

Phytochemical analysis and toxicity of *Annona muricata* stem bark and leaf extracts on *Anopheles gambiae* larvae.

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Abstract

Global efforts to control mosquitoes have relied heavily on the use of synthetic insecticides which have negatively impacted the ecosystem. With the continuous campaign on sustainability and the need to preserve the ecological balance, it has become imperative to develop alternative measures to contain the spread of mosquitoes which are efficient, sustainable and less impactful on the environment. It is in light of these, that laboratory investigations were conducted to determine the phytochemical components present in *A. muricata* stem bark and leaf extracts. The toxicity effects of various concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/mL) of *A. muricata* stem bark and leaf extracts to *An. gambiae* complex (Diptera: Culicidae) were evaluated by exposing the mosquito larvae at ambient temperature of $29 \pm 2^\circ\text{C}$ and $29.5 \pm 5\%$ r.h and photoperiod 12:12 light and dark hours. Three replicates of each of the concentrations were used to treat 20 active larvae of *An. gambiae* in a completely randomized design. Mortality resulting from eclosion inhibition was monitored at 3 hourly intervals for 48 hours, post-treatment and adult emergence were recorded. Data were analysed using log-probit regression and analysis of variance. Results of the qualitative phytochemical analysis revealed the presence of saponins, flavonoids, phenols, cardiac glycosides and alkaloids in both stem bark and leaf extracts. The toxicity result showed that the different concentrations of the extracts resulted in considerable mortality of the larvae. The stem bark and leaf extracts caused significant ($p < 0.05$) mortalities of 100% and 70% respectively at highest concentration of 200 mg/mL while at lowest concentration of 25 mg/mL caused mortalities of 30% and 20% respectively. The percentage inhibition of emergence for stem bark and leaf extracts at highest concentrations were respectively 100% and 70.00%. The LC_{50} and LC_{90} are (36.64 and 79.82 mg/mL) and (67.03 and 570.96 mg/mL) respectively for stem bark and leaf extracts. This study suggests that the stem bark and leaf extracts of *A. muricata* could perhaps be good alternatives to synthetic pesticides that adversely contaminate the environment while also suggesting that the active phytochemicals present in these plant parts could be saponins, flavonoids, alkaloids acting individually or synergistically.

Keywords: Phytochemicals, Toxicity, *Anopheles gambiae*, Leaf extracts, Pesticides.

Abbreviations: r.h: Relative Humidity; s.s: SENSU Stricto; LC_{50} and LC_{90} : Lethal Concentration; LT_{50} and LT_{90} : Lethal Time

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Introduction

Mosquitoes are of public health importance because they vector diseases such as malaria [1]. There are about 300 mosquito species globally with *Anopheles gambiae* being endemic in the tropical regions [2]. Climatic conditions in the tropics favours growth and survival of Anopheles mosquitoes and therefore have led to high prevalence of malaria within the tropics [3]. To reduce prevalence and mortality due to malaria, strategies that either prevents or reduces contact with vectors are encouraged [4]. One such strategy is the eradication of vectors which might include the larval stages [5]. Vector control relies heavily on the use of synthetic pesticides which are not biodegradable and not eco-friendly and therefore pose threats to the environment and non-target organisms [5,6]. Studies have revealed the genetical and physiological resistance of mosquitoes to conventional

pesticides [7,8]. Current research efforts are now focused on ecologically tolerable control measures which include the use of inert materials, plant powders, oil and extracts from plants [9,10]. While plants are reported to possess chemicals that may naturally protect them from pests and pathogens, The tropics are endowed with various plants that can be explored in the control of vectors. Crude extracts of *Agerantum conyzoides* exhibited both larvicidal effect and inhibition of emergence effects on *An. gambiae* s.s [11]. The effect was attributed to imbalance of developmental hormone [11]. *Annona muricata* commonly called "soursop" has been reported to have insecticidal activities against *Ae. aegypti* larvae [9]. More so, amongst the plants investigated for pesticidal activity, *Annona muricata* showed potential as a repellent against *Ae. aegypti* [12]. Phytochemicals such as alkaloids were extracted and isolated from *Annona*

squamosa and it showed larvicidal growth regulatory activities against *Anopheles stephensi* [13]. In view of this, we conducted qualitative and quantitative phytochemical screening on both stem bark and leaf extracts of *A. muricata* and further evaluated the effects of different concentrations of the plant parts on *An. gambiae*.

Materials and Methods

Experimental materials

Larval sampling was carried out on stagnant water found within Egbeagu and Okwukwa communities in Amansea. A town in Awka North L.G.A which lies between latitude 6°15'13, 17" N and longitude 6°55'02.93"E. Identification of mosquito larvae was conducted at the research laboratory of the Department of Parasitology and Entomology. Larvae were reared in the laboratory at 29 ± 2°C and 29.5 ± 5% r.h. The *Annona muricata* Stem bark and leaves were collected from *A. muricata* tree found in a farm in the community. The leaves were identified and authenticated at Botany Department, Nnamdi Azikiwe University, Awka, Nigeria. About 500 g of *A. muricata* leaves and stem bark were dried under shade and pulverized using an electric blender to obtain fine powder. The powdered sample was extracted by soaking 200 g in 600 mL of methanol in a Winchester bottle for 24 hours. This was filtered using Whatman filter paper No. 4 and was kept in a screw cap vial sealed with aluminum foil over the mouth.

This was vigorously shaken to dissolve the material on the solvent; the extract was concentrated over a water bath and was stored at 4°C for further analysis.

Phytochemical screening

The phytochemical components of the stem and leaf extracts of *A. muricata* were screened using standard analytical methods to determine Phenols, Total Flavonoids, Tannin, Alkaloids, Saponin, and Cardiac Glycosides.

Toxicity study

The different concentrations of the technical materials obtained from diluting in acetone were used. Appropriate aliquots of 1ml in ml/ml of the formulations were added in plastic cups containing 200 mL of distilled water for both *A. muricata* stem bark and leaf extracts. Subsequently, 20 wild-caught larvae of *An. gambiae* were introduced into the cups. Each treatment was replicated 3 times and each bioassay repeated twice. The bioassay was carried out in the laboratory at photoperiod of 12L, 12D, temperature of 29 ± 2°C and 29.5 ± 5% of relative humidity.

Observation

Mortality/inhibition of emergency assessments was made every 3 hours interval for 24 hours and 48 hours post monitoring for adult emergence.

Dead larvae were counted and recorded. The inability of the larvae to wriggle (moribund) after probing with five forceps was counted as dead. All adults that emerged were counted and recorded.

Data analysis

Log-probit analysis was carried out [14] for determining

(LC₅₀ and LC₉₀) and LT₅₀ and LT₉₀ or level of inhibition of emergence (IE%). IE was calculated using the following formula; IE (%)=100-(T × 100/C), where T=percentage survival or emergence in treated batches and C=percentage survival or emergence in the control. One-way Analysis of variance (ANOVA) was also performed on the mortality data in order to determine the level of significance on the effect of both concentration and time.

Results

Phytochemical components

The qualitative phytochemical analysis of the stem bark and leaf extracts showed the presence and absence of certain phytochemicals in these extracts (Table 1).

The phytochemicals present are mainly Saponin, Tannins, Alkaloids, cardiac Glycosides, phenol, and flavonoids. Anthraquinone was not present.

The quantitative phytochemical analysis of the stem bark and leaf extracts showed that Phenols are more in quantity in both the stem bark and leaf extracts than other phytochemicals. More so, Alkaloids and Tannins were also abundant in the two plant extracts. The result showed that the phytochemicals were found to be more in quantity in the leaf extract than in the stem bark extract Table 2.

Toxicity of *A. muricata* to the larvae of *An. gambiae* complex

Tables 3 and 4 shows the mortality responses of *An. gambiae* exposed to different concentration of *A. muricata* stem bark and leaf extracts at 48 hours post-treatment. The result showed that *A. muricata* exhibited significant (p<0.05) levels of toxicity to *An. gambiae*. There were dose-dependent mortality responses to both plant parts. At the highest concentration of 200 mg/mL of *A. muricata* stem bark and leaf, mortalities of 100% and 70% were recorded. These tables also show that at the lowest concentration of 25 mg/mL, mortalities of 30% and 20% respectively were

Table 1. Results of the qualitative phytochemical analysis of *A. muricata* stem bark and leaf extracts.

Phytochemical component	Plant Parts	
	Stem bark	Leaf extract
Saponin	+	++
Tannins	++	++
Alkaloids	++	++
Cardiac Glycoside	+	+
Phenol	+++	+++
Flavonoids	+	++
Anthraquinone	-	-

Presence of component: +; Absence of component: -

Table 2. Quantitative phytochemical analysis of *A. muricata* Stem bark and leaf extracts.

Phytochemical components	Plant Parts	
	Stem bark	Leaf
Alkaloids (%)	2.4	1.3
Flavonoids (mg/100 g)	51.28	60
Phenol (mg/100 g)	412.68	667.58
Tannin (mg/100 g)	35.58	3.6
Saponin (%)	2.2	3.6
Cardiac glycosides (%)	25	20

Table 3. Mortality effect of different concentration of *A. muricata* stem bark extract on *Anopheles gambiae* larvae.

Concentration (mg/ml)	Exposure Time (Hours)						Mean \pm se	Mortality %	Probit
	3	6	9	12	24	48			
200	0	2	7	9	12	20	8.33 \pm 2.95	100	8.72
100	0	1	5	7	10	18	6.83 \pm 2.70	90	7.33
50	0	0	2	3	5	11	3.50 \pm 1.69	55	5.13
25	0	0	0	1	2	6	1.50 \pm 0.96	30	4.48
Control	0	0	0	0	0	0	0.00 \pm 0.00		
Mean \pm se	0.00 \pm 0.00	0.75 \pm 0.48	3.50 \pm 1.56	5.00 \pm 1.83	7.25 \pm 2.29	13.75 \pm 3.22			
% mortality	0	3.75	17.5	25	36.25	68.75			
Probit	-	3.23	4.07	4.33	4.65	5.49			

Table 4. Mortality effect of different concentrations of *A. muricata* leaf extract on *Anopheles gambiae* larvae.

Concentration (mg/ml)	Exposure Time (Hours)						Mean \pm se	% Mortality	Probit
	3	6	9	12	24	48			
200	0	2	5	6	8	14	5.83 \pm 2.01	70	5.52
100	0	0	2	2.33	4	12	3.39 \pm 1.83	60	5.25
50	0	0	0	1.33	2	8	1.89 \pm 1.27	40	4.75
25	0	0	0	0	1	4	0.83 \pm 0.65	20	4.16
Control	0	0	0	0	0	0	0.00 \pm 0.00		
Mean \pm se	0.00 \pm 0.00	0.50 \pm 0.50	1.75 \pm 1.18	2.42 \pm 1.29	3.75 \pm 1.55	9.50 \pm 2.22			
% mortality	0	2.5	8.75	12.1	18.75	47.5			
Probit	-	3.06	3.65	3.83	4.11	4.94			

Means of three replicates (\pm s.e); p-value for concentration: 0.047; p for time: 0.001; LSD for concentration: 3.99; LSD for time: 3.95

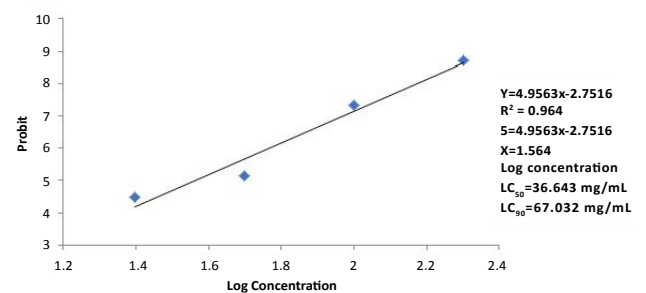
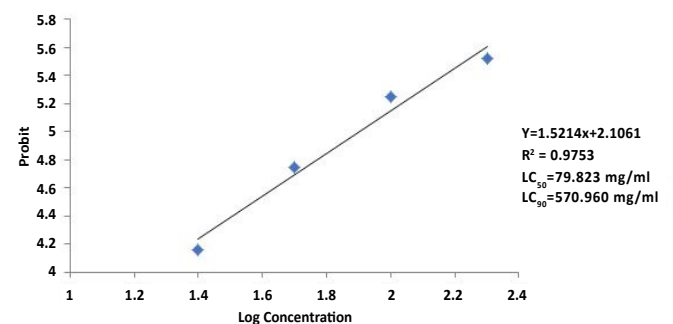
recorded. Mortality was also significant ($p < 0.05$). Figures 1 and 2 show the graph of probit against log-concentration of stem bark and leaf extracts of *A. muricata*. The LC_{50} and LC_{90} derived from the graph showed that the stem bark had 36.64 mg/mL and 67.03 mg/mL respectively, while the leaf extract had 79.82 mg/mL and 570.96 mg/mL respectively.

Effect of exposure time of *A. muricata* stem bark and leaf extracts on *An. gambiae* treated larvae

From the results (Tables 3 and 4), exposure time has an effect on various concentrations of *A. muricata* stem bark and leaf extracts on *An. gambiae* treated larvae. After 6 hours exposure of the extracts, mortalities were respectively 3.75% and 2.50%. However, as time progressed to 48 hours, mortalities were 68.75% and 47.50% for stem bark and leaf extracts respectively. Figures 3 and 4 show the graph of probit against log-time of stem bark and leaf extracts of *A. muricata*. LT_{50} of stem bark and leaf extracts derived from the graph were 29.11 hours and 56.34 hours respectively. This shows that exposure time was significant ($p < 0.05$).

Effect of the plant extracts on adult emergence of *An. gambiae*

The adults of *An. gambiae* that emerged from the larvae treated with stem bark and leaf extracts of *A. muricata* is presented in Tables 5 and 6. The results showed that at the highest concentration of 200 mg/mL of stem bark and leaf extracts, percentage adult emergence was 0% and 30% respectively. However, at the lowest concentrations of these two plant extracts, the stem bark extract had percentage emergence of 70% while the leaf extract had percentage emergence of 80%. These were not significantly different ($p > 0.05$) when compared with control which recorded 100% emergence. The time of exposure also had effect on the number of emerged adults. At 72 hours of exposure, only 1.25 adults emerged in both stem bark

**Figure 1.** Graph showing probit versus log-concentration of *A. muricata* stem bark extract.**Figure 2.** Probit against log-concentration of *A. muricata* leaf extracts on *An. gambiae* treated larvae.

and leaf extracts respectively (Tables 5 and 6). At 168 hours of exposure up to 31.25% of the adult emerged from water treated with stem bark extract while 25.0% emerged from water treated with leaf extract.

Percentage inhibition of emergence (IE%)

The result of inhibition of the emergence of *An. gambiae* treated with stem bark and leaf extracts of *A. muricata* is represented in Tables 7 and 8. The results showed that at the

Table 5. Adult emergence of *An. gambiae* treated larvae on various concentration of *A. muricata* stem bark extract.

Concentration (mg/ml)	Exposure Time (Hours)					Mean ± se	% Emergence
	72	96	120	144	168		
200	0	0	0	0	0	0.00 ± 0.00	0
100	0	0	1	2	2	1.00 ± 0.45	10
50	0	0	2	4.00	9	3.00 ± 1.67	40
25	1	4	6	8	14	6.60 ± 2.18	70
Control	3	6	8	10	20	9.40 ± 2.89	100
Mean ± se	0.25 ± 0.25	1.00 ± 1.00	2.25 ± 1.32	3.50 ± 1.71	6.25 ± 3.22		
%emergence	1.25	5	11.25	17.5	31.25		

Means of three replicates (± s. e); LSD for concentration: 5.29; LSD for time: 5.41; p value for concentration: 0.007; p value for time: 0.195

Table 6. Adult emergence of *An. gambiae* treated larvae on various concentration of *A. muricata* leaf extract.

Concentration (mg/ml)	Exposure Time (Hours)					Mean ± se	% Emergence
	72	96	120	144	168		
200	0	0	0	1	3	0.80 ± 0.58	30
100	0	0	0	1	4	1.00 ± 0.77	40
50	0	1	2	3.00	5	2.20 ± 0.86	50
25	1	1	3	3	8	3.20 ± 1.28	80
Control	2	4	5	7	10	5.60 ± 1.36	100
Mean ± se	0.25 ± 0.25	0.50 ± 0.28	1.20 ± 0.75	2.00 ± 0.57	5.00 ± 1.08		
%emergence	1.25	2.5	6.25	10	25		

Means of three replicates (± s. e); LSD for concentration: 3.00; LSD for time: 2.00; p value for concentration: 0.021; p value for time: 0.001

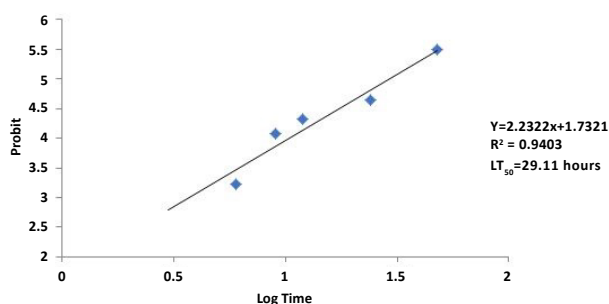


Figure 3. Probit against log-time of *A. muricata* stem-bark extracts on *An. gambiae* treated larvae.

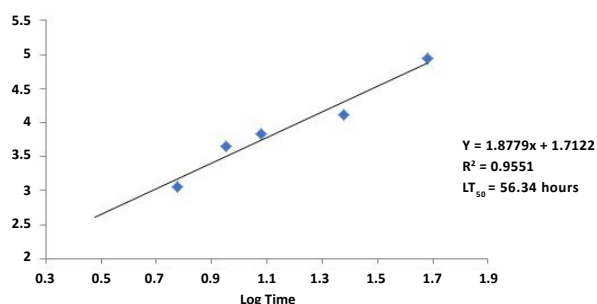


Figure 4. Probit against log-time of *A. muricata* leaf extracts on *An. gambiae* treated larvae.

highest concentration of 200 mg/mL of stem bark and leaf extracts, inhibition of emergence (IE%) were 100% and 70% respectively. However, at the lowest concentration of the two plant extracts, the stem bark extract had inhibition of emergence (IE%) of 30% while the leaf extract had inhibition of emergence (IE%) of 20%.

Discussion

The phytochemical analysis and toxicity of stem bark and leaf extracts of *Annona muricata* to larvae of *Anopheles gambiae*

Table 7. Percentage emergence and inhibition of emergence of *A. muricata* stem bark extract on *An. gambiae* complex.

Concentration (mg/ml)	Percentage Emergence (%)	Inhibition of Emergence (IE)%
200	0	100
100	10	90
50	40	60
25	70	30
Control A (Acetone)	100	0
Control B (Water)	100	0

Table 8. Percentage emergence and inhibition of emergence of *A. muricata* leaf extract on *An. gambiae*.

Concentration (mg/ml)	Percentage Emergence (%)	Inhibition of Emergence (IE)%
200	30	70
100	40	60
50	50	50
25	80	20
Control A (Acetone)	100	0
Control B (Water)	100	0

complex were studied in the laboratory. The studies have shown that each plant extract contained at least one of the important phytochemical constituents and that the extracts could be used as independent control method in reducing the impact of mosquitoes. Identification of various plant extracts that have larvicidal potential against mosquitoes could be of advantage in reducing the problem of resistance and environmental contamination [6].

Mosquitoes in their larval stage are attractive targets for pesticides because mosquitoes breed in water, which makes it easy to control them in this habitat [15,16]. The use of conventional pesticides in the water sources, however, introduces many risks to people and the environment [17,18].

Therefore, natural pesticides especially those derived from plants are more promising in this aspect.

Many plant chemicals produce larvicidal effects. This agreed with [19,20] who reported some paramount bioactive constituents of plants that have larvicidal activity. Also, it has been reported that leaf extracts of *Aegie marmelos* contained saponins, flavonoids, tannins and other phytochemicals [21]. This finding is in line with the present study that reported that the stem bark and leaf extracts of *Annona muricata* contained these phytochemical components such as flavonoids, alkaloids, saponins and known for their toxicity to harmful insect. Saponins isolated from *Achyranthes aspera* possess larvicidal activity against *Ae. Aegypti* and *Cu. quinquefasciatus* [22]. Also, flavonoids isolated from seed extracts of *A. squamosa* were reported to be effective as insecticides against the red flour beetle [13]. It is therefore suggested that the pesticidal actions of *A. muricata* stem bark and leaf extracts may be attributed to the presence of the phytochemicals present in the plant (flavonoids, tannins, saponins, and alkaloids).

The different responses induced by phytochemicals on various species of mosquitoes could have been influenced by extrinsic and intrinsic factors such as the plant species, plant part, the solvent used for extraction, the geographical location where the plants were grown and the methods employed for extraction [23]. Phytochemicals can be extracted from either the whole plant or specific parts of the plants depending on the activity of the derivatives [6]. Plants accumulate bioactive chemicals differentially in the various parts of the plant such as leaves, fruits, flowers, roots and bark and the effectiveness of chemicals derived from them varies with the mosquito species [6]. The isolated compound saponin from ethyl acetate extract of *Achyranthes aspera* was effective against the larvae of *Ae. aegypti* and *Culex quinquefasciatus* with LC_{50} value of 18.20 and 27.24 ppm respectively [22]. In the present study, the crude extracts of stem bark and leaf of *A. muricata* caused mortality of 100% and 70% respectively at 200 mg/mL and caused inhibition of emergence of 100% and 70% respectively.

Crude extracts of many plants showed larvicidal activity against Anopheles mosquito and other mosquito species and the results of the present study were comparable with those reports. For instance, it was reported that ethanolic leaf extract *Nerium oleander* exhibited larvicidal activity against *Ae. aegypti* with LC_{50} value of 197.97 mg/L [24]. More-so, aqueous leaf extract and stem bark extracts of *Annona muricata* were reported to have resulted in 65% and 87.1% mortality of *Aedes aegypti* at the highest concentrations [9] which were consistent with the present study and suggest that the stem bark exerts better larvicidal effect than the leaf extracts. In the present study also, the crude extracts of the stem bark and leaf of *A. muricata* exhibited larvicidal activity against the larvae of *An. gambiae* with LC_{50} and LC_{90} values of (39.5 mg/mL and 75.12 mg/ml) and 8.02 and 807.2 mg/mL respectively. In another study, [25] reported LC_{50} and LC_{90} of 10 mg/mL and 40 mg/mL respectively after 48 hours of exposure of *Ae. aegypti* to plant leaf extracts. Recent studies on the larval and pupal mortality of *An. Stephensii* after treatment with methanolic extract of *clerodendron inerme* leaf extract showed 22% mortality of larvae at 20 ppm [26]. This finding contradicts the present study where the leaf extract

of *A. muricata* at 200 mg/mL caused up to 70% of mortality of larvae of *An. gambiae*. In this study the toxic effect of the extracts was high, as the mortality and the rate of inhibition of emergence increased with increasing concentration and time of exposure. In another study, [27] it was observed that fraction of *melia volkensii* fruit kernel extracts had growth inhibition activity at very low concentrations, whereas two other fractions had acute toxic effects on the mosquito larvae. Thus, in the present studies, only a few surviving larvae were left in some treated samples, and they were able to mature to adult stage.

It was also reported by Pushpalatha E [10] that leaf extracts of *vitex negundo* at very low concentrations had larvicidal activity against *Cu. quinquefasciatus* and *An.stephensi* and also extended the derivation of larval pupation.

The botanical pesticide, *A. muricata* has proved to be very effective in mosquito larval control as demonstrated in the present studies. The activity extended over a longer period and its effectiveness increased with increasing exposure time. The extracts probably exhibited their effects on the mosquito larvae as growth regulators, as well as blocking the respiratory organs. It has been reported that some plant essential oil block the octopamine neuroreceptors that regulate the movement, heart rate, behaviour, metabolism, and pupation of insects [28,29]. Thus, the regulatory activity of *A. muricata* plant parts was exhibited in the mortality of the larval and inhibition of their eclosion into adult stage. Further investigations are therefore needed to elucidate their activity against a wide range of all stages of mosquito species and if possible, to isolate the active ingredient(s) of the extracts responsible for larvicidal activity.

Conclusion

The study on the phytochemical analysis and toxicity of methanolic extracts of stem bark and leaf of *A. muricata* to *An. gambiae* complex showed that the stem bark and leaf extracts of *A. muricata* contain alkaloids, flavonoids, phenol, saponins, and tannins. These compounds are known to possess insecticidal and larvicidal activities on insects and other animals.

The crude extracts of *A. muricata* plant parts showed larvicidal activity to *An. gambiae* complex larvae which are manifested by high percentage mortality and inhibition of emergence compared to those in the control groups. The mortality of mosquito larvae between the various concentrations of the plant extracts and the control group is also significantly different. Furthermore, the stem bark of *A. muricata* extract shows the most effective larvicide compared to the leaf extract with percentage mortality of 100% at the highest concentration of 200 mg/mL and 68.75% after 48 hours of exposure.

The present study therefore, demonstrate that *A. muricata* could be used as mosquito larvicide for the control of *An. gambiae* complex. This would help to reduce the mortality and morbidity due to malaria transmitted by this mosquito. It would be necessary to increase campaign on the growth of this plant in all ecological zones of Nigeria, as an invaluable tool for the control of mosquito vector.

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