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Larvicidal and ovicidal activities, characterization and stability of *Anacardium occidentale* (Cashew) shell wastes

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Abstract

Dengue and zika continue to be the most rapidly emerging febrile diseases and pose a negative impact on social and economic activity of the country. Despite of recent innovation in dengue vaccine, eradication of the vector, *Aedes aegypti*, is still the best way to inhibit dengue and zika outbreaks. In this study, we evaluated the larvicidal and ovicidal activities and characterized the shell wastes of *Anacardium occidentale* including its stability for a period of two (2) years. The shell wastes were extracted using 95% EtOH and n-hexane and were bio-assayed for larvicidal and ovicidal activities against *A. aegypti* following the WHO standard bioassay method. The mortality was observed 24 and 48 hours after treatment and data were subjected to probit analysis to determine lethal concentrations (LC_{50} and LC_{90}). The ethanol extract was characterized by thin-layer chromatography (TLC) and phytochemical analysis.

Both ethanol and hexane extracts exhibited significant larvicidal activity with LC_{50} and LC_{90} of 3.29 mg/L and 8.13 mg/L; and 7.31 mg/L and 13.55 mg/L, respectively. The ovicidal activity of the ethanol extract was 8.06 mg/L and 15.53 mg/L, respectively. Phytochemical screening of the crude ethanol extract of cashew shell wastes indicated the presence of phytochemical constituents such as unsaturated steroids and triterpenoids, free fatty acids, fats and oils, flavonoids, leucoanthocyanins, anthraquinones, and tannins. TLC showed bioactive components of the extract. The larvicidal activity of the ethanol extract decreased slightly after two (2) years with an LC_{50} of 4.20 mg/L and LC_{90} of 9.87 mg/L. The ethanol extracts of *A. occidentale* showed promising potential as an alternative source to control the spread of the dengue and zika vector, *A. aegypti*.

Introduction

Dengue is a severe, often fatal, most rapidly emerging febrile disease transmitted by female *Aedes aegypti* mosquitoes. In the Philippines, dengue fever and dengue hemorrhagic fever are widespread in all its regions according to World Health Organization. A total of 117, 658 dengue cases and 433 deaths were reported in the year of 2013 [1]. Zika is caused by a virus transmitted primarily also by *A. aegypti*. This is a mosquito-borne flavivirus that was first identified in Uganda in 1947. Zika virus infection during pregnancy is a cause of congenital brain abnormalities, including microcephaly. Zika virus is a trigger of Guillain-Barre syndrome. There are 57 reported cases of zika from six (6) regions in the country (DOH, 2017). However, there were no reported deaths until this time. These are the reasons behind the efforts of many institution and research bodies to find a solution in controlling the vector of dengue and zika, *A. aegypti*. Use of chemical control is said to be an effective way used generally by most of the people [2] but these controls have negative drawbacks [3] like insecticide resistance [4], environmental pollution, toxic hazards to human and other non-target organisms [5]. This results in the increasing attention and consideration to natural products [6]. Natural products in various forms have been used for the treatment of pathological conditions [7]. Green larvicides are now being considered because most plants are said to be non-toxic and biodegradable. Unlike the conventional insecticide which is based on single active ingredient, green insecticides comprise of bioactive chemical variations which have behavioral and physiological activities [8]. The researches about the interactions between plants and insects revealed the potential use of plants in fundamental pest control programs [9].

Anacardium occidentale commonly known as "cashew" is a tree in the family Anacardiaceae which produces a seed that is harvested as the cashew nut. The cashew nut is a popular snack and food source. Nigeria was the world's largest producer of cashew nuts with shell in 2010 [10]. The Philippines is actually sixth among the top 10 producers of cashew nuts with shell as of 2010.

Our main focus is now on the research study of *A. occidentale* as a potential vector control measure against *A. aegypti* that is cost effective and more importantly, safe for the environment. The use of natural alternatives such as plant extracts can provide the best option as environmentally safe natural larvicides. Therefore, the objectives of the study were to evaluate the larvicidal and ovicidal toxicities of the ethanol and hexane extracts of the shell wastes of *A. occidentale* against *A. aegypti* including its stability and to characterize the ethanol extract by qualitative phytochemical analysis and thin-layer chromatography (TLC).

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Experimental

Botanical material

The cashew shell wastes were collected in Puerto Princesa City, Palawan, Philippines during rainy season in the months of July and August. Specimen label was placed in the plant. Herbarium specimen of the plant collected was prepared and was sent to the National Museum of the Philippines for necessary identification and authentication.

Sample preparation

The cashew shell wastes were ground with Wiley mill (Thomas Scientific, Model 3379-K05-99). About 300 grams of ground plant material were macerated with 900 mL of 95% EtOH and n-hexane (Chemline Manila, Philippines) for 48 hours. Then, the mixture was filtered with a coarse filter paper. The filtrate was concentrated under vacuum at 55°C to a syrupy consistency. This was further evaporated in a water bath at the same temperature to semi-solid extract.

Rearing of *Aedes aegypti* larvae

The 3rd and early 4th instars larvae of *A. aegypti* used in the study were reared in the Insectary of the Industrial Technology Development Institute – Department of Science and Technology, Taguig City, Metro Manila, Philippines at 27 ± 2°C, 80 ± 5% humidity and a photoperiod of 12 h light followed by 12 h dark (12L: 12D).

Preparation of stock solutions and serial dilutions

A 1% or 10,000 mg/L stock solution was serially diluted in dechlorinated water to different concentrations used in the assay.

Larvicidal Bioassay against 3rd and 4th instars larvae of *A. aegypti*

The larvicidal bioassay was conducted following the WHO method [11]. The test larvae were exposed to a wide range of test concentrations from 5 to 50 mg/L and a control to determine the effective dose. After determining the mortality of the larvae in this wide range of concentration, a narrower range from 2 to 25 mg/L was re-evaluated to determine the lethal concentrations level. The appropriate volume of each extract used in each concentration was added to dechlorinated water to make a 100mL volume of solution starting with the lowest concentration. Homogenous batches of twenty 3rd or 4th instars larvae were added by means of droppers to each vessel containing 100 mL volume of solution concentration under test. Four replicates were set up for each concentration and an equal number of controls were set up simultaneously with dechlorinated water, to which 1 mL EtOH was added. For negative control, dechlorinated water with 1% ethanol was used. The test containers were held at 27 ± 2°C and a photoperiod of 12 h light followed by 12 h dark (12L: 12D). Each test was run three times on different batches of larvae. The results were recorded where the LC_{50} and LC_{90} values and slope and heterogeneity analysis were also noted. Stability of the ethanol extract was determined after two (2) years.

Ovicidal Bioassay against 3rd and 4th instars larvae of *A. aegypti*

The *Aedes aegypti mosquito eggs* used in the ovicidal study were reared in the Insectary of Standards and Testing Division, Industrial Technology Development Institute, Department of Science and Technology at a laboratory condition of 27 ± 2°C temperature and 80 ± 10% relative humidity. About 3-5 days old batches of eggs laid in filter papers and dried were used in the test. Batches of 20 eggs were transferred by means of a camel brush to 250 mL beakers containing 100 mL volume of solution concentration under test. Four replicates were set up at each concentration with an equal number of controls set up simultaneously. For negative control, dechlorinated tap water with 1% ethanol was used. The test containers were held at 25–28°C and relative humidity of 80 ± 10%. After 72 hours exposure, the egg emergence was recorded.

Data analysis

Data from all replicates were pooled for linear regression probit analysis and calculated using SPSS for Windows including 95% confidence limit. A test series is valid if the relative standard deviation (or coefficient of variation) is less than 25% or if confidence limits of LC_{50} overlap (significant level at $P < 0.05$).

Phytochemical analysis

The qualitative phytochemical analysis of the extract was performed [12] to detect the presence of secondary metabolites such as alkaloids, tannins, saponins, free fatty acids, unsaturated steroids, triterpenoids, flavonoids, leucoanthocyanins, and anthraquinones.

Thin-layer chromatographic (TLC) fingerprinting/profiling

Characterization of the *A. occidentale* extracts was done by thin-layer chromatographic fingerprinting and profiling to determine presence of bioactive compounds. The TLC was carried out in a 20cm x 20cm pre-coated silica gel $60F_{254}$ TLC plate (Merck, Inc. Darmstadt, Germany) which was cut into squares (8 x 11 cm). The solvent systems used were hexane:EtOAc (3:7) and toluene:EtOAc (93:7). The developed chromatogram was visualized by inspecting under ultraviolet light at 254 nm and 366 nm and exposing to iodine vapors in a glass jar. The chromatogram was photographed and documented. The Rf values of the spots were then calculated.

Results and discussion

Both ethanol and hexane extracts exhibited significant larvicidal activity with LC_{50} and LC_{90} of 3.29 mg/L and 8.13 mg/L; and 7.31 mg/L and 13.55 mg/L, respectively. The ethanol extract also exhibited toxicity against *A.aegypti* eggs at LC_{50} of 8.06 mg/L and LC_{90} of 15.53 mg/L. The larvicidal activity of the ethanol extract after two (2) years decreased slightly with an LC_{50} of 4.20 mg/L and LC_{90} of 9.87 mg/L. Toxicities exhibited by both ethanol and hexane extracts are comparable to the commercially available biolarvicide, Ph Big R with LC_{50} and LC_{90} of 1.71 mg/L and 8.49 mg/L, respectively.

The extract was reflected to be bioactive since they presented lethal concentrations (LC_{50} and LC_{90}) that are extremely lower than 1000 mg/L [13]. The solvent used can contribute to the variation between the fractions since it has been reported that the extraction of active biochemical compounds from plants depends upon the polarity of the solvents used [8]. A number of studies have reported the larvicidal potential of *A. occidentale* supporting the results gathered in the study [14,15].

Phytochemical analysis of the crude ethanol extract of cashew shell waste indicated the presence of free fatty acids, flavonoids, leucoanthocyanins, hydrolyzable tannins, unsaturated steroids and triterpenoids, fats and oils, and anthraquinones (Table 1).

Thin layer chromatographic profiling of the bioactive extracts showed characteristic profile for the plant studied as indicated by the presence of different spots in the chromatogram. These spots represent

Table 1. Qualitative phytochemical analysis of the ethanol extract of *A. occidentale* shell wastes. + Presence of compounds; - Absence of compounds.

Phytochemical constituent	Qualitative Test	Indication
Alkaloids	Mayer's Test Dragendorff's Test	- -
Saponins	Froth Test	-
Free fatty acids	Sodium carbonate Test	+
Leucoanthocyanins	Bate-Smith & Metcalf Test	+
Flavonoids (γ-benzopyrone nucleus)	Wilstatter "Cyanidin" Test	+
Hydrolyzable tannins	Ferric Chloride Test	+
Unsaturated steroid/ triterpenoids	Liebermann-Burchard Test	+
Anthraquinones	Borntrager's Test	+
Fats and oils	Spot Test	+

Table 2. Results of the TLC of the ethanol extract of cashew shell wastes developed in n-hexane: EtOAc (3:7).

Visualizing Agent	Spot Characteristic	h*Rf* values
Naked Eye	Purple	66.67
UV Light (254 nm)	Purple	66.67
	Light purple	75.00
Iodine Crystals	Orange	66.67
	Light Orange	75.00

a measure of the components present in the plant. The chromatogram showed that the extract is UV-active which may indicate the presence of aromatic components and compounds with double bonds that have extended conjugation [16].

Based on literature, more than 2000 plant species are reported to have insecticidal properties [17]. Several studies have reported the potential of *A. occidentale* against *A. aegypti*. A study was conducted on the antioxidant, larvicidal and anti-acetylcholinesterase activities of cashew nut shell liquid constituents namely, cardol, cardanol and anacardic acid. Results of study indicated that these constituents showed good larvicidal activity against *A. aegypti* [15]. Another study reported that the *A. aegypti* was found to be highly susceptible to cashew nut shell liquid against the immature stages of two mosquitos, *A. aegypti* and *Anopheles subpictus*. In addition, sodium anacardate, a bioactive constituent isolated from *A. occidentale* was found to be non-toxic and reported to have good insecticidal activity [18].

The remarkable toxicity effects exhibited by the ethanol and hexane extracts of *A. occidentale* shell wastes against *A. aegypti* indicate their promising use as natural larvicides for the control of dengue and zika vector. This finding fully supports the results gathered from the present study and suggests that the utilization of *A. occidentale* shell wastes as a new green larvicidal may be considered as a new alternative to combat spreading of dengue and zika virus. Larvicidal products can be developed from the isolated constituents of the *A. occidentale* shell wastes.

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