

Brine Shrimp Lethality Test of *Kleinhovia hospita* stem and bark from Agusan del Sur, Philippines

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Abstract. Plants with medicinal importance are now widely studied because of their major contribution to healthcare practices. Several pharmacological evaluations are employed to detect the bioactivity of these medicinal plant species on test organism. In this study, mortality effects and Median lethal concentrations (LC₅₀) of *Kleinhovia hospita* extracts on *Artemia salina* were determined through the Brine Shrimp Lethality Test. The results showed weak to moderate toxicity of the extracts on the brine shrimps with LC₅₀ values of 698.54 µg/mL for the decoction and 452.03 µg/mL for the ethanolic extract. The bioactivity of *Kleinhovia hospita* extract was more apparent in ethanol than in decoction extract which indicates that extraction with ethanol is effective in obtaining bioactive components of the plant species being studied. The bioactivity of the extracts supports the uses of this plant in traditional medicine.

Key Words: bioactivity, ethanolic extract, LC₅₀, Medicinal plant, toxicity.

Rezumat. Plantele cu importanță medicinală sunt acum studiate pe larg datorită contribuției lor majore în practicile medicale. Diferite evaluări farmacologice sunt efectuate pentru a detecta activitatea biologică a acestor specii de plante asupra unui organism test. În acest studiu, efectele letale și concentrațiile letale mediane (LC₅₀) ale extractelor de *Kleinhovia hospita* asupra speciei de *Artemia salina* au fost determinate prin *Testul de Mortalitate al Artemiei*. Rezultatele au prezentat o toxicitate a extractelor asupra crevetelui *Artemia* de la slabă la moderată cu valori LC₅₀ de 698.54 µg/mL pentru decoct și 452.03 µg/mL pentru extract alcoolic. Bioactivitatea extractului de *Kleinhovia hospita* a fost mai vizibilă în extractul alcoolic decât în decoct, ceea ce indică faptul că extracția cu etanol este eficientă în a obține componentele bioactive ale plantei studiate. Bioactivitatea extractelor susține utilizarea acestei plante în medicina tradițională.

Cuvine cheie: bioactivitate, extract alcoolic, LC₅₀, plantă medicinală, toxicitate.

Introduction. Medicinal plants are now more widely known and used worldwide because of their potential as pharmaceutical products. Countless plants have been reported to have medicinal properties. These plants are not only ornamental, they also exhibit antiviral, antifungal, antibacterial, antioxidant and anti-inflammatory functions (Okwu & Uchenna 2009). They are sources of drugs and are used in herbal medicine as remedy and cure to various types of diseases. Undeniably, medicinal plants have a big contribution on healthcare practices. For this reason, laboratory investigations have been employed on medicinal plants for their active components and potential (Ignacimuthu et al 2006). Through pharmacological evaluations of plant substances, principal compounds are identified which leads to the development of novel and safe medicinal agents (Rana et al 2012).

One of the plants which has been reported of having medicinal property is *Kleinhovia hospita*, a plant species under family Sterculiaceae. *K. hospita* is an evergreen, tropical tree native to Indonesia, Malaysia and other parts of tropical Asia (Young-soo 2009). It is the only species in its genus and is common in secondary forests, shrubberies, and deserted clearings at low and medium altitudes throughout the Philippines (Pancho & Gruezo 2006). It is a plant which has been traditionally used in Indonesia as therapy to cure liver disease (Soekamto et al 2008). Bark of *Kleinhovia hospita* is also used in Papua New Guinea to relieve cough and tuberculosis (Holdsworth

1977). In the Philippines, *Kleinhovia hospita* was documented as treatment for relapse (Miano et al 2011).

Natural products from the extracts of medicinal plants such as *Kleinhovia hospita* provide infinite opportunities for new drug leads because of the unmatched availability of chemical diversity (Sasidharan et al 2011). Bioactive compounds from plant species are almost always toxic in high doses (Moshafi et al 2009) thus, it is necessary to evaluate the bioactivity of plant species for their possible cytotoxic effects.

Brine shrimp lethality assay (BSLA) is a simple, high throughput cytotoxicity test of bioactive chemicals and natural products (Meyer et al 1982). It is based on the killing ability of test compounds on a simple zoological organism, the brine shrimp (*Artemia salina*) (Harwig & Scott 1971) and is also a convenient monitor for screening and fractionation in the discovery of bioactive natural product (McLaughlin & Rogers 1998).

This study aims to investigate the toxicity level of the stem and bark of the *Kleinhovia hospita* which is commonly used for wound cleansing and wound healing by the Manobo tribe living in Talacogon, Agusan del Sur. Using the brine shrimp lethality test, the Medium Lethal Concentrations (LC₅₀ values) were determined to assess the bioactivity of the plant extract being administered as therapeutic agent and to distinguish the presence of any potential sources of novel cytotoxic compounds.

Materials and Methods

Plant collection. Fresh bark and stem of *Kleinhovia hospita* were collected randomly from Desamparados, Talacogon, Agusan del Sur. This plant was selected because of its medicinal usage as wound cleansing and wound healing as gathered from interview with Mr. Lucesio Durango, Tribal chieftain of Manobo tribe in Desamparados, Talacogon, Agusan del Sur. Plant samples were identified by Professor Edgardo Aranico from the Department of Biological Sciences, Mindanao State University - Iligan Institute of Technology, Philippines.

Plant decoction preparation. Extraction is the most important step in the analysis of constituents present in botanicals and herbal preparations (Sasidharan et al 2011). About 450 grams of fresh and clean samples of the *Kleinhovia hospita* were cut into pieces and boiled in about 900 mL distilled water corresponding to a 1:2 ratio of water and sample for five minutes. The mixture was filtered and was freeze dried to remove traces of water, cooled and stored in vials until needed for the lethality testing.

Crude extracts preparation. One to two kilograms of fresh samples (stem and bark) were properly washed with tap water and rinsed with distilled water. The rinsed samples were air-dried for two weeks. The dried samples were pulverized using a sterile electric blender.

The powder was weighed, and was stored in glass containers. Enough absolute ethanol was then used to saturate the powdered samples for three days. The solution was filtered using Whatman filter paper and collected in a glass container. The filtered solution was then concentrated in a rotary evaporator to get the crude plant extract.

Brine shrimps hatching. Brine shrimp eggs were obtained from Mindanao State University-Naawan. The eggs were rehydrated with distilled water for 30 minutes and were transferred to a glass container containing filtered sterile seawater. The hatching chamber has two partitions: dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber. The other side was lighted with lamp to attract the hatched nauplii. The nauplii were subjected to strong aeration until needed for the toxicity test.

Brine Shrimp Lethality Test. To obtain 10,000 µg/mL stock solution, 30 milligrams of each of the extracts were dissolved in 3mL sterile seawater. For emulsification of the ethanolic extract, a few drops of dimethyl sulfoxide (DMSO) were also added. In triplicate, serial dilutions (1000 µg/mL, 500 µg/mL, and 100 µg/mL) were made for each test tube.

Ten nauplii were collected and transferred to the test tubes using glass dropper; sterile seawater were then added to each test tube to produce a 5mL total volume. The number of nauplii survivors was counted after 24 hours.

Statistical analysis. Brine shrimps were exposed to different concentrations of the two extracts: decoction and ethanolic, to determine the relative toxicity. The relationship between the concentration of the extracts and mortality of the brine shrimps was shown by plotting the concentration log (x-axis) versus mortality (y-axis).

The Medium Lethal Concentrations (LC₅₀ values) of *Kleinhovia hospita* stem extracts after 24 hours were determined using trendline fit linear regression analysis in Microsoft Excel, with the dose-response data transformed into a straight line. Equation was obtained from the best-fit line and LC₅₀ was calculated.

Results and Discussion. Results showed that the level of toxicity was observed to be directly proportional to the concentration of the extracts. Mortality rate of the brine shrimp exposed for 24 hours increased with increasing concentration of the extracts. The highest mortality was observed at 1000 µg/mL specifically with ethanolic extract while the lowest mortality was observed at 100 µg/mL (Table 1).

In the present study, both the decoction and ethanolic extracts exhibited LC₅₀ values < 1000 µg/mL, indicating the presence of cytotoxic compounds responsible for the observed toxicological activities (Ngutaa et al 2012). Cytotoxic activity is considered weak when the LC₅₀ is between 500 and 1000 µg/mL, moderate when the LC₅₀ is between 100 and 500 µg/mL, as strong when the LC₅₀ ranges from 0 to 100 µg/mL (Clarkson et al 2004) and designated as non-toxic when the LC₅₀ value is greater than 1000 µg/mL (Oketch-Rabah et al 1999). Result showed that decoction had weak cytotoxic activity since it has LC₅₀ values between 500µg/mL and 1000µg/mL.

Table 1
Mortality Rate and Chronic LC₅₀ Values of *Kleinhovia hospita* extracts on *Artemia salina*

Extract (µg/mL)	Mortality, %	Chronic LC ₅₀ , µg/mL
Decoction		
1000 µg/mL	70%	
500 µg/mL	33%	698.54
100 µg/mL	17%	
Ethanol		
1000 µg/mL	77%	
500 µg/mL	77%	452.03
100 µg/mL	13%	

In traditional medicine, most of the extracts are prepared as decoctions, which suggest that the traditional way of obtaining the extract poses no threat of acute toxicity since the LC₅₀ is >500 µg/mL (Moshi et al 2010). On the other hand, the ethanolic crude extract of *K. hospita* has LC₅₀ values between 100µg/mL and 500µg/mL, and can be categorized as having moderate cytotoxicity.

The ethanolic extract of *Kleinhovia hospita* (stem and bark) was more active against the organisms studied having 452.03 µg/mL LC₅₀ value as compared to the decoction with 698.54 µg/mL LC₅₀ value. Thus, extraction with ethanol was a better way of obtaining bioactive compounds from the stem and bark of *K. hospita*.

Bioactive compounds are naturally produced in plants (Castillo et al 2012). The effects of bioactive compounds may be positive or negative depending on the substance, dose or the bioavailability (Guaadaoui et al 2014). Initial screening of medicinal plants for

possible bioactivity typically starts by using crude aqueous or alcohol extraction and can be followed by various organic extraction methods (Vileges et al 1997). In many studies, it had been reported that various plants and plant-based foods have been subjected to extraction of antioxidant compounds by using ethanol (Sultana et al 2009).

Previous related study showed that coumarin and steroid compounds isolated from stem bark of *Kleinhovia hospita* L. from Indonesia also exhibited moderate activity to *Artemia salina* (Soekamto et al 2008). Investigation on the leaves of *Kleinhovia hospita* extract was also reported to have antitumor activities in animal model (Latiff et al 1997) as well as potent antioxidant activity and moderate cytotoxicity on HepG2 liver cancer cells (Arung et al 2009). The brine shrimp test represents a rapid, inexpensive and simple bioassay for testing the plant extract lethality which in most cases correlates reasonably with cytotoxic properties (Baravalia et al 2012) and is also very useful in providing a preliminary screening that can be supported by a more specific bioassay, once active compounds are isolated (Olowa & Nuñez 2013). *In vitro* study of medicinal plants showed that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms which can also be an economic and safe alternative to treat infectious diseases (Mathur et al 2010). Plant extracts that show moderate to weak toxicity on *A. salina* at the concentrations tested may have low toxicity towards human cell lines and therefore may have potential value as antiseptic and cleaning agents (Cock 2007). These results may validate the medicinal use of *Kleinhovia hospita* in wound cleansing and wound healing.

Conclusion. The bioactivity of the *Kleinhovia hospita* extracts could be of high importance for further pharmacological studies. The results of the present work provide useful baseline information in the search for new active compounds and potential uses of *Kleinhovia hospita* extract. Nevertheless, further studies for more elaborate assays are needed to verify the suitability of these extracts for their specific purpose in pharmacology.

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