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Larvicidal activity of fractions of *Sphaeranthus indicus* Linnaeus (Asteraceae) ethyl acetate whole plant extract against *Aedes aegypti* Linnaeus 1762, *Anopheles stephensi* Liston 1901 and *Culex quinquefasciatus* Say 1823 (Diptera: Culicidae)

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Abstract
Mosquito control, in view of their medical importance, assumes global importance. In the context of ever increasing trend to use more powerful synthetic insecticides to achieve immediate results in the control of mosquitoes, an alarming increase of physiological resistance in the vectors and its increased toxicity to non-target organism are noteworthy. This has led to intensified search for tools that demonstrate eco-friendliness and target specificity. Phytochemicals are botanicals which are naturally occurring insecticides obtained from floral resources. In the quest for alternative natural biological control agents against mosquito larvae, the present paper reports on the larvicidal activity of fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract against vector mosquitoes *viz*., *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*. Nine fractions *viz*., A, B, C, D, E, F, G, H and I were obtained from the residue of ethyl acetate extract by column chromatography. Standard WHO protocols with minor modifications was adopted for the larvicidal bioassay. Larvicidal activity was evaluated at concentrations of 25, 50, 75 and 100 ppm. Larval mortality was observed 24 hours after treatment. Amongst the fractions tested, fraction 'F' showed one hundred per cent mortality against third instar larvae of *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* at 100 ppm and LC_{50} values were 36.76, 26.85 and 32.60 ppm respectively. In conclusion, the bioassay result of the present study indicated the larvicidal property against vector mosquitoes of fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract, especially for the 'F' fractionated group. Future research to extract a pure compound of the active fractionated group should be explored to find a new highly efficient larvicidal substance.

Keywords: *Sphaeranthus indicus,* ethyl acetate fractions, larvicidal activity, *Aedes aegypti*, *Anopheles stephensi*, *Culex quinquefasciatus*

Introduction
Man could land on Mars but failing to outwit a tiny creature i.e. mosquito over centuries. Unfortunately, it is possible to say that, presently, in the battle between mosquitoes and man, the mosquitoes have proven to be the great winners. In the history of the world, more people would have died from diseases transmitted by mosquitoes than from all the fighting in the wars. The world's most dangerous creature is in fact the mosquito. Mosquitoes referred to as "flying syringes" can transmit more diseases than any other group of arthropods. WHO [1] has declared the mosquitoes as "public enemy number one". These tiny assassins have the potential and lethal capacity to affect and kill millions of people throughout the world [2]. Several mosquito species belonging to genera *Aedes*, *Anopheles* and *Culex* are vectors for the pathogens of various diseases like dengue, chikungunya, yellow fever, malaria, filariasis and Japanese encephalitis [3-5]. Mosquitoes (Class Insecta: Order Diptera: Family Culicidae), classified into two subfamilies Anophelinae and Culicinae, are cosmopolitan insects. A number of members of this very diverse family are considered medically important as vectors of viruses and parasites associated with diseases that have been emerging as a threat in relation to global warming and environmental change [6].
Mosquito control, in view of their medical importance, assumes global importance. Vector

control is by far the most successful method for reducing incidences of mosquito-borne diseases [7]. Chemical pesticides are proved to be effective in mosquito control program. In the context of ever increasing trend to use more powerful synthetic insecticides to achieve immediate results in the control of mosquitoes, an alarming increase of physiological resistance in the vectors and its increased toxicity to non-target organism are noteworthy [8]. However, high cost of synthetic pyrethroids, environment and food safety concerns, unacceptability and toxicity of many organophosphates and organochlorines, and a global increase in insecticidal resistance, have argued for stimulated research towards the development of potential insecticides of botanic origin [9, 10]. Thus, the Environmental Protection Act in 1969 has framed a number of rules and regulations to check the application of chemical control agents in nature [11]. Many developed and developing countries are searching environmentally safe products for vector control program. This has led to intensified search for tools that demonstrate eco-friendliness and target specificity and this has been found with plant extracts otherwise known as botanicals. The use of plant products is one of the best alternatives for mosquito control and many plant products have been tried in earlier days before the discovery of chemical pesticides [3]. Hence, the search for herbal preparations and pure compounds that do not produce adverse effects in the non-targeted organisms, along with the benign environmental characteristics, remain a top priority research for scientists associated with the development of alternative vector control measures [12, 13].

Many plant species are known to possess biological activity that is frequently assigned to the secondary metabolites [14]. Phytochemicals are naturally occurring insecticides obtained from floral resources. The active toxic ingredients of the plant extracts are secondary metabolites endowed to protect them from herbivores. Some of their functions include the blockage of calcium channels in the cell membrane, hormonal imbalance and disruption of molecular events of morphogenesis. Applications of plant phytochemicals in the control of mosquitoes have been in use since 1920's [15]. The efficacy of phytochemicals against mosquito larvae can vary significantly depending on plant species, Plant parts used, age of plant parts (young, mature or senescent) and solvent used during extraction affect the efficacy of plants used against vector species. Several researchers reported that plant phytochemicals provide multiple modes of action on target organisms such as larvicides, insect growth regulators, repellents and oviposition attractants or deterrents [16-18].

Many plant natural products have been tested as insecticides against mosquitoes [19-21] as they are nontoxic to mammals and are promising candidates to replace conventional insecticides [22-25]. In the majority of these studies, although larvicidal activity has been described for the extracts and the presence of a range of compounds sometimes detected, very few have actually identified the compounds responsible for activity together with their structure [26]. Members of the plant families Solanaceae, Asteraceae, Cladophoraceae, Labiatae, Miliaceae, Oocystaceae and Rutaceae have various types of larval, adulticidal or repellent activities against different species of mosquitoes [23]. A brief delve into the literature reveals many investigations have been made towards the biological screening of botanical extracts and the activity of many plant derived components against mosquitoes [23, 27-44] and in the current scenario, several researchers are searching locally available plant materials in order to find out eco-friendly products to manage different mosquito species [45-65]. In the quest for alternative natural biological control agents against mosquito larvae, the present paper reports on the larvicidal activity of fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* (Diptera: Culicidae).

Sphaeranthus indicus Linnaeus. (Asteraceae) (Figure 1) commonly called as mundi in Hindi and Sanskrit and kottai karantai in Tami [66] is distributed throughout the plains and wet lands in India, Sri Lanka and Australia [67, 68]. The plant is cultivated all over India for its medicinal values [69]. Traditionally the plant is used for treatment of rheumatic arthritis [70, 71] and several tribal population in Northern India use the plant to cure diabetes [72]. In folk medicine, the plant is used for treating epileptic convulsions, mental illness and hemicranias [73]. The juice of the plant is styptic and said to be useful in liver and gastric disorders [74]. Further, the plant is also used in homeopathic medicine for the treatment of insomnia, epilepsy, tetanus and muscle spasms [75, 76]. It is used indigenously in Indian system of traditional medicine to treat tuberculosis, spleen diseases, anaemia, bronchitis, elephantiasis, piles, asthma, leucoderma and pain of uterus and vagina [68, 77-79]. The paste of the plant is used as an external application for treating oedema, arthritis, filariasis, gout and cervical adenopathy [79]. Besides, the plant is used to treat jaundice, cough, hepatopathy, gastropathy, hernia, haemorrhoids, helminthiasis, dyspepsia, skin diseases, hepatitis, indigestion, dysentery, bowel complaints and also serves as a nerve tonic [76, 79].

The plant also possesses antimicrobial [80, 81], antiviral [82], antibacterial and antifungal [83], anthelmintic [84], neuroprotective [85], hepatoprotective and antioxidant [86], antiulcer [87], antihyperlipidemic [88], wound healing [89], anti-inflammatory [90, 91], antidiabetic [92], immunomodulatory and immunosuppression [93], antiallergic [70,71], analgesic, antipyretic [94], antioxidant [95] and anticancer [96] properties. Some of the phytochemical constituents present in the plant are tannins, ocimene, terpinene, citral, geraniol, stigmasterol, β-sitosterol, sesquiterpene lactone, sesquiterpene glycoside, flavones, isoflavone glycosides, isoflavonoid, sterol glycoside, ocimene, geraniol, methylchavicol, sphaeranthanolide, lactones, camphene, myrcene, limonene, cubenol, indipone, guaiol, borneol, dihydroagarofuran, caryophyllene oxide, eugenol, geranyl acetate, peptide alkaloid and an alkaloid sphaeranthine [97-103].

Sphaeranthus indicus possess insecticidal property. The aqueous extract of whole plant was proved toxic to cockroach *Periplaneta americana*, pulse beetle *Callosobruchus chinensis* and rice weevil *Sitophilus oryzae* [104]. Patole *et al*. [105] reported the extracts of this plant to possess ovicidal and ovipositional activity against *Callosobruchus chinensis*. The crude extracts of *Sphaeranthus indicus* whole plant showed mortality against *Callosobruchus maculatus* [106]. The crude hexane, diethyl ether, dichloromethane and ethyl acetate extracts of *Sphaeranthus indicus* whole plants were screened for ovicidal [107], oviposition [108] and antifeedant [109] properties against *Spodoptera litura*. In addition, *Sphaeranthus indicus* also exhibited mosquitocidal activity [47-50, 55, 56, 110-116]. In view of the mosquitocidal property reported by the above mentioned researchers, the present study was focused to test the fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract for larvicidal activity against the vector mosquitoes *viz*., *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*.

2. Materials and Methods

2.1. Plant collection and preparation of crude extract

Sphaeranthus indicus whole plants were collected in and around Chennai, Tamil Nadu, India (12.9213° N, 80.1220° E). Taxonomical identity of the plant was confirmed at the Department of Plant Biology and Biotechnology, Loyola College, Chennai, Tamil Nadu, India. The whole plants brought to the laboratory were shade dried under room temperature and powdered using an electric blender. Dried and powdered whole plants (1 kg) was subjected to extraction using 3 L of ethyl acetate for a period of 72 hours to obtain the crude extracts using rotary vacuum evaporator which was then refrigerated at 4 °C.

2.2. Isolation and fractionation of crude extracts by column chromatography

The residue from the crude extract of *Sphaeranthus indicus* (38.642g) was mixed with silica gel (60-120 mesh, 120g) as admixture, subjected to column chromatography (si gel, 100-200 mesh 400g) to obtain nine fractions by increasing polarity of eluents *viz*., hexane and ethyl acetate in the ratio of 100:0; 90:10; 80:20; 60:40; 40:60; 20:80; 0:100 finally ethyl acetate and acetone in the ratio of 50:50 and 0:100 respectively.

2.3. Test mosquitoes

Tests were carried against laboratory reared vector mosquitoes *viz*., *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* free of exposure to insecticides. Cyclic generations of vector mosquitoes were maintained at 25-29 ºC and 80-90% relative humidity in the insectarium. Larvae were fed on larval food (powdered dog biscuit and yeast in the ratio 3:1) and adult mosquitoes on ten per cent glucose solution. The eggs laid were then transferred to enamel larval trays maintained in the larval rearing chamber. The larvae on becoming pupae were collected, transferred to plastic bowls and kept inside a two feet (2'x2'x2') mosquito cage for adult emergence.

2.4. Larvicidal bioassay

Standard WHO [117] protocol with minor modifications was adopted for the study. The tests were conducted in glass beakers. Mosquito immatures particularly third instar larvae were obtained from laboratory colonized mosquitoes of F_1 generation. Larvicidal activity at test concentrations of 25, 50, 75 and 100 ppm were assessed. Twenty five healthy larvae were released into each 250 ml glass beaker containing the required test concentration and quantity of test solution. Larval mortality was observed 24 hours post treatment. Larvae were considered dead when they showed no signs of movement when probed on their respiratory siphon with a needle. A total of five trials with three replicates per trial for each concentration were carried out. Distilled water as control was run simultaneously. The larval per cent mortality was calculated and when control mortality ranged from 5-20% it was corrected using Abbott's formula [118]. SPSS 11.5 version package was used for the determination of LC_{50} and LC_{90} values [119]. The percentage data obtained was angular transformed. Data from mortality and effect of concentrations were subjected to ANOVA to determine the difference in larval mortality between concentrations. Results with $P<0.05$ level were considered to be statistically significant.

3. Results

Results revealed that nine fractions (A, B, C, D, E, F, G, H and I) obtained from *Sphaeranthus indicus* ethyl acetate whole plant extract when tested against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* showed larvicidal activity. Amongst them, fraction 'F' showed one hundred per cent mortality against the larvae of vector mosquitoes at 100 ppm. Other fractions showed less than one hundred per cent mortality. No mortality was observed in control. The larval mortality observed in fraction 'F' at lowest dose (25 ppm) was 44.8, 55.2 and 46.4% in *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* respectively and at highest dose (100 ppm) it was 100.0% against all the vector mosquitoes (Table 1, 2 and 3; Figure 2, 3 and 4). The fraction 'F' exhibited LC_{50} and LC_{90} values of 36.76 and 82.51; 26.85 and 84.06; 32.60 and 74.74 ppm after 24 hours exposure respectively (Table 4). Analysis of variance of larval mortality in different concentrations was found to be statistically significant at $P<0.05$ level in all the fractions. Amongst the vector mosquito species studied, *Anopheles stephensi* was more susceptible followed by *Culex quinquefasciatus* and *Aedes aegypti*.

Table 1: Per cent larvicidal activity of fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract against *Aedes aegypti*

Concentration (ppm)	Fractions								
	A	B	C	D	E	F	G	H	I
25	24.8 ±1.78 (29.9)^{b}	12.8 ±3.34 (20.9)^{a}	08.0 ±2.82 (16.4)^{a}	23.2 ±3.34 (28.8)^{b}	26.4 ±3.57 (30.9)^{b}	44.8 ±1.78 (42.0)^{d}	36.0 ±2.82 (37.2)^{c}	08.8 ±1.78 (17.3)^{a}	10.4 ±2.19 (18.5)^{a}
50	35.2 ±5.93 (36.4)^{b}	18.4 ±3.57 (25.4)^{a}	13.6 ±2.19 (21.6)^{a}	37.6 ±4.56 (37.8)^{bc}	44.0 ±4.0 (41.6)^{cd}	54.4 ±2.19 (47.5)^{e}	50.4 ±4.56 (45.2)^{de}	12.0 ±2.82 (20.3)^{a}	14.4 ±2.19 (22.3)^{a}
75	48.8 ±4.38 (44.3)^{cd}	22.4 ±4.56 (28.3)^{b}	14.4 ±4.56 (22.3)^{a}	42.4 ±2.19 (40.6)^{c}	55.2 ±3.34 (47.9)^{e}	83.2 ±1.78 (65.8)^{g}	64.0 ±2.82 (53.1)^{f}	15.2 ±1.78 (22.9)^{a}	18.4 ±3.57 (25.4)^{ab}
100	56.8 ±5.93 (48.9)^{d}	33.6 ±4.56 (35.4)^{c}	17.6 ±4.56 (24.8)^{a}	52.8 ±1.78 (46.6)^{d}	64.8 ±3.34 (53.6)^{e}	100.0 ±0.0 (90.0)^{g}	81.6 ±2.19 (64.6)^{f}	20.8 ±1.78 (27.1)^{ab}	26.4 ±4.56 (30.9)^{bc}
Control	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}

Values are mean (%) of five-replicates of three trials ±standard deviation. Figures in parentheses are angular transformed. Different superscript alphabets in the column indicate statistical significant difference at $P<0.05$ levels by two way ANOVA followed by Tukey's test performed.

Table 2: Per cent larvicidal activity of fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract against *Anopheles stephensi*

Concentration (ppm)	Fractions								
	A	B	C	D	E	F	G	H	I
25	21.6 ±2.19 (27.7)^{b}	11.2 ±1.78 (19.6)^{a}	09.6 ±2.19 (18.1)^{a}	26.4 ±3.57 (30.9)^{bc}	28.8 ±3.34 (32.5)^{c}	55.2 ±3.34 (47.9)^{e}	35.2 ±1.78 (36.4)^{d}	08.0 ±2.82 (16.4)^{a}	07.2 ±3.34 (15.6)^{a}
50	34.4 ±2.19 (36.0)^{b}	17.6 ±3.57 (24.8)^{a}	12.0 ±2.82 (20.3)^{a}	36.8 ±3.34 (37.4)^{b}	44.8 ±5.21 (42.0)^{c}	63.2 ±3.34 (52.7)^{d}	48.0 ±2.82 (43.9)^{c}	14.4 ±3.34 (22.3)^{a}	17.6 ±4.56 (24.8)^{a}
75	43.2 ±1.78 (41.1)^{c}	24.0 ±2.82 (29.3)^{b}	13.6 ±3.57 (21.6)^{a}	44.8 ±3.34 (42.0)^{c}	56.8 ±3.34 (48.9)^{d}	80.0 ±2.82 (63.4)^{f}	65.6 ±2.19 (54.1)^{e}	19.2 ±3.34 (25.9)^{ab}	20.0 ±2.82 (26.6)^{b}
100	52.8 ±3.34 (46.6)^{d}	30.4 ±2.1 (33.5)^{c}	16.0 ±2.82 (23.6)^{a}	53.6 ±2.19 (47.1)^{d}	66.4 ±3.57 (54.6)^{e}	100.0 ±0.0 (0.0)^{g}	84.0 ±2.82 (66.4)^{f}	23.2 ±3.34 (28.8)^{b}	25.6 ±5.36 (30.4)^{bc}
Control	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}

Values are mean (%) of five-replicates of three trials ±standard deviation. Figures in parentheses are angular transformed. Different superscript alphabets in the column indicate statistical significant difference at $P<0.05$ levels by two way ANOVA followed by Tukey's test performed.

Table 3: Per cent larvicidal activity of fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract against *Culex quinquefasciatus*

Concentration (ppm)	Fractions								
	A	B	C	D	E	F	G	H	I
25	28.8 ±3.34 (32.2)^{cd}	14.4 ±2.19 (22.3)^{b}	11.2 ±1.78 (19.6)^{ab}	28.0 ±2.82 (31.9)^{c}	32.8 ±1.78 (34.9)^{d}	46.4 ±2.19 (42.9)^{f}	39.2 ±1.78 (38.8)^{e}	07.2 ±1.78 (15.6)^{a}	12.8 ±1.78 (20.9)^{b}
50	37.6 ±3.57 (37.8)^{c}	19.2 ±3.34 (25.9)^{b}	16.8 ±1.78 (24.2)^{ab}	39.2 ±3.34 (38.8)^{c}	48.0 ±2.82 (43.9)^{d}	62.4 ±3.57 (52.2)^{f}	54.4 ±2.19 (47.5)^{e}	12.8 ±1.78 (20.9)^{a}	20.8 ±1.78 (27.1)^{b}
75	51.2 ±1.78 (45.7)	27.2 ±3.34 (31.4)^{c}	19.2 ±3.34 (25.9)^{ab}	48.0 ±2.82 (43.9)^{d}	60.8 ±1.78 (51.0)^{e}	89.6 ±3.57 (71.2)^{g}	72.0 ±2.82 (58.1)^{f}	18.4 ±3.57 (25.4)^{a}	25.6 ±4.56 (30.4)^{bc}
100	55.2 ±4.38 (47.9)^{d}	38.4 ±2.19 (38.3)^{c}	21.6 ±3.57 (27.7)^{a}	58.4 ±4.56 (49.8)^{d}	69.6 ±3.57 (56.5)^{e}	100.0 ±0.0 (0.0)^{g}	92.4 ±3.34 (74.0)^{f}	25.6 ±2.19 (30.4)^{ab}	28.8 ±4.38 (32.5)^{b}
Control	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}	0.0 ±0.0 (0.0)^{a}

Values are mean (%) of five-replicates of three trials ±standard deviation. Figures in parentheses are angular transformed. Different superscript alphabets in the column indicate statistical significant difference at $P<0.05$ levels by two way ANOVA followed by Tukey's test performed.

Table 4: Probit analysis of larvicidal activity of fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract against vector mosquitoes

Vector mosquitoes	*Aedes aegypti*						*Anopheles stephensi*						*Culex quinquefasciatus*					
Fractions	LC_{50}	95% CL		LC_{90}	95% CL		LC_{50}	95% CL		LC_{90}	95% CL		LC_{50}	95% CL		LC_{90}	95% CL	
	(ppm)	LL	UL	(ppm)	LL	UL	(ppm)	LL	UL	(ppm)	LL	UL	(ppm)	LL	UL	(ppm)	LL	UL
A	82.25	76.87	88.79	192.76	172.91	220.89	91.40	85.01	99.72	206.94	184.09	240.01	80.84	74.68	88.80	213.87	187.29	254.25
B	149.71	132.27	177.77	288.59	244.47	361.12	153.57	135.35	183.53	292.70	247.24	368.09	130.75	118.29	149.31	254.05	220.71	305.21
C	262.66	200.79	424.56	491.43	358.97	840.46	347.63	237.62	829.49	667.32	434.19	1693.29	240.76	186.31	379.09	479.47	352.69	804.43
D	91.52	84.51	100.92	219.53	192.65	259.98	89.13	82.01	98.70	224.76	195.77	269.35	79.14	73.43	86.09	202.21	179.07	236.28
E	67.44	63.17	71.92	164.63	150.40	183.89	64.19	59.83	68.63	162.99	148.76	182.34	56.70	52.02	61.09	156.73	143.08	175.33
F	36.76	30.76	41.66	82.51	76.06	91.23	26.85	16.29	34.25	84.06	75.86	96.14	32.60	27.73	36.65	74.74	69.87	80.91
G	48.78	44.72	52.44	127.20	118.85	137.40	49.46	45.83	52.78	120.38	113.27	129.21	41.72	38.28	44.81	101.23	96.20	107.29
H	216.14	176.54	297.56	396.37	310.45	574.68	181.44	155.14	228.16	331.72	272.44	438.36	161.65	142.88	191.57	284.21	242.39	351.88
I	178.92	152.99	225.01	333.78	273.82	441.81	165.74	138.98	220.05	303.20	241.47	431.16	170.24	144.62	217.59	345.36	279.28	469.64

Figure 1: *Sphaeranthus indicus*

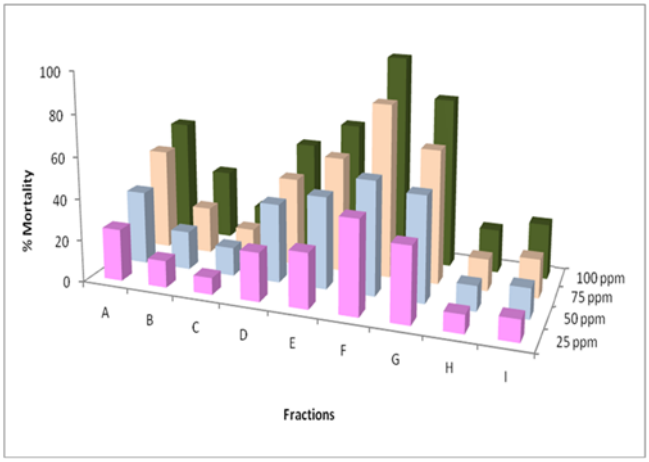

Figure 2: Larvicidal activity of fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract against *Aedes aegypti*

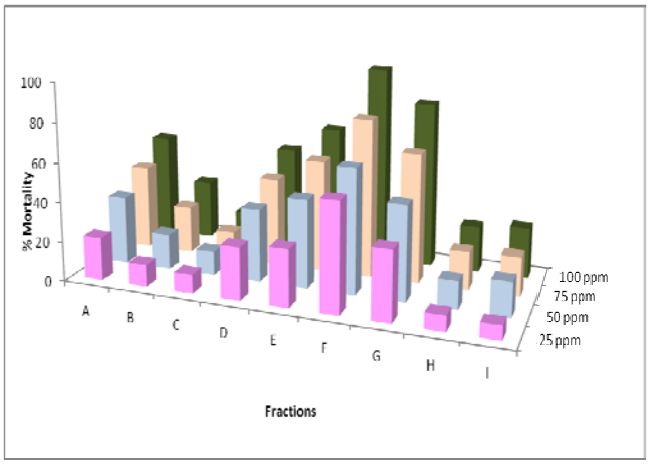

Figure 3: Larvicidal activity of fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract against *Anopheles stephensi*

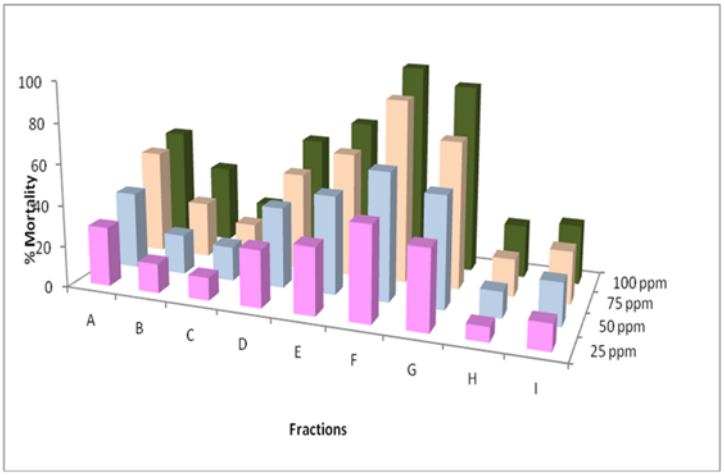

Figure 4: Larvicidal activity of fractions of *Sphaeranthus indicus* ethyl acetate whole plant extract against *Culex quinquefasciatus*

4. Discussion

Man suffers extensively due to the nuisance of insect populations both in agriculture and health. In agriculture, insects affect directly on growing part of the crop and cause severe damage, resulting in revenue loss. In health point of view, insect vectors especially mosquitoes directly transmit diseases [120]. Human vector-borne diseases account for 17% of the estimated global burden of all infectious diseases. The major part and most widely distributed of these diseases are transmitted by mosquitoes [121]. Mosquitoes are nuisance and annoyance insects that transmit various diseases from organism to human and animal. Prevention and control of mosquitoes are important to reduce the vector-borne disease incidence. Many control measures have been applied to reduce mosquito menace in which larvae are decimated at different stages to prevent the establishment of mosquito population. Mosquito larval control commonly referred to as Larval source management (LSM) is particularly valuable in regions where the primary mosquito vectors are exophilic and/or bite before people are in bed, so rendering indoor residual spraying less effective [122, 123]. Therefore, LSM involves the management of aquatic habitats that are potential larval habitats for mosquitoes, in order to prevent the completion of development of the immature stages [124].

Synthetic chemicals are proved to be effective, but they cause adverse effects on the environment and human health [125]. In this situation, ecofriendly alternatives are important for safer control of mosquitoes. One of the most effective alternative approaches under the biological control programme is to explore the floral biodiversity and enter the field of using safer insecticides of botanical origin as a simple and sustainable method of mosquito control [38]. The results of pesticidal and phytochemical screenings of a number of plants based on traditional knowledge strongly indicate that plants are endowed with pesticidal properties that can be harnessed cheaply for use in agriculture and related fields. The need to use plant-based products arises from the fact that the synthetic pesticides are harmful to humans, and the entire ecosystem due to high toxicity and persistence [126]. Natural product literature provides a growing research on plant derived mosquitocidal agents [36]. The search for natural and benign environmental mosquitocides is ongoing worldwide [127-129]. The phytochemicals from plant origin were proved to be effective due to multiple modes of action [16-18]. Running after

controlling mosquitoes, many efforts have been paid to obtain active ingredients from *Sphaeranthus indicus*. From the foregoing mosquitocidal property of *Sphaeranthus indicus* reported elsewhere particularly larvicidal activity, it was justifiable to take *Sphaeranthus indicus* whole plant ethyl acetate extract to further analysis. Hence, to complement in this research program, the fractions of ethyl acetate extract of *Sphaeranthus indicus* whole plants were further studied for larvicidal activity against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*.

In the present study, the ethyl acetate fractions of *Sphaeranthus indicus* whole plants caused significant larval mortality on vector mosquitoes. Samuel and Arivoli [47] in their preliminary investigation tested different solvent extracts (hexane, diethyl ether, dichloromethane and ethyl acetate) of *Sphaeranthus indicus* whole plants against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* for larvicidal activity and found the ethyl acetate extract to be active. The results of the present study strongly corroborates with the reports of Samuel and Arivoli [47] by confirming the presence of active principles to be present in the 'F' fractionated group of ethyl acetate extract, indicated by higher toxicity and the lowest LC_{50} value reported. The findings of the present study are in line with the high potential of mid-polar solvent *viz*., ethyl acetate that mainly extracts steroids, alkaloids, etc. For instance, Sun *et al*. [130] screened ethyl-acetate (polarity index of 4.4), n-butyl alcohol (polarity index of 3.9) and aqueous fractions of alcoholic extract of leaves and stems of *Vanilla fragrans* against *Culex pipiens* larvae. Both n-butyl alcohol and ethyl acetate fractions were active in bioassays, while the aqueous fraction appeared to contain no substances.

Basheer [131] tested the *Ricinus communis* hexane, ethyl acetate and ethanolic leaf extracts for larvicidal activity against *Anopheles arabiensis* and found ethyl acetate to be effective. Seven fractions (F1-F7) were obtained from ethyl acetate extract and fraction F3 showed the highest effect with a LC_{50} value of 107 µg/mL on 24 hours of exposure. This fraction was found to contain: linalool, eugenol in addition to small quantities of cinoele estragol, limonine and methyl chavicol. Famuyiwa and Adebajo [132] reported that the fractions (A, B_1-B_5) of *Eugenia uniflora* methanolic leaf extract when tested for larvicidal activity against *Aedes aegypti*, fraction 'B_5' was effective and LC_{50} value was <10 mg/mL after 24 hours of exposure. Thongwat *et al*. [133] tested the fractions (E-Gr3 - E-Gr5) of *Pereskia bleo* fruit endocarp ethanol extract against *Aedes aegypti* and found fraction E-Gr3 to exhibit high larvicidal activity with LC_{50} value of 707.94 ppm after 24 hours. da Silva *et al*. [134] stated that *Copaifera reticulate* oil resin hexane (CRH_1 and CRH_5) and methanol extract fractions (CRM_1 and CRM_5) exhibited larvicidal activity against *Aedes aegypti* and LC_{50} values were 2.3, 0.8, 13.9 and 10.5 ppm after 24 hours. Samidurai and Mathew [135] reported the crude extracts of ethyl acetate latex extract of *Euphorbia lactea* to possess larvicidal activity on 24 hours of exposure against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* and LC_{50} values were 21.01, 25.65 and 49.69 respectively. Further, out of four fractionations (A1, A2, B1 and B2) obtained from the ethyl acetate latex extract of *Euphorbia lactea*, fraction B2 elicited 100% larval mortality against *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi* while fraction A1 and A2 was also reported for 100% larval mortality against *Anopheles stephensi* and A2 for *Culex quinquefasciatus* after 24 hours exposure. Arivoli *et al*. [58] reported that the isolated fractions (A-H) of *Citrullus colocynthis* dichloromethane whole plant extract when evaluated for larvicidal activity against the vector mosquitoes *viz*., *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*, the fraction 'C' showed 94.4, 96.0 and 98.4% mortality against third instar larvae of *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* at 100 ppm and LC_{50} values were 18.57, 23.48 and 19.26 ppm respectively after 24 hours. Arivoli *et al*. [59] also reported that the isolated fractions (A-F) of *Murraya koenigii* hexane leaf extract when evaluated for larvicidal activity against the vector mosquitoes *viz*., *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*, the fraction 'D' showed 100.0, 99.2 and 97.6% mortality against third instar larvae of *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* at 100 ppm and LC_{50} values were 35.06, 42.51 and 27.20 ppm respectively after 24 hours.

The secondary compounds of plants make up a vast repository of compounds with a wide range of biological activities. In the present study, ethyl acetate fractions were toxic to the larvae of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*. It is found that botanical derivatives possessing mosquitocidal properties in general, directly attack on the nervous system and damage it, primarily affect the mid-gut epithelium and secondarily affect the gastric caeca and the malpighian tubules in mosquito larvae [136], act as mitochondrial poison [137], and work by interacting with cuticle membrane of the larvae ultimately disarranging the membrane which is the most probable reason for larval death [138]. This could be due to the presence of alkaloids, flavonoids, steroids, tannins, terpenes and terpenoids within the fraction and it is said that several groups of the above mentioned phytochemicals from different plants have been reported for their insecticidal activities [23]. Plant parts containing alkaloids, coumarins, flavonoids, quinines, saponins, steroids and triterpenoids (terpenoids) [139, 140] may be toxic to the immature mosquitoes. The fraction 'F' of *Sphaeranthus indicus* ethyl acetate whole plant extract in the present study might certainly contain one or more phytochemical compounds thereby confirming that the larvicidal activity might be due to the presence of phytoconstituents.

Liu *et al*. [141] considered alkaloids among the active molecules against mosquito larvae. Alkaloids are nitrogenous compounds that show insecticidal properties at low concentration and the mode of action on insect vectors varies with the structure of their molecules, but many are reported to affect acetylcholinestrase (AChE) or sodium channels as inhibition of acetylcholinesterase activity is responsible for terminating the nerve impulse transmission through synaptic pathway [142]. Alkaloids work by constricting blood vessels and depressing autonomic nervous system activity thereby contributing to the insecticide's effectiveness in killing the larvae of mosquitoes and disrupting the life cycle of the mosquito [143]. The flavonoids, poncirin, rhoifolin and naringin isolated from *Poncirus trifoliata*, showed larvicidal activity against *Aedes aegypti* with LC_{50} values of 0.082–0.122 mg/L after 24 hours [144]. The isoflavonoids, neotenone and neorautanone isolated from *Neorautanenia mitis* displayed activity against adult *Anopheles gambiae* mosquitoes with LD_{50} values of 0.008 and 0.009 mg/mL, respectively [145]. The lactones isolated from *Hortonia floribunda*, *Hortonia angustifolia* and *Hortonia ovalifolia*, exhibited potent larvicidal activity against the second instar larvae of *Aedes aegypti* [146]. The 3-n-butyl-4,5-dihydrophthalide isolated from seeds of *Apium graveolens* showed 100% mortality at 25 µg/mL [147] and sedanolide

isolated from seeds of same species exhibited 100% mortality at 50 µg/mL against fourth instar larvae of *Aedes aegypti* [148]. The dehydrocostus lactone and costunolide identified from essential oil of *Saussurea lappa* exhibited strong larvicidal activity against *Aedes albopictus* with LC_{50} values of 2.34 and 3.26 µg/mL respectively [149]. Sesquiterpene lactone, isolated from a petroleum ether extract of *Sphaeranthus indicus* was screened for its effects on the hatching of eggs and metamorphosis of larvae of *Culex quinquefasciatus* at concentration of 50 mg/L. Rates of fecundity and fertility were found to be affected in the larval treated adult females. Egg hatching was also significantly lowered. Mortality in the larvae, pupae and adults produced a marked decrease in mosquito populations [110]. The sesquiterpene lactones isolated from leaves, stem bark, flowers and fruits of *Magnolia salicifolia* exhibited significant toxicity against *Aedes aegypti* larvae [150]. The β-selinene isolated from seeds of *Apium graveolens* shows 100% mortality against fourth instar larvae of *Aedes aegypti* at 50 µg/mL [147]. The pregeijerene, geijerene and germacrene-D isolated from leaves of *Chloroxylon swietenia*, possessed larvicidal activity against *Anopheles gambiae*, *Culex quinquefasciatus* and *Aedes aegypti*. The sesquiterpenes, elemol, β -eudesmol, carotol and patchoulol occurring in plants *Amyris balsamifera* and *Daucus carota* showed >90% larval mortality against *Culex pipiens pallens* at 0.1 mg/mL [151]. The guanine type sesquiterpenes, 9-oxoneoprocurcumenol and neoprocurcumenol isolated from *Curcuma aromatica* exhibited significant toxicity on mosquito larvae of *Culex quinquefasciatus* [152]. A major sesquiterpene lactone isolated from petroleum ether fraction of *Sphaeranthus indicus* flowers showed acetylcholine esterase inhibitory activity [153].

According to a research, tannins and alkaloids in *Pistia stratiotes*; tannins, alkaloids and steroid glycosides in *Typha latifolia*; tannins, saponins and steroid glycosides in *Leucas martinicensi*; alkaloids, saponins and tannins in *Cynodon dactylon* and saponins and tannins in *Nymphaea lotus* have been reported to be responsible for larval toxicity of *Anopheles* mosquitoes [154]. In addition, triterpenoids and saponins in chloroform; saponins in hexane; steroids, saponins, tannins and alkaloids in methanol extracts of *Adansonia digitata* had revealed their toxicity against *Aedes aegypti* and *Culex quinquefasciatus* larvae [155]. Saponins and alkaloids had been reported by Mousumi *et al*. [156] to be responsible for toxicity of seed coat of *Cassia sophera* on all instar larvae of *Culex quinquefasciatus*. The compound stigmasterol isolated from *Uvariodendron pycnophyllum* and many other plant species, exhibit larvicidal activity at different levels with LC_{50} value of 46 ppm in 24 hours [157] and β-sitosterol-3-O-β-D-glucoside isolated from *Acanthus montanus* resulted in 100% mortality against adult *Aedes aegypti* at 1.25 µg/mL [158].

Plant bioactive components may serve as a suitable alternative to chemical insecticide as they are relatively safe and available everywhere in the world. The efficacy of botanicals however, generally depends on the plant part [159], extract concentration, age of plant or location found, solvent used and species of larvae tested [160-162]. The solvent used contribute to the variation since it has been shown that the extraction of active biochemical from plants depends upon the polarity of the solvents used [38]. Shaalan *et al*. [23] reported that screening involves mosquitocidal bioassay guided fractionation to identify highly active fractions and compounds isolated from the crude extract. The crude extract contains a complex mixture of biocidal active compounds. Hence, crude plant extracts have played an important role in this aspect. If an exceptionally low lethal concentration is detected, the extract may be fractionated in order to locate the particular chemical constituent causing the lethal effect. The purpose of fractionation is thus to produce several simple mixtures of compounds to reduce the number of compounds which may be identified in further analyses. Fractions isolated from the same extract always have different larvicidal activity because they contain different phytochemicals. Once a fraction has proved to be effective, compounds can be extracted to isolate the active ingredient. However, some compounds loose efficacy when separated since many synergistic relations potentially exists in botanical preparations which may promote killing activity.

The plant world comprises a rich untapped pool of phytochemicals that may be widely used in place of synthetic insecticides in mosquito control programme. Screening of mosquitocidal potentials by the isolation of natural products seems to be an attractive approach, which can result in the efficient elucidation of new lead compounds [37]. Kishore *et al*. [36] reviewed the efficacy of phytochemicals against mosquito larvae according to their chemical nature and described the mosquito larvicidal potentiality of several plant derived secondary metabolites, *viz*., alkanes, alkenes, alkynes and simple aromatics, lactones, essential oils and fatty acids, terpenes, alkaloids, steroids, isoflavonoids, pterocarpans and lignans. Several studies have documented the efficacy of plant extracts as the reservoir pool of bioactive toxic agents against mosquito larvae. Though several compounds of plant origin have been reported as insecticides and larvicides, there is still a wide scope for the discovery of more effective plant products [163]. Identification and isolation of bioactive compounds of plant origin against mosquito menace are imperative for the management of mosquito-borne diseases. Further, Tehri and Singh [164] stated that the successful results of preliminary studies on mosquitocidal potential of plant extracts encourage further effort to investigate the bioactive compounds in those extracts that might possess good larvicidal properties when isolated in pure form. In addition, novel drug delivery system of plant based active substances is the need of the hour. In conclusion, the bioassay result of the present study indicated the larvicidal property against vector mosquitoes of isolated fractions of *Sphaeranthus indicus* whole plant ethyl acetate extract, especially the 'F' fractionated group. Future research to extract a pure compound of the active fractionated group should be explored to find a new highly efficient larvicidal substance.

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