OVICIDAL AND LARVICIDAL PROPERTIES OF *PERGULARIA EXTENSA* AND *SPERMACOCE HISPIDA* ETHANOL ROOT EXTRACTS ON *ANOPHELES STEPHENSI* LISTON

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Abstract. Mosquitoes are the most critical group of insects in the context of public health as they transmit key parasites and pathogens, causing millions of deaths annually. Larvicidal and ovicidal activities of ethanol root extracts of *Spermacoce hispida* and *Pergularia extensa* against malaria vector *Anopheles stephensi* were evaluated. Complete ovicidal activity was obtained with 350 and 300 mg/l *P. extensa* extract after 24 and 48 hours exposure respectively, and with 450 mg/l *S. hispida* extract after both 24 and 48 hours exposure. *S. hispida* extract had an LC_{50} value of 18., 33, 37, and 44 mg/l and LC_{90} of 93, 119, 125, and 133 mg/l at 24 hours against the first to fourth instar larvae, respectively; similarly *P. extensa* extract had an LC_{50} value of 30, 49, 63, and 8 mg/l, and LC_{90} value of 144, 160, 143, and 170 mg/l, respectively. These results indicate the potential of ethanolic extracts of *S. hispida* and *P. extensa* as new eco-friendly larvicides and ovicides against the malaria vector *An. stephensi*.

Keywords: *Anopheles stephensi*, *Pergularia extensa*, *Spermacoce hispida*, larvicide, ovicide

INTRODUCTION

Mosquitoes transmit blood-borne pathogens causing dengue, filariasis, malaria, and yellow fever. These diseases result significantly to morbidity and mortality in developing tropical countries (Jang *et al*, 2002). *Anopheles*, genus *Culicidae*, contain approximately 460 recognized species, of which over 100 can transmit human malaria parasites but only 30-40 transmit *Plasmodium* spp (Abriamai *et al*, 2011). In India are found *An. stephensi*, *An. culicifacies, An. fluviatilis, An. minimus, An. sundaicus* and *An. philippinensis* (Mittal *et al*, 2005).

Anopheles stephensi Liston is the major human malarial mosquito vector prevalent in several countries including the Middle East and South Asia (Bian *et al*, 2005). *An. stephensi* is recognized as a major vector for urban malaria in India (Mittal *et al*, 2005). This species prefers to breed in small water collection sites and is responsible for frequent outbreaks of malaria, particularly at construction sites in urban areas (Mittal *et al*, 2005).

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Phytochemicals are botanicals, which are naturally occurring insecticides obtained from floral resources. Applications of phytochemicals in mosquito control have been in use since the 1920s (Shahi *et al*, 2010), but the discovery of synthetic insecticides such as DDT in 1939 side tracked the application of phytochemicals in mosquito control programs. After facing several problems due to injudicious and over-application of synthetic insecticides, there is a re-focus on phytochemicals that are easily biodegradable and have no ill-effects on non-target organisms. The search for new bioactive compounds from the plant kingdom, and efforts to determine their structure and commercial production have gained global attention. At present phytochemicals make up 1% of the world pesticide market (Isman *et al*, 1997).

Botanicals are basically secondary metabolites, which serve as a means of defence mechanism of the plants to withstand the continuous onslaughts from herbivore predators and other environmental menace. Several groups of phytochemicals, such as alkaloids, steroids, terpenoids, essential oils and phenolics from different plants have been reported for their insecticidal activities (Shaalan *et al*, 2005). Insecticidal effects of plant extracts vary not only according to plant species, mosquito species, geographical varieties and parts used, but also to extraction methodology adopted and the polarity of the solvents used during extraction. A wide selection of plants from herbs, shrubs and large trees was used for extraction of mosquito toxins (Ghosh *et al,* 2012). Phytochemicals have been extracted either from the whole body of small herbs or from various parts, *viz*, fruit, leaf, stem, bark, and root, of large plants and trees. When the most toxic insecticidal substances are found, they invariably are extracted and utilized for mosquito control.

Spermacoce hispida L. (Rubiaceae) ("Nattaiccuri" in Tamil or "Shaggy button weed") (Narayan and Kumar, 2003) is widely distributed in the Western Ghats of Kerala (Pushpangandan and Atal, 1984) and in Maruthamalai forest, which is an extension of Western Ghats (Sekar and Francis, 1999) in Tamil Nadu, India. The seed extract of the plant has been used as a remedy for the treatment of internal injuries of nerves and kidney. Also it is suggested that it can remove signs of old age, purify blood and improve vitality, and has been used by tribal people living in the forest regions in Western Ghats of Kerala since ancient times (Pushpangandan and Atal, 1984). It has been reported that *S. hispida* is an effective natural drug for treatment of hypertension (Arnold and Schmidt, 2004). *S. hispida* is one of the five plants that contain the maximum amount of flavonoids among 25 plants analyzed (Sekar and Francis, 1999). Plants containing flavonoids have been reported to possess strong antioxidant and hypolipidemic properties (Aviram, 2004; Sudheesh and Vijayalakshmi, 2005; Sweedy *et al*, 2007). Methanol extract of the whole plant exhibits strong antioxidant activity (Surveswaran *et al*, 2007).

Pergularia extensa (family Asclepiadaceae) ("Veliparuthi" in Tamil and "Utranajutuka" in Hindi) is a hispid perennial herb that grows along the roadsides of India and other tropical and subtropical regions (Khare, 2007). Traditionally the whole plant is used as anthelmintic, antipyretic, laxative and expectorant and to treat infantile diarrhea and malaria fever (Kirtikar and Basu, 1999). The root of the plant is effective in treating convulsion, asthma, poisoning, mental

disorder, anemia, leprosy and piles (Yoganarasimhan, 2000). Dried leaf of this plant is used as an emetic agent and is effective in treating bronchitis (Mittal *et al*, 1962) and asthma (Elango *et al*, 1985). Fresh roots and shoots were found to be useful in treating whooping cough (Kokwaro, 1981). *Pergularia* spp are widely distributed in the old world tropics and subtropics, ranging from southern and tropical Africa to Asia, and have multiple applications in different folk medicine, including the Indian Ayurvedic system, and have been documented for use as antifertility agent (Golam *et al*, 2001), wound healing (Kumar *et al*, 2006), antidiabetic (Wahi *et al*, 2002), hepato-protective effect (Sureshkumar and Mishra, 2006), beneficial cardiovascular effect (Dhawan *et al*, 1973), and antibacterial (Senthilkumar *et al*, 2005). Terpenoids, flavonoids, sterols and cardenolides are among the chemicals from this plant that have been used as an emetic, laxative, antipyretic and expectorant, besides as treatment of infantile diarrhea, malarial fever, toothache and cold (Dokosi, 1998; Hebbar *et al*, 2004). Studies have shown hepato-protective, anti-fertility, anti-diabetic, analgesic, antipyretic and anti-inflammatory properties of substances in its aerial parts (Bhaskar and Balakrishnam, 2009).

Hence, the aim of this study is to evaluate the ovicidal and larvicidal properties of *S. hispida* and *P. extensa* root extracts against malarial vector, *An. stephensi*.

MATERIALS AND METHODS

Plant materials

S. hispida and *P. extensa* roots were collected from in and around Coimbatore Districts in Tamil Nadu, India. Roots of *S. hispida* and *P. extensa* were washed first with tap water, then with distilled water and dried under shade at room temperature (28 ± 2°C) for 10-20 days. The roots were ground to a fine powder using an electric blender, and 500 g of powder were macerated with 1.5 l of ethanol for 72 hours and filtered. One gram of the plant residue was dissolved in 100 ml of acetone [1% (w/v) stock solution]. From this stock solution, 20, 40, 60, 80 and 100 mg/l solutions were prepared.

Qualitative analysis of *S. hispida* and *P. extensa* root extracts for the presence of alkaloids, anthroquinones, flavonoids, cardiac glycosides, coumarins, phenols, saponins, steroids, and tannins were performed using the methods described by Nkere and Iroegbu (2005).

Mosquito culture

Eggs of *An. stephensi* were collected from National Centre for Disease Control (NCDC) field station of Mettupalayam, Tamil Nadu, India and transferred to the laboratory to hatch. Larvae were reared (and adult mosquitoes held) at 27 ± 2°C and 75-85% relative humidity in a 14:10 hours (light: dark) photoperiod. Larvae were reared on ground dog chow and brewer's yeast (3:1 ratio). Pupae were transferred to 500 ml of water and placed inside a screened cage (90 cm x 90 cm x 90 cm). Emerging adults were provided with 10% (w/v) sucrose in water. On day 5 post emergence, mosquitoes were provided access to a rabbit host for blood feeding overnight. Glass Petri dishes lined with filter paper containing 50 ml of water were subsequently placed inside the cage for oviposition by female mosquitoes.

Ovicidal bioassay

Freshly laid eggs were collected by providing ovitraps in mosquito cages after the female mosquitoes were given a blood meal. The eggs were laid on filter paper lining provided in the ovitrap. After scoring,

100 gravids were placed in a screen cage where ten oviposition cups were introduced for oviposition 30 minutes before the start of the dusk period. Of these ten cups, nine were each filled with test solutions and one was filled with 100 ml of distilled water containing acetone and polysorbate 80, which served as control. A minimum of 100 eggs were used for each treatment and each experiment was performed in quintuplet. Following treatment, eggs were sieved through muslin cloth, rinsed with tap water and placed in plastic cubs filled with dechlorinated water for assessment of hatching after counting the eggs under a light microscope (Su and Mulla, 1998). Percent egg mortality is calculated on the basis of non-hatchability of eggs with unopened opercula (Kuppusamy and Murugan, 2008). Hatching rate of eggs is assessed after 98 hours post treatment (Rajkumar and Jebanesan, 2009).

Larval toxicity test

Larvicidal activity was assessed by the procedure of WHO (2005) with some slight modification and as per the method of Kovendan *et al* (2012c). Twenty-five specimens each of 1st to 4th instar larvae were placed into a 500-ml glass beaker containing 249 ml of dechlorinated water and 1 ml of 100, 200, 300, 400 and 500 mg/l root extract. Larval food also was provided. Two to five trials were conducted in quintuplicate. Positive and negative control solution contained 1 ml of acetone plus 249 ml of dechlorinated water and dechlorinated water alone, respectively. Corrected mortality is calculated using the following formula (Abbott, 2009):

$$\text{Percent corrected mortality} = \frac{\text{Observed mortality in treatment - Observed mortality in negative control}}{\text{100 - negative control mortality}} \times 100$$

$$\text{Percent mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

Table 1
Qualitative analysis of chemical constituents of *Pergularia extensa* and *Spermacoce hispida* roots.

Constituent	Plant	
	S. hispida	*P. extensa*
Alkaloids	-	+
Anthroquinones	-	+
Flavonoids	+	+
Cardiac glycosides	+	+
Coumarins	+	+
Phenols	+	+
Saponins	+	+
Steroids	-	-
Tannins	+	-
Triterpenoids	+	+

+, present; -, absent.

Data analysis

The average larval mortality data were subjected to probit analysis (Finney, 1971) for calculating LC_{50} (concentration causing 50% mortality) and LC_{90} values, and other 95% upper confidence limit (UCL) and lower confidence limit (LCL). SPSS software package version 16.0 (SPSS, Chicago, IL) was used for all analyses. A *p*-value <0.05 is considered statistically significant.

RESULTS

All major phytochemical types were present in ethanolic root extracts of *P. extensa* and *S. hispida*, except for the absence of alkaloids and anthroquinones in *S. hispida*, steroids in both plants, and tannins in *P. extensa* (Table 1).

Ovicidal effects against *An. stephensi* of the ethanolic root extracts of *S. hispida* and *P. extensa* were concentration and exposure time dependent; and exerted 100%

Table 2
Ovicidal activity of ethanolic root extracts of *Pergularia extensa* and *Spermacoce hispida* against *Anopheles stephensi.*

Extract	Exposure period (hour)	Percent eggs hatched (mean ± SD)								
		Concentration of extract (mg/l)								
		150	200	250	300	350	400	450	500	Negative control
S. hispida	24	99 ± 5	71 ± 4	58 ± 5	41 ± 5	29 ± 6	19 ± 3	0	0	100
	48	82 ± 4	43 ± 4	27 ± 4	21 ± 3	17 ± 6	5 ± 3	0	0	100
P. extensa	24	90 ± 5	60 ± 4	28 ± 4	9 ± 3	0	0	0	0	100
	48	68 ± 4	41 ± 2	14 ± 2	3 ± 1	0	0	0	0	100

ovicidal activity was obtained with 300 and 350 mg/l after 24 and 48 hours exposure respectively to *P. extensa* extract, and with 450 mg/l after both 24 and 48 hours exposure to *S. hispida* extract (Table 2).

After exposure to the root extracts, the treated larvae exhibited restlessness, sluggishness, tremor and convulsion, followed by paralysis at the bottom of the bowl. Larvicidal LD_{50} and LD_{90} values for *S. hispida* extract were in the range16-44 and 77-135 mg/l, respectively (Table 3), and 16-84 and 64-170 mg/l, respectively for *P. extensa* extract (Table 4).

DISCUSSION

Vector control is facing a threat due to the emergence of resistance to conventional synthetic insecticides in vector mosquitoes, warranting both counter measures and development of new insecticides (Chendre *et al*, 1998). In the present study we demonstrated that ethanolic root extracts of *S. hispida* and *P. extensa* have ovicidal and larvicidal activities against *An. stephensi*, with the former extract being better of the two. These biological properties could be attributed to the presence of the major types of phytochemicals, except the steroids that were absent in the extracts.

In ovicidal activity, exposure to freshly laid eggs was more effective than to the older eggs. It has been shown that the age of the embryos at the time of treatment played a crucial role with regard to the effectiveness of the chitin synthesis inhibitor, dimilin, to *Cx. quinquefasciatus* (Miura *et al,* 1976). Smith and Salkeld (1966) reported differences in susceptibility to ovicides are due to differential rates of uptake, penetration through the chorion, conversion to the active form, and detoxification, in addition the failure of the toxicant to reach the target. Freshly laid *An. stephensi* were used in the current study.

Rajkumar and Jebanesan (2004) reported 100% ovicidal activity of *Moschosma polystachyum* leaf extract against *Cx. quinquefasciatus* at 100 mg/l. Zero hatchability was observed at 400 mg/l for methanol leaf extract and 625 mg/l for methanol seed extract of *P. dulce* against *An. stephensi* and *Ae. aegypti,* respectively (Govindarajan *et al*, 2013). The percent hatchability was inversely proportional to the concentration of extract and directly

Table 3
Larvicidal activity of ethanolic root extract of *Spermacoce hispida* against *Anopheles stephensi*.

Mosquito life stage	Exposure period (hour)	Percent larval mortality (mean ± SD) Concentration (mg/l) 20	40	60	80	100	LC_{50}[a] LC_{90}[a] (mg/l)	Regression equation	95% Confidence limit LCL LC_{50} LC_{90}	UCL LC_{50} LC_{90}	χ^2
First instar	24	51 ± 3	64 ± 2	77 ± 3	83 ± 3	93 ± 2	18	Y= -0.311 +0.017χ	3	83	0.84*
							93		28	111	
	48	55 ± 2	69 ± 2	82 ± 2	89 ± 2	98 ± 1	16	Y= -0.331 +0.021χ	3	69	1.24*
							77		25	89	
Second instar	24	46 ± 2	54 ± 2	61 ± 2	72 ± 2	91 ± 2	33	Y= -0.500 +0.015χ	21	103	5.09*
							119		42	146	
	48	51 ± 2	62 ± 2	75 ± 2	88 ± 3	97 ± 2	22	Y= -0.431 +0.020χ	10	77	1.27*
							87		30	100	
Third instar	24	43 ± 3	51 ± 2	59 ± 2	69 ± 2	87 ± 2	37	Y= -0.550 +0.015χ	25	108	3.90*
							125		46	154	
	48	48 ± 2	58 ± 3	70 ± 2	84 ± 2	90 ± 2	25	Y= -0.435 +0.017χ	12	89	0.65*
							101		34	119	
Fourth instar	24	40 ± 2	48 ± 3	57 ± 6	65 ± 2	75 ± 2	44	Y= -0.610 +0.014χ	32	52	2.73*
							135		116	169	
	48	44 ± 2	54 ± 2	66 ± 2	75 ± 2	86 ± 2	32	Y= -0.480 +0.015χ	19	102	0.50*
							117		41	143	

Nil mortality was observed in negative controls. LCL, lower confidential limit; UCL, upper confidential limit. LC_{50}, LC_{90}, concentration required for 50 or 90% lethality. χ^2, chi-square value. *p-value <0.05.

Table 4
Larvicidal activity of ethanolic root extract of *Pergularia extensa* against *Anopheles stephensi*.

Mosquito life stage	Exposure period (hour)	Percent larval mortality (mean ± SD) Concentration (mg/l) 20	40	60	80	100	LC_{50}[a] LC_{90}[a] (mg/l)	Regression equation	95% Confidence limit LCL LC_{50} LC_{90}	UCL LC_{50} LC_{90}	χ^2
First instar	24	47 ± 2	54 ± 2	62 ± 3	70 ± 2	80 ± 2	30 144	Y= -0.332 +0.11χ	9 41	119 196	0.39*
	48	65 ± 3	7 ± 2	85 ± 2	96 ± 1	100 ± 0	16 64	Y= -0.426 +0.027χ	5 23	58 73	3.46*
Second instar	24	38 ± 2	44 ± 2	57 ± 2	61 ± 3	74 ± 2	49 160	Y= -0.566 +0.012χ	37 59	133 216	0.76*
	48	56 ± 2	62 ± 3	71 ± 3	84 ± 2	95 ± 2	18 94	Y= -0.311+0.017χ	3 28	83 111	3.90*
Third instar	24	26 ± 2	33 ± 3	46 ± 2	61 ± 2	73 ± 3	63 143	Y= -1.015+0.016χ	56 71	125 174	0.56*
	48	51 ± 3	59 ± 2	67 ± 2	79 ± 2	91 ± 2	24 107	Y= -0.362 +0.056χ	8 33	93 130	2.48*
Fourth instar	24	19 ± 2	21 ± 2	38 ± 2	50 ± 2	58 ± 2	84 170	Y=-1.240 +0.015χ	75 96	145 213	1.54*
	48	44 ± 2	58 ± 2	60 ± 2	71 ± 2	87 ± 1	32 123	Y=-0.454 +0.014χ	18 41	106 153	3.54*

Nil mortality was observed in negative controls. LCL, lower confidential limit; UCL, upper confidential limit. LC_{50}, LC_{90}, concentration required for 50 or 90% lethality. χ^2, chi-square value. *p-value <0.05.

proportional to the eggs. Mortality of 100 % of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* eggs methanol extract of *Acalypha alnifolia* was exerted at 125 and 300 ppm, respectively (Kovendan *et al*, 2013).

Murugan and Jayabalan (1999) reported 90% larval mortality was exhibited at 4% concentration of *Leucas aspera* leaf extract against fourth instar larvae of *An. stephensi*. The ethanol extract of *L. aspera* whole plant against the first to fourth instar larvae and pupae had LC_{50} of 9.70, 10.27, 10.82, 11.30, and 12.73 mg/l, respectively against *An. stephensi* (Kovendan *et al*, 2012b).

Sivapriyajothi *et al* (2014) reported *Leucas aspera* methanol leaf extract having LC_{50} values ranging from 148.93 to 417.07 mg/l and the LC_{90} values ranging from 449.72 to 912.94 mg/l against *An. stephensi* first to fourth instar larvae and pupae, respectively. Methanol leaf extract of *Vitex negundo*, *V. trifolia*, *V. peduncularis*, and *V. altissima* possesses LC_{50} value of 212.57, 41.41, 76.28 and 128.04 mg/l, respectively against *Cx. quinquefasciatus* and *An. stephensi*, (Pushpalatha and Muthukrishnan, 1995).

Sharma *et al* (2005) reported that acetone extract of *Nerium indicum* and *Thuja orientelis* has LC_{50} values of 200.87, 127.53, 209.00, and 155.97 ppm against III instar larvae of *An. stephensi* and *Cx. quinquefasciatus*, respectively. Chowdhury *et al* (2009) reported that chloroform and methanol extracts of mature leaves of *Solanum villosum* showed LC_{50} values for all instar stages of *An. subpictus* of between 24.20 and 33.73 mg/l after 24 hours and between 23.47 and 30.63 mg/l after 48 hours exposure. These larvicidal effects are due to the secondary metabolites present in the flower extract. Secondary metabolites present in plants exert their insecticidal properties through damaging insect membrane integrity, in particular the lipid and aqueous components of the gill membrane (Nivsarkar *et al*, 2001).

Aqueous extract of *Chrysanthemum indicum* has LC_{50} value of 78.22, 110.63, 181.85, 298.79 and 339.11 mg/l and LC_{90} value of 417.30, 531.31, 675.76, 973.54 and 996.50 mg/l against first to fourth instar larvae and pupae, respectively of *An. stephensi* (Arokiyaraj *et al*, 2015).

LC_{50} and LC_{90} values of *Morinda citrifolia* methanol extract after 24 hours exposure against the first to fourth instar larvae and pupae of *An. stephensi* are 146.08, 159.07, 172.16, 185.08 and 202.68 mg/l, and 322.12, 363.48, 388.56, 436.51 and 513.56 mg/l, respectively (Kovendan *et al*, 2012a).

Crude hexane extract of *Nerium oleander* flowers have exhibited larvicidal activity against the filarial vector *Cx. quinquefasciatus* with LC_{50} value of 102.54 and 61.11 mg/ml after 24 and 48 hours exposure, respectively (Raveen *et al*, 2014).

LC_{50} value of hexane, diethyl ether, ethyl acetate and acetone extract of *Abutilon indicum* against *An. culicifacies* third instar larvae after 24 hours exposure is 1,031.65, 949.18, 833.58, and 673.68 mg/l, respectively and LC_{90} value of 2,215.87, 2,234.39, 2,152.97, and 2,455.10 mg/l, respectively; LC_{50} value of the respective solvent extract of *Hyptis suaveolens* is 423.00, 347.50, 236.58, and 217.24 mg/l, respectively and LC_{90} value of 1,431.91, 1,292.15, 1,138.49, and 1,049.27 mg/l, respectively; and LC_{50} value of the respective solvent extract of *Leucas aspera* is 559.77, 401.56, 299.71, and 263.01 mg/l, respectively and LC_{90} of 1,400.80, 1,549.31, 1,157.96 and 1,108.72 mg/l, respectively (Kovendan *et al*, 2014).

Aloe vera-synthesized AgNPs extract is toxic against *An. stephensi* first to fourth instars larvae and pupae, with LC_{50} value of 48.79, 59.09, 70.88, 83.58, and 152.55 mg/l, respectively (Dinesh *et al*, 2015).

Pizarro *et al* (1999) estimated LC_{50} and LC_{90} of saponin from *Agave sisalana* against third instars of *Cx. quinquefasciatus* to be 183 and 408 mg/l, respectively. Saponin-induced mortality of mosquitoes may result from alterations to the larval cuticle (Morrissey and Osbourn, 1999; Farid *et al*, 2002).

A piperidine alkaloid from *Piper longum* fruit was found to be active against larvae of *Cx. pipiens* (Lee, 2000). Leaf extract of *Phyllanthus niruri* is toxic against all four larval instar stages and pupae of *Ae. aegypti*, with LC_{50} value of 158.24, 183.20, 210.53, 210.53, and 358.08 mg/l, respectively (Suresh *et al,* 2015).

In summary, crude ethanolic root extracts of *P. extensa* and *S. hispida* demonstrate larvicidal and ovicidal properties against *An. stephensi*. Their activities were more potent than organic solvent extracts from a variety of plants against a number of important mosquitoes responsible for vector-borne diseases as reported in the literature. We suggest that these extracts could be better alternatives to the hazardous synthetic chemical insecticides and bio-pesticides currently in use, and could contribute to a healthier environment.

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