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Time-course effects of Vitex schiliebenii (Varbenaceae) solvent extracts on Anopheles gambiae giles s.s. larvae under simulated semi-field conditions

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ABSTRACT

***Objectives:** To determine the growth-disrupting effects of polar phyto-extracts of V. schilibenii on Anopheles gambiae larvae in a simulated semi-field condition and to undertake phytochemical screening of constituents present.*
***Materials & Method:** 3rd and early 4th instars of An. gambiae larvae were exposed to acetone and methanol extracts of stem bark and leaves of V. schiliebenii and their effects on larval, pupal and adult stages recorded. Phytochemical screening of the extracts was undertaken using standard methods.*
***Results:** The results revealed that Anopheles gambiae larvae were susceptible to V. schiliebeinii extracts with less than 20 % adult emergence at concentrations ≥ 25 ppm except for methanol extract of stem bark. About 11 % pupae emerged in V. schiliebenii acetone leaf extract (VSL 1) between day 6 and 10 but they did not transform into viable adults. Phytochemical screening revealed the presence of flavonoids, terpenoids, steroids, alkaloids, saponins and tannins in the extracts.*
***Conclusion:** Eco-friendly polar extracts of V. schiliebenii show potential for mosquito control in small breeding habitats, which may be a useful component in integrated control of malaria vectors. Characterization of the active constituents of the extracts of the plant is in progress.*

KEYWORDS: *Anopheles gambiae* s.s., *Vitex schiliebenii*, Verbenaceae, larvicidal effects, Photochemical

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INTRODUCTION

Over two billion people in tropical countries are at risk from mosquito-borne diseases such as dengue fever, hemorrhagic fever, malaria and filariasis. It is estimated that US$ 2 billion is spent on malaria control and treatment programmes in Africa annually[1]. The problem has become increasingly difficult to manage because of the spread of resistance to anti-malarial drugs by the parasites resulting in increased severity of the disease[2]. Personal protection using

synthetic and plant repellents from mosquito bites and control of mosquitoes using mosquito nets impregnated with synthetic insecticides are currently the dominant measures of reducing mosquito bites[3]. However, vector resistance to synthetic insecticides is a recurring problem[4]. A number of studies have shifted focus to botanical substances for either personal protection and/or controlling mosquitoes at immature stages[5-11]. Promising plant families studied for larvicidal effects include Meliaceae, Rutaceae, Labiatae, Piperaceae, Verbenaceae, Asteraceae, Cladophoraceae, Oocystaceae, and Annonaceae[12].

Besides their popular use in traditional medicines in many countries, *Vitex* species have been reported to exhibit activities against a variety of mosquito species, such as *Culex trataeniorhnchus*, *Anopheles gambiae*, *Culex quinquefasciatus*, *Plutella xylostella*, and *Callosobruchus maculates*[9, 13-15] *V. schiliebenii* is a branched shrub with multiple square-shaped stems and low height (4-8 m). The leaves have 3-leaflets with a smooth surface, about 10-12 cm long. In Kenya, it grows in the coastal region at Watamu located in a low altitude, semi-arid area[16-17]. In an initial laboratory study, polar extracts of *V. schiliebenii* showed interesting growth-disrupting activities on immature of *An. gambiae* (Mokua *et al*., submitted). In the present study, the potential of the extracts to control the vector was investigated under simulated semi-field conditions.

MATERIAL AND METHODS

Plant material collection

The leaf and stem bark of *V. schiliebenii* were collected from the North Coast of Kenya (at Kenya Forest Research Institute , KEFRI) near Gede along Mombasa-Malindi road 18 km from Malindi town. The plant species was authenticated at the field by Simon Mathenge of the National Museum of Kenya (NMK) and a voucher specimen Ref. No. GMN/22 deposited at the NMK herbarium.

Preparation of plant extracts

The plant materials were air-dried under shade at room temperature (25±2^{o}C) for three weeks, ground into powder in an electric miller and soaked in different solvents to obtain the extracts. Powdered leaves (400 g) and stem bark (800g) were separately soaked in acetone (2.0 and 4.0 L, respectively) for 24 h with occasional stirring, and then filtered. This was repeated three times. The process was repeated with similar quantities of methanol for both plant materials. Each set of solvent extracts were pooled together and concentrated to dryness using a rotary evaporator at 40^{o}C.

Mosquito species

Larvae of *An. gambiae* s.s. Giles used in bioassays were obtained from a colony maintained at the Insect Mass Rearing Unit of International Centre of Insect Physiology and Ecology (ICIPE). This strain of mosquitoes originated from ICIPE's Thomas Odhiambo Campus (Mbita Point) near Lake Victoria in 2003. Larvae were allowed to emerge from eggs in plastic containers filled with distilled water and were transferred to larger pans (37 × 31 × 6) at densities of 200-300 at 2nd instar stage. They were fed on Tetramin® fish food (Terta GmbH, Germany) and the water temperature was maintained at 28±2^{o}C throughout larval development.

Simulated field assays

Bioassays were conducted in accordance to World Health Organization procedure[18] in 1000 ml chlorine-free water in white plastic containers of internal diameter 21.5 cm and 13 cm. These containers were placed in holes (10 cm deep) in a screen house arranged in three rows and 13 columns with a distance of 50 cm in between. The containers were filled with 400 ml

of water and left for 24 h for conditioning and then batches of 50 freshly molted late 3rd and early 4th instars larvae of *Anopheles gambiae* were added together with food and left for 2 h to allow the larvae get acclimatized with the environment. Five milligrams of each extract was dissolved in 1 ml distilled water containing 5 % dimethyl sulphoxide (DMSO), which gave a homogenous solution. Stock solution of each plant extract was successively diluted to give concentrations of 12.5, 25, 50, 100, 250, and 500 ppm in 500 ml. The effect of each test solution together with a negative control (treated with DMSO-distilled water) was replicated three times. Sampling was carried out using a 350 ml dipper at different sides of the containers. Three dips were taken for each test sequenced in such a way that triplicates of similar concentrations were sampled consecutively with the same dipper. One dip was taken from each container at a time returning to it after completion of the cycle for that particular treatment until all the four samples were taken. Mortality was recorded after every 24 hours until the death of the last larva or emergence of adult[19]. Mortality in control treatments was corrected using Abbott's[20] formula when it ranged between 5-10 percent. All the treated and control containers containing pupae were kept separately in a netted cage to prevent successfully emerged adult from escaping into the environment. The percent adult emergence inhibition (% EI) was based on the number of moribund and dead larvae, pupae that did not develop successfully into viable adults and pupal-adult intermediates. The average screen house temperature was 32 ± 4°C and the humidity was 65 ± 10 % RH. The larvae were fed on Tetramin® fish food (Terta GmbH, Germany) and the adults 10 % glucose solution.

Analyses

The average number of larvae or pupae collected per dip for each replicate of each treatment and the control were recorded after 24 h. Percentage inhibition of adult emergence (% IE) from the data on mortality at all stages was estimated for each treatment according to the formula [18]:

$$\% \text{ IE} = 100 - (T / C \times 100)$$

Where T = number of adults emerging in treated test and C = number of adults emerging in control test.

Where mortality in control tests ranged between 5-10 %, the corrected mortality was calculated using Abbott's formula [19]:

$$\% \text{ mortality} = \left[\frac{\text{mortality in treated - mortality in control}}{100 - \text{mortality in control}}\right] \times 100$$

However, where control tests showed more than 10 % mortality (two cases out of sixteen), they were discarded and the whole experiment repeated.

Phytochemical screening

The following phytochemical tests [20] were carried out on the acetone and methanol extracts to identify the major classes of constituents.

Alkaloids: about 20 ml of Dragendorff's reagent was added to about 20 mg of the extract; the formation of orange red precipitate suggested the presence of alkaloids;

Tannins: a small amount of the extract was mixed with water, heated on the water bath, filtered and ferric chloride added; a dark green solution suggested the presence of tannins;

Saponins: about 200 mg of the extract was shaken with 5 ml distilled water heated until it boiled, transferred into a test tube and shaken vigorously and left to stand for about 10 minutes; a thick persistent froth suggested presence of saponins;

Flavonoids: Extract of about 200 mg was dissolved in NaOH followed by addition of HCl; yellow solution that turned colorless suggested the presence of flavonoids;

Terpenoids (Salkowski test): About 200 mg of the extract was mixed with 2 ml of chloroform and 3 ml concentrated sulphuric acid was carefully added; a reddish brown coloration at the interface suggested the presence of terpenoids;

Steroids: About 2 ml of acetic anhydride was added to about 200 mg of the extract followed by 2 ml of sulphuric acid; change from violet to green indicated the presence of steroids.

RESULTS

Yields and phytochemical profiles

Acetone and methanol crude extracts of *V. schiliebenii* produced semi-solid materials of varying yields between 2.48-4.11 % w/w. The percentage yield obtained from acetone extracts (3.67-4.11) was generally higher than from methanol extracts (2.48-3.05). Phytochemical screening revealed the presence of flavonoids, terpenoids, steroids, alkaloids, saponins and tannins in both sets of extracts.

Developmental disruption and mortality

Prolonged developmental time was observed in the treated cohorts as compared with control tests. The total larval period lasted 9-10 days (6-8 days in control) and pupal period lasted 2-3 days (1 day in control) resulting in total developmental period (larval + pupal development) of 11-13 days (7-9 days in control).

Most of the mortality was in larval and pupal stages and only a few were dead at the adult stage. Cumulative mean percentage mortality of larvae exposed to different extracts at 12.5 ppm ranged between 23-52 %, and that of the pupae and pupal-adult intermediates ranged between 2-15 %. At 25 ppm, % larval mortality ranged between 43-90 % while that of pupae and pupal-adult intermediates ranged between 4-14 %. There were no significant differences ($P < 0.05$) in mortalities between treatments at 50 and 100 ppm with different extracts. Larvae exposed to *V. schiliebenii* methanol stem bark extract and *V. schiliebanii* acetone leaf extract at 100 ppm and above produced no live or dead pupae. The few adults that emerged from treated cohorts appeared weak and unable to fly effectively. They rested for longer periods on the surface of the water compared with the control adult mosquitoes and died within 48 h after emergence. In control treatments, during the four-day exposure mortality increased from 2 to 8 % and the rest developed into pupae and then adults within 2-4 days.

Inhibition of adult emergence

The main indicator of the treatment response was the percentage inhibition of emerged adults. At 12.5 ppm, acetone extracts of *V. schilebenii* stem bark and leaves were found to be most effective, inhibiting adult emergence by 56-57 %, At 25 ppm, no adult emerged in treatments involving both *V. schiliebenii* acetone and methanol leaf extracts (88 and 86 % larval mortality and 12 and 14 % pupal/adult mortality, respectively; Fig 1).

DISCUSSION

In the present study, the effects of leaf and stem bark extracts of *V. schiliebenii* on *An. gambiae* s.s. larvae were evaluated for their potential as sources of botanicals for the control of malaria vectors under semi-field conditions. Prolonged developmental time was observed at the lowest concentration (12.5 ppm), suggesting a subtle physiological effect of the phytochemicals. Suppression in pupation was also observed at 12.5 ppm and in some cases resulting in larval-pupal intermediates. This could be attributed to hormonal balance disruption with internal levels of ecdysone. One of the main effects of ecdysteroids on insects

is their interference with development and the important role they play in the transition from one developmental stage to another, especially during metamorphosis.At higher concentrations (≥25 ppm), remarkable larval mortality was observed suggesting that *V. schilibenii* can be used as an insecticidal agent. Results revealed that *V. schiliebenii* is rich in phytochemicals and the observed biological activity might be due to the presence of these bioactive compounds which could synergistically, antagonistically or independently contribute to the activity of the crude extracts.

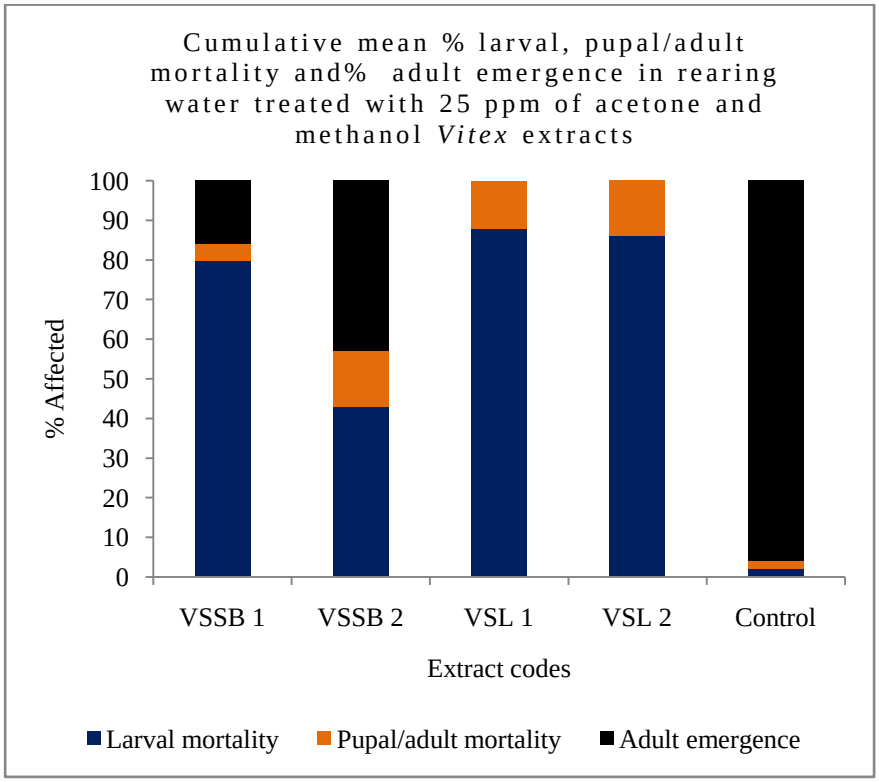

Figure 1: Percent affected at different stages
VSL 1: acetone leaf extract; VSL 2: methanol leaf extract;
VSSB 1: acetone stems bark extract, VSSB 2: methanol stem bark extract

Interestingly, a similar study conducted by[21] with plant sterols on the larvae of *Dermestes maculates* reported that the phytosterols interfere with the utilization of cholesterol by the insect. In the family *Verbeneceae*, the genus *Vitex* has been identified as one of the best sources of phytoecdysteroids [22]. Phytochemical investigation of the plant in the current study also revealed the presence of phytoecdysteroids in the extracts. Significant disruptions of the insect hormonal system by exogenic ecdysteroids ingested with food and eventual death have also been reported by[23]. Some of the *Vitex* species like *V. negundo*, *V. trifolia*, *V. rotundifolia* and *V. agnus-castus* and their bioactive constituents have been reported to exhibit larvicidal activity against various mosquito species such as *Anopheles subpictus*, *Culex tritaeniorhynchus*, *Culex quinquefasciatus*, *Anopheles stephensi*,[7, 13-14, 24-27,]. Bioactive constituents like methyl-*p*-hydroxybenzoate isolated from the leaves of *V. trifolia*[28], lauric acid, palmitic acid, steric actid and oleic acid extracted from *V. altissima*, *V. negundo* and *V. trifolia*[29] have been reported to be responsible for their potential as larvicides. However, in the present study the specific constituents or blends associated with the observed effects on the mosquito larvae are yet to be unveiled.

In conclusion, *V. schiliebenii* showed promising larvicidal activity and hence may be considered as a source of a botanical agent which can be developed into eco-friendly chemicals for control of vector-borne diseases and more particularly, *An. gambiae*. Identification of the principle constituents responsible for the observed effects is underway to help in throwing further light on the mode of action.

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