Larvicidal and repellent activity of *Vetiveria zizanioides* L, *Ocimum basilicum* Linn and the microbial pesticide spinosad against malarial vector, *Anopheles stephensi* Liston (Insecta: Diptera: Culicidae)

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ABSTRACT

Use of synthetic pesticides causes some unfortunate consequences such as environmental pollution, pests/ vector resistance and toxicity to other non-target organisms including human beings, biological pesticides from plant, microbial origin are environmentally safe pesticides. Microbial insecticides are especially valuable because their non-toxicity to non-target animals and human beings. Laboratory investigation using the plants such as, *Vetiveria zizanioides* (Linn.) (Poaceae), *Ocimum basilicum* (Linn.) (Lamiaceae) and the microbial pesticide spinosad against the malarial vector *Anopheles stephensi* Liston showed 85% mortality. The observed mortality rate suggests the above extract can be used as biopesticides. The LC_{50} of second, third and fourth instar larvae of *A. stephensi* were 0.276%, 0.285% and 0.305%, respectively.

Key words: *Vetiveria zizanioides, Ocimum basilicum, microbial* pesticide, spinosad.

INTRODUCTION

Malaria is transmitted by different *Anopheles* species depending on the the region and the environment (Burfield and Reekie, 2005). Mosquitoes are a major threat for over 2 billion people in the tropics (Odalo *et al*., 2005). They are nuisance to human beings and spread dreadful disease like malaria, filariasis, dengue haemorrhagic fever and Japanese encephalitis etc., *Anopheles stephensi* was the vector for malarial fever. It developed resistance to a variety of insecticides. These factors have created a search for biodegradable and target-specific insecticides for the mosquitoes. Plant products have been used by traditionally human communities in many parts of the world against the vectors and species of insects. The phyto-chemicals derived from plant sources can act as larvicides, insect growth regulators, repellents, ovipositional attractants and have deterrent activities (Babu and Murugan, 1998). Murugan *et al*. (2003) studied the interactive effect of botanicals, Neem, Pongamia and *Leucas aspera*, *Bacillus sphaericus* against *Culex quinquefasciatus.*

Spinosad is a natural fermentation product produced by an actinomycete, *Saccharopolyspora spinosa* Mertz and Yao. This compound is a mixture of Spinosyns A and D (Christos *et al.,* 2008). Structurally, Spinosad can be described as a macrocyclic lactone containing a unique tetracyclic ring to which two different sugars are attached. Spinotoram is a powerful neurotoxin against certain arthropods (Steven *et al.,* 2007). Spinosad exhibits stomach and contact poisoning properties and affects specifically the function of ã-aminobutyric acid (GABA) receptors and nicotinic acetylcholine receptors of the target insects. This product has been widely tested against injurious insects in a variety of crops, such as cotton, wheat and tobacco (Thavara *et al.*, 2009). It has shown activity against Lepidoptera, Thysanoptera, and other insect orders such as Diptera. This naturally derived insecticide has been reported to have no adverse effects on predatory insects such as ladybirds, lacewings, big-eyed bugs, or minute pirate bugs (Williams *et al*., 2003). Spinosad acts as a stomach and contact poison and degrades rapidly in the environment (Cisneros *et al.*, 2002). Bond *et al.* (2004) reported that it is naturally derived insecticide having high toxicity to *Aedes* and *Anopheles* mosquito larvae. Darriet *et al.* (2005); Romi *et al*. (2006) studied bioinsecticidal activity of Spinosad against mosquitos. Spinotoram an analogous compound can be used in cotton, citrus, pome fruit pest management. The aim of this work was to evaluate the larvicidal and repellent potential of *V.zizanioides, O. basilicum* and microbial pesticide spinosad against malarial vector, *A. stephensi*.

MATERIALS AND METHODS

Collection and Maintenance of mosquitoes

The eggs of *A. stephensi* were collected from in and around Coimbatore districts (drinking water bodies, water stored container) with the help of 'O' type brush. These eggs were brought to the laboratory and transferred to 18 X 13 X 4 cm size enamel trays containing 500 ml of water for larval hatching. The mosquito larval and pupal culture was maintained in the laboratory. The plastic jars will be kept in 90 X 90X 90 cm size mosquito cage for adult emergence. The cage is made up of wooden frames and covered with polythene sheets on four sides (two laterals, one back and other one upper) and the front part as covered with a muslin cloth bottom of the cage is fitted with 10% sugar solution for a period of three days before they will be provided with animal for blood feeding. The adult female mosquitoes were allowed to feed on the blood of a rabbit (exposed on the dorsal side) for two days to ensure adequate blood feeding for 5 days. After blood feeding enamel trays with water from the culture trays will be placed in the cage for the adults to lay eggs.

Collection and preparation of Phyto extract

Vetiveria zizanioides and O. basillicum were collected from Bharathiar University, Coimbatore. The plants were identified at BSI, Coimbatore (Botanical Survey of India) and the specimens were deposited at Zoology Department, Bharathiar University, Coimbatore, India. The roots of *V. zinzanoides* and leaves of *O. basillicum* leaves were washed with double distilled water and shade dried at room temperature for 7-10 days. The dried parts were chopped into small pieces of approximately 1 cm size by a falcon stem cutter and powdered using electric blender. The dried powder was subjected to methanol in a Soxhlet apparatus (Borasil, Mumbai, India) for 72 h (Vogel, 1978). After removing the solvents from the plant extracts in a vacuum rotary evaporator, stock solution of 1% was prepared with 200 mg residue in 20 mL ethanol and was kept in a screw-cap vial with aluminum foil over its mouth. The stock solution was then serially diluted ten-fold in methanol (2 mL solution to 18 mL solvent) and test concentrations were obtained by adding 0.1–1.0 mL of the appropriate dilution to 100 mL distilled water (WHO 2005). One gram of the plant residue was dissolved in 100 ml of methanol (stock solution) considered as 1% stock solution. From this stock solution different concentrations were prepared ranging from 2 to 10%, respectively.

Preparation of Spinosad

Spinosad was purchased from Stanes and Co, Coimbatore, Tamil Nadu, India. Required quantity of Spinosad was thoroughly mixed with distilled water to prepare various concentrations like 0.1 ppm, 0.1 ppm, 0.2ppm, 0.4 ppm and 0.5 ppm, ranging from 0.001 to 0.008 ppm, where 1 ppm is equivalent to 1mg\liter.

Test for larvicidal activity

Anopheles stephensi was used for the larvicidal and pupicidal activity. It was maintained at 27 ± 2 °C, 75–85% RH and 14L: 10D photoperiod cycles. The larvae were fed with dog biscuits and yeast at 3:1 ratio. Twenty five II, III and IV instar larvae of *A. stephensi* were kept in 500ml glass beaker containing 249 ml of dechlorinated water and 1.0ml of desired plant extract concentration such as 2, 4, 6, 8 and 10 ppm. Three replicates for each concentration were made set up. A control was maintained with 1.0 ml of acetone in 249 ml of dechlorinated water. The control mortality was corrected by Abbott's formula (Abbott, 1925) and LC_{50}, LC_{90} regression equation and 95% confidence limit of lower (LCL) and upper confidence limits (UCL) were calculated by using probit analysis (Finney, 1971).

Smoke Toxicity Test

The mosquito coils were prepared using *V. zizanioides* root and *O. basilicum* leaf by following the method of Saini *et al.* (1986) with minor modifications by using 4 grams of coconut shell, charcoal powder as burning material. These ingredients were thoroughly mixed with distilled water to form a semisolid paste. A mosquito coil (0.6 cm thickness) was prepared manually and shade dried. The control coils will be prepared by without the plant ingredient. The experiments were conducted in glass chamber measuring 140 X 120 X 60 cm. A window measuring 60 X 30 cm was situated at mid bottom of one side of the chamber. Three or four day's old blood starved hundred adult female mosquitoes, fed with sucrose solution, were released in the chamber. A belly shaven pigeon was kept tied inside the cage in immobilized condition. The experimental chamber was tightly closed. The experiment was repeated five times on separate days including control, using mosquitoes of same age groups. The data were pooled and average values were subsequently used for calculations. Control was maintained in two sets. One set was run with coil lacking the active ingredient of plant powder (control 1) another one was a commercial coil (control 2), which was used for positive control to compare the effectiveness of plant coils. After the experiment was over, the fed, unfed (active and dead) mosquitoes were counted. The protection given by the smoke from plant samples against the biting of *A. stephensi* was calculated in terms of percentage of unfed mosquitoes due to treatment. Data were analyzed using analysis of variance (ANOVA) and means separated by Duncan's multiple range tests.

Field trial

The field trials were conducted at Bharathiar University by using required concentration of plant extracts and bacterial pesticide in different breeding habitat such as overhead tank, cement tank and cement container (0.5x 0.5 m and 1cm depth). Selection of the localities was decided on the basis of the breeding potential and operational convenience. Field application of the plant extracts and bacterial pesticides were done with the help of a knapsack sprayer (or) hands sprayer at 11.1 Percentage. Biopesticides were sprayed uniformly at the surface of the water in each habitat. The mean larval density was calculated on the basis of 5 dips per each habitat. Prior to the experiment the surface area of the breeding habitat were measured along with the pre-spray density of larvae. After the treatment the post–spray density of larvae were recorded after 24 hours. Successive observations were made at an interval of one day. The percentage reduction was calculated by the following formula (Mulla, 1971, Murugan *et. al.*, 2003). Per cent réduction = $100 – (C_1 / T_1 \times T_2 / C_2)$. Where C_1 and T_1 are pre-treatment density and T_2 and C_2 are the post-treatment density of larvae per dip in the control and treated habitats, respectively.

RESULTS

Table 1 illustrates the larval (II to IV) mortality after the treatment of spinosad at different concentrations. Results revealed that mortality was does dependent one (43 and 71 % for 0.2 ppm and 0.5 ppm, respectively) for second instar larva of *A. stephensi*. Similar trend has been noticed in third and fourth instar larvae the malarial vector at different concentration of spinosad treatment. The LC_{50} values of II, III and IV instar larva was 0.276 %, 0.285 %, and 0.305 %, respectively. Similar trend has also been observed for LC_{90} (0.574 %, 0.535 %, and 0.535% for II, III and IV instar larva, respectively). The considerable mortality was evident after the treatment of *O. basilicum* for II to IV instars. Mortality was increased as the concentration was increased, for instant 78%, 81%, and 84% mortality were noted for II instar larva by the treatment of *O. basilicum* leaf extract at 2%, 4 %, and 6% 8 % and 10%, respectively. The larval mortality of *A. stephensi* after the treatment of methanolic extract of *V. zizanioides* root extract respectively.

Table 1. Impact of *Spinosad* against the malarial vector *Anopheles stephensi* Larval mortality (in %)

Larval instars	Concentration (mg /ml) 0.1	0.2	0.3	0.4	0.5	LC_{50}	LC_{90}
Spinosad							
II	19 [b]	37 [a]	49 [c]	66 [a]	85 [ab]	6.249	11.019
III	16 [b]	34 [b]	47 [c]	63 [b]	82 [b]	5.898	10.369
IV	12 [c]	30 [b]	43 [d]	61 [b]	80 [b]	6.520	11.634
O. basilicum leaf extract							
II	16 [b]	35 [a]	44 [c]	62 [a]	84 [ab]	5.785	10.761
III	13 [b]	32 [b]	45 [c]	61 [b]	81 [b]	5.967	10.731
IV	10 [c]	29 [b]	40 [d]	58 [b]	78 [b]	6.369	10.960
V. zizanioides root extract							
II	12 [b]	30 [a]	41 [c]	59 [a]	80 [ab]	0.276	0.574
III	11 [b]	32 [b]	47 [c]	62 [b]	84 [b]	0.285	0.535
IV	14 [c]	25 [b]	43 [d]	54 [b]	75 [b]	0.305	0.535

Table 2 provides the results of smoke toxicity effect of *V. zizanioides, O. basilicum* on biting activity of *A. stephensi.* Two gram of plant ingredients from *V. zizanioides, Ocimum basilicum* leaf plant used for smoke toxicity. The control was maintained without plant ingredients. It acts as negative control. The commercially available (Mortein) mosquito coil used as positive control. One hundred 4-3 days starved *A. stephensi* were used. After the treatment of the plant, the fed and unfed mosquitoes were counted. There were 22 fed and 78 unfed mosquitoes counted after the treatment of *Vetiveria zizanioides, Ocimum basilicum* root and leaf. The comparisons of positive control to other plant product efficacy very high, but the combined effect of each plant showed good smoke toxicity effect on *A. stephensi.*

Table 3 shows the field trail after using *Vetiveria zizanioides, Ocimum basilicum* and spinosad alone and it combination against malarial vector, *Anopheles stephensi.* The field study conducted by Bharathiar University Campus, Coimbatore, India. Field trail have been conducted by using the *Vetiveria zizanioides, Ocimum basilicum* seed extract and spinosad against malarial vector, *Anopheles Stephensi* (Overhead tank). The spinosad were prepared required concentration and sprayed by using knapsack sprayer. Bioefficacy of plant extract and spinosad have been noted based on the lethal concentration of plants the LC_{90} value has been double for *Vetiveria zizanioides, Ocimum basilicum* and spinosad sprayed individually at different breeding sites of malarial vector. The percentage of larval reduction was noticed during 24hrs, 48hrs and 72 hrs at the breeding sites. The *V. zizanioides* and *O. basilicum* extract treatment at 24hrs the larval reduction was 73.9% at 48hrs it was 84.8% and at 72hrs it was increased to 94.2%, respectively on malarial vector *A. stephensi*. Spinosad also showed very high percentage of larval reduction at the same breeding sites.

DISCUSSION

Many approaches have been developed to control mosquito menace. One such approach to prevent

Table 2. Smoke toxicity effect of *V. zizanioides* and *O. basilicum* against *A. stephensi.*

Plants used	No. of mosquitoes tested	No. of Fed mosquitoes	Unfed mosquitoes		Total	% Unfed over control
			Alive	Dead		
O. basilicum leaf	100	20^{bc}	52^{ab}	28^{a}	80^{b}	51^{ab}
V. zizanioides root	100	22^{b}	47^{a}	31^{c}	78^{bc}	49^{ab}
O. basilicum + *V. zizanioides*	100	18^{bc}	38^{c}	44^{a}	82^{ab}	53^{a}
Control 1	100	75^{a}	23^{d}	2^{d}	25^{a}	0^{c}
Control 2	100	14^{c}	40^{cd}	46^{a}	86^{d}	0^{c}

Within column means followed by the same letter(s) are not significantly different at 5% level by DMRT; Control 1 = Negative control—blank without plant material; Control 2 = Positive control—Mortein coil.

mosquito borne disease is by killing mosquito at larval stage. The current mosquito control approach is based on synthetic insecticides. Even though they are effective they created many problems like insecticide resistance (Liu *et al*., 2005), pollution, toxic side effect on human beings (Lixin, 2006). Plant extracts, especially botanical insecticides, are currently studied more and more because of the possibility of their use in plant protection. The genus *Ocimum* comprises more than 150 species and is considered as one of the largest genera of the *Lamiaceae* family (Evans, 1996). *Ocimum basilicum* L. (sweet basil) is an annual herb which grows in several regions all over the world. Traditionally, basil has been used as a medicinal plant in the treatment of headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunction (Simon *et al*., 1999). Aroma chemicals identified in the basil extract were 30 monoterpenes, 14 sesquiterpenes, 20 aromatic compounds, 8 alcohols, 4 aldehydes, 7 ketones and esters, and 3 miscellaneous compounds. *Vetiveria zizanioides* comes under the family *poacea* and the scientific reports do however exist of repellent compounds present in vetiver oil extracted from roots of vetiver grass. Vetiver oil is a complex essential oil that consist of several hundreds of compounds of which six are reported to possess insect repellent properties (Jain *et al*., 1982). The

Table 3. Effect of plant extract against larval density of mosquito vectors at the breeding sites of *A. stephensi*

Sl. No	Larval Density			
	Before treatment	After Treatment (in hours)		
		24	48	72
1.	35	6	2	–
2.	35	8	5	–
3.	23	4	3	–
4.	10	3	2	–
5.	12	3	3	–
6.	8	2	1	–
Total	123	26	16	–
Average	20.5	4.3	2.6	–
Reduction	–	81.5 %	88.7%	100%

latter authors, in bioassays with vetiver oil, found it to have topical irritant activity on cockroaches and flies. Research on insect - vetiver grass interactions and the possible role of vetiver as trap crop in IPM systems was stimulated by the recent development of a novel pest management strategy for stem borers in East Africa (Khan *et al*., 1997; Midega *et al*., 2005). Trap cropping as pest management tool has received a lot of attention over the past decade (Hokkanen, 1991; Van den Berg, 2006; Glas *et al*. 2007). Criss Juliard (personal communication) informed the author that a man in Senegal invented a mosquito repellent by mixing vetiver root and groundnut shell into a small ball which he tested in a rainy season as a smoker against mosquitoes. It was evident that there was a significant drop in malaria in the village he tested. The naturally derived insecticide spinosad (Dow Agrosciences LLC) represents a new generation of biorational products developed for the agricultural industry that have a reduced spectrum of toxicity compared with the synthetic insecticides that were developed previously (Williams *et al*., 2003). Spinosad is a mixture of two neurotoxic macrolide compounds: spinosyn A and spinosyn D that are active mainly by ingestion. Spinosyns are produced by fermentation of the actinomycete *Saccharopolyspora spinosa* Mertz and Yao isolated from a Caribbean soil sample (Bret *et al*., 1997). Spinosyns A and D are highly toxic to Diptera, Lepidoptera, Thysanoptera, and some species of Coleoptera (Thompson *et al*., 2000). They further reported that it has extremely low toxicity for mammals; therefore, spinosad is classified by the U. S. Environmental Protection Agency as a reduced- risk material. Spinosad acts on the post-synaptic nicotinic acetylcholine and GABA receptors, resulting in tremors, paralysis, and death. Our results showed that spinosad is highly toxic to *A. stephensi* and by others that tested other mosquito species (Darriet *et al*., 2005, Darriet and Corbel, 2006; Romi *et al*., 2006; Cetin *et al*., 2005), including populations with known resistance to synthetic insecticides (Liu *et al*., 2004b). Moreover, this kind of plant derived product does not cause any ill-effect

to other beneficial organism (Murugan, 2004). Further, Cisneros *et al*. (2002) said that spinosad acts as a stomach and contact poison and degrades rapidly in the environment. Earlier, Bond *et al*. (2004) have reported that the spinosad was most effective at the lowest concentrations (0.024 to 0.025). Romi *et al*. (2006) studied the efficacy of a Spinosad-based product (Laser® 4.8% emulsifiable concentrate) was evaluated in laboratory bioassays against laboratory-reared mosquito strains of 3 species of medical importance: *Aedes aegypti, Anopheles stephensi,* and *Culex pipiens.* Spinosad was particularly effective against larval *Aedes* and *Culex*, with a less marked activity against *Anophelines* (24-h median lethal concentration = 0.0096, 0.0064, and 0.039 mg/liter, respectively), showing a persistence of the insecticide action of about 6 wk in laboratory containers. Results of Kamaraj *et al*. (2008) suggest that the chloroform and methanol extract of *C. sinensis,* ethyl acetate flower extracts of *O. canum and* acetone extract of *O. sanctum* have the potential to be used as an ideal ecofriendly approach for the control of the medically important vector *A. stephensi*. The lethality varied in adults and plant extracts of mixture; *Eucalyptus globulus*, *Cymbopogan citratus*, *Artemisia annua*, *Justicia gendarussa*, *Myristica fragrans*, *Annona squamosa*, and *Centella asiatica* were found to be most effective against *Anopheles stephensi* (Senthilkumar *et al.,* 2009). In the present study also spinosad greatly affected the larval stages of *A. stephensi* and brought out considerable mortality after the spinosad at the ppm concentrations. In the present study also spinosad and plant extracts significantly increased the toxicity and brought out considerable mortality on mosquito larvae. This may due to the presence of active compounds from plants interacted with spinosad and exhibited the mortality of larvae. Thus, the investigation suggests this combination of extract can be considered as a potent biopesticides.

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