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BIOASSAY GUIDED FRACTIONATION OF *SPHAERANTHUS INDICUS* EXTRACT AGAINST MOSQUITO VECTORS

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ABSTRACT: Phytochemicals with mosquitocidal potential are now recognized as potent alternative insecticides. This study aims at exploring the mosquitocidal potential of the extract of the medicinal plant *Sphaeranthus indicus*. The soxhlet extracts of flowers, leaves and aerial parts of *S. indicus* with four solvents *viz*., hexane, chloroform, ethyl acetate, and methanol were screened against the larvae of the vector mosquitoes *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*. The commercially available synthetic insecticide temephos was used as positive control. The LC_{50} values for the hexane extract of the flowers, leaves and aerial parts were 75.62, 48.22, 70.23 mg/L; 18.61, 53.34, 33.04 mg/L and 191.9, 71.58, 116.21 mg/L respectively for *A. aegypti*, *A. stephensi* and *C. quinquefasciatus*. Bioassay-guided fractionation was carried out for the hexane extract of the leaves of *S. indicus* using a silica gel column with eluents hexane followed by 5% and 10% ethyl acetate in hexane. Fractions were examined by thin layer chromatography (TLC) using silica gel, 60 F_{254} plates using hexane-ethyl acetate (95:5), pooled the similar fractions and screened for mosquito larvicidal activity. The bioassay-guided fractionation showed that the fractions F3-F7 eluted with hexane showed 100% mosquito larvicidal activity against all the three species of mosquitoes at 10 mg/L. The FT-IR spectrum of this fraction revealed the presence of long-chain alkanes. Phytochemical analysis showed the presence of terpenes. Hence the active fraction consists of long-chain alkanes and terpenes. *S. indicus* extract may be developed as a botanical insecticide for mosquito larval control.

INTRODUCTION: The awareness of the harmful side effects on human and deleterious effect on the environment and development of resistance among vectors made concern over the use of conventional synthetic insecticides for vector control and this paved the way for the search for alternative control agents based on phytochemicals.

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Phytochemicals with mosquitocidal potential are now recognized as potent alternative insecticides to reinstate synthetic insecticides under the integrated mosquito management programs due to their notable mosquitocidal properties [1].

Many medicinally important plant extracts have been studied for their efficacy as a mosquitocidal agent against different species of vector mosquitoes [2]. In recent years, use of conventional synthetic insecticides has been restricted due to their high cost, concern for environmental pollution, detrimental effect on human health, and other non-target populations, and increasing insecticide resistance on a global scale.

There is, therefore, a clear requisite for the development of alternative mosquito control agents with a different mode of action.

Sphaeranthus indicus Linn. (Asteraceae) is widely used in Ayurvedic system of medicine to treat vitiated conditions of epilepsy, mental illness, jaundice, hepatopathy, diabetes, leprosy, fever, cough, hernia, hemorrhoids, helminthiasis and skin diseases [3]. *S. indicus* is effective in the treatment of type II diabetes mellitus owing to its ability to decrease insulin resistance [4]. *S. indicus, Cleistanthus collinus,* and *Murraya koenigii* leaf extracts caused significant mortality of *Cx. quinquefasciatus* larvae [5]. However, no study has been reported for the identification of the active fraction responsible for the mosquito larvicidal activity of *S. indicus.* This study aims at the evaluation of the mosquitocidal efficacy of *S. indicus* against three species of mosquito vectors *viz., Anopheles stephensi*, *Cx. quinquefasciatus* and *Aedes aegypti* and an attempt to isolate the active fraction/active principle and the phytochemical analysis of the active fraction.

MATERIALS AND METHODS: Laboratory grade hexane, chloroform, ethyl acetate, methanol (s. d. fine-chem., Mumbai, India) and ethanol (Hayman, England) and all other chemicals mentioned (Sisco Research Laboratory, Mumbai, India) were used for the study. Silica gel 60 F_{254} TLC aluminum sheets were from Merck, Germany. The Fourier transform infrared (FT-IR) spectrum was recorded on a Shimadzu FT-IR model 8300 (Shimadzu Corporation, Kyoto, Japan).

All the plant materials were identified by a botanist, and voucher specimens were kept for future studies. Leaves, flowers and aerial parts of *S. indicus* were collected from a nearby village Kilyannur in Tamil Nadu, India. The collection was done in April 2013. The plant parts were cleaned and separated as each part. They were shade dried at room temperature (26 ± 2 ºC). The dried parts were powdered in a Remi laboratory blender. 100gm of the *S. indicus* powder of leaf, flower and aerial parts were filled separately in a thimble and extracted in a Soxhlet extractor using four different solvents (750 ml) of varying polarities such as hexane, chloroform, ethyl acetate, and methanol. Each extraction was done for six hours. All the extracts were concentrated using rotary vacuum evaporator.

The residues obtained after removal of the solvent were weighed and dissolved in ethanol to get a 10% (100000 mg/L) stock solution. Bioassay for the larvicidal activity was carried out using ICMR common protocol [6]. From the stock solution, different concentrations were prepared by proper dilution. Initial screening was done at 500, 400, 300, 200, and 100mg/L. Mosquito larvae were collected from the rearing and colonization laboratory of Vector Control Research Centre, Pondicherry, India. Twenty-five late 3rd instar larvae were introduced into 150 ml paper cup containing 100ml of water with each concentration. A total of four replicates kept for each concentration. An equal number of control cups was kept with solvent alone (without the extract). The positive control was set up with commonly used mosquito larvicide Temephos (Technical grade). Mortality was recorded after 24 h.

The moribund and dead larvae in four replicates were combined and expressed as a percentage of larval mortality for each concentration. The test cups were held at 27 ± 2 ºC and 80-90 relative humidity and a photoperiod of 12 h light followed by 12 hrs dark (12L: 12D). For slow-acting insecticides, 48 h reading may be required. In cases where the control mortality is between 5-20%, the observed percentage of mortality was corrected using Abbott's formula [7]. The experiment was repeated three times with proper doses to get the median lethal dose LC_{50}. Data from all replicates were pooled for analysis. LC_{50} and LC_{90} values were calculated from a log dosage–probit mortality regression line using computer software program SPSS yielding a level of effectiveness at 50% and 90% mortality and 95% confidence intervals (95% CI).

Part of the crude hexane leaf extract of *S. indicus* was subjected to bioassay-guided fractionation. Crude hexane leaf extract was fractionated using column chromatography using silica gel 230-400 mesh size. Thirty-five gm silica was used for making the slurry with n-hexane and loaded in a glass column of length 30cm and internal diameter 2cm. Column chromatographic elutions were carried out with hexane followed by 5% ethyl

acetate in hexane and 10% ethyl acetate in hexane. Fractions were collected in separate quantified beakers and examined by thin layer chromatography (TLC). This was done on silica gel plates (Merck, $60F_{254}$) using hexane/ethyl acetate in 95:5 ratio as the mobile phase. Visualization and identification of spots that indicate constituents of each fraction was done using an Ultra Violet lamp at a wavelength of 254nm and by keeping in Iodine chamber. Finally, fractions having similar spots were pooled and concentrated. Each pool was screened for mosquito larvicidal activity at 100 mg/L against all the three species of mosquitoes. The characterization of the pooled fractions was done using by FT-IR for the presence of functional groups. Chemical tests were carried out on the plant extract and the powdered specimens using standard procedures to identify the constituents as described earlier [8-9].

RESULTS AND DISCUSSION: The Soxhlet extraction of powdered leaves with different solvents with varying polarity based on the polarity index (PI) [10] yielded different quantities of residues. The PI is a measure of the relative polarity of a solvent. The polarity index increases with polarity. The soxhlet extraction of 100 gm of the leaves with nonpolar solvent - hexane (polarity index (PI-0.1) yielded 4.25 gm and moderately nonpolar solvents such as chloroform (PI-4.1) extraction yielded 3 gm and ethyl acetate (PI-4.4) extraction yielded 2.5 gm. Extraction with polar solvent methanol (PI-5.1) yielded 5.12gm. The soxhlet extraction of 100gm of the flower with hexane yielded 0.825 gm and with chloroform 1.0gm and extraction with ethyl acetate and methanol yielded 1.25 gm and 1.56 gm respectively. Extraction of 100 gm of aerial part with hexane yielded 4.16 gm of residue.

The crude extracts of leaf, flower and aerial parts were screened for larvicidal activity initially against early third instar larvae of the vector mosquitoes *viz., C. quinquefasciatus, A. aegypti, A. stephensi* at 500 mg/L. During the test period, no control mortality was observed for all the three mosquito species tested. 00% mortality was observed in the hexane extract of *S. indicus* leaf and flower against all the three species of mosquito larvae at 48 h. All the other extracts were also effective with >75% mortality.

The results of the LC_{50} and LC_{90} values and their 95% upper and lower confidence limits and Chi-square (χ^2) values of the extracts of *S. indicus* in different solvents for 48 hrs of exposure of all the three species of mosquito larvae are given in the **Table 1** along with the values for the positive control Temephos observed for 24 h.

TABLE 1: DOSE RESPONSES OF *S. INDICUS* EXTRACTS AGAINST MOSQUITO LARVAE USING PROBIT ANALYSIS

Plant part & extract	Mosquito species	Lethal Concentration (mg/L)				
		LC_{50}	95% CL	LC_{90}	95% CL	(χ^2)
Flower						
Hexane	*C. quinquefasciatus*	70.23	64.97-75.05	110.39	101.97-123.50	0.26
	n. stephensi	48.22	37.99-57.73	127.08	108.56-160.21	0.61
	A. aegypti	75.62	60.54-88.13	196.34	172.62-234.47	1.92
Chloroform	*C. quinquefasciatus*	219.22	195.3-242.0	425.82	388.08- 478.66	2.46
	A. stephensi	196.77	164.2-224.9	445.50	405.46-499.99	0.11
	A. aegypti	213.24	185.3-238.0	455.31	409.04-525.97	1.66
Ethyl acetate	*C. quinquefasciatus*	248.99	225.3-273.5	315.30	427.43-563.60	2.59
	A. stephensi	314.4	287.6-612.8	547.10	503.20-612.87	0.05
	A. aegypti	191.37	122.6-240.3	479.51	250.60-430.21	4.99
Methanol	*C. quinquefasciatus*	248.43	230.0-268.9	385.62	356.25-423.71	1.17
	A. stephensi	464.79	427.1-522.1	783.67	682.72-968.11	2.32
	A. aegypti	242.42	225.8-258.5	375.04	352.12-404.53	3.21
Leaf						
Hexane	*C. quinquefasciatus*	33.04	12.03-48.28	162.82	140.00-199.10	2.60
	A. stephensi	53.34	30.6-70.9	223.19	188.8-281.6	3.27
	A. aegypti	18.61	10.64-24.8	72.6	62.90-87.30	2.99
Chloroform	*C. quinquefasciatus*	160.03	147.5-173.0	264.19	244.7-289.3	1.66
	A. stephensi	205.92	157.6-242.8	528.68	475.7-607.9	0.34
	Ae. aegypti	383.57	356.5-413.2	638.13	583.0-719.2	3.30
Ethyl acetate	*C. quinquefasciatus*	299.8	277.3-323.4	468.15	435.1-510.0	1.35
	A. stephensi	228.60	202.8-253.1	434.71	401.1-477.3	2.44

	A. aegypti	183.42	167.8-199.9	325.27	297.7-362.5	1.81
Methanol	*C. quinquefasciatus*	246.44	221.0-273.4	483.05	436.5-547.6	2.27
	A. stephensi	240.5	208.3-271.1	541.83	473.1-658.7	1.68
	A. aegypti	298.09	262.8-333.1	632.83	564.6-735.6	0.03
Arial part						
Hexane	*C. quinquefasciatus*	116.21	64.47-150.9	383.14	340.35-451.31	0.08
	A. stephensi	71.58	51.68-88.34	208.85	181.48-251.53	0.14
	A. aegypti	191.90	162.9-219.8	457.69	408.32-528.09	2.08
	C. quinquefasciatus	0.015	0.01-0.02	0.016	0.016-0.018	4.37
	A. stephensi	0.27	0.03-0.03	0.034	0.030-0.055	4.55
	A. aegypti	0.123	0.12-0.13	0.163	0.163-0.154	1.63

The larvicidal activity of all the extracts of *S. indicus* was analyzed to find out the most effective extract. The results of the comparison of the different extracts of flower extracts are given in **Fig. 1.** Among the extracts of the flower, the hexane extract was found to be more effective than other solvent extracts with LC_{50} values of 75.62, 48.22 and 70.23 mg/L respectively for *A. aegypti*, *A. stephensi* and *C. Quinque fasciatus*. The hexane extract was more effective in killing the *A. stephensi* larvae than the other two species as indicated by the low LC_{50} value of 48.22 mg/L.

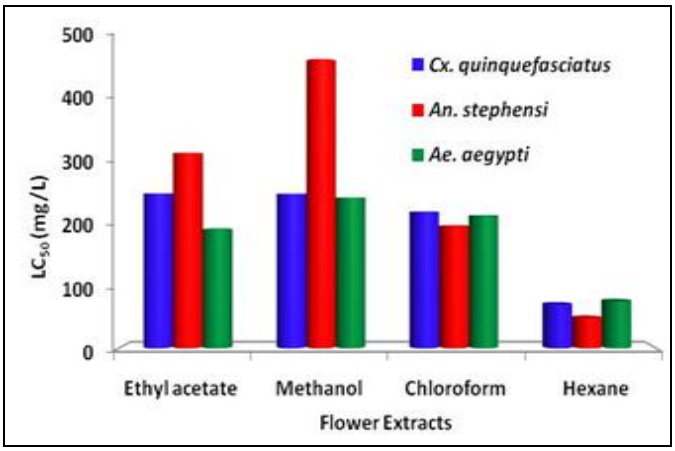

FIG. 1: COMPARISON OF LARVICIDAL ACTIVITY OF DIFFERENT EXTRACTS OF *S. INDICUS* FLOWER

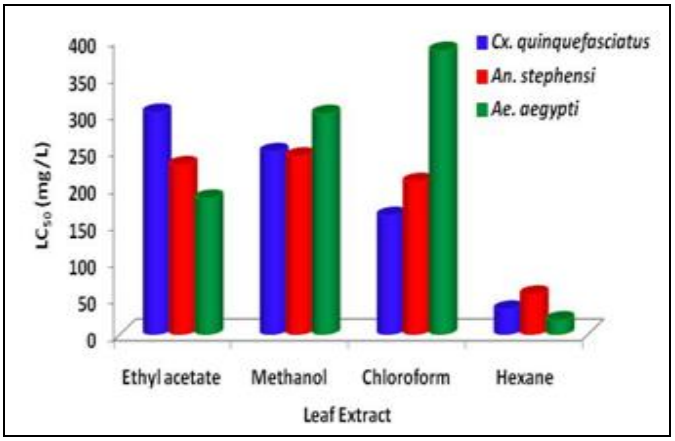

FIG. 2: COMPARISON OF LARVICIDAL ACTIVITY OF DIFFERENT EXTRACTS OF *S. INDICUS* LEAVES

The results of the comparison of the leaf extracts are given in **Fig. 2**. Among the extracts of the leaf, the hexane extract was found to be more effective than other solvent extracts with LC_{50} values of 18.61, 53.34 and 33.04 mg/L respectively for *A. aegypti*, *A. stephensi* and *C. quinquefasciatus*. The hexane extract was more effective in killing the *A. aegypti* larvae than the other two species as indicated by the low LC_{50} value of 18.61 mg/L.

The results of the comparison of the hexane extracts of leaf, flower, and aerial parts are given in **Fig. 3**. Among these extracts, the leaf extract was found to be more effective than the flower followed by the aerial parts with LC_{50} values of 18.61, 53.34, and 33.04 mg/L respectively for *A. aegypti*, *A. stephensi* and *C. quinquefasciatus*.

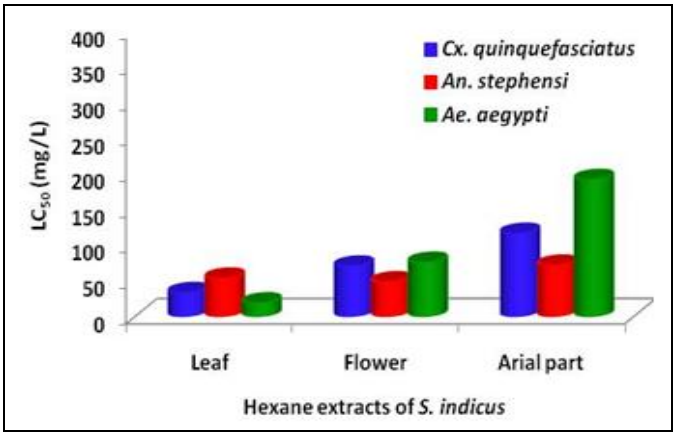

FIG. 3: COMPARISON OF LARVICIDAL ACTIVITY OF HEXANE EXTRACTS OF LEAF, FLOWER AND ARIAL PARTS OF *S. INDICUS*

Among the different extracts of *S. indicus* the hexane extract of the leaf was found to be the most promising. This extract was subjected to Column chromatographic fractionation. Each fraction was analyzed by TLC and fractions with similar spots were pooled, and bioassay was conducted. The pooled fractions were F1-F2, F3-F7, F8-F9, F10-F12, and F16-19. Among the tested fractions, F3-

F7 exhibited 100% larvicidal activity for all the three species of mosquitoes at 10 mg/L. The experiment was repeated three times and showed the same results confirming the activity of fractions F3-F7. Fractions F3-F7 showed the presence of UV absorbing spots as well as Iodine absorbing spots. This pooled fraction was subjected to FT-IR and the spectrum **Fig. 4** showed the presence of major peaks at the following wavelengths *viz.*, 2918, 2649, 1464 and 1377cm^{-1}. The sharp bands at 2918, 2649 cm^{-1} indicate the presence of C-H stretching. The band at 1464 cm^{-1} shows the C-H bending and 1377 cm^{-1} is for rocking vibrations. These absorbances are characteristics of alkyl groups.

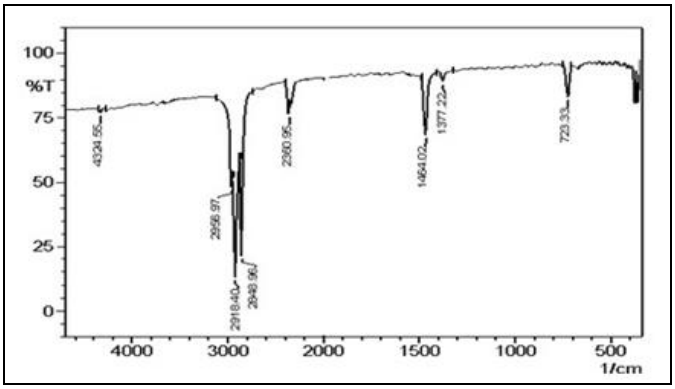

FIG. 4: FT-IR SPECTRUM OF FRACTIONS F3-F7 IN KBR

The results of the phytochemical analysis of the plant parts of *S. indicus* are given in **Table 2**. The qualitative study carried out with the hexane extract of the leaf, flower, and arial part of *S. indicus* showed the presence of carbohydrate, tannin, and flavonoids. The hexane extract of flower and aerial parts showed the presence of phenolic compounds also. Carbohydrate, tannin, flavonoids or phenols were absent in the active fraction F3-F7. However, it showed the presence of terpenes along with other crude extracts as shown by the Salkowski test.

TABLE 2: QUALITATIVE ANALYSIS OF THE PHYTOCHEMICALS OF *S. INDICUS* EXTRACTS AND ACTIVE FRACTION

Phyto-chemicals	Arial part extract	Leaf extract	Flower extract	Fractions F3-F7
Alkaloids	-	-	-	-
Carbohydrate	+	+	+	-
Tannin	+	+	+	-
Saponin	-	-	-	-
Phenolic compounds	+	-	+	-
Flavonoids	+	+	+	-
Terpenes	+	+	+	+

Sphaeranthus indicus Linn. is well known for its medicinal properties such as hepatoprotective activity [11], anti-hyperlipidemic property [12], antipyretic [13], anti-bacterial [14], antiviral [15], anti-inflammatory [16], anthelminthic [17,] and anti-filarial activity [18]. A large number of constituents have been isolated from the extracts of whole herb, leaves, and flowers. The essential oil obtained by steam distillation of the whole herb, contains ocimene, α-terpinene, methyl-chavicol, α-citral, geraniol, αionone, ß-ionone, d-cadinene, p-methoxycinnamaldehyde [19]and an alkaloid sphaeranthine [20]. Two potent anticancer compounds β-Sitosterol and 7-hydroxyfrullanolide were isolated from petroleum ether extract of *S. indicus* [21]. A major sesquiterpene lactone isolated from petroleum ether fraction of *S. indicus* flowers showed acetylcholine esterase inhibitory activity [22].

The mosquito larvicidal activity of *S. indicus* extracts against *C. quinquefasciatus* [5] was reported recently. However, no attempt has been made to isolate the active fraction responsible for the larvicidal activity of this plant extract. This study examined the larvicidal activity of *S. indicus* against the three mosquito species such as *A. stephensi, C. quinquefasciatus* and *A. aegypti*. The leaves, flowers and the aerial parts of *S. indicus* were extracted with different organic solvents of varying polarity such as hexane, chloroform, ethyl acetate, and methanol and screened against all the three species of mosquitoes. The results showed that the extract with the highly nonpolar solvent hexane exhibited the maximum larvicidal activity against all the three species of mosquitoes. This shows that the active principle is a lipophilic molecule or combination of nonpolar molecules.

This was evidenced by the bioassay-guided fractionation where the fractions F3-F7 eluted with hexane showed 100% mosquito larvicidal activity against all the three species of mosquitoes tested at 10 mg/L. FT-IR spectroscopy of this active fraction showed the presence of C-H stretching, C-H bending and C-H rotating bands indicating the presence of long-chain alkanes. No other functional group was shown by the FT-IR spectrum of the active fraction. The Salkoswski test showed the presence of terpenes. The TLC analysis showed the presence of three major spots visualized by UV 254 nm lamp and Iodine absorption. Hence, the

active fraction is a combination of a minimum of three components as evidenced by the FT-IR and TLC analysis consisting of long-chain alkanes and terpenes.

The efficacy of phytochemicals against mosquito larvae according to their chemical nature and described the mosquitocidal potentiality of several plant-derived secondary materials such as alkanes, alkenes, alkynes and simple aromatics, lactones, essential oils, and fatty acids, terpenes, alkaloids, steroids, isoflavonoids, pterocarpans, and lignans had been reviewed [23]. The high rate of biodegradation exhibited by most phytochemicals makes them environmentally acceptable substitutes for synthetic chemicals as pest control agents. In this study, also the active fraction contains long-chain alkanes and terpenes.

Several studies have documented the efficacy of plant extracts as the reservoir pool of bioactive toxic agents against mosquito larvae. But only a few have been commercially produced and extensively used in vector control programme[24]. The main reason for the failure in the laboratory to field movements of bioactive toxic phytochemicals is poor characterization and inefficiency in determining the structure of active toxic ingredients responsible for the larvicidal activity. The isolation of the active component could be useful as a biomarker in quality checking of each extract before moving to the field from the laboratory.

CONCLUSION: In conclusion, among the extracts of flowers, leaves and aerial parts of *S. indicus* with four different solvents such as hexane, chloroform, ethyl acetate and methanol, the hexane extract of the leaves was found to have promising mosquito larvicidal activity against all the three mosquito species tested *viz.*, *A. aegypti*, *A. stephensi* and *C. quinquefasciatus* with LC_{50} values 18.61, 53.34 and 33.04 mg/L respectively. Bioassay-guided fractionation of the hexane extract of the leaves revealed that the fractions F3-F7 eluted with hexane contains the active principles responsible for the mosquito larvicidal activity. The FT-IR analysis and phytochemical analysis showed the presence of long chain alkanes and terpenes in the active fractions. A further investigation of this fraction by ^{1}HNMR, ^{13}CNMR, and GC/MS analysis will reveal the chemical composition of this active fraction. This may pave the way for the development of an environmentally safe botanical insecticide for the control of mosquito larvae.

Targeting larvae, particularly in human-made habitats, can significantly reduce the mosquito vector population, particularly when applied in conjunction with indoor residual spraying (IRS) and other adulticidal measures. The practical advantage of improved operational efficiency of dengue vector control was reported by the combined use of biocontrol agent (larvicide) and adulticide when applied together [25]. Botanical insecticides, in combination with microbial biocontrol agents and insect growth regulators, would be a good option for environmentally friendly, toxicologically safe and community acceptable mosquito vector control programs.

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CONFLICT OF INTEREST: Nil

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