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Evaluation of larvicidal potential of flavonoid extracted from *Sphaeranthus indicus* Linn (Asteraceae) for controlling mosquito *Culex quinquefaciatus* (Culicidae) Diptera

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Abstract

The entire world is facing the problem of vector borne diseases, which are one of the leading causes of deaths in developing countries. This is not because of any lapse or failure of disease control operations but due to enormous population growth of various types of vectors, among which mosquitoes are playing a major role. Their control is a difficult task due to a variety of factors including the development of insecticide resistance in the insects and environmental pollution. No doubt, the chemical insecticides will be continuously used for mosquito control but a cheap, less harmful and eco-friendly alternative is badly needed. The chemicals derived from plants, have been projected as weapons in future mosquito control programs. They act as general toxicants, growth inhibitors, reproduction inhibitors, repellents and oviposition deterrents. So far, more than thousand plants have been searched for biopesticides, not only to control the vectors, but also other pests of agricultural importance. Plants have evolved a variety of secondary metabolic compounds, some of them for providing protection from phytophagous insects. Flavonoids are phenolic secondary metabolites found in a wide range of plant tissues, possessing a common aromatic ring, bearing one or more hydroxyl substituents. Flavonoids are most commonly known for their antioxidant activity. In the present piece of research work it was observed that the flavonoid extract of *Sphaeranthus indicus* proved to be a very effective mosquito larvicide and can provide eco-friendly alternative to synthetic insecticides. The findings suggest that plant extracted bio-pesticides may play a major role in designing an integrated vector control program in near future.

Key-Words: *Culex quinquefasciatus, Sphaeranthus indicus,* Flavonoid, Eco-friendly, Biopesticide

Introduction

Entire world is facing the problem of resurgence of vector borne diseases such as Malaria, Filaria, Japanese Encephalitis and Dengue fever etc. This is not because of any lapse or failure of the disease control operations but mainly due to enormous population growth, rapid industrialization, and fast urbanization. This provides comfort zone to increase of various types of vectors among which mosquitoes are playing major role in transmitting various vector borne diseases. There are various technologies and tools for the control of vector borne diseases, but no single method is successful in all situations. One of the methods available for the control of mosquito vector borne diseases is the use of chemical insecticides but a major drawback in it is that they are non-selective and could be harmful to human and other non target organisms also.

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Due to the harmful effects of chemical pesticides an alternate method for vector control is essentially preferred and that is undoubtedly the biological control. It is generally cheaper due to its biological origin and eco-friendly leaving no by or end product in the last. Plants have evolved a variety of secondary compounds, some of them for providing protection from phytophagous insects. These plants extracts with insecticidal properties are used in the form of biological ecofriendly pesticides. So far more than five thousand plants have been searched for biopesticides to control not only the vectors but also other pests of agricultural importance. The best known and widely used bio-insecticide is pyrethrum which is extracted from the flower of *Chrysanthemum*. A number of plants have been tested for larvicidal properties such as castor, Vasudevan *et al.* (1989), Kalyansundaram and Das (1985).

In last few years, petroleum ether extract of 41 indigenous plants found in India have been studied for the larvicidal activity against Culicine mosquitoes, Latha *et al.* (1999).The chemicals derived from plants have been projected as weapons for future mosquito control programs. They function as general toxicants, growth and reproductive inhibitors, repellents and deterrents, Sukumar *et al.* (1991).Irritant effect, prevention of blood feeding and toxicity of nets impregnated with different pyrethroids on *Anopheles stephensi* was reported by Hodjati *et al.* (2003).Das *et al.* (2003) analysed repellent properties of extracts of two plants viz. *Zanthoxylym limonella and Citrus aurantifolia*.Larvicidal and mosquito repellent activity of plant extracts obtained from *Pinus longifolia* was reported by Ansari *et al.* (2005).Larvicidal efficacy of *Capsicum annum* against *Anopheles stephensi* and C*ulex quinquefasciatus* was observed by Madhumathy *et al.* (2007).The larvicidal activities of the oil of *Zanthoxylum armatum* against mosquito vector was reported by Tiwari *et al.* (2007). Six copepod species as biological control agents against *Aedes aegypti* and *Mesocyclops aspericornis* have been reported by Phong *et al.* (2008).

In the present work flavonoidal compounds of *Sphaeranthus indicus* were tested for mosquito control. The term flavonoid refers to a class of plant secondary metabolities. Flavonoids are most commonly known for their antioxidant activity. Besides this, the health benefits they provide are protection against cancer and heart diseases. The flavonoids are chemically polyphenolic compounds possessing 15 carbon atoms; two benzene rings joined by a linear three carbon chains.

Material and Methods

Collection of plant material

In the present study an indigenous plant *Sphaeranthus indicus* of family Asteraceae was selected to obtain biologically active flavonoid compounds from it. Collection was done in winter season. Identification of the collected plants were carried out at the Department of Botany of Govt. Model Science College, Rewa.

***Sphaeranthus* is also known by various local names in various places**

Hindi : Gorakhmundi
Sanskrit : Mahamundi
Gujrati : Mundi

It is a much branched, strongly scented annual herb with winged stem. Leaves are compound oblong, narrowed to the base dentate or serrate. They grow throughout India and are abundant in moist shady places.The juice of the leaves is used for gargles and the extract of herb is antifungal and antibacterial Chopra *et al.*(1956) and Akhtar *et. al* (1992).It's juice is used in gastric and hepatic disorders. Decoction is used in cough and chest troubles and is antitubercular. Flowers are used as tonic.

For phytochemical analysis of flavonoids of plant, the collected material after proper identification was shade dried and powdered. This material was soxhlated in 90% alcohol, rectified spirit and water respectively.The extract thus obtained was kept in a glass vials and stored in the refrigerator, percentage yield was calculated and recorded. Percentage loss in weight after drying of the plant material was also recorded which showed 78.4% loss in weight. The cold percolation method mentioned by Harborne (1984) was followed for fresh plants. Crude extracts obtained from the plant were subjected to purification process by different chromatographic techniques.

Chemical analysis and identification of the compound

First the crude extract of plant was defatted in n-hexane and extracted with acetone, chloroform and petroleum ether. The concentrated solution was allowed to stand when a green yellow deposit was obtained. The deposit was repeatedly crystallized from a mixture of acetone and methanol, till a single spot was obtained by paper and thin layer chromatography. This yellow compound melted at 236^0C to 248^0C. This solution was expected to be of flavonoidal nature which was further confirmed by UV spectrum as it gave dull green colour with ethanolic $FeCl_3$ and various shades of red colour with HCl. The compound obtained after fractionation by column chromatography was acid hydrolyzed, methylated and tested by related chemical tests.

5-10 ml of each fraction from column chromatography was mixed with 2 ml of ethnolic HCl and refluxed for four hours by evaporation. This mixture was diluted with 3 ml of water and again evaporated to remove ethanol. The aqueous solution was extracted with $CHCl_3$. After evaporation the aqueous layer was neutralized with NaOH and concentrated under reduced pressure.

Methylation: Purified fractions were separately dissolved in MeOH. In this process compound was washed by MeOH until it got converted into crystal or powder form.

Test for Flavonoid

Alkaline reagent test: To the test compound solution few drops of NaOH solution were added. Intense yellow colour was obtained, which turned to colourless by addition of a few drops of dilute HCl. This indicated the presence of flavonoids.

Zinc Hydrolysis test: To the test compound solution mixture of zincdust and conc. HCl was added which produced red colour after a few minutes.

For further characterization and structural elucidation of plant extract the purified samples of selected plant fraction was sent to SAIF, CDRI Lucknow for following spectral analysis:

IR Spectrum: The IR spectra were recorded on Perkins – Elmer model 783 spectrophotometer Graph.

UV Spectrum: The UV spectra were recorded on Schimadzu U-160 spectrophotometer and it was scanned in the range between 200-400 nm. Graph.

NMR: The H^1 NMR and C^{12} NMR spectra were recorded on F + NMR spectrophotometer (90 MHZ) in $Cdcl_3$ using TMS as an internal standard and the chemical shifts were determined in ppm value (s) from TMS as internal standard Graph.

Mass Spectrum: The mass spectra were recorded on a JEOL JMS 300 spectrometer with accelerating potential of 3kv Graph.

On the bases of spectral data obtained from SAIF CDRI Lucknow and on comparing the data with authentic markers available finally the flavonoidal compound was obtained. The isolation and structure elucidation of the compound Sf2 was carried out by colmn chromatography and successive elution with benzene removes fatty materials, carotenoids and phytosterols respectively. After elucidation with ethyl acetate yielded contaminated fraction with chlorophyll which was removed by the treatment with active charcoal. The fraction was rechromatographed on small glass colmn (30x20cm) of silica gel, yield fraction Sf2.

The compound Sf2 was purified by the chromatographic procedure and structure was elucidated using spectroscopic methods such as IR, UV, NMR and Mass Spectra. After comparison with the standard sample it was suggested that the structure of compound Sf2 lead to flavonoid structure as Quercetin.

Laboratory colonization of mosquitoes

The aquatic stages of the larvae were collected with the help of long hand dippers and other manual methods. The fourth instar stages were sorted out and transferred to enamel trays of size (30 × 25 × 5 cm) and fed finely ground Brewer's yeast and dog biscuits (3:1). The adults, which emerged, were transferred to insect iron cages covered with muslin cloth. For mass production, standard wooden as well as iron insect cages of (30×30×30 cm) size were used. Adults were held in these cages for egg production. Hatching of eggs started within 48 hrs and newly hatched larvae were held in the enamel trays. The larvae were maintained instar-wise in different rearing trays.

Pupae that emerged from the larvae were separated in beakers containing water and kept in iron cages. The adults emerged were maintained till 25^{th} generation.

Population of test insect exposed to the insecticidal activity of plant extract of *Sphaeranthus indicus* for bioassay experiments were II^{nd} and IV^{th} instar larvae and adult female mosquitoes. Mortality was observed after 24 hrs intervals at each level of concentration. There were four replicates of each concentration along with one control. The experiment was performed at $27+ 2^0$ C and R.H. about 80% $\pm$ 5%.

Dead larvae, pupae, partly emerged and precocious adults were regularly removed and counted. LC_{50} value of each concentration of the plant extract was calculated.

Toxic larvicidal activity

The larvicidal potency of plant extracts (crude and purified) were evaluated by exposing II^{nd} and IV^{th} instar *Culex quinquefasciatus* larvae of laboratory origin in batches of 25. The larvae were strained with the help of insecticide free strainer and delivered into 500 ml beaker containing 250 ml of water. The required volume of the stock solution was added in the beaker to give the desired test concentration. Each concentration was tested at four replicates with one control. Routine food schedule to larvae was followed for both treated and untreated beakers following Ansari et *a1*. (1978). Observations were recorded at an interval of 24 hrs till the emergence of adults. Dead and moribond larvae, pupae and partly emerged adults were regularly removed. LC_{50} values were determined by plotting the dosage of mortality curve on a log-probit paper.

Results and Discussion

Plant extract was tested against laboratory colonized II^{nd} and IV^{th} instar larvae of *Culex quinquefasciatus.* 25 larvae of each instar were taken in four replicates, each along with one control. The experiments were performed in 500 ml glass beaker, taking 499 ml distilled water and 1 ml. of test concentration.

The mortality was counted after every 24 hrs interval till complete mortality or pupation was seen. The mortality was analyzed statistically using probit analysis method of Finney and following parameters for each crude extract were worked out. The parameters were regression equation ($Y = a \pm bx$), Chi-square, LC_{50} value, Variance as well as upper & lower fiducial limits.

Table No. 1 reports the statistical analysis of experimental data of acetone crude extracts of *Sphaeranthus indicus.* The mortality ranges for IInd instar larvae between 32% to 60% which gave LC_{50}

306.902 ppm. The X^2 value came to be 0.16. The regression equation (Y = a ± bx) was found to be 2.513 ± 1.0x. These values were at the variance 0.01448. The standard deviation was calculated to be 0.12034. The lower and upper fiducial limit was found to be 145.926 and 432.380 ppm. The mortality of IVth instar larvae was found to be between 36% to 72%, which gave LC_{50} value of 219.845 ppm. The X^2 values came to be 0.651 and regression equation (Y = a ± bx) came to be 1.892 ± 1.327x. The Standard deviation for these values was calculated 0.0857. In both the cases control mortality never exceeded 5%. The result indicates that the IVth instar larvae prior to pupation were more susceptible to the crude extract rather than IInd instar larvae. The results were found quite significant over the control at 1% level of significance ($p < 0.001$). Standard error for IInd and IVth instar larvae were 0.5382 and 0.03833.

Experimental statistical data for petroleum ether crude extract of *Sphaeranthus indicus* are given in **Table No.2**. As mentioned in the table the mortality ranges between 28% to 68%, which gave LC_{50} 311.3 ppm and Chi-square value came to be 1.791. The regression equation (Y = a ± bx) was found to be 1.083 ± 1.571x. These values were derived at the variance 0.0074.. The Standard deviation was calculated to be 0.08602. Upper and lower fiducial limits were found to be 523.6 and 240.43 ppm respectively for IInd instar larva.The mortality of IVth instar larvae was found to be between 28% to 60%, which gave LC_{50} value 319.529 ppm. The Chi-square value was calculated to be 3.58. Regression equation (Y = a ± bx) was found to be 1.391 ± 1.441x. The Standard deviation for these values was calculated to be 0.07956. The upper and lower fiducial limits were 452.832 and 220.832 ppm. It was observed that 500 ppm concentration caused high mortality rate.

Table No.3 shows the statistical data of larvicidal activity of crude chloroform extract of *Sphaeranthus indicus*. The results depict that this crude extract was less effective as compared to other two crude extracts of *Sphaeranthus indicus*. Concentrations ranging from 100 to 500 ppm gave LC_{50} values for 24 hrs duration as 446.461 and 306.902 for IInd and IVth instar larvae respectively. For IInd instar larvae the statistical analysis such as regression equation, Chi-square, log LC_{50} , Standard error and standard deviation of the data were found to be 2.541 ± 0.928x, 0.250, 0.05608 and 0.12541 with variance of 0.01572 and the upper and lower fiducial limits were equal to 701.157 and 226.039.

The same analysis for IVth instar larvae was found as, Regression equation (Y = a ± bx) 2.513 ± 1.00x, Chi-square 0.160 and LC_{50} was found to be 306.902 ppm with variance of 0.01448. The standard error and standard deviation calculated was 0.05382 and 0.12034 for IVth instar larvae. The upper and lower fiducial limits came to be 432.380 and 145.926. The level of significance indicated that these results were more significant than the control ($P < 0.001$).

Results indicate that IVth instar larvae were more susceptible to the crude extract than IInd instar larvae.

The purified fraction of *Sphaeranthus indicus* Sf2 which was found to be biologically active was tested against. IInd and IVth instar larvae in five concentrations 50, 100, 150, 200 and 250 ppm. The results are mentioned in **Table No.4** depicting the mortality of IInd instar larvae in 24 hrs. ranging between 48 to 80%. LC_{50} value was 64.58 ppm.

For IVth instar larvae, the same concentrations cause 60 to 88% mortality. The LC_{50} value came to be 35.619 ppm. The Standard deviation, Standard error, Variance, Chi-square and Regression equation are mentioned in the table.

Awolaola (2007) has emphasized the need of some alternative vector control methods because malaria vector *Anophelese gambiae*, have developed resistance against pyrethroids. It was noticed in the present experiment that IVth instar larvae are more susceptible than IInd instar larvae. Singh *et al.* (2008) have reported the larvicidal properties of *Mimordica charantia*. They reported LC_{50} value for IVth instar larvae to be 1.29% concentration. According to them *Anopheles* larvae were more susceptible as compared to *Culex* and *Aedes* larvae. Present findings are similar to the findings of Singh *et al.* (2008). Krishna *et al.* (2008) and Saxena *et al.* (2008) concluded that purified fraction of the plant extract was more effective than the crude extract,taking into consideration three different plants *Mimordica charantia*, *Annona squamosa* and *Tegetes erectus*.

Innocent *et al.* (2008) studied the larvicidal effect of *Lantana viburnoides* of family Verbenaceae, where the crude extract gave LC_{50} value for 72 hours as 7.70 ppm for *Anopheles gambiae*. They isolated triterpenoid cumeric acid from the plant which gave 72 hours LC_{50} value as 6.19 ppm. It was significant over untreated control group.Tiwari *et al.* (2007) have noticed the larvicidal activity of essential oil of *Zanthoxylum armatum* against three mosquito species and reported that the plant contained oxygenated monoterpenes which were responsible for larvicidal activity. *Zanthoxylum* plant extract has higher larvicidal activity in the pure compound form than the crude extract.

Madhumathi *et al.* (2007) isolated capasicin from

Capsicum annum against *Anopheles* and *Culex*. They have also emphasized on the need of a principle to formulate the insecticidal compounds from the plants. Mosquitoes exploit all most all type of lentic aquatic habitat for breeding. The immature stage of mosquitoes lives in water bodies along with their cospecies and heterospecies. The predators and competitors present in the habitat devour populations of mosquito larvae. So any larvicidal activity against mosquito vector should be planned keeping this fact in mind.

George and Vincent (2005) have traced comparative efficacy of *Annonna squamosa* and *Pongamia glabra* against mosquito. They have reported the LC_{50} value which was 59.75 for *Anopheles stephensi* and 27.28 for *Aedes aegypti*.The point emerged from the discussion of recent literature that various species of mosquitoes show differential susceptibility to different extracts.The use of botanical pesticide against mosquito vectors will not only reduce our dependence on chemical pesticides, but also promote eco-friendly and sustainable means of vector control.

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Table 1: Stastical data of acetone extract of *S. indicus* against Culicine mosquitoes (*Culex quinquefasciatus)*

Instar	Concentration (ppm)	24 hr. larval mortality (%)	Regression equation (Y=a±bx)	Ch-square $x^2(n-1)$	LC_{50} (ppm)	Variance (V)	S.E.	S.D.	Fiducial limits (ppm)
	100	32							
	200	42							M_1=145.926
II	300	48	2.513±1.0x	0.16	306.902	0.01448	.05382	0.12034	
	400	54							M_2=432.380
	500	60							
	Control	04							
	100	36							
	200	44							M_1=191.956
IV	300	54	1.892±1.327x	0.651	219.849	0.007346	0.03833	0.0857	
	400	62							M_2=313.413
	500	72							
	Control	04							

25 each second and fourth instar larvae were taken in average of four replicates.
Values are significantly different than the control ($p<0.001$)

Table 2: Stastical data of Petroleum ether extract of *S. indicus* against Culicine mosquitoes (*Culex quinquefasciatus)*

Instar	Concentration (ppm)	24 hr. larval mortality (%)	Regression equation (Y=a±bx)	Ch-square $x^2(n-1)$	LC_{50} (ppm)	Variance (V)	S.E.	S.D.	Fiducial limits (ppm)
	100	28							
	200	34							M_1=240.43
II	300	44	1.083±1.571x	1.791	311.3	0.0074	0.0496	0.08602	
	400	54							M_2=523.6
	500	68							
	Control	04							
	100	28							
	200	34							M_1=220.832
IV	300	46	1.391±1.441x	3.58	319.529	0.00633	0.03558	0.07956	
	400	56							M_2=452.832
	500	64							
	Control	04							

25 each second and fourth instar larvae were taken in average of four replicates.
Values are significantly different than the control ($p<0.001$)

Table 3: Statistical data of Chloroform extract of *S. indicus* against Culicine mosquitoes *(Culex quinquefasciatus)*

Instar	Concen tration (ppm)	24 hr. larval mortality (%)	Regression equation (Y=a±bx)	Ch-square $x^2(n-1)$	LC_{50} (ppm)	Variance (V)	S.E.	S.D.	Fiducial limits (ppm)
	100	30							
	200	36							M_1=226 .039
II	300	42	2.541±0.928 x	0.250	446.461	0.01572	0.05608	0.125 41	
	400	48							M_2=701 .157
	500	56							
	Control	04							
	100	32							
	200	42							M_1=145 .926
IV	300	48	2.513±1.00x	0.160	306.902	0.01448	0.05382	0.120 34	
	400	54							M_2=432 .380
	500	60							
	Control	04							

25 each second and fourth instar larvae were taken in average of four replicates.
Values are significantly different than the control ($p<0.001$)

Table 4: Statistical data of the purified fraction of *S. indicus* (Sf_2) against Culicine mosquitoes (C*ulex quinquefasciatus)*

Instar	Concen tration (ppm)	24 hr. larval mortality (%)	Regression equation (Y=a ±bx)	Chi-square $x^2(n-1)$	LC_{50} (ppm)	Variance (V)	S.E.	S.D.	Fiducial limits (ppm)
	50	48							
	100	54							M_1=41.353
II	150	66	2.748 ±1.244x	0.669	64.58	0.00876	0.04186	0.09361	
	200	72							M_2=96.268
	250	80							
	Control	04							
	50	60							
	100	66							M_1=19.331
IV	150	72	3.222±1.143x	1.97	35.619	0.1189	0.04876	0.10904	
	200	80							M_2=51.728
	250	88							
	Control	04							

25 each second and fourth instar larvae were taken in average of four replicates.
Values are significantly different than the control ($p<0.001$)