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Ezemuoka LC
Nnamdi Azikiwe University, Awka, Nigeria

Nwankwo EN
Nnamdi Azikiwe University, Awka, Nigeria

Ogbonna CU
Federal University Ndufu Alike Ikwo, Nigeria

Okonkwo NJ
Nnamdi Azikiwe University, Awka, Nigeria

Ojukwu KC
Nnamdi Azikiwe University, Awka, Nigeria

Correspondence
Ezemuoka LC
Nnamdi Azikiwe University, Awka, Nigeria

Toxicity of the aqueous leaf and Stem-bark extracts of *Annona muricata* to the 4th instar larvae of *Aedes aegypti*

Ezemuoka LC, Nwankwo EN, Ogbonna CU, Okonkwo NJ and Ojukwu KC

Abstract
The crude aqueous extracts were tested against the fourth instar larvae of *Aedes aegypti* (Diptera: Culicidae) at five concentrations of the technical material ranging from 12.5-200 µg/ml, including control. The bioassays were carried out in the laboratory at temperature of 29 ^{0}C ± 2 and 29.5 ± 5% relative humidity. Four replicates of each concentration of the respective toxicants in a Completely Randomised Design (CRD) were introduced with ten fourth instar larvae of *Ae. Aegypti.* Mortality resulting from eclosion inhibition was monitored at 3-hours interval for a period of 3, 6, 9, 12- and 24-hours post treatment. Data collected were analyzed using log-probit regression and analysis of variance. Results showed that mortality increased with increase in time and concentration and these were significantly different (P<0.05). Results indicated 65% and 87.1% mortality at the highest concentration (200 µg/ml) of the leaf and stem-bark aqueous extract respectively while the least concentration (12.5 µg/ml) resulted in 19.4% and 38.7% respectively. The LC_{50} values were 92.5 µg/ml and 26.5 µg/ml for the leaf and stem-bark aqueous extract respectively while the LT_{50} values were 29.7hr and 53.9hr respectively for the leaf and the stem-bark aqueous extract. The study suggests that *Annona muricata* leaf and stem-bark aqueous extract has shown promise as biopesticide for *Ae. Aegypti* larvae control. However, the stem-bark was found to be more effective compared to the leaf aqueous extract.

Keywords: Toxicity, *Annona muricata*, *Ae. Aegypti*, biopesticide

Introduction
Mosquito species are well known vectors for transmission of vector borne diseases affecting human beings particularly malaria and lymphatic filariasis [1]. Yellow fever, dengue fever, Chikungunya fever, encephalitis, West Nile Virus infection is also transmitted by mosquitoes. There are over 3000 different species of mosquito throughout the world, about 1,900 species occur in humid tropics and subtropics where the climatic conditions are favourable for rapid larval development and adult survival [2]. These vectors occur mainly in tropical countries were more than two billion people live in endemic regions with about one million deaths caused yearly due to malaria and filariasis [3] Yellow fever and dengue haemorrhagic fever transmitted by *Aedes aegypti* are common in Africa and tropical areas of America and South –East Asia.
To prevent proliferation of mosquito borne diseases and improve quality environment and public health, mosquito control is essential. Eradication of larvae is the key strategy of vector control programmes around the world [4]. Past control efforts towards mosquitoes have relied heavily on the use of very toxic synthetic insecticides mostly organ chlorines and organophosphates which have culminated in the loss of effectiveness due to resistance acquisition in several populations and adverse effects to non-target organisms and environment [5]. To control mosquito population various pesticides are being used widely. Recent reports state that mosquitoes have become genetically and physiologically resistant to many conventional insecticides [6]. These factors have created the need for environmentally safe, biodegradable and target specific insecticides against mosquitoes. The search for such compounds has been directed extensively to the plant kingdom [7]. Consequently, limitation in the use of synthetic insecticides in mosquito control programme is due to lack of novel insecticides, high cost of synthetic insecticides, concern for environmental sustainability, harmful effect on human health and other non-target populations, non-biodegradable nature, higher rate of biological magnification through ecosystem and increasing insecticide resistance on a global scale [8]. Thus, the environmental Protection Act in 1969 has formed a number of

rules and regulations to check the application of chemical control agents in nature. These have prompted researchers to look for alternative approaches ranging from provision of or promoting the adoption of effective and transparent mosquito management strategies that focus on public education, monitoring and surveillance, source reduction and environmentally friendly larval control. In addition, it has resulted in the search for environmentally friendly, cost-effective, biodegradable and target specific insecticides against mosquito species. In consideration, this application of eco-friendly alternatives such as biological control of vectors has become the central focus of the control programme in lieu of the chemical insecticides.

Several extract and compounds from different plant families have been evaluated to show new and promising larvicides [9]). Up to 300 plants from South Western USA have been investigated for pesticidal activity [10]. However, very few plants products have been developed for controlling mosquitoes. Annonaceae (custard apple family) plants have been intensively studied since they were discovered to contain compounds with important biological properties. These properties include cytotoxic, Antitumour, Antiparasitic, antifungal, antispasmodic, repellent and insecticidal activities [11]. Annona ceous plant species have been studied for their mosquito Larvicidal properties with extracts from the genus *Annona* (commonly called soursop), only four species have been studied for their Larvicidal activities [12].

Acetogenin compounds from Annonaceae type were reported to have toxicity that is effective against insects of several orders such as Lepidoptera, Coleoptera, Homoptera and Diptera [13]. Other studies reported that Annonaceae family contains acetogenin that are larvicidal. Acetogenin also acts as an insecticide, acaricide, Antiparasitic and bactericidal [14]. *A. muricata* (Soursop) seed extract contain announcing, bulletin, annonin VI, goniothalamin and sylvatic which act as insecticides [15]. All parts of *A. muricata* are used in natural medicine in the tropics including the bark, leaves, and root and fruit seeds. Much of the recent research on *A. muricata* has been on a novel set of phytochemicals *(Annona ceous acetogenin*) that are found in the leaves, seeds and stem which are cytotoxic. However, pest control potential of *A. muricata* remained largely untapped due to the advent of DDT and other broad-spectrum synthetic insecticides [16]. Due to this, the present studies evaluated the toxicity of the aqueous leaf and stem-bark extract of *Annona muricata* to the 4th instar larvae of A*edes aegypti.*

Materials and Methods

Experimental materials

Mosquito eggs were collected and identified at the Federal Ministry of Health, Department of Public Health National Arbovirus and Vector Research Centre, 33 Park Avenue, G.R.A, Enugu, Nigeria. They were reared in the laboratory at 28± 2 ^{0}C and 29.5±5 r.h. Fresh leaves and stem-barks of *A. muricata* were collected from *Annona* plant from a farm in Igboukwu, Aguata Local government Area, Anambra State in August, 2014.

Five hundred grammes of *A. muricata* fresh leaves and stem barks were thoroughly washed with tap water and air dried at room temperature, 25 ^{0}C for one week. One hundred grammes of the air-dried plant parts were grounded into powder using manual grinder. The powdered leaves and stem-bark were macerated in distilled water and extracted twice on each occasion with 600 ml of distilled water at room temperature for 48 hours (with occasional shaking). The filtrate containing the extract was filtered over a filter paper plugged in a funnel into a container. The combined aqueous extract was concentrated in water bath for 6 hours at 60 ^{0}C. The crude aqueous extract obtained was refrigerated and subsequently used in the study [17].

Treatment

Serial dilutions of the aqueous extract of both the leaf and stem-bark of *A. muricata* were prepared in acetone. The extracts were taken as 100% concentration which were then diluted serially to 20%, 10%, 5%, 2.5%, 1.25% of the extract yielding 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml and 12.5 µg/ml respectively. Appropriate aliquots 1ml in ml/ml of the formulations was added in plastic containers containing 200ml of distilled water for both the leaf and stem-bark aqueous extracts of *A. muricata.* Ten cohorts of fourth instar larvae of *Ae. Aegypti* were added in each container accordingly [18]. Each treatment and control was replicated 4-times and each bioassay repeated twice. The bioassay was carried out in the laboratory temperature of 29±2 ^{0}C, 29.5±5% r.h.

Observations

Mortality inhibitions of emergence assessments were made every 3-hours intervals for 24 hours. Dead larvae were counted and recorded. Those unable to wriggle (that is moribund larva) were counted as dead.

Data analysis

The data collected was analysed using GENSTAT Package (9th Edition). Analysis of variance was run at 95% confidence level. Percentage mortality was calculated and control mortality was also corrected using Abbott's formula. Log probit analysis was carried out [19] for determining $LC_{50,}$ Analysis of variance (ANOVA) was also performed on the mortality data and means separated using Least Significant Difference, LSD.

Results

Toxicity of various concentrations of A. *muricata* leaf aqueous extract to 4th instar larvae of *A. aegypti.*

The mortality values of 4th instar larvae of *Ae. Aegypti* exposed to the aqueous leaf of *A. muricata* at 3-hours intervals is presented in Table 1. The analysis of variance showed that the concentrations were significantly different (P<0.05), While the time intervals were not significantly different (P>0.05). No mortality was recorded in the control. Highest concentration of (200 µg/ml) recorded 65% mortality while the least concentration (12.5 µg/ml) recorded 19.4 µg/ml. The mortality recorded in the highest concentration with a mean value of 5.3 is significantly different from that recorded in the least concentration (12.5 µg/ml) with a mean mortality of 0.8 but not with the 100 µg/ml concentration with a mean value of 4.1. All concentrations were significantly different from the control except the least concentration (12.5 µg/ml).

Table 1: The mean mortality effects of different concentrations of *A. muricata* leaf aqueous extract on 4th instar larvae *of Ae. Aegypti* after 24 hours interval.

Consent-ration µg/ml	Time interval (hr) 3 6 9 12 24					Mean mortality	% mortality	LSD
200.0	3.0	4.3	6.3	6.8	6.5	5.3± 0.85	65.0	1.4
100.0	2.3	3.3	5.0	4.4	5.4	4.1± 0.63	54.0	1.4
50.0	1.3	2.5	4.0	4.4	3.55	3.1± 0.76	35.5	1.4
25.0	0.5	1.8	2.8	2.7	2.9	2.1± 0.71	29.0	1.4
12.5	0	0.3	1.0	0.9	1.94	0.8± 0.69	19.4	1.4
0.0	0	0	0	0	0	0.0±0.0	0	1.4
Mean mortality	1.4±0.57 2.4±0.68 3.8± 0.91 3.82±0.84 4.1±0.59							
% mortality	14 24 38 38.2 40.5							

Mean of four replicates ± s. e, $P<0.05$
P value of concentration= 0.001
P value of time = 0.139

Effects of various concentrations of *A. muricata* stem-bark aqueous extract on 4th instar larvae of *A. aegypti*

The mortality values of 4th instar larvae of *Ae. Aegypti* exposed to the aqueous stem-bark of *A. muricata* at 3-hours interval is presented in Table 2. The analysis of variance showed that both the concentrations and time intervals were significantly different ($P<0.05$). No mortality was recorded in the control. Highest mortality was recorded in the 200 µg/ml concentration level which had the mean mortality of 5.6 compared to the least concentration (12.5 µg/ml) which had a mean mortality of 1.4. The highest concentration of 200 µg/ml is significantly different from the least concentration (12.5 µg/ml) but not with the 100 µg/ml concentration with a mean value of 4.6. All concentrations were significantly different from the control except least concentration (12.5 µg/ml).

Table 2: The mean mortality effects of different concentrations of *A. muricata* stem-bark aqueous extract on 4th instar larvae of *A. aegypti* after 24 hours interval.

Consent-ration µg/ml	Time interval (hr) 3 6 9 12 24					Mean mortality	% mortality	LSD
200.0	2.5	4.0	6.5	6.5	8.7	5.6± 1.2	87.0	2.4
100.0	2.0	3.3	4.8	5.3	7.7	4.6± 1.1	77.0	2.4
50.0	1.0	2.3	3.8	4.1	4.52	3.1± 0.87	45.2	2.4
25.0	0.3	0.8	3.0	4.7	5.48	2.8± 1.2	54.8	2.4
12.5	0	0	1.0	2.4	3.87	1.4± 1.0	38.7	2.4
0.0	0	0	0	0	0	0.0±0.0	0	2.4
Mean mortality	1.15±0.48 2.05±0.75 3.8±0.91 4.95±0.57 0.06±0.72							
% mortality	11.5 20 38 38.2 40.5							

LSD 2.3 2.3 2.3 2.3 2.3
Mean of four replicates ± s. e, $P< 0.05$
P value of concentration= 0.001
P value of time = 0.002

Probit against log dose of *A. muricata* leaf and stem-bark aqueous extracts

The log-probit analysis revealed that after 24 hours of exposure, the LC_{50} of *A. muricata* aqueous leaf treated 4th instar larvae was 92.46 µg/ml and stem-bark extract was 26.49 µg/ml (Figure 1).

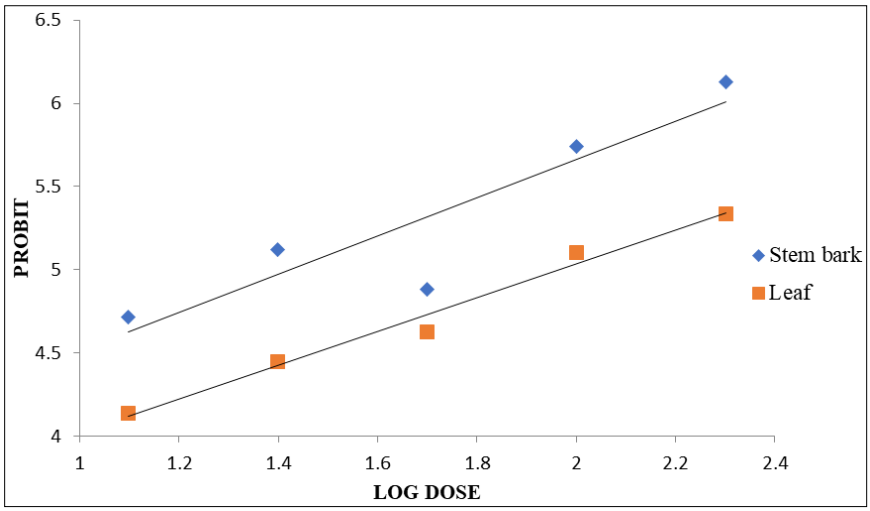

Fig1: Graph showing Probit against log dose of *A. muricata* leaf and stem-bark aqueous extracts

For the leaf extract

From the graph above, the regression equation is = 1.0136logdose + 3.0073

$R^2 = 0.9837$

Log of LC_{50}=1.98, therefore LC_{50} = 92.5 µg/ml

For the stem bark extract

From the graph above, the regression equation is = 1.1476logdose + 3.3669

$R^2 = 0.8312$

Log of LC_{50} = 1.42, therefore LC_{50} = 26.4 µg/ml

Probit against log time of *A. muricata* leaf and stem-bark aqueous extracts

The log-probit analysis revealed that after 24 hours of exposure, the LT_{50} values of *A. muricata* aqueous leaf treated 4th instar larvae was 29.72 hr and stem-bark extract were 53.9 hr (Figure 2).

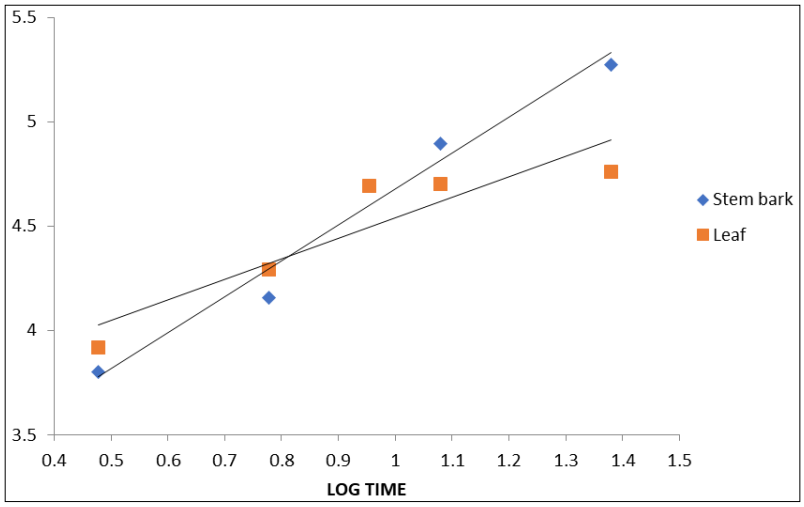

Fig 2: Graph showing Probit against log time of *A. muricata* leaf and stem-bark aqueous extracts

Log time of the leaf extract

The regression equation from the above graph is = 0.9836log time + 3.5551

$R^2 = 0.8419$ Log of LT_{50} =1.47 therefore LT_{50} = 29.7 hr

Log time of *A. muricata* stem-bark extract\

From the graph above, the regression equation is = 1.7181log time + 2.96

$R^2 = 0.9716$

Log of LT_{50} =1.73, therefore, LT_{50} = 53

Discussion

The present study showed that both the leaf and stem-bark aqueous extracts of *A. muricata* have toxic effect on *Ae. Aegypti* larvae, this may be due to the active ingredients contained in the extracts. This is in consistent with the work done by Abubakar *et al*. [20], the *A. muricata* aqueous leaf extract was tested against the larvae of *Ae. Aegypti*, the Gas chromatography and mass spectroscopy (GC-MS) analysis revealed the presence of major bioactive compound methyl ester of hexadoconic acid. As this compound might have been responsible for the mortality recorded in this work. Also, Komansilan *et al.* [21] reported that the soursop seed extracts contain secondary metabolites compounds of saponin, alkaloid and triterpenoid groups in their study, 'the isolation and identification of larvicide bioactive from soursop, *A. muricata* seeds against the larvae of *Aedes aegypti* mosquito'. These also might have caused the mortalities recorded in the present study.

Annona muricata leaf and stem-bark aqueous extracts showed high mortality effects on *Aedes* larvae, which were 65% and 87% respectively for both extracts. This is in agreement with the work done by Gonzalez-Esquinca *et al*. [22], where the ethanolic and aqueous extracts of stems and leaves of *Annona muricata* was tested against larvae of *Anastrepha ludens*, results of larvicidal activity after 72hour of exposure was 63-74%. The leaf aqueous extract has LC_{50} value of 92.5 µg/ml while the stem-bark has LC_{50} value of 26.5 µg/ml, these means that 92. 5 µg/ml of the leaf aqueous extract and 26.5 µg/ml of the stem-bark aqueous extracts are needed to cause 50% mortality of the 4th instar larvae of *Ae. Aegypti*. This implies that the stem bark aqueous extract is more lethal than the leaf aqueous extract. This may be due difference in chemical constituents contained in different parts of the *Annona* plant. In the work of Nayak [23] where he evaluated the efficacy of crude extracts of the leaf of *Annona reticulata* and leaf and stem-bark of *Pongamia pinnata* on the 4th instar larvae of *Culex quinquefasciatus*, he reported that the bark of *Pongamia pinnata* showed highest larvicidal activity against *Cx. quinquefasciatus* mosquito larvae within a short time of 12 hrs.

Treatment of the *Ae. Aegypti* larvae with *A. muricata* leaf and stem-bark aqueous extract in this study, resulted to a delayed larval development and caused some morphological abnormalities such as formation of larval-pupal intermediates. This is consistent with the study done by Spielman and Skaff [24], butanol extract of the soapberry plant, *Phytolacca dodecandra*, induced morphogenetic aberrations in *Aedes aegypti, Culex pipens* and *Anopheles quadrimaculatus*. Similarly, Supavarn *et al.* [25] using methanol extract of plant species from 17 families, reported that in acute toxicity, compounds from these plants significantly lengthened the larval period in *Ae. Aegypti* and *An. stephensi* when 5 and 10 mg/l concentrations of *Mentha longifolia, Acorus calamus* and *Ageratum conyzoides* were applied.

In this study, the toxicity of the leaf and stem-bark aqueous extract were high as the mortality and the rate of inhibition of emergence increased with increased concentration and time. There was increased significant difference (P<0.05) for the concentration. This is in line with the study carried out by Nwankwor *et al*. [26] on the toxicity of Novaluron (Mosquiron 100EC), a new chitin synthesis inhibitor type of insect regulator and *Annona muricata* seed oil (AMSO) against the

second and fourth instar larvae of *Aedes aegypti* (Diptera: Culicidae), results showed that the dosage-related mortality responses noted at different time interval were significant ($P < 0.05$) for both instars..

At the highest concentration (200 µg/ml), both the leaf and the stem-bark aqueous extracts produced a rapid mortality on the 4th instar larvae of *Ae. Aegypti* while the least concentration (6.25 µg/ml) was slow and continuous. This shows that if the treatment was left for a longer period, it might produce higher mortality, since increase in time led to higher mortality. Higher concentration caused higher mortality as seen in the present study compared to the least concentration, this may be due to the higher active ingredient contained in the highest concentration. This is in agreement with the work done by Nayak [23] where he evaluated the efficacy of crude extracts of the leaf of *Annona reticulata* and leaf and stem-bark of *Pongamia pinnata* on the 4th instar larvae of *Culex quinquefaciatus*, the highest concentration (200 ppm) of the leaf of *Pongamia pinnata* gave 100% mortality while the least concentration (5 ppm) yielded 40% mortality.

The LC_{50} of the leaf and stem-bark aqueous extracts were 92.5µg/ml and 26.5µg/ml respectively, this indicate that *A. muricata* leaf and stem-bark aqueous extract could be highly effective larvicides for mosquito control. The highest concentration (200 µg/ml) is more acceptable since it caused the highest rate mortality of *Ae. Aegypti* larvae compared to the lower concentrations. *Aedes aegypti* larvae is more susceptible to the stem bark aqueous extract than the leaf extract since the stem-bark extract caused 87% mortality while the leaf caused 65% mortality. The time of exposure affects the mortality of both aqueous extracts to the *Aedes* larvae hence mortality increases with increase in time of exposure.

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