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Brine shrimp lethality assay of whole plant extracts of *Eleusine indica*

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Abstract. Brine shrimp lethality assay (BSLA) is an important method in the evaluation of plant bioactivity for the subsequent isolation of bioactive compounds from the plant extracts which may lead to the development of new drugs. In this study, BSLA was employed to evaluate the potential cytotoxic properties of the whole plant extracts of *Eleusine indica. E. indica* has been widely used for the treatment of several diseases like kidney problems, diabetes, gastro-intestinal diseases and is used as diuretic. The most common methods of extraction (decoction, absolute ethanol, 50% water - 50% ethanol) were tested to determine cytotoxic effects against the brine shrimp nauplii. Results showed that the best extraction method for the whole plant samples of *E. indica* is through the use of 50:50 ethanol-water mixture extract. The acute lethal concentration (LC_{50}) of *E. indica* after 6 h exposure to mixture extract was 153.99 ppm, while the ethanolic extract obtained an LC_{50} of 409.73 ppm. After 24 h exposure, increased mortalities of brine shrimps were recorded in all prepared extracts. Maximum mortalities (100%) were observed in the three concentrations of 100-1000 ppm in the mixture extract of *E. indica.* In this respect, *E. indica* possesses cytotoxic behavior suggesting the presence of potential bioactive chemical components in the plant's extract.
Key Words: Bioactivity, cytotoxic, decoction, lethal concentration, medicinal.

Introduction. Many of the essential needs of humans, including life-saving pharmaceutical agents have been provided by plants. Historically, plants have formed the basis of traditional medicine practices that have been used for thousands of years by many countries led by China and India (Singh 2006). Plants are still important sources of medicines especially in developing countries that still rely on plant-based traditional medicine for their healthcare (Ramesh & Okigbo 2008). There is a perception that more than two billion people all over the world may be heavily reliant on medicinal plants (Smith-Hall et al 2012). According to World Health Organization, traditional medicine has maintained its popularity worldwide since the 1990s until its use has surged in many developed countries. As an important source of nutrition and substances which produce physiological action on the human body (Olowa & Nuñeza 2013), plants are recommended for their therapeutic values which can be used in drug development and synthesis (Ramawat & Merillon 2008).

Pharmacologically active compounds present in medicinal plants are responsible for their efficacy (Salim et al 2005). These are largely secondary metabolites which include alkaloids, essential oils, tannins, resins, and many others, which could be used either in the original form or in their semi-synthetic form (Olowa & Nuñeza 2013; Rasool Hassan 2012). Secondary metabolites are used as raw materials for the extraction of active components in the synthesis of different drugs (Salim et al 2005). Thus, the evolving commercial importance of these secondary metabolites as a source of pharmaceuticals has in recent years resulted in a greater interest (Hussain et al 2012). Although, these active compounds are essential for therapeutic potential, these substances including the lack of sufficient knowledge of other detrimental components from local plants have been reported to have negative effects in the body when

consumed largely (Melanie 1999). Hence, there should be a powerful and proper deep assessment on the pharmacological qualities of herbal-derived remedies (Firenzuoli & Gori 2007).

In Philippine traditional medicine, *E. indica* is one of the medicinal plants that has been widely used for the treatment of several diseases like kidney problems, diabetes, gastro-intestinal diseases and is used as diuretic (Castillo et al 2005). This plant is commonly known as “Bila-bila”, “Bakis-bakisan” and “Parag-is”, which is native in the tropics and subtropical regions (Haber & Semaan 2007). *E. indica* is an abundant weed in waste places and along river banks, roads, and settled areas in the Philippines (Stuart 2013). It has a broad tolerance to a wide range of environmental conditions, but its vegetative growth is significantly reduced during dry seasons (Leach et al 1995).

The whole plant of *E. indica*, especially the root has been reported in Malaysia as depurative, diuretic, febrifuge and laxative, and hence is used for the treatment of influenza, hypertension, oliguria and urine retention (Al-Zubairi et al 2011). It is also used for kidney ailments in Trinidad and Tobago (Lans 2006). In the Philippines, a survey on the Research Information Series of Ecosystems (RISE) by Castillo et al (2005) showed that decoction of fresh plants of *E. indica* is used as a diuretic and for dysentery. The entire plant is also mixed with coconut extract to be used as anti-dandruff and prevent the loss of hair. The extracted juice from the leaves is known to be used after childbirth for placenta elimination (Gbadamosi & Otobo 2014). The whole plant, particularly the root, may be used for fever, liver complaints and for treating cough. Moreover, this plant is used as poultice in sprains and is used as anti-helmintic (Ettebong et al 2012).

At present, therapeutic benefits of this medicinal plant are often attributed to its antioxidant (Al-Zubairi et al 2011; Iqbal & Gnanaraj 2012) and antiplasmodial and antidiabetic properties (Okokon et al 2010). However, ethnopharmacological and scientific reports on this plant are still scarce, especially on its phytochemical observation and cytotoxic properties. Thus, further investigation of this medicinal plant is needed in the country to uphold more of its significance as herbal-derived remedies. To date, no scientific investigation has been reported on evaluating the cytotoxicity of *E. indica* using animal models. Therefore, this study was conducted in order to evaluate the toxicity of *E. indica* whole plant extracts using Brine Shrimp Lethality Assay (BSLA). The most common methods of extraction (decoction, absolute ethanol, 50% water - 50% ethanol) were tested in the present study for cytotoxic effects of extracts against the brine shrimp nauplii and related toxicity results with *E. indica’s* known traditional uses. Specimens of the brine shrimp, *Artemia* sp., a marine microcrustacean, are used as target organisms to detect bioactive compounds in plant extracts in which the toxicity test against these animals has shown to have a good correlation with antitumor activity (Arcanjo et al 2012). Thereby, this type of bioassay may prove advantageous in the evaluation of plant bioactivity for the subsequent isolation of bioactive compounds from the plant extracts, leading to the possible development of new drugs.

Material and Method

Plant collection. Three kilograms of whole plant samples (including roots) of *E. indica* were collected in Barangay Ditucalan, Iligan City in January, 2014. Documentation and labeling procedure were done according to the protocol of Guevara (2005). Plant identification was based on Castillo et al (2005).

Preparation of the plant extract. In this study, *E. indica* whole plant extracts were obtained by using three types of extraction solvent systems: decoction, mixture of water and ethanol (50:50), and ethanolic extraction. To prepare the decoction extracts, fresh and clean plant samples were cut into small pieces and boiled in distilled water in 1:2 ratio for 5 minutes. It was subsequently cooled to 25^{o}C before filtration then freeze-dried to remove excess amount of water. For the (50:50) mixture and ethanolic extracts, fresh samples were washed in tap water and then rinsed in sterile water to remove contaminants. The rinsed samples were air dried for one week or when the samples were already crispy enough upon crumpling. The dried samples of each plant were pulverized

using a sterile electric blender. The powder was weighed, divided into two equal parts and stored in glass containers; one was percolated with enough 95% ethanol and the other one was soaked with 50:50 proportion mixture of water and ethanol for three days (72 h). Each solution was then filtered using Whatman filter paper and collected in a glass container. Enough amount of the filtered ethanol solution was subjected to rotary evaporator to obtain the crude plant ethanol extract. The 50:50 mixture extract was concentrated *in vacuo* and subsequently freeze-dried to obtain the crude mixture extract.

Brine shrimp lethality assay (BSLA). Brine shrimp eggs were provided by the Chemistry Department of MSU-IIT. Filtered sterile seawater was put in a 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 of the chamber attracted the hatched shrimps. The nauplii larva brine shrimps were used for the bioassay after two days.

Four concentrations of the three extract (decoction, mixture and ethanol) of *E. indica* were prepared: 10 μg/mL, 100 μg/mL, 500 μg/mL and 1000 μg/mL. To make the stock solution, weighed amounts of the three extracts (decoction, mixture and ethanol), 36.5 mg, 25.18 mg and 35.4 mg were dissolved separately with sufficient amount of solvent to obtain their respective 10,000-ppm stock solution. Ethanol was used as solvent for the alcohol-based extracts and allowing it to evaporate for two days. After the evaporation of ethanol, DMSO, a surfactant was added to the two extracts, except for the decoction. From these stock solutions, 10 ppm, 100 ppm, 500 ppm and 1000 ppm concentrations were derived through serial dilution. Three replicates were prepared for each extract and 5 mL of filtered sterile seawater served as control. Subsequently, five brine shrimps were introduced into each tube. Thus, there were a total of 30 brine shrimps per dilution. Then, the volume was adjusted with sterile seawater and the tubes were left uncovered under the lamp. After 6 h and 24 h, the number of dead and surviving nauplii in each tube were counted and recorded.

Statistical analysis. The Reed-Muench method (Reed & Muench 1938) was used to determine the relative toxicity of the various concentrations of the extracts to *Artemia salina.* LC_{50} represents the dose lethal to half of the *A. salina* population. This was determined by plotting the % mortality (y-axis) versus log dose (x-axis) with the dose which rendered 50% mortality as the LC_{50}.

Results and Discussion. Table 1 shows the percent mortalities and LC_{50} values after 6 h (acute) and 24 h (chronic) exposure to various extracts of *E. indica*. The least percent mortalities were recorded for the decoction extract which ranged from 0-27.77%, while high percent mortalities were recorded for the alcohol-based extracts (mixture and ethanol). Results also showed that the degree of mortality was directly proportional to the *E. indica* concentration in all types of extracts. After 24 h exposure, increased mortalities of brine shrimps were recorded in all prepared extracts. Maximum mortalities (100%) were observed in the three concentrations of the mixture extract tested (100, 500, 1000 ppm). Among the three extracts of *E. indica,* the most cytotoxic to the brine shrimp after both 6 h and 24 h exposures was the water-ethanol mixture as indicated by its lowest LC_{50} value of 154 ppm and <10 ppm, respectively. Thus, the best extraction method for the whole plant samples of *E. indica* is through the use of a mixture of ethanol and water as indicated by the percent mortality and LC_{50} values.

According to Meyer et al (1982), LC_{50} lower than 1000 ppm is considered cytotoxic (active) on the evaluation of plant extracts by BSLA, while non-toxic (inactive) if it is greater than 1000 ppm. The results of the decoction plant preparation exhibited inactivity wherein very low mortality among the brine shrimp nauplii was observed. This would mean that usage of this plant extract as therapeutic agent poses no high risk of harm upon administration especially when prepared as decoction which is the traditional way of preparation. However, alcohol-based extracts (water-ethanol mixture and ethanol) of *E. indica*, were potent or active which caused high mortalities against the brine shrimps.

Table 1

LC_{50} values of the *Eleusine indica* plant extracts against the brine shrimp, *Artemia salina*

Type of extract	*Concentration (ppm)*	*Brine shrimp mortality (%)*		*LC_{50} (ppm)*	
		After 6 h	*After 24 h*	*Acute (After 6 h)*	*Chronic (After 24 h)*
Decoction	10	0	0	>1000	>1000
	100	0	0		
	500	3.33	5.56		
	1000	6.25	27.27		
50:50 Water-ethanol mixture	10	1.69	86.67	154.00	<10
	100	42.00	100		
	500	68.85	100		
	1000	86.11	100		
Ethanol	10	0	0	409.73	290.07
	100	8.33	0		
	500	58.14	84.38		
	1000	83.64	96.49		

Our study has demonstrated that *E. indica* possess cytotoxic behaviour, suggesting the presence of potential bioactive chemical components in the plant's extract. As mentioned, plants produce a large number of naturally occurring secondary metabolites which have many unique pharmacologic activities (Salim et al 2005*).* A phytochemical screening of the extract of *E. indica* showed that the extract contains secondary metabolites like alkaloids, terpenes, flavonoids, tannins, anthraquinones, saponins and cardiac glycosides (Okokon et al 2010). These phytochemicals have been implicated on the cytotoxicity activity demonstrated by the alcohol-based extracts of *E. indica*.

E. indica produced physical signs of toxicity in albino wistar rats depending on the dose given ranging from writhing, decreased respiration and death 24 h after administration of the ethanolic leaf extract of *E. indica* (Okokon et al 2010). Moreover, Hansakul et al (2009) had examined the antiproliferative and cytotoxic effects of the hexane and butanolic extracts of *E. indica* on human lung and cervical cancer cells. Results showed that both hexane and butanolic extracts of *E. indica* demonstrated cytotoxic effects against lung and cervical cancer cells via apoptosis. On the other hand, a cytotoxicity study by Al-Zubairi et al (2011) revealed that the hexane, dichloromethane, ethyl acetate and methanol extracts of *E. indica* were found to be non-effective in inducing cell death towards the MCF-7 human breast cancer cells, HT-29 human colon carcinoma cells and Human T4-lymphoblastoid cell line, following guidelines of the American National Cancer Institute.

Although *E. indica* was conferred as non-effective in inducing cell death towards some of the cancer cell lines tested, this plant has been effective as an antibacterial, anti-diabetic and anti-oxidants (Iqbal & Gnanaraj 2012; Okokon et al 2010). Some phytochemical compounds such as polysaccharides, terpenes and tannins and steroids have been implicated in the antidiabetic activities of this plant (Okokon et al 2010). It has been reported that phenolic compounds have strong and significant correlation to the antioxidant activities of this plant (Iqbal & Gnanaraj 2012). Even though different phytochemicals have been already correlated to the biological activities of this plant, the precise mechanisms of these biological activities of *E. indica* are still incompletely

understood. Thus, further investigations are needed to verify the cytotoxic effect and other biological properties of this plant.

Conclusions. Alcohol based extracts (ethanol and water-ethanol) of *E. indica* have caused high mortalities against the brine shrimps, unlike the decoction plant preparation in which very low mortality among the brine shrimp nauplii was observed. The best extraction method for the whole plant samples of *E. indica* is through mixture of ethanol and water as it demonstrated maximum mortalities (100%) and high LC_{50} values against the brine shrimps.

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