Hep-2 cells obtained from human epithelioma of the larynx[9]. Thus, the results on *Euphorbia hirta* support its use in traditional medicine as well as that of *Lantana camara* and *Chromolaena odorata*.

Conclusion

The leaf extracts of *Lantana camara* and *Chromolaena odorata* and whole plant extract of *Euphorbia hirta* exhibited cytotoxic activity against the brine shrimp and considered as containing active or potent components. This is because their LC_{50} values are less than 1000 ppm or µg/mL. The ethno-pharmacological activities of these plant species are due to the different bioactive compounds present in these plants. Although, BSLA is inadequate in determining the mechanism of action of the bioactive substances in the plant, it is very useful by providing a preliminary screen that can be supported by a more specific bioassay, once the active compound has been isolated. Thus, some useful drugs of therapeutic importance may develop out of the research work.

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