Journal of Applied Pharmaceutical Science Vol. 3 (05), pp. 091-095, May, 2013
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2013.3517
ISSN 2231-3354

Larvicidal and Brine Shrimp Activities of *Vitex Schiliebenii* Extracts and Isolated Phytoecdysteroids on *Anopheles gambiae* Giles S.S Larvae

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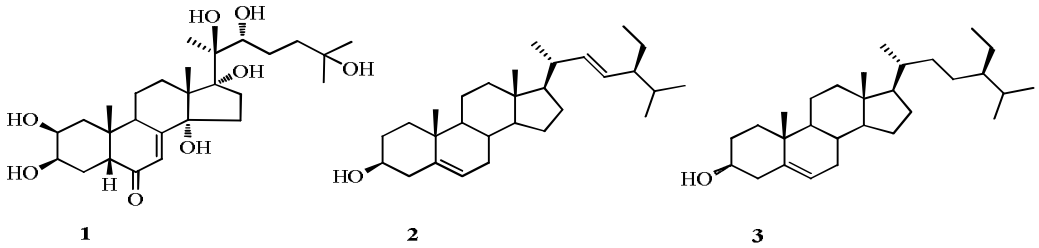
ARTICLE INFO

Article history:
Received on: 12/04/2013
Revised on: 29/04/2013
Accepted on: 15/05/2013
Available online: 30/05/2013

Key words:
Vitex schiliebenii, *Verbenaceae*, *Anopheles gambiae*, phytoecdysteroids, 20-hydroxyecdysone, stigmasterol, γ-sitosterol, Larvicide

ABSTRACT

Acetone, methanol and aqueous extracts of the leaves, stem bark and root bark of *Vitex schiliebenii* belonging to the family Verbenaceae were evaluated for their larvicidal activity against late 3rd/early 4th *Anopheles gambiae* Giles s.s. larvae (Diptera: Culicidae). The extracts of the acetone leaves and stem bark were active with LC_{50} values of 14.6 and 17.4 ppm respectively at 24 hrs. These extracts exhibited low toxicity to brine shrimps with LC_{50} values of 180.9 and 154.4 ppm respectively. The constituents in these extracts were isolated and evaluated and the phytoecdysteroids 20-hydroxyecdysone (**1**) and stigmasterol (**2**) were identified as the active principles in the acetone stem bark while γ-sitosterol (**3**) was the active principle of the acetone leaf extract. The methanol leaf extract, the stem bark aqueous extract and the acetone root bark also showed potency against the mosquito species.



1 **2** **3**

Fig. 1: Isolated Phytoecdysteroids.

INTRODUCTION

Several mosquito species belonging to the genera *Anopheles*, *Culex* and *Aedes* thriving in the tropical and sub-tropical climates are vectors for pathogens transmitting diseases that affect the health of both man and livestock. A*nopheles* species, the malaria vector (Sinka et al., 2010), *Aedes aegypti*, the vector for yellow fever and dengue (Reiter, 2010) and *Culex pipiens*, the vector of West Nile virus (Diaz-Badillo et al., 2011) are responsible for most of tropical diseases. Malaria alone kills over half a million people each year (WHO, 2011).

Most of these insects are controlled using conventional chemical insecticides which are harmful to the environment, humans and many beneficial organisms. In addition, the chemicals have developed resistance to these synthetic insecticides. Consequently, this has indicated a need for additional or alternative approaches for controlling the proliferation of mosquito population. In response to this, several efforts have been made to identify alternative insecticides that are target-specific, biodegradable, environmentally safe, and botanicals in origin for integrated pest management (IPM) programmes. Presently, several products of botanical origin, especially the secondary metabolites, have received significant renewed attention as potentially bioactive

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agents used in insect vector management (Baraza *et al*., 2008; Kiran and Devi, 2007; Ndungu et al., 2004; Maniafu *et al*., 2009; Ribeiro, *et al*., 2009). Plant derived insecticides comprise of an array of chemical compounds which act in concert on both behavioral and physiological processes. Hence, the chances of pests developing resistance to such substances are less likely. Moreover, botanical insecticides are less likely to bioaccumulate as they are biodegradable (Saxena, 1987).

Among the botanical insecticides, phytoecdysteroids are present in the genus *Vitex* belonging to the family Verbenaceae and may be used as taxonomic markers. An extensive literature search on the genus *Vitex* revealed the presence of ecdysteroids in a number of *Vitex* species (Sena *et al*., 2008) found in tropical and subtropical regions (Correa, 1926).

The genus *Vitex* has been reported to exhibit larvicidal activities against a number of mosquito species (Rahman and Talukda, 2006; Yuan et al., 2006; Kannathasan et al., 2007; Rodriguez-Lopez et al., 2007; Karunamoorthi et al., 2008). In the current study, larvicidal and brine shrimp activities of *V. schiliebenii* extracts and isolated phytoecdysteroids on *An. gambiae* are presented.

MATERIALS AND METHOD

Plant materials

The leaves, stem bark and root bark of *V. schiliebenii* used in the study were collected from Watamu (Malindi County) along the Kenyan coast in 2009. The plant species was authenticated at the field by a botanist from the National Museum of Kenya (NMK) and a voucher specimen Ref. No. GMN/22 deposited at the NMK herbarium. The plant materials were dried under the shade to constant weight and ground into powder in an electric miller.

Chemicals and instruments

All solvents used were analytical grade (E-Merck,D-6100 Darmastadt, F.R. Germany). Analytical thin layer chromatography (TLC) was performed on 60 F_{254} plates 5 X 10 cm, 0.20 mm thickness) with fluorescence indicator. The spots were viewed using a multi-band UV- 254/366 nm lamp (UV GL-58). The TLC plates were then sprayed with *p*-anisaldehyde reagent (solution of anisaldehyde, 0.6% ethanol and 5% sulphuric acid) (reagents obtained from Sigma Aldrich, Germany) and kept in the oven at 110^{o}C until the spots appeared. Compounds containing unsaturated bonds especially those with conjugated systems became visible as quenching spots under UV light at 254 nm. Spots of organic compounds gave specific colors with this reagent after heating at 110^{o}C for 2-5 minutes. Column chromatography was carried out using silica gel mesh 0.06 mm (230-400 Merck-Germany) and eluted with varying concentrations of dichloromethane, acetone and methanol mixture. Melting points of the isolated crystallized compounds were determined on Sanyo GallenKamp electronic melting point apparatus. ^{1}H NMR spectra were run on Varian Gemini 300, 500 MHZ in $CDCl_3$ and CD_3OD and ^{13}C NMR spectra in Varian-Germini/Bruker 600 MHz in $CDCl_3$ and CD_3OD.

Extraction of plant materials

Each powdered material was extracted three times in acetone (5-fold volume) for 24 h with occasional stirring. The extracts were filtered and concentrated to dryness using a rotary evaporator at 40^{o}C and the combined extract stored at 4^{o}C. This procedure was repeated with methanol in the same proportion and for the same periods. The aqueous extracts were obtained using soxhlet extraction. The extracts were filtered and then freeze dried to obtain the dry powder which was then stored at 4^{o}C for further chemical and biological analysis.

Larvicidal assay

Larvae of *An. gambiae* Giles *s.s.* used in bioassays were obtained from a colony maintained at the International Centre of Insect Physiology and Ecology (ICIPE) Insect Mass Rearing Unit. This strain of mosquitoes originated from ICIPE's Thomas Odhiambo Campus (Mbita Point) near Lake Victoria in 2003. The larvae were fed on Tetramin® fish food (Terta GmbH, Germany) at about 1mg per beaker every 24- h and the water temperature was maintained at 28±2^{o}C throughout larval development. Larvicidal and insect growth regulatory (IGR) activities were conducted in accordance to the World Health Organization method (WHO, 1996). Batches of twenty freshly moulted late 3rd and early 4th instar larvae of *An. gambiae* s.s. were transferred by means of dropper to glass beakers containing 100 ml of tap water. Appropriate volume of stock solution where the crude or pure compounds were dissolved in 5% dimethylsuphoxide (DMSO) was added to 100 ml water in the glass beakers to obtain 25, 50, 100, 250 and 500 ppm for the crude extracts and 1, 5, 10 ppm dose levels for the pure compounds. Three replicates were set up for each concentration and two negative controls (treated with DMSO-distilled water) were set up simultaneously. Larval mortality, abnormal behavior and/or morphological deformations were recorded at 24-h intervals until the death of the last larva or emergence to adult. The bioassay room was kept at a temperature of 30^{o}C, an average humidity of 78 % and a photo period of 12 hours of light and 12 hours of darkness.

Brine shrimp test (BST)

In vitro brine shrimp lethality test of the extracts was used to detect larval toxicity. Brine shrimp eggs were purchased from Aquaculture Innovations (Grahamstown 6140, South Africa) and sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast. Brine shrimp eggs were placed in sea water (3.8 g of sea salt in one liter of distilled water) and incubated infront of an electric lamp. Eggs were hatched within 48 h, providing a large number of brine shrimp larvae (nauplii). Stock solutions (40 mg/ml) of each extract were prepared by dissolving them in DMSO. Different concentration levels were prepared by serial dilution and 10 nauplii were added into 10 ml vials. The volume was then adjusted

to 5ml with artificial sea water (3.8% w/v sea salt in distilled water). Each concentration was tested in triplicates with two negative controls running simultaneously containing 10 nauplii, sea water and 5% DMSO only for comparison. The vials were incubated for 24 h under the same conditions used for rearing the brine shrimp larvae.

Isolation of compounds from the acetone stem barks and leaves of *V. schiliebenii*

Sixteen grams (16 g) of the acetone stem bark extract was subjected to column chromatography on silica gel with Dichloromethane: Acetone gradient (100:0 - 0:100). The 50% acetone eluent gave a mixture of two compounds (47 mg), which were separated by repeated column chromatography followed by preparative thin layer chromatography (PTLC). The fraction yielded two compounds 20-hydroxyecdysone (**1**) (16 mg) isolated as a crystalline solid melting point 234-236 ^{o}C (lit. 230-233 ^{o}C, (Kavel *et al.*, 1998) which was soluble in methanol and stigmasterol (**2**) (25 mg of white crystals with a melting point of 165-7^{o}C (lit. 163-6^{o}C (Greca *et al.*, 1990). The acetone leaf extract (10 g) was subjected to column chromatography on silica gel (eluting with 100% dichloromethane and gradually increasing acetone to 100%). The 40% acetone eluent gave a mixture of two compounds, which were subjected to repeated column chromatography using DCM: MeOH to yield γ sitosterol (**3**) (6 mg with a melting point of 142-144^{o}C) which was then re-crystallized using the same solvent into a white star shaped crystal soluble in DCM. The other compound was too little to be analyzed.

Statistical analysis

The average larval mortality (± standard error) resulting from each dose of each extract/compound was calculated and the data was subjected to probit analysis for calculating the lethal concentrations of the crude plant extracts/pure compounds at LC_{50} at 95% confidence limit of upper and lower levels. The values were calculated using GenStat Discovery Edition 4. Results with $p<0.05$ were considered to be statistically significant.

RESULTS

Acetone, methanol and aqueous extracts of the leaves, stem bark and root bark of *V. schiliebenii* were tested for lethality against the late 3rd and early 4th instar larvae of *An. gambiae*. Acetone leaves and stem bark extracts showed significant activities ($p<0.05$), with LC_{50} values of 14.6 and 17.4 ppm respectively at 24 h with the former extract causing 100% mortality at all doses tested while the latter achieved 90% mortality at the lowest dose at 72 h (Table 1).

The two extracts exhibited low toxicity to the brine shrimp larvae with LC_{50} values of 180.9 and 154.4 ppm respectively.

Other active extracts included methanol leaf, aqueous stem bark and acetone root bark extracts with LC_{50} values of 136.3, 182.6 and 252.1 ppm respectively while methanol stem bark exhibited relatively weak activity (LC_{50} = 522.6 ppm) at 24 h. The control cohorts showed a maximum percentage mortality of 8.3±1.7 at 72 h. The larvae developed into pupae and then adults within 48–72 h.

Table. 1: Laboratory activity of crude extracts of *Vitex schiliebenii* against 3rd/4th Instar larvae of *Anopheles gambiae* s.s Giles after 24h, 48h and 72h exposure

Extract code	Timee (Hr)	Cumulative mean mortality (% ± SE)/Concentration (ppm)					Lethal concentration values (ppm)	
		500 ppm	**250 ppm**	**100 ppm**	**50 ppm**	**25 ppm**	**LC_{50}**	**95% CL**
VSRB-221	24	96.7±1.6	43.3±3.3	3.3±1.6	-	-	252.1	225.0-281.8
	48	100.0±0.0	96.6±3.3	15.0±0.0	0.3±0.0	0.3±03	136.0	120.1-154.0
	72	100.0±0.0	100.0±0.0	100.0±0.0	83.3±4.4	4.7±1.7	38.5	33.9-43.6
VSRB-222	24	56.7±1.6	15.0±2.9	5.0±2.0	-	-	444.0	392.0-505.0
	48	71.7±3.3^{ab}	38.3±1.6	18.3±1.6	-	1.7±0.0	295.9	260.8-335.7
	72	100.0±4.4^{a}	100.0±0.0	68.3±4.4	10.0±1.7	5.0±2.9	80.6	71.6-90.9
VSRB-223	24	-	-	-	-	-	3652.6	50.2-284895
	48	-	-	-	-	-	-	-
	72	10.00±0.0^{a}	28±1.6	-	-	-	307.5	256.9-370.7
VSSB-221	24	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	78.3±6.0	17.4	14.6-20.3
	48	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	81.7±4.4	15.0	12.3-18.1
	72	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	90.0±5.8	11.9	9.1-15.1
VSSB-222	24	43.3±1.7	6.7±1.7	-	-	-	522.6	462.8-594.9
	48	100.0±0.0	98.3±1.7	50.0±1.7	6.7±3.3	10.0±2.7	103	91.7-117.1
	72	100.0±0.0	100.0±0.0	90.0±5.8	65.0±5.7	30.0±0.0	39.9	35.2-45.2
VSSB-223	24	100.0±0.0	57±3.3	30±3.3	-	-	182.6	161.8-206
	48	100.0±0.0	88±1.7	86±2.7	-	-	84	67.9-104
	72	100.0±0.0	100±0.0	100±0.0	50.8±1.7	-	49.1	41.2-58.2
VSL-221	24	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	96.7±1.7	14.6	11.9-17.6
	48	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	13.1	10.4-16.2
	72	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	10.6	7.9-13.9
VSL-222	24	100.0±0.0	100.0±0.0	31.6±3.3	10.0±2.9	6.7±1.7	136.3	120.8-154.8
	48	100.0±0.0	100.0±0.0	60.0±5.8	48.3±1.7	10.0±0.0	63.1	56.0-71.1
	72	100.0±0.0	100.0±0.0	95.0±6.0	63.3±2.9	28.3±1.7	40.1	17.0-28.2
VSL-223	24	3.0±3.3	-	-	-	-	1672.9	1222.1-2389
	48	15.0±3.3	-	-	-	-	-	-
	72	25.0±1.7	-	-	-	-	-	-
Control	24	2.3±3.3	-	-	-	-	-	-
	48	8.3±1.7	-	-	-	-	-	-
	72	8.3±1.7	-	-	-	-	-	-

Chromatographic separation of the acetone stem bark extract resulted in the isolation and identification of two phytoecdysteroids [20-hyrdoxecdysteroid (**1**) and stigmasterol (**2**)] and γ-sitosterol (**3**) was isolated from the acetone leaves. The identification was done by physical and spectroscopic techniques and also comparison with literature data (Table 2). These compounds were evaluated for larvicidal activities where compounds **1** and **2** were found to be responsible for the activity of the stem bark and compound **3** was responsible for the activity of the leaves. Among the three compounds, 20-hyrdoxecdysteroid (**1**) showed the highest activity with LC_{50} value of less than 1 ppm followed by stigmasterol (**2**) with LC_{50} = 8.145 ppm and then γ-sitosterol (**3**) with LC_{50} = 8.609 ppm at 72 h.

Table. 2: ^{13}CNMR spectral data for compounds 1, 2, and 3.

Compounds	**1**		**2**		**3**	
Position	$\delta_{(obs.)}$	$\delta_{(lit.)}$	$\delta_{(obs.)}$	$\delta_{(lit)}$	$\delta_{(obs.)}$	$\delta_{(lit.)}$
1	36.0	37.3	37.3	37.2	37.3	37.0
2	67.3	68.6	31.9	31.8	29.4	29.5
3	67.1	68.5	71.8	71.5	71.8	71.8
4	31.5	32.7	42.3	42.2	42.3	42.3
5	50.4	51.7	140.8	140.7	140.8	140.8
6	205.1	206.6	121.7	121.6	121.7	121.7
7	120.7	122.1	33.9	33.6	31.7	31.9
8	166.5	168.1	29.7	29.6	29.2	29.2
9	33.7	35.0	50.1	50.1	50.2	50.2
10	37.9	39.3	36.5	36.4	36.5	36.5
11	20.1	21.5	21.1	21.1	21.1	21.1
12	30.4	32.4	39.7	39.7	26.1	26.1
13	47.4	48.6	42.2	42.2	45.9	45.9
14	83.4	84.2	56.8	56.1	56.8	56.7
15	31.1	31.7	24.3	24.1	24.3	24.1
16	20.1	21.5	28.4	28.3	39.8	39.8
17	49.1	50.5	56.7	56.0	56.1	56.1
18	16.7	18.1	12.0	12.1	11.9	12.2
19	23.0	24.4	19.4	19.4	18.8	18.8
20	76.5	78.0	40.5	40.3	34.0	34.0
21	19.7	21.1	19.8	20.5	19.1	19.1
22	77.0	78.4	138.3	138.5	37.3	37.3
23	25.9	27.3	129.3	129.4	26.2	26.6
24	41.0	42.3	51.2	51.2	51.6	50.1
25	69.9	71.4	31.7	31.9	28.2	28.3
26	28.3	29.1	21.1	21.2	19.4	19.4
27	27.6	29.7	19.0	19.8	19.8	19.8
28			26.1	25.4	23.3	23.3
29			11.9	11.9	11.8	12.0

*- signals may be interchanged
Compounds 1 and 2: (500 MHz, MeOD); Compound 3: (300 MHz, $CDCl_3$);

DISCUSSION

Currently, numerous products of botanical origin have received considerable renewed attention as potentially bioactive agents used in mosquito control (Marimuthu et al., 2012; Kovendan et al., 2012; Paneerselvan & Murugan, 2013). More than 2000 plant species have been known to produce chemical factors and metabolites of value in pest control programmes. Members of the plant families-Solanaceae, Asteraceae, Cladophoraceae, Labiatae, Miliaceae, Oocystaceae, Verbenaceae and Rutaceae have various types of larval, adulticidal or repellent activities against different species of mosquitoes (Anupam et al., 2012). In addition, the genus *Vitex* (Verbenaceae) has been reported to have a plethora of ethnopharmacological uses (Padmalatha et al., 2009). This suggests that natural products from plant species in the genus have low human toxicity but can still be used as potential agents against mosquitoes. This is confirmed by the observation made in the current study where the two active acetone extracts were found to exhibit good larvicidal activity but very low toxicity to the brine shrimp larvae. It is also worth noting that although acetone and methanol extracts produced encouraging results, they are difficult to produce and use by resource-poor people in rural Africa particularly. The aqueous extract is more applicable to rural situations where malaria causes the greatest burden. The stem bark aqueous extracts displayed good larvicidal potential with LC_{50} values decreasing from 182.6 ppm to 49.1 ppm at 24 h and 72 h (Table 1).

Previously, Vasanth Raj et al (2009) had evaluated aqueous extract of *V. negundo* against mosquito larvae of *Cx. quinquefasciatu, An. stephensis* and *Ae. aegypti* and the extract was found to be effective with LC_{50} values of 167.88 ppm, 167.88 ppm and 231.17 ppm respectively. Hence in the absence of *V. schiliebenii*, *V. negundo* can be used. Other than phytoextracts being used as larvicides, several groups of phytochemicals have been reported for their insecticidal activity and among them are the phytocdysteroids (Zolotar et al 2001). In the present study, the high larvicidal activity noted in compound **1** is in agreement with those of other phytoecdysteroids having a long side chain with hydroxyls (Zolotar et al 2001, Radi et al., 2011). According to literature, the steroidal side chain with hydroxyls is necessary in producing high insect hormonal activity in ecdysteroids (Zolotar et al 2001).

CONCLUSION

The results of the present study indicate a potent effect of phytoextracts from *V. schiliebenii* and the isolated phytoecdysteroids on *An. gambiae*. The plant therefore holds great promise as a locally available larvicidal plant. Further work in this area should focus on the exploration of the efficacy of the isolated phytoecdysteroids, under field conditions, with the aim of formulating a botanical larvicide.

ACKNOWLEDGEMENTS

The authors are grateful to the International Centre of Insect Physiology and Ecology (ICIPE-Kenya) for providing the necessary bioassay requirements. Muhimbili University of Health and Allied Sciences is also acknowledged for providing the necessary chromatographic equipment for fractionation and purification of the crude plant materials. The authors also acknowledge DAAD-NAPRECA for funding the work reported in this paper.

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How to cite this article:

Mokua G. Nyamoita, Innocent Ester, Mbwambo H. Zakaria, Lwande Wilber, Bwire J. Ochola, Hassanali Ahmed., Larvicidal and Brine Shrimp Activities of *Vitex Schiliebenii* Extracts and Isolated Phytoecdysteroids on *Anopheles gambiae* Giles S.S Larvae. J App Pharm Sci, 2013; 3 (05): 091-095.