Evaluation of the cytotoxicity potential of ethanolic extract of *Chrysophyllum cainito* using the brine shrimp lethality bioassay

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Abstract

The 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 25.85 μg/ml and 84.14 μg/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 World Health Organization, 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 & Le, 2020). *C. cainito Linn* is a tree mostly found in the tropics including Southeast Asia (Rymbai *et al*., 2015). This tropical tree belongs to the family Sapotaceae and is native to Greater Antilles and the West Indies (Luo *et al*., 2002; (Oranusi *et al*., 2015). *C. cainito* tree has a spreading crown, growing to a height of 15 meters. Branches are numerous and slender, the young tips are copper-colored and covered with appraised hairs. The leaves of *C. cainito* can be characterized as leathery ovate or oblong with approximately 7.5 *to* 13 centimeters long with pointed tip, blunt or rounded at the base and has covering underneath with silky, golden-brown, and soft hairs (Shailajan and Gurjar, 2014).

C. cainito has several health benefits and has been chemically tested and proven (Doan & Le, 2020). Various studies were conducted through extractions of its fruit, pulp, seeds, and leaves. In 2002, Luo found polyphenolic antioxidants present in a *C. cainito* fruit. Fruit extracts were tested not only for their antioxidant activity but also for their antidiabetic effect (Hegde et al., 2016) and gastroprotective activity (Da Rosa et al., 2019). Moreover, (Oranusi (2015) tested the pulp and seed extracts for antimicrobial activity. 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 antidiabetic 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 in the treatment of sterility, sexual asthenia, and asthma; while seeds were mostly used to cure intestinal worms and hemorrhoid. The bark has been used for treating cough, icterus, yellow fever and the fruits against avitaminosis and dental decay. The treatment of ulcer and varicella is not related to specific plant part (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). Brine shrimp lethality assay is an indispensable tool 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* (Olowa and Nuneza, 2013), *Kleinhovia hospita* (Morilla et al., 2015), *Acmella grandiflora* (Elias et al., 2014), *Ficus nota* (Arquion et al., 2015), *Phyllanthus niruri* and *Passiflora foetida* (Juario et al., 2015).

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 cytotoxic property against the brine shrimp nauplii and correlated with the known pharmacological activities 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

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. In this study, extracts from *C. cainito* leaves were prepared by using three types of extraction solvent systems, namely: decoction, hydro-ethanol (50:50), and ethanolic extraction. The decoction extracts were prepared by cutting fresh and clean plant leaves into small pieces and boiled in distilled water in 1:2 ratios for 5 minutes. Plant samples were gradually cooled to 25^{o}C prior to filtration then freeze-dried succinctly to remove excess amount of water. For the hydro-ethanol 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 until the samples were already crispy enough upon prickling. The dried samples were ground using a sterile electric blender. The powdered plant samples were weighed, divided into two equal parts and stored in glass containers; one was percolated with enough absolute ethanol and the other one was soaked with 50:50 water-ethanol mixtures for three days (72 h). The prepared solutions were filtered using Whatman filter paper and collected in a glass container. Sufficient amount of the filtered ethanol solution was subjected to rotary

evaporation to obtain the ethanol extract. The hydro-ethanol mixture extract was concentrated *in vacuo* and subsequently freeze-dried to obtain the hydro-ethanolic extract.

Brine Shrimp Lethality Test.Brine shrimp eggs were obtained from the Chemistry Department of MSU-IIT. The filtered sterile seawater was decanted in a hatching chamber with a partition for dark (covered) and light areas. Shrimp eggs were put into the dark portion 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 right after two days. Four concentrations of the three extracts (decoction, hydro-ethanolic mixture, and ethanol) of *C. cainito* were prepared: 10 μg /mL, 100 μg /mL, 500 μg /mL and 1000 μg /mL. To prepare the stock solution, exact amounts of the three extracts (decoction, hydro-ethanolic, and ethanolic), 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 allowed to evaporate for two days. After the evaporation of ethanol, dimethyl sulfoxide (DMSO) was added to the two extracts, except for the decoction. From the prepared stock solutions, 10 ppm, 100 ppm, 500 ppm and 1000 ppm concentrations were prepared through serial dilution. Three replicates were prepared for each extract and 5 mL of filtered sterile seawater served as control. Ten nauplii were added to each of the prepared extracts whereas another 10 nauplii were added to the sterile seawater. The test tubes were examined and the number of dead (non-motile) nauplii in each test tube was recorded after 6 hours and 24 hours.

Data analysis

Reed-Muench statistical method was used to assess the relative toxicity of the C. *cainito* extracts to living organisms. The response of *A. salina* was tested under various

concentrations of the extract. The LC_{50} represents the dose lethal to the half population of the *A. salina*. To determine the lethality, the mortality (y-axis) was plotted versus log of concentration (x-axis). The concentration that triggered 50% mortality is 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 different 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 hydro-ethanolic extracts were both 16.67 % at 10 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 252 µg/ml. Based on the pattern of mortality rates, it can be inferred that the cytotoxic property of *C. cainito* is dose-dependent, as the concentration of the extract increased, the percentage of mortality rates and cytotoxicity of the extract towards the brine shrimp also increased. The cytotoxicity activity of the extracts can be assessed based on (Meyer et al. (1982) where a crude plant extract is toxic (active) if it has an LC_{50} value of less than 1000 ppm while non-toxic (inactive) if it is greater than 1000 ppm. The results indicated that the three prepared extracts of *C. cainito leaves* are potent or active against brine shrimp.

Table 1: The effects of the different 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 decoction of the leaves of *C. cainito* is used to treat various diseases of digestives systems such as constipation, diarrhea, stomach ulcer, and rectal inflammation (Balinad and Chan, 2017). The pharmacological properties of *C. cainito* may be rooted on its rich phytochemical contents such as flavonoids, anthraquinones, triterpenoids (Guererro et al. (2017) and notable elevated content of phenols (Li et al., 2015et al, Guererro et. al, 2017).

C. cainito has been reported to possess antioxidant properties (Li et al. (2015) 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 *E. coli*, *S. aureus*, *Shigella spp*., and *Salmonella typhimurium* (Duyilemi and Lawal, 2009).

Accordingly, the antimicrobial potential of *C. cainito* could be due to its ability to bind to the cell wall of the bacteria, thereby inhibiting 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* Posses rich phytochemicals such as saponins, carbohydrates, flavonoids, quinones, cardiac glycosides, fatty acids, and terpenoids (Oputah et al., 2016) while the aqueous extracts of the fruit of *C. albidum* possess antioxidant properties attributed to its high phenolic compounds.

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 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 in traditional medicine. In addition, the present study proves the utilization of the 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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