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EVALUATION OF THE CYTOTOXICITY POTENTIAL OF ETHANOLIC EXTRACT OF *CHRYSOPHYLLUM CAINITO* USING THE BRINE SHRIMP LETHALITY BIOASSAY

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ABSTRACT

Philippines has rich floral biodiversity accompanied with an abundant source of medicinal plants easily accessible in the locality. In terms of ethno-medical properties, *Chrysophyllum cainito* has been used to treat various diseases. In this study, *C. cainito leaves* were collected and evaluated for cytotoxicity using the Brine Shrimp Lethality Bioassay. The *C. cainito* leaves were extracted with water, 50:50 ethanol-water, and absolute ethanol to produce the decoction, hydro-ethanolic, and ethanolic extracts respectively. Four concentrations (10, 100, 500, 1000 µg/ml) of the extracts were prepared and tested. The mortality rates of the brine shrimp were observed after 6 and 24 hours. The results showed that all the prepared extracts exhibited active biological activities with the ethanolic and hydro-ethanolic extracts exhibiting greater activities compared to the decoction. The ethanolic and hydro-ethanolic extracts showed toxicity effects after 24-h exposures with LC_{50} values of 25.85µg/ml and 84.14µg/ml, respectively. The results indicate that the use of absolute ethanol and 50:50 ethanol-water may have successfully extracted the bioactive compounds in the *C. cainito* that have acted on the brine shrimp. The presence of active components in the extracts indicated the potential of *C. cainito* as an alternative medicine and hence requires further tests to qualitatively identify the bioactive compounds.

Keywords: Alternative medicine, bioactive, ethno-medical, extraction, mortality rate

INTRODUCTION

The emergence of new diseases calls for new antidote that is effective, safe, and easily accessible for immediate treatments. Most populations around the world are highly dependent on traditional medicine such as the usage of herbal plants for primary health care needs (Boerma et. al. 2015). In fact, in developing countries such as the Philippines particularly people, who live in rural areas, resort to traditional healer when they get sick (Debas et. al. 2006). Previous works reported the presence of chemical compounds and biological activities of plants which have yielded novel compounds with therapeutic agents which further support the viability of plants as an alternative medicine (Ji et. al. 2009).

One of the locally known plants which has medical importance but poorly explored scientifically is the *Chrysophyllum cainito* Linnaeus commonly known as star apple or "caimito" depending on its location **(**Doan and Le, 2020). *C. cainito* Linn is a tree mostly found in the tropics including Southeast Asia (Rymbai et. al. 2020). This tropical tree is affiliated to the family sapotaceae and is homegrown to the places of West Indies and Greater Antilles (Oranuse et. al. (2015); Luo et. al. (2002). *C. cainito* tree has a wide spreading crown, *ito* can

reaching to a height of 15 meters. Branches are usually few and slender; the young tips are conspicuously copper-colored and hid with appraised hairs. The leaves of *C. cainito* can be characterized as leathery ovate or oblong with an estimated 7.5 to 13 centimeters long appeared with a pointed tip, edgeless or rounded at the base and has covered underneath with golden-brown, silky and soft hairs (Shailajan and Gurjar, 2014).

C. cainito has several health benefits and has been chemically tested and proven (Doan and Le, 2020). Various studies were conducted through extractions of its fruit, pulp, seeds, and leaves. In 2002, polyphenolic antioxidants were isolated in the *C. Cainito* fruit (Luo et. al. 2002). Fruit extracts were tested not only for their antioxidant activity but also for their anti-diabetic effect (Hegde et. al. 2016) and gastro-protective activity (Da Rosa et. al. 2019). Moreover, a study tested the pulp and seed extracts for antimicrobial activity (Oranusi et. al. 2015). The tests have proven that *C. cainito* pulp and seed extracts also have great potential as antimicrobial agent for treating enteric bacterial infections and other selected pathogens. In addition, various studies also reported on the extraction of the leaves which lead to successful evaluations of the anti-diabetic activity (Koffi et. al. 2009), anti-inflammatory, anti-hypersensitivity effects (Meira et. al. 2014), and also for wound healing (Shailajan and Gurjar, 2016). From all these assessments, the medicinal benefits of *C. cainito* are supported.

Furthermore, the root of *C. cainito* has been utilized as alternative medicine in curing sterility, sexual asthenia, and asthma; while seeds were predominantly used to alleviate hemorrhoid and intestinal worms. The bark has been wielded for treating cough, icterus, yellow fever while the fruits are used to treat dental decay and avitaminosis. Moreover, the entire plant can potentially treat ulcer and varicella (Houessou et. al. 2012).

To further scientifically assess the medicinal importance of *C. cainito* Linn, a simple test on its cytotoxicity potential is highly desirable. Accordingly, cytotoxicity potential can be assessed by brine shrimp lethality bioassay (BSLT). The Brine shrimp lethality (BSLT) assay is an indispensable technique on the initial assessment of bioactive compounds present in plant extract (Sarah et. al. 2017). This assay has been effective as a bioassay template in prescreening of active cytotoxic and antitumor agents (Meyer et. al. 1982). Moreover, brine shrimp lethality assay provides a comprehensive analysis on the degree of cytotoxicity. It is a simple test with no aseptic techniques required. It can easily process a large quantity of organisms for statistical validation with relatively minimal quantity of sample (Sarah et. al. 2017).

The test uses *Artemia salina* (Leach) reaction towards the extracts. The mortality is quantified and the lethality assay is computed. The assay is highly capable to detect wide spectrum of bioactivity in crude extracts. The method has been proven to be predictive of cytotoxicity and pesticide activity (Mentor et. al. 2014). Previously, the brine shrimp lethality assay has been utilized in determining the cytotoxicity potential of various medicinal plants such as *Lantana camara*, *Chromolaena odorata*, and *Euphorbia hirta* (Balinado and Chan, 2017), *Kleinhovia hospital* (Morilla et. al. 2015), *Acmella grandiflora* (Elias et. al. 2014), *Ficus nota* (Arquion et. al. 2015), *Phyllanthus niruri* and *Passiflora foetida* (Balinado and Chan, 2017).

In this study, the *C. cainito* Linn leaves were collected in Iligan City and their decoction, hydro-ethanolic, and ethanolic extracts were prepared and tested for their cytotoxicity potential against the brine shrimp and correlated with the known medicative properties of the plant. The data generated from the present work serve as baseline information in targeting specific bioactive compounds present in the *C. cainito* extracts.

MATERIALS AND METHODS

The protocols on performing the experiment were adapted from the modified protocol from the previous works of Olowa and Nuñeza (2013).

Botanical Source and Preparation of Extracts

C. cainito Linnaeus, particularly the leaves, was selected because of its known ethno-pharmacological uses based on a previous survey and interview with local folks

and traditional practitioners in the communities. The fresh leaves of the plant were collected on February 2014 from a local source in Iligan City. The extracts from *C. cainito* leaves were prepared with various extraction solvent namely: ethanol, hydro-ethanol (50:50) and decoction. The decoction were prepared by cutting clean and fresh plant leaves into miniature sizes and heat to boil in distilled water in a 1:2 ratios for a minimum of 5 minutes. Plant samples were gradually chilled at 25°C before subjected to the process of separation then allow to freeze-dried succinctly to dissipate excess amount of moisture. In preparing the mixture of ethanolic extracts and hydro-ethanol, unwilt samples were cleaned in clean water and the dirt and other foreign matter then removed by final rinsing using the sterile water. The cleaned samples were air-dried in a week or until the samples were already fragile upon picking. The dried samples were ground using a sterile electric blender. The powdered plant samples were weighed, and divided evenly into two parts and placed into a glass container; it was then filtered with sufficient quantity of absolute ethanol while the other samples was immersed in a 50:50 mixture of water-ethanol for three consecutive days (72 h). The prepared solutions were purified using the whatman filter paper and decanted in storage glassware. An ample quantity of the purified ethanol solution was transferred into rotary evaporation to prepare the ethanolic extract. The blended hydro-ethanol extract was accumulated in a *vacuo* and afterwards freeze-dried to formulate the hydro-ethanolic extract.

Brine Shrimp Lethality Test

The eggs of the Brine shrimp test organism eggs were acquired from MSU-IIT Chemistry Department. The sterile purified seawater was decanted in a devise compartment which simulated the light and dark areas. The eggs of the test organism were exposed into the dark portion of the compartment while the lamp above on the other boundary of the compartment captured the attention of the hatched shrimps. The larva of brine shrimps was employ for the cytotoxicity bioassay right after two days. From the three extracts (decoction, blended extract of hydro-ethanolic and ethanolic extract) with four concentration of *C. cainito* were prepared: 10 µg /mL, 100 µg /mL, 500 µg /mL and 1000 µg /mL. By using the same amounts of the three extracts, the stock solutions were prepared. Then, 36.5 mg, 25.18 mg and 35.4 mg were weighed and separately dissolved with plenty of solvent to prepare the 10,000-ppm stock solution. For the extraction of alcohol-based extracts, ethanol was used as extraction solvent and allowed to standby to be evaporated for two days. The same amounts of Dimethyl sulfoxide (DMSO) were added to the extracts except for the decoction. From the prepared stock solutions, 1000 ppm, 500 ppm, 100 ppm and 10 ppm concentrations from the stock solution were prepared by serial dilutions. For each extract, three replicates were prepared while the 5 mL sterile filtered seawater served as the control set up. The Ten nauplii were added to every prepared extracts whereas another 10 nauplii were added to the control set up (sterile seawater). The test tubes were then observed and examined the number of dead (non-motile) nauplii in each test tube and was documented after 6 hours and 24 hours.

Data Analysis

To analyze the result, the Reed-Muench statistical method was employed to assess the degree of toxicity of the *C. cainito* extracts to the test organisms. The response of *A. salina* was tested under various concentrations of the extract. The Lethality concentration (LC_{50}) constitutes the dose lethal to the 50 % of the population of the *A. salina*. To determine the lethality, the number of observed mortality (y-axis) was plotted versus log of concentration (x-axis). The concentration that inhibited 50% mortality constitutes the LC_{50}.

RESULTS AND DISCUSSION

The results showed that extraction with absolute ethanol and 50:50 ethanol-water successfully extracted the bioactive compounds in the *C. cainito* leaves. The effects of the various levels of concentrations of *C. cainito* Linnaeus extracts on the mortality of brine shrimp Nauplii were shown in table 1. The brine shrimp mortality rates treated with the ethanolic and blended extract of hydro-ethanol was both 16.67 % at10 µg/ml and 100% at 1000 µg/ml. Meanwhile, the decoction extracts only

brought about 16.67% and 96.67% mortality rates at 10 and 100µg/ml, respectively. Consequently, the LC_{50} range of the three extracts was 25.85 to 252µg/ml. Based on the pattern of mortality rates, it can be inferred that the cytotoxic property of *C. cainito* is highly dose-dependent, as the concentration of the extract increased, the percentage of mortality rates and cytotoxicity of the extract towards the brine shrimp also increases. The cytotoxicity property of the extracts can be assessed based on the activity of the crude extract, it is highly toxic (active) if the value of LC_{50} has less than 1000 ppm while non-toxic (inactive) if it is greater than 1000 ppm (Meyer et. al. 1982). The results indicated that the three prepared extracts of *C. cainito leaves* have shown potential cytotoxic activity against brine shrimp.

Table 1: The effects of the various concentrations of *C. cainito* Linnaeus extracts on the mortality of brine shrimp Nauplii

Extracts	Concentration (µg/ml)	Mortality of brine Shrimp (%)		LC_{50} of Extract,(µg/ml)	
		After 6 H	After 24 H	Acute	Chronic
Ethanolic	1000	100	100	51.58	25.85
	500	86.67	100		
	100	76.67	93.33		
	10	16.67	16.67		
Hyrdo-ethanolic	1000	90	100	230.41	84.14
	500	100	100		
	100	16.67	53.33		
	10	0	16.67		
Decoction	1000	63.33	96.67	578.76	252.64
	500	53.33	73.33		
	100	6.67	23.33		
	10	10	16.67		

It was previously reported that decocted leaves of *C. cainito* is used to treat various diseases of digestives systems such as constipation, diarrhea, stomach ulcer, and rectal inflammation. The pharmacological properties of *C. cainito* may be rooted on its rich phytochemical contents such as flavonoids, anthraquinones, triterpenoids and notable elevated content of phenols (Li et. al. 2015; Chel-Guerero et. al. 2017).

C. cainito has been reported to possess antioxidant properties (Li et. al. 2015; Chel-Guerero et. al. 2017) and wide range of antibacterial activities as it inhibits the growth of *Staphylococcus aureus*, *Micrococcus varians*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Proteus vulgaris* (Oputah et. al. 2016). Moreover, the ethanolic extract of *C. cainito* leaves was shown to have an antimicrobial activity against *S. aureus*, *E. coli*, *Salmonella typhimurium*, and *Shigella* spp. (Duyilemi and Lawal, 2009).

It is highly plausible that the, the antimicrobial property of *C. cainito* could be attributed to the ability to bind to the bacterial cell wall, thereby blocking its synthesis probably because of the saponins, flavonoids, tannin, steroid, and cardiac glycoside (Oranusi

et. al. 2015).The other species of Chrysophyllum like the seed extracts of *C. albidum* possesses rich phytochemicals such as carbohydrates, cardiac glycosides, fatty acids, flavonoids saponins, quinones, and terpenoids while the aqueous extracts of the fruit of *C. albidum* possess antioxidant properties attributed to its high phenolic compounds (Oputah et. al. 2016).

Moreover, the bark of other species of *Chrysophyllum*, the bark of *C. pruniforme* is also abundant with phytochemicals such as flavonoids, saponins, tannins, reducing sugars, polyphenols, and anthraquinones (Angone et. al. 2013). So the cytotoxic activity of the ethanolic extracts can be attributed to its rich content of phytochemicals, antioxidants, and antimicrobial activities. The brine shrimp lethality essay proved to be a useful tool in the initial screening of potential bioactive compounds present in the plants. Moreover, to fully utilize the medicinal importance of *Chrysophyllum cainito*, further studies on the determination specific medicinal properties are highly desirable.

CONCLUSION

The result of the study demonstrated the toxicity of the ethanolic extract of *C. cainito*, which is very useful in the utilization of the species for further studies. The results indicated the presence of bioactive compounds which can be attributed to the plant's toxicological effects. Moreover, the results support the use of this plant species to be subjected for further pharmacological study to explore its specific medicinal properties. In addition, the present study supports the use of brine shrimp (*Artemia salina*) bioassay as a reliable, simple, and convenient method in the initial screening of bioactive compounds in medicinal plants.

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