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Full Length Research Paper

Mosquito larvicidal and cytotoxic activities of 3 *Annona* species and isolation of active principles

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Cytotoxicity and larvicidal properties of the leaf extracts of 3 Annonaceous plants, *Annona muricata* L., *A. senegalensis* Pers and *A. squamosa* L. were tested against brine shrimp larva and the late 3rd instar of *Culex quinquefascintus* Say. The larval mortality was observed 24 h post-exposure. Based on the probit analysis, the LC_{50} value for crude extracts of *A. muricata*, *A. senegalensis* and *A. squamosa* were observed to be 20.87, 0.67 and 0.64 µg/mL respectively for shrimp larvae and 56.47, 23.42 and 11.01 µg/mL respectively for *C. quinquefascintus*. Bioassay-guided fractionation of crude extracts indicated high activity of the ethyl acetate fractions with mortality of ≥ 90% at 50 µg/mL for both *A. senegalensis* and *A. squamosa*. However, further bioassay of sub-fraction of the 2 ethyl acetate fractions from the 2 plant species showed close dependent and a decreased activity (p ≥ 0.05) at the same concentration levels. 2 known aporphine alkaloids, (-)-roemerine and annonaine were identified as active principles from the ethyl acetate extracts of *A. senegalensis* and *A. squamosa* respectively. With the larvicidal properties of *A. senegalensis* being described here for the first time, its value together with that of *A. squamosa* may prove to be the best natural source of larvicidal agents.

Key words: *Annona* species, cytotoxic and larvicidal activities, isolation, aporphine alkaloids.

INTRODUCTION

Mosquito species are well known vectors for transmission of vector-borne diseases affecting human beings particularly malaria and lymphatic filariasis (Hubalek and Halouzka, 1999). These vectors occurs mainly in tropical countries where more than two billion people live in endemic regions with about one million deaths been claimed yearly from malaria and filariasis (WHO report, 2005; Southgate, 1984). In the absence of an effective preventive measures or vaccine, the best approach should be the interruption of disease transmission by either killing, preventing mosquitoes from biting people or by killing the larva at the breeding sites of vectors. The discovery and use of conventional pesticides like DDT and malathion against adult mosquitoes in the last five decades demonstrated a successive move. However, this success was short lived due to the development of resistant to many mosquito strains, ecological imbalance and harm to mammals. This has necessitated the continued effort for search and development of environmenttally safe, biodegradable and low cost larvicides and adultcides for killing larva and adult mosquitoes respecttively from natural sources (ICMR Bulletin, 2003).

Natural products are the best option because of their less harmful nature to environment and non-targeted organisms. Several extracts and compounds from different plant families have been evaluated to show new and promising larvicides (Markouk et al., 2000; Prabakar et al., 2004; Mohan and Ramaswamy, 2007; Innocent et al., 2008a and 2008b), however very few plant products have been developed for controlling mosquitoes. In the case of the family Annonaceae, the literature search indicated that only seven annonaceous plant species have been studied for their mosquito larvicidal properties with extracts from the genus *Annona* showing strong activities

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Table 1. Annonaceous plant extracts with larvicidal activity against mosquito vectors

S/N	Plant species	Country of collection	Part*	Solvent *	LC_{50} (µg/mL)	Mosquito species tested	Ref.
1	*Annona crassiflora*	Brazil	SB	H	192.57	*Aedes aegypti*	Rodrigues et al. (2006)
			RW	E	26.89		
			RB	E	23.06		
			RW	H	154.02		
			RB	H	264.15		
			RB	E	0.71		Omena et al. (2007)
			RW	E	8.94		
2	*Annona glabra*	Brazil	SB	E	0.06	*A. aegypti*	Omena et al. (2007)
3	*Annona muricata*	Thailand	Se	E	67.4	*A. aegypti*	Promsiri et al. (2006)
4	*Annona squamosa*		Whole plant			*A. stephens*	Saxena et al. (1993)
		India	L	M	20.26	*A. albopictus*	Das et al. (2007)
			L	E	20.70		
			L	M	17.70	*C. quinquefascintus*	
			L	E	6.96		
5	*Duguetia furfuracea*	Brazil	RW	H	56.6	*Aedes aegypti*	Rodrigues et al. (2006)
6	*Polyalthia longifolia*		L			*C. quinquefascintus*	Murty et al. (1997)
7	*Xylopia aromatica*	Brazil	L	E	384.37	*Aedes aegypti*	Rodrigues et al. (2006)

*SB = Stem bark; RB = Root bark; RW =Root wood; L = Leaves; H = Hexane; M = Methanol; E = Ethanol: Se = Seed.

(Table 1). However, with about 150 plant species known from the genus *Annona* only 4 species have been studied for larvicidal activities (Das et al., 2007; Promsiri et al., 2006; Rodrigues et al., 2006; Saxena et al., 1993).

Annona muricata L. is planted throughout in Tanzania but commonly in coast, Zanzibar and Pemba. Leaves of this plant are used as Insecticidal and fish poisoning with powder or oil from the seeds being used to kill lice and bedbugs (Nyambo et al., 2005). *Annona senegalensis* Pers. grows in Tanzania mainly in the Coast, Usambara mountains and along the lake Victoria basin (Ruffo et al., 2002). Ethnomedically, leaves and stem barks are used to treat colds and pneumonia while the fruits are used against diarrhoea, dysentery and vomiting. Ruffo et al., 2002). *Annona squamosa* L. is planted in Tanzania, mostly along the coast and Zanzibar. Leaves and unripe fruits in combination with garlic are used for biological control of insects (Nyambo et al., 2005). Branches of the 3 *Annona* species reported under this investigation are depicted (Figure 1).

Cytotoxic and larvicidal activities of the present plant materials collected in Tanzania have not been studied. Hence this study was aimed to test the leaf extracts of *A. muricata*, *A. senegalensis* and *A. squamosa* for their larvicidal activity against *C. quinquefascintus* and brine shrimp larvae.

MATERIALS AND METHODS

Plant collection and identification

Leaves of *A. muricata*, *A. senegalensis* and *A. squamosa* were collected in February 2008 from Bunju village, 25 km Northwest of Dar es Salaam city, Tanzania and identified by an ethnobotanist, Dr Joseph N. Otieno of the Department of Medical Botany, Plant Breeding and Agronomy, Muhimbili University of Health and Allied Sciences, Tanzania. The voucher specimen numbers OT #00351, OT #00352 and OT #00353 have been deposited for verification purposes at the herbarium of the Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences, Tanzania.

Extraction and concentration

Leaves of each plant were dried in shade for 14 days and later milled to obtain pulverised powder through a sieve of 60-mesh. The powdered leaves of *A. muricata* (200 g), *A. senegalensis* (250 g) and *A. squamosa* (165 g) were soaked each separately in ethanol for 48 day (2 x 5 L) and extracted by the maceration process. The crude extracts were obtained after evaporation of the solvent under reduced pressure at 40°C on a rotary evaporator. The crudeethanol extracts were measured resulting into 17.2 g (*A. muricata*), 21.0 g (*A. senegalensis*) and 15.6 g (*A. squamosa*).

Bioassay-guided fractionation of crude extracts

Each crude plant extract was fractionated separately using the

Figure 1. (a) Leaves of *A. squamosa*, (b) Leaves of *A. muricata*, (c) Leaves of *A. senegalensis.*

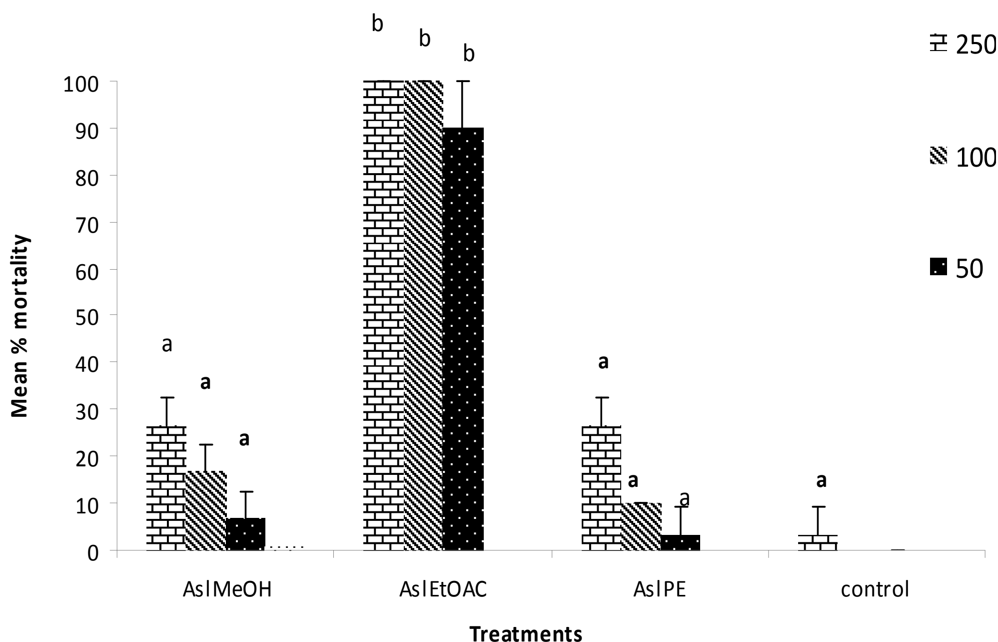


Figure 2. Mosquito larvicidal effect of crude extracts from *A. senegalensis.*
Mean values with the same letters are not significantly different at $p > 0.05$ by student-Newman-Kuels (SNK).

Vacuum Liquid Chromatography (VLC) technique. The solvents used were Petroleum ether (PE), Ethyl acetate (EtOAc) and Methanol (MeOH). The resulting weights of fractions after VLC were recorded as 2.6 g (PE), 6.0 g (EtOAc) and 8.1 g (MeOH) for *A. muricata*, 3.8 g (PE), 5.8 g (EtOAc) and 11.4 g (MeOH) for *A. senegalensis* and 2.6 g (PE), 3.9 g (EtOAc) and 9.10 g (MeOH) for *A. squamosa*. The VLC fractions were tested for larvicidal activities and the results are presented in Figures 2 and 3 with high activity being noted in the EtOAc extracts of both *A. senegalensis* and *A. squamosa.* In order to identify the active compound, each of the EtOAc fraction was subjected to column chromatography under silica gel eluting with PE:EtOAc [7:3, 150 mL, Se-1 (15.0 mg)], PE:EtOAc [3:7, 150 mL, Se-2 (11.0 mg)] and finally with EtOAc [100%, 150 mL, Se-3 (28.1 mg)] for *A. senegalensis* while the yield for the EtOAc subfractions of *A squamosa.* were PE:EtOAc [7:3, 150 mL, Sq-1 (9 mg)], PE:EtOAc [3:7, 150 mL, Sq-2 (15 mg)] and EtOAc [100%, 150 mL, Sq-3 (36.2 mg)]. The amount obtained was tested for their larvicidal activities against larva of *C. quinquefascintus* with Se-3 and Sq-3 showing higher activities (Table 4).

Phytochemical screening of EtOAc sub-fractions and isolation of active principles

The phytochemical screening was achieved by employing different chemicals (Table 3). This gives a prior clue of the class of compounds present in the fractions under investigation. This was followed by subjecting (each separately) the Se-3 and Sq-3 fractions on column chromatography under silica gel eluting with

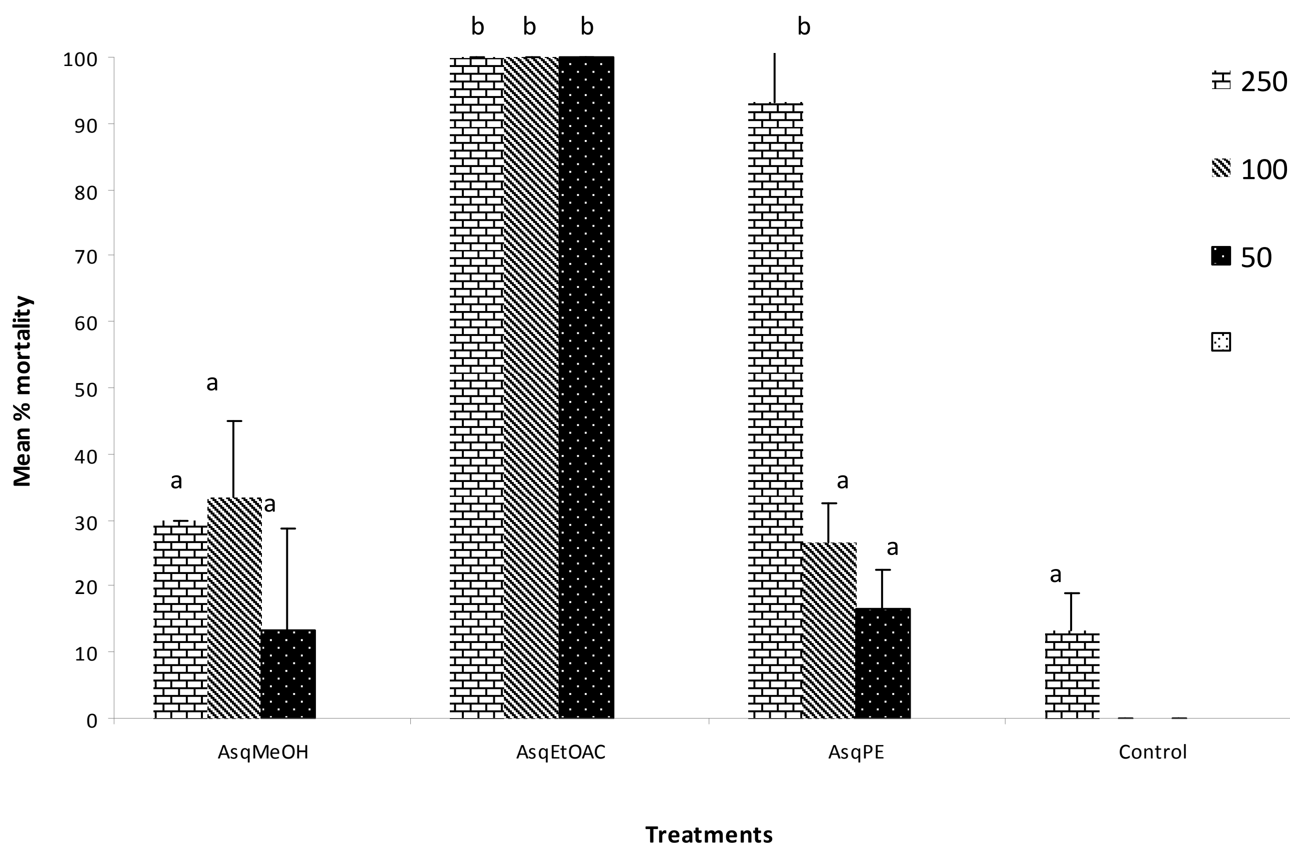


Figure 3. Mosquito larvicidal effect of crude extracts from *A. squamosa.*
Mean values with the same letters are not significantly different at $p > 0.05$ by student-Newman-Kuels (SNK).

PE:EtOAc (1:4). Fraction Se-3 gave Se-A (6.0 mg) as a pure sample while fraction Sq-3 gave Sq-B (4.6 mg) as a pure sample.

NMR analysis of samples Se-A and Sq-B

The 2 samples were analysed at the Department of Chemistry, University of Botswana. Application of physical, chemical and available spectral analysis, pure samples Se-A and Sq-B were identified as known aporphine alkaloids, (-)-roemerine (1) (You et al., 1995) and annonaine (2) (Miski et al., 1995) respectively (Figure 4).

Brine shrimp testing

The brine shrimp lethality test (BST) was used to predict the presence of toxins in the extracts. The crude extracts were subjected to this assay to determine if they possess some toxicity properties. Artificial seawater was prepared by dissolving previously generated sea salt in distilled water (38 g/L) and poured into a 23 x 3 cm glass tank divided into two unequal compartments by a perforated polystyrene wall. Brine shrimp eggs (0.5 g) were sprinkled into the larger compartment and kept dark by covering it on top. The smaller compartment was illuminated with an electric bulb. After 48 h the hatched, mature phototropic nauplii, were collected from the illuminated compartment using a micropipette. Solutions of the extracts/pure compounds were made in DMSO, at concentrations of 8, 24, 40, 80 and 240 µg/mL. Triplicate aliquots (2 mL) of each of the solutions were put in vials and made up to 10 mL with sea water. Ten brine shrimp larvae were transferred into each of the triplicate vials. The vials are maintained under illumination. Controls were placed in a mixture containing seawater and DMSO only. After 24 h the nauplii were examined against a lighted background and the average numbers of survived larvae in each triplicate were determined. The mean percentage mortality was plotted against the logarithm of concentrations and the concentration killing 50% of the larvae (LC_{50}) was determined from the graph (Meyer et al., 1982).

Larvicidal testing

250 mg of each plant extracts was dissolved in 5 mL of dimethyl sulphoxide (DMSO) to make a stock solution. Different dilutions with concentrations of 250, 100 and 50 µg/mL were prepared from the stock solution of each plant extract. The larvicidal bioassay was conducted according to standard WHO techniques (WHO, 1996 and 2003) at which a 100 mL glass beakers having 50 mL of water and 10 numbers of the third late instar mosquito larvae for different concentrations. Three concentrations of each plant extract were carried out with three replicates. A control experiment (blank) was provided for each set of experiment. All tests were carried out under controlled temperature (27 ± 2°C) and relative humidity of 75 - 85% against the laboratory reared *C. quinquefascintus* larvae. The mortality was determined after 24 h, at which larvae with motionless

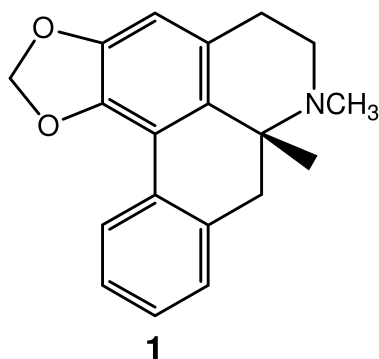
Table 2. Brine Shrimp Lethality (BST) test and Larvicidal activities of crude extracts against larva of *C. quinquefascintus.*

Plant extracts/Activity (µg/mL)	Brine Shrimp lethality test *(BST)*			Larvicidal activity against larvae of *C. quinquefascintus*		
	LC_{50}	LFL	UFL	LC_{50}	LFL	UFL
A. muricata leaves	20.87	14.03	27.71	56.47	35.92	77.03
A. senegalensis leaves	0.67	0.29	1.05	23.42	17.05	29.78
A. squamosa leaves	0.64	0.21	1.07	11.01	7.43	14.59

Table 3. Phytochemical Screening of Se-3, Sq-3, Se-A and Sq-B*.

	Class	Test used	Extracts		Pure samples	
			Se-3	Sq-3	Se-A	Sq-B
1	Terpenoids	Vanilin-sulphuric acid	-	-	-	-
2	Phenolics	Ferric chloride	-	-	-	-
3	Alkaloids	Dragendorff reagent	+	+	+	+
4	Flavonoids	Aluminium chloride	-	-	-	-

* +: present; -: absent



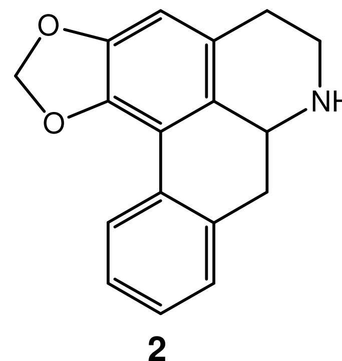


Figure 4. Aporphine alkaloids isolated from *A. senegalensis* (compound 1) and *A. squamosa* (Compound 2).

were considered dead (Macedo et al., 1997). The obtained mortality values were subjected to ANOVA and probit analysis and means were separated by Student Newmann Keuls test (SAS, 2000).

RESULTS

The results of the phytochemical analysis of the EtOAc sub-fractions show that only alkaloids are present in the extract (Table 3).

The cytotoxicity levels of the two crude extracts from *A. muricata*, *A. senegalensis* and *A. squamosa* against the brine shrimp larvae were high with LC_{50} values of 20.87, 0.67 and 0.64 µg/mL respectively (Table 2). The toxicity effect against *C. quinquefascintus* larvae was high for *A. squamosa* extract with LC_{50} value of 11.01 and 23.42 µg/mL for *A. squamosa* and *A. senegalensis* respectively. Larvicidal activity for *A. muricata* was however much low (LC_{50} value 56.47 µg/mL) as compared to the other two plant species (Table 2). This formed a basis for subjecting the extracts for larvicidal activity. Thus, the extract of *A. muricata* was not investigated further.

Each of the crude extract of *A. squamosa* and *A. senegalensis* was fractionated using a Vacuum liquid chromatography (VLC) technique and the resulting extracts were again tested for larvicidal activities.

Phytochemical screening of Se-3 and Sq-3 EtOAc sub-fractions gave a positive result with Dragendorff reagent confirming the presence of an alkaloid compounds. Two alkaloids namely (-)-roemerine (1) and annonaine (2) were isolated and identified from fractions Se-3 and Sq-3 respectively. However the two alkaloids were not tested further due to the small amount of pure samples obtained.

Table 4. Mean percentage mortality of *Culex quinquefascintus* due to treatment with different EtOAc Sub-fractions*.

	Conc (µg/ml)		
Treatment	**250**	**100**	**50**
100% Sq-3	86.67± 8.82^{aA}	56.67± 3.33^{bA}	40.0 ± 10^{cA}
70% Sq-2	20.0 ± 5.77^{aC}	10.0 ± 0^{aB}	10.00 ±5.67^{aB}
30% Sq-1	0 ± 0^{aC}	0 ± 0^{aB}	0± 0^{aB}
100% Se-3	90.0 ± 5.67^{aA}	60.0 ± 5.67^{bA}	46.67 ±3.33^{cA}
70% Se-2	56.67 ± 6.67^{aB}	56.67± 6.67^{aB}	13.33 ± 8.82^{bB}
30% Se-1	13.33 ± 8.82^{aC}	6.67 ± 3.33^{aB}	0 ± 0^{aB}

*Mean values with the same capital letters within the same concentration level and mean values with the same small letters for a particular treatment are not significantly different at p > 0.01 by student-Newman-Kuels (SNK).

DISCUSSION

The significant activity demonstrated by extracts of *A. squamosa* and *A. senegalensis* suggest that the two plants may have strong killing effects against insects particularly mosquitoes, hence giving a promising source of larvicidal agents. The EtOAc fractions of *A. squamosa* and *A. senegalensis* were the most active achieving 100 and 90% mortality at 50 µg/mL. (Figures 2 and 3). In order to determine the active principles in the EtOAc fractions further larvicidal testing of the three sub-fractions Sq-1, Sq-2 and Sq-3 for *A. squamosa* and Se-1, Se-2 and Se-3 for *A. senegalensis* showed a dose dependant ($p \geq 0.05$) but also significantly a decreased activity from its parent fraction at the same concentration levels (Table 4). This observation indicates that, several medium polar compounds in the extract are acting synergesitically or competitively at the active site.

Previously, a collection of *A. squamosa* plant materials from Brazil indicated larvicidal effect against *Aedes abopictus* and *C. quinquefascintus* (Das et al., 2007) and against *Anopheles stephensi* (Saxena et al., 1993). In this study, apart from testing the leaf extracts of *A. squamosa* grown in Tanzania against *C. quinquefascintus* we included the *A. senegalensis* and *A. muricata* whose larvicidal activity has not been reported. Present cytoctoxic and larvicidal activity results, supports other reports (Rodriques et al., 2006) and demonstrated that extracts of *Annona* species are potential anti-mosquito agents.

Conclusion

It has been revealed that the leaf extracts of *A. squamosa and A. senegalensis* possess cytotoxic and larvicidal activities. Their potential application in managing mosquito larvae would therefore be a promising undertaking. However, further work is required to isolate the active constituents in good amounts in order to test them for their potential cytotoxic and larvicidal potentials.

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