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Brine Shrimp Lethality Assay of the Ethanolic Extracts of *Antidesma ghaesembilla* Gaertn

Reizl P. Jose[1], Kay P. Ramos[1], Maricel L.Gelsano[1], Olga M. Nuñeza[1*], Mylene M. Uy[2]
1-Department of Biological Sciences, College of Science and Mathematics, Mindanao State University-Iligan Institute of Technology, Iligan City, Philippines.
2-Department of Chemistry, College of Science and Mathematics, Mindanao State University-Iligan Institute of Technology, Iligan City, Philippines.
*Corresponding author's email: olgamnuneza@yahoo.com

ABSTRACT
Many plants have been used as traditional medicine. The Antidesma ghaesembilla Gaertn, locally known as "bugnai", which is also found in Bohol, Philippines is believed to have medicinal properties. Brine Shrimp Lethality Assay (BSLA) of the ethanolic extract of Antidesma ghaesembilla Gaertn was conducted in order to determine whether this medicinal plant has potential pharmacological activities. Three extracts were prepared (decoction, ethanol extract, and mixture extract) at four concentrations of 1000 ppm, 500 ppm, 100 ppm, and 10 ppm in three replicates. The study showed 100% mortality of brine shrimp in all concentrations at six hours and 24 hours exposures in all extract preparations. Among the different types of extracts, the 50-50 ethanol extract had the most toxic concentration with less than 10 ppm toxic in both acute and chronic LC_{50} values. The result indicated potential bioactive component of the plant extract since it revealed cytotoxic activity against the brine shrimp. This can be accounted to its pharmacological effect that supports the traditional uses of this plant.
Keywords: *decoction, ethanol extract, pharmacological, traditional medicine.*

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INTRODUCTION

There is growing interest in exploiting plants for medicinal purposes especially in Asia [1]. Medicinal plants have assumed greater importance in recent days, due to the tremendous potential they offer in formulating new drugs against many diseases and illness that affect the human kind [2]. According to the World Health Organization (WHO), 75-80% of the world's population relies on plants either in part or entirely for their primary health care [3]. In recognition of the deep seated practice of traditional medicine as an alternative modality for treating and preventing diseases in the Philippines, the Department of Health (DOH) launched the Traditional Medicine Program in 1992. The program was set to promote and advocate traditional medicine in the Philippines [4]. There are many medicinal plants in the Philippines and there are probably a great deal more with properties that produce a physiological action on the human body as yet undiscovered scientifically.

The Euphorbiaceae is an extensive family of plants that includes about 300 genera and 5000 species and is mainly distributed in tropical areas. For a long time this family has been recognized and reported for its anti-cancer components, anti-hepatitis B components, and carcinogenic factors. In the literature of ancient traditional Chinese medicine (TCM), 33 species of plants from 17 genera of Euphorbiaceae have been mentioned as medicine [5].

Antidesma ghaesembilla Gaertn is a small deciduous tree belonging to family Euphorbiaceae. Its leaves and fruits are edible, nutritious, and this plant has been reported to provide various culinary and medicinal properties [6]. The plant is used by local people as a medicine to treat cough, rheumatism, diarrhea, and headache [7, 8]. In India, leaves are used to treat fever, headache and swollen abdomen[9]. It is also used for breast milk production for lactating women [2]. Its stem is used as medicine to stimulate menstrual flow as well as dysmenorrhea [10]. The ripe fruits of *A. ghaesembilla* are used as seasoning agent in fish and meat preparations [11]. Aside from being eaten locally, the fruit is also used as purgative

[2]. Moreover, its fruits may be potential source of nutrients and antioxidants [12]. In Bohol, *A. ghaesembilla* Gaertn or known as "bugnai" is believed as treatment for urinary tract infection or any kidney problem according to locals through personal communication.

A. ghaesembilla Gaertn is an important source of bioactive compounds. It was found to contain glycosides, esteroids, flavonoids, and terpenoids. Pharmacological evaluation of methanol extracts of its fruit showed neuropharmacological activity. Using behavioral pharmacology models, plant extracts have significant therapeutic utility for the treatment of anxiety and related neuropsychiatric disorders [13]. Sakunpak and Panichayupakaranant [14] reported that leaf extracts of this plant exhibit antimicrobial activity against gram negative pathogens of the gastrointestinal tract. In the study of Gargantiel and Ysrael [15], ethnobotanical information indicates that more than 800 plants including *A. ghaesembilla* Gaertn are used as traditional remedies for the treatment of diabetes. The available information should be subjected to further scientific investigations to corroborate these findings.

There have been several scientific studies conducted to determine the toxicity of *A. ghaesembilla* plant. A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in plant crude extracts is the Brine Shrimp Lethality Assay (BSLA) [16]. BSLA is used for testing cytotoxic potential of the plant extract [17]. It is further used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticides compounds. The low cost and ease of performing the assay and the commercial availability of inexpensive brine shrimp egg makes BSLA a very useful bench top method [18]. Further, this assay has been noted as a useful tool for the isolation of bioactive compounds from plant extracts [19].

The *A. ghaesembilla* Gaertn has a long history of use in traditional medicine, and even today many locals rely on the administration of plant-derived preparations for the treatment of diversity of ailments. Considering that a major challenge today is the discovery of plants with promising activities, we applied the brine shrimp test (BST) for general activity screening of several extracts of the plant.

MATERIALS AND METHODS

Collection of Plant Material

Three kilos of fresh leaves of *A. ghaesembilla* Gaertn were collected from Bilar, Bohol during the month of February 2014. This plant is commonly found in grassland areas but very rare along the road side.

Preparation of the Plant Extract

The fresh leaves were cleaned with tap water and then with distilled water. One kg of the leaves was subjected to decoction in 2:1 water proportion in five minutes. Then the liquid was filtered and freeze dried. The remaining 2 kg of the sample were dried in an oven at 40^0C. The oven was set at the optimum temperature needed for the compounds. Dried leaves were ground to coarse powder and soaked in absolute ethanol and ethanol: water mixture (50% EtOH: 50% H_2O). The liquids were filtered after three days and were subjected to rotary evaporator under reduced pressure at temperature below 39^0C. Three extracts were produced for the sample: decoction, ethanol extract, and mixture extract. In the sample preparation, 30 mg of each extract was dissolved with 3 mL ethanol (10,000-ppm stock solution). From the 10,000-ppm stock solution, four concentrations were obtained through serial dilution method. These are 1000, 500, 100 and 10 ppm and three replicates were done for each concentration. DMSO was added to each concentration as surfactant. There were three replicates in each concentration and a control test with pure dimethyl sulfoxide (DMSO) was also prepared.

Brine Shrimp Lethality Assay (BSLA)

The brine shrimp eggs were provided by the Chemistry Department of MSU-IIT. A small tank was filled with filtered sterile seawater that was divided into two compartments. The hatching chamber was covered with black material and the other half of the tank was lighted to attract and separate the hatched shrimp from the eggs. The lighting is also important to stimulate the temperature of the natural habitat of the shrimp. After two days, shrimp larvae of *A. salina* were added to each test tube. Then the volume was adjusted to 5mL by adding filtered sterile seawater in each test tube. The test tubes were let uncovered under the lamp. The number of surviving shrimps were observed, counted, and recorded after 6 and 24 hours.

Statistical Analysis

The Reed-Muench method was used to determine the relative toxicity of the extracts to living organisms. Using this analysis, the lethality concentration (LC_{50}) was assessed at 95% confidence intervals. LC_{50} of less than 100ppm was considered as potent (active). LC_{50} value of less than 1000 µg/mL is considered toxic while LC_{50} value of greater than 1000 µg/mL is considered non-toxic [20]. The percentage mortality was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%.

RESULTS AND DISCUSSION

Herbal medicines have received great interest as an alternative to clinical therapy, and the demand for these therapies has currently increased rapidly [21]. The use of *Artemia* sp. is essential in this study as a test species in toxicity, screening hepatotoxic cyanobacterial strains [22], and natural products [23].

Table 1 shows the percent mortality of brine shrimp nauplii after 6 hours exposure to the three preparations of *A. ghaesembilla* extracts. The different types of extracts at 1000ppm, the 100% ethanol concentration and 50-50 ethanol-water extracts indicated 100% mortality both in 6 hours and 24 hours exposure, while the decoction had 97.56% mortality in 6 hours but achieved 100% mortality at 24 hours. For the 500 ppm concentration of the three different extracts, the percentage mortality is 100% at 24 hours and at 6 hours exposure the percentage is still high with 98.33% for the 100% ethanol, 98.75 % for the 50-50 ethanol-water and 88.14 for the decoction. Moreover, at 100ppm at 6 hours exposure, the mortality is also high with 96.77% in 100% ethanol, 94.34% in 50-50 ethanol-water and 72.97% in decoction. However, at 24 hours exposure in 100ppm concentration it achieved 100% mortality in 100% ethanol and 98.31% in 50-50 ethanol-water extract and 94.12% in decoction. Further, at 10ppm concentration in 6 hours exposures of both 100% ethanol and decoction, there were no mortality achieved but in 50-50 ethanol-water it achieved 66.67% mortality and had higher mortality at 24 hours with 93.55%. In addition, 20% mortality was achieved in 24 hours in 100% ethanol and 12.50% in decoction. According to Carballo *et al.* [24], toxic effect to brine shrimp has potential pharmaceutical applications. This conforms to the present study which had high toxicity to brine shrimp as exposed to different *A. ghaesembilla* extracts even at 1000ppm and even below 10 ppm concentrations at different types of extracts. Habib *et al*. [6] and Patil *et al.* [2] reported that the plant *A. ghaesembilla* extract has a high amount of bioactive substances and may contain compounds like tannin, resin, glycosides flavonoids, phenol alkaloids, xanthoprotein, cysteine, and oil that possess lethality effects to brine shrimp. This plant demonstrated qualitative phytochemical tests which exhibited the presence of common phytocompounds including alkaloids, terpenoids, antraquinones, cardiac glycosides, saponins, and volatile oils as major active constituents [25].

Table 1. Percent Mortality of Brine shrimp nauplii at 6 hours and 24 hours to various concentrations of *A. ghaesembilla* Gaertn extracts.

Type of Extract (ppm)	Percent Mortality	
	6 Hours	4 Hours
100%Ethanol		
1000	100.00	100.00
500	98.33	100.00
100	96.77	100.00
10	0	20.00
50-50 Ethanol Water		
1000	100	100.00
500	98.75	100.00
100	94.34	98.31
10	66.67	93.55
Decoction		
1000	97.56	100.00
500	88.14	100.00
100	72.97	94.12
10	0	12.50

Table 2 shows the acute and chronic LC Values in all types of extracts with less than 10 ppm in 50-50 ethanol-water type of extract and exhibited low LC values of 34.48 in acute and 21.13 chronic LC values in 100% Ethanol and 48.7 in acute and 27.38 chronic LC values in decoction in 10-1000 ppm concentrations. According to Nguta *et al.* [26], LC_{50} values < 1000 µg/ml indicates the presence of cytotoxic compounds. In the study of Sahgal *et al.* [21] on seed extracts, LC_{50} was 0.68 mg/ml indicating a mild toxicity. In addition, the lethality in acute oral toxicity of *Swietenia mahagoni* (Linn.) Jacq. Seed methanolic extract showed only to be moderately toxic to brine shrimp at LC_{50} with 1.1 mg/ml at 12 hours. In other studies, LC values are greater than 100 µg/ml, which exhibited very low toxicity [27] and the LC_{50} values below 100 µg/ml have potential for toxicity [28]. However, in the study of Baravalia *et al.* [29], the crude extract showed 73% mortality at 1000 µg/mL concentration and its LC_{50}-value was 763.34 µg/mL, which was considered moderately toxic. The *E. guineensis* extracts screened for toxicity against brine shrimp with 50% lethal concentration (LC_{50}) values of more than 1.0 mg/mL (9.00 and 3.87 mg/mL, at 6 and 24 h,

respectively), confirmed that the extract was not toxic [30]. The LD_{50} is 887.75 ± 9.2 (n = 4) mg/kg is safe up to 100 mg/kg [31].

Table 2. LC_{50} Values of the *A. ghaesembila* Gaertn Extracts against the Brine Shrimp *A salina.*

Type of Extract	Concentration	LC_{50} (ppm)	
		Acute	Chromic
100% Ethanol	10-1000 ppm	34.38	21.13
50-50 Ethanol-Water	10-1000 ppm	<10	<10
Decoction	10-1000 ppm	48.70	27.38

In the study of Krishnarajua *et al.* [32], *Polygonum cuspidatum* and *Syzygium cumini* had the most promising aqueous extracts which have been used in the traditional medicine for the treatment of antitumor and anti-inflammatory diseases and the lethality (LC_{50}) values were 13.5 and 20 ug/mL respectively. In addition, Sahgal *et al.* [21] mentioned that brine shrimp lethality on *Swietenia mahagoni* (Linn.) Jacq. Seed methanolic extract in LC_{50} had only moderate toxicity in 0.68 mg/ml at 24 hours of exposure.

In the present study, *A. ghaesembilla* Gaertn is far better in toxicity to brine shrimp at LC_{50} in 100% concentration, 50-50 ethanol-water, and decoction which revealed a chronic toxicity and comparably better in terms of lethality to brine shrimp. Hence, the concentration of *A. ghaesembilla* Gaertn in all types of extracts is potentially toxic and the most prominent is in the 50-50 ethanol extract since the acute and chronic LC values are less than 10 ppm.

CONCLUSION AND RECOMMENDATION

The leaf extract of *A. ghaesembilla* Gaertn (bugnai) in 100% percent, 50-50 ethanol extracts and decoction revealed cytotoxic activity against the brine shrimp. The leaf plant toxicity against brine shrimp is chronically toxic. This finding supports the traditional medicinal alternative use of this plant. Based on the possible relationship between brine shrimp lethality and plant bioactivity, this study could serve for further ethnobotanical and phytochemical research. Moreover, this study recommends Isolation through Gravity Column Chromatography (GCC) and High Performance Liquid Chromatography (HPLC) with Nuclear Magnetic Resonance (NMR) for the specific organic compounds determination.

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