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Brine Shrimp Lethality Assay on *Citrus microcarpa* Bunge (kalamansi)

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ABSTRACT
The extract of *Citrus microcarpa* Bunge leaves was evaluated for brine shrimp lethality in different concentrations (1000 µg/mL, 500 µg/mL, 100 µg/mL, and 10 µg/mL). All experiments were done in triplicate, and the mean result was noted. The lethal concentration LC_{50} of the test samples after 6 hours and 24 hours was obtained. Using probit analysis, the lethality concentration (LC_{50}) was assessed at 95% confidence intervals. LC_{50} of less than 100 µg/mL was considered as potent (active). Absolute ethanol Extract has the highest value of being potent or having the highest value for having bioactive components compared to the other two extracts. It has the highest percent mortality relative to the other two extracts, and it also has the highest acute toxicity value for 6 hours of exposure to *Artemia salina* with a value of 434.01 µg/mL, while the chronic toxicity is 29.01 µg/mL. The result indicates that the prepared extract was rich in bioactive compounds. Thus, Brine Shrimp Lethality Test is a convenient monitor for screening and fractionation in the discovery and monitoring of bioactive natural products.

KEYWORDS: bioassay; bioactive compound; Citrus microcarpa; medicinal plant; plant extract

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1. Introduction

Research on medicinal plants has witnessed a global surge, necessitating the collection of evidence to showcase the immense potential of these plants in traditional systems and tribal communities. Individuals from ancient and modern cultures have extensively utilized medicinal plants to treat a wide array of ailments. It is noteworthy that a single plant, when processed into different formulations, can effectively address a diverse range of diseases [1]. The continued utilization and reliance on medicinal plants by a significant portion of the world's population underscore their universality and efficacy. Nevertheless, it is crucial to recognize that the use of medicinal herbs for disease treatment, prevention, and pharmacological development does not guarantee

their safety when employed without proper knowledge or control by the public [2]. In fact, the misuse or contamination of medicinal plants can result in injuries and even fatalities [3].

A commonly employed bioassay for assessing the broad spectrum of bioactivity in plant extracts is the brine shrimp lethality bioassay (BSLT). This technique is simple to master, cost-effective, and requires only small quantities of test material. Its purpose is to serve as an initial screening tool, which more specific and costly bioassays can subsequently complement once the active compounds have been isolated [4].

Kalamansi, scientifically known as *Citrus microcarpa* Bunge, belongs to the Rutaceae family and is indigenous to the Philippines, where it is extensively cultivated. This plant species is endemic to the Philippine archipelago and is not naturally found elsewhere. It is a smooth plant with slight spines, reaching a height of 3 to 5 meters. The leaves are elliptic to oblong-elliptic, measuring 4 to 8 cm in length. The petioles are narrow and barely winged, approximately 1 cm long. The flowers are solitary, axillary, occasionally paired, and white, with short stalks. When ripe, the fruit is yellow, nearly spherical, 2 to 3.5 cm in diameter, with 6 to 7 cells and thin skin [5]. While the fruit is known for its effectiveness in treating coughs and colds, the leaf extract is believed to have antihypertensive properties [6].

The use of herbal preparations in their unrefined state, combined with the lack of specificity or precision in traditional medicine practices, may lead to the risk of overdosing on herbal medicines. This can result in the accumulation of essential and non-essential plant components in the human body, potentially reaching toxic levels. Individuals who heavily rely on unrefined herbal products are particularly susceptible to this problem, which can have severe consequences on their biochemical and genetic systems. However, this adverse condition can be prevented by early detection of the genotoxic/cytotoxic effects caused by the plant extract and identifying the potentially lethal dosage levels. The Brine Shrimp Lethality Test can be utilized to determine the cytotoxic and/or genotoxic effects of *C. microcarpa* [7].

This study aims to evaluate the genotoxic potential of decoction, mixture (50:50) water and ethanol, and absolute ethanol leaf extract of *Citrus microcarpa* Bunge (kalamansi leaves) using Brine Shrimp Lethality Test. It also aimed to determine the relation between toxicity results with their known ethnopharmacological activities.

2. Materials and Methods

For botanical identification, small branches or twigs with reproductive structures, healthy leaves, stipules, bark, and wood samples from each plant are collected in duplicates following proper documentation and labeling protocols [7]. Specimens are sent to a local botanist for confirmation of identification and voucher specimens are deposited at the local herbarium.

For the materials of study to be used in the preparation of crude extracts, about 2-3 kg of fresh samples of the plants/plant parts are properly washed in tap water and then rinsed in distilled water. The rinsed samples are air-dried for one week. The dried samples of each plant are pulverized using a sterile electric blender, weighed and percolated with enough 95% ethanol for three days. Each solution is then filtered,

concentrated in vacuo at temperatures not exceeding 40°C and weighed to give the crude ethanol extract. A portion of the crude ethanol extract is then sequentially partitioned in hexane:water and chloform:water solutions. The hexane-soluble, chloroform-soluble and aqueous soluble portions are individually concentrated in vacuo and weighed to give the crude hexane, chloroform, and aqueous extracts, respectively.

For the preparation of the plant decoctions, about 1 kg of fresh and clean samples of the plants are cut into pieces and boiled in a sufficient amount of distilled water (1:2 ratio) for 5 min. The mixture is then filtered, cooled, and stored in glass containers until required.

Brine shrimp eggs were obtained from the Department of Chemistry of Mindanao State University. Sea water was filtered and sterilized 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 to the dark side of the chamber while the lamp above the other side (light) will attract the hatched shrimp. The shrimp were allowed to hatch two days and mature as nauplii (larva). After two days, when the shrimp larvae were 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. The dilution were: 1000 µg/mL, 500 µg/mL, 100 µg/mL and 10 µg/mL. Dimethyl sulfoxide (DMSO) was added with 150 µL volume to 1000 µg/mL and 500 µg/mL and 75 µL DMSO volume to 100 µg/mL and 10 µg/mL. 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 was counted and recorded after 24 hours. The lethality concentration (LC_{50}) was assessed using probit analysis at 95% confidence intervals. The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number and multiplying by 100%.

3. Results

The summary of acute toxicity and chronic toxicity or the lethal concentration LC_{50} of the test samples after 6 hours and 24 hours against sample concentration (toxicant concentration) is shown in Table 1.

The values of the percent mortality with the corresponding dosage of the decoction for 6 hours and 24 hours exposure of *Artemia salina* were lower compared to the other extract doses. The Acute and Chronic toxicity for this is greater than 1000 µg/mL. Since it has an LC_{50} value of greater than 1000 µg/mL, it is considered non-toxic.

On the other hand, the percent mortality with the corresponding dosage of the mixture (50:50) water and ethanol Extract for 6 hours and 24 hours exposure of *Artemia salina* was higher compared to the decoction extract. Acute toxicity has a value of greater than 1000 µg/mL and is also considered non-toxic at such level. However, chronic toxicity for this is 39.02 µg/mL, indicating that it has potent or may have a bioactive component.

Comparing the three extracts, Absolute ethanol Extract has the highest value of being potent or having the highest value for having bioactive component. It has the highest percent mortality relative to the other two extracts. The acute toxicity for 6

Table 1. LC_{50} values of the *Citrus microcarpa* Bunge 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	9.38	26.5	>1000	>1000
	100	3.39	7.14		
	500	0	1.22		
	1000	0	0		
50:50 Water-Ethanol Mixture	10	28.13	100	>1000	39.02
	100	3.77	98.04		
	500	0	63.64		
	1000	0	4.76		
Ethanol	10	93.88	100	434.01	29.01
	100	57.58	100		
	500	58.14	88.57		
	1000	0	14.71		

hours exposure of *Artemia salina* to this extract is 434.01 µg/mL while the chronic toxicity is 29.01 µg/mL.

4. Discussion

Citrus microcarpa Bunge fruits are known to cure cough and colds, while the leaf extract is thought to lower hypertension [6]. The extract of *Citrus microcarpa* Bunge leaves was evaluated for brine shrimp lethality in different concentrations. All experiments were done in triplicate and the mean result was noted. The lethal concentration LC50 of the test samples after 6 hours and 24 hours was obtained. 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). As mentioned by Meyer and others, an LC_{50} value of less than 1000 µg/mL is toxic, while an LC_{50} value of greater than 1000 µg/mL is non-toxic. The death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.

As reported previously, bioactive compounds are almost always toxic in high doses [8]. Pharmacology is simply toxicology at a lower dose, and toxicology is simply pharmacology at a higher dose. Thus, *in vivo* lethality in simple zoologic organisms such as *Artemia salina* can be used as a convenient monitor for screening and fractionation in discovering and monitoring bioactive natural products. The evaluation of the pharmacological component of substances from plants is an established method for identifying lead compounds that can direct the path to developing novel and safe medicinal agents. The result of this investigation can be added as a clue in its connection to the pharmacologic and cytotoxic activity of the plant extract.

The study of the phytochemical composition of extracts from citrus plants revealed that citrus species have bioactive compounds [9]. The phytochemical analysis of five varieties of citrus species: sweet orange (*Citrus sinensis*), tangerine (*Citrus*

reticulta), lemon (*Citrus limonum*), lime (*Citrus aurantifolia*), and grape (*Citrus grandis*) revealed the presence of bioactive compounds comprising alkaloids (0.22-1.60%), saponin (0.30-0.98%), flavonoids (0.30-0.89%), phenols (0.02-0.64%) and tannins (0.23-1.45%). The content of alkaloids was low in the leaves compared to the peels. This observation was also made in the results of the phenolic content, where the values of phenol in the peels are higher than those in the leaves. Alkaloids, phenolic, and other bioactive compounds exhibit inhibitory actions on microorganisms [9]. This could explain the claim of the people using the crude extract of the plant that they have been treated with the disease they have after using the plant extract. Since extracts and essential oils of plants are mainly characterized by their complex chemical mixture, more bioassay, and more study must be conducted with *Citrus microcarpa* Bunge. In a preliminary assay, the results can be diffuse and not well bio-directed; however, continuing the phytochemical fractionation and testing the sub-fractions using the protocol described previously can identify potential bioactive natural products [10].

4. Conclusion

The findings of this study indicate that the leaf extract of *Citrus microcarpa* Bunge has potential bioactive natural products. Although crude extracts from various parts of *Citrus microcarpa* Bunge have had medicinal applications from time immemorial, modern drugs can be developed after extensive investigation of their bioactivity, mechanism of action, pharmacotherapeutics, and toxicity after proper standardization and clinical trials. Extensive research is required on *Citrus microcarpa* Bunge and its product for therapeutic utilization.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Author Contributions: All authors have contributed equally. They have approved the final version of this manuscript.

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