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Larvicidal activity and GC-MS analysis of flavonoids of *Vitex negundo* and *Andrographis paniculata* against two vector mosquitoes *Anopheles stephensi* and *Aedes aegypti*

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ABSTRACT

Background & objectives: Development of insect resistance to synthetic pesticides, high operational cost and environmental pollution have created the need for developing alternative approaches to control vector-borne diseases. In the present study, larvicidal activity of flavonoid extracts of different parts of *Vitex negundo* (Linnaeus) and *Andrographis paniculata* (Nees) have been studied against the late III or early IV instar larvae of *Aedes aegypti* and *Anopheles stephensi* (Liston).

Methods: Flavonoids were extracted from different parts of the selected plants using standard method. Bioassay test was carried out by WHO method for determination of larvicidal activity against mosquitoes. Different compounds of the most active extract were identified by the gas chromatography-mass spectrometry (GC-MS) analysis.

Results: Flavonoid extract of whole aerial part of *A. paniculata* was found to be inactive against the selected larvae of *Ae. aegypti* even at the concentration of 600 ppm, whereas it caused 70% mortality in *An. stephensi* at the concentration of 200 ppm. Flavonoid extract of flower-buds produced highest mortality (100%) at the concentration of 600 ppm for the late III or early IV instar larvae of *Ae. aegypti* and at the concentration of 200 ppm for the larvae of *An. stephensi*. GC-MS analysis of the most active flavonoid extract from flower-buds of *Vitex* showed 81 peaks. Phenol (26.83% area), naphthalene (4.95% area), 2,3-dihydrobenzofuran (6.79% area), Phenol-2,4-Bis (1,1-dimethyl) (4.49% area), flavones 4'-OH,5-OH,7-di-O-glucoside (0.25% area) and 5-hydroxy-3,6,7,3',4'-pentamethoxy flavones (0.80% area) were present in major amount.

Conclusion: Flavonoid extracts from different parts of two selected plants possess larvicidal activity against two selected mosquito species, hence, could be utilized for developing flavonoid-based, ecofriendly insecticide as an alternative to synthetic insecticides.

Key words Flavonoids; GC-MS analysis; larvicidal activity; vector-borne diseases

INTRODUCTION

Prevalence of mosquito-borne diseases is one of the world's most health hazardous problems. Several mosquito species belonging to genera *Anopheles*, *Culex* and *Aedes* are vectors for the pathogens of various diseases like malaria, filariasis, Japanese encephalitis, dengue and dengue hemorrhagic fever, yellow fever and chickungunya[1]. The recent World Health Organization (WHO) Malaria Report[2] estimates that 3.3 billion people were at the risk of malaria in the year 2010. Many approaches have been developed to control mosquito menace. In the absence of an effective vaccine/antiviral therapy, at present vector control is the only way to limit these mosquito-borne diseases[3–5]. Conventional pesticides such as malathion, DDT and pyrethroids that are generally used for mosquito control are known to cause the problems such as environmental pollution, residual effects and resistance in mosquito species. Development of resistance in *Cx. quinquefasciatus* and *Ae. aegypti* has been noted by WHO[6] and by other studies[7–9]. DDT, malathion and synthetic pyrethroids are used to control malaria throughout India, especially in rural areas. However, the development of insecticide resistance threatens to halt these once effective methods of control and prevention. In particular, growing insecticide resistance in the predominant malaria vectors such as *An. culicifacies* and *An. stephensi* is a major concern[10]. Furthermore, the chemical insecticides used can have adverse effects on human health and the environment. These problems forced to search for new, alternative and safer control measures especially from plant sources as plant-derived molecules are eco-friendly, biodegradable and target specific[11]. Moreover, the development of resistance by vectors against plant-derived molecules has not been reported so far. In the present study, mosquito larvicidal activity has been investigated, using

flavonoid extracts of different parts of two selected plants, i.e. *Vitex negundo* Linn. and *Andrographis paniculata* Nees.

Vitex negundo Linn. belonging to family Verbenaceae (which comprises 75 genera and nearly 2500 species), is commonly known as 'Five leaved chaste tree (Eng)'. Although, all parts of the *V. negundo* are used as medicine in the indigenous system of medicines, the leaves are the most potent for medicinal use. The decoction of leaves is used for treatment of inflammation, eye-disease, toothache, leucoderma, enlargement of the spleen, ulcers, cancers, catarrhal fever, rheumatoid arthritis, gonorrhea, sinuses, scrofulous sores, bronchitis and as tonics, vermifuge, lactagogue and emmenagogue. It is also used as an antibacterial, antipyretic, antihistaminic, analgesic, insecticidal, ovicidal, feeding deterrent, growth inhibitor and morphogenetic agent[12–21].

Andrographis paniculata is a plant that has been effectively used in traditional Asian medicines for centuries. The perceived "blood purifying" property of this plant results in its use in diseases where blood "abnormalities" are considered causes of disease, such as skin eruptions, boils, scabies, and chronic undetermined fevers. Aerial part of the plant, used medicinally, contains a large number of chemical constituents, mainly lactones, diterpenoid, diterpene glycosides, flavonoids, and flavonoids glycosides. *Andrographis paniculata* has been reported to have antibacterial, antifungal, antiviral, choleretic, hypoglycemic, hypercholesterolemia, and adaptogenic effects[22].

The present investigation was undertaken to study the larvicidal effects of flavonoid extracts of different parts of both the selected plants against late III or early IV instar larvae of *Ae. aegypti* and *An. stephensi.*

MATERIAL & METHODS

Plant collection and authentication

Different parts of *V. negundo* (leaf, stem, root and flower-buds) and *A. paniculata* (whole aerial parts and roots) were collected in the month of September–October and January–February 2010 respectively, from the western parts of India (Jaipur, Rajasthan). Plants have been identified by the senior taxonomist of the Department of Botany, University of Rajasthan, Jaipur and the voucher specimen No. RUBL20838 and RUBL20873, had already been deposited to the 'Herbarium' of the Botany Department, University of Rajasthan.

Preparation of extracts: Flavonoids extraction

Collected plant parts were separately washed with water, shade-dried, and powdered finely using a blender. Each sample was subjected to extraction, following the method of Subramanian and Nagarjan[23]. Using soxhlet apparatus 100 g of each sample was extracted with 80% hot methanol (500 ml) on a water bath for 24 h and filtered. Filtrate was re-extracted successively with petroleum ether (fraction I), ethyl ether (fraction II), and ethyl acetate (fraction III) using separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances, whereas ethyl ether and ethyl acetate fractions were analyzed for free and bound flavonoids respectively. Ethyl acetate fraction of each of the samples was hydrolyzed by refluxing with 7% H_2SO_4 for 2 h (for detaching bound sugars) and the filtrate was then extracted with ethyl acetate in separating funnel. Ethyl acetate extract thus obtained was washed with distilled water to neutrality. Ethyl ether (free flavonoids) and ethyl acetate fractions (bound flavonoids) were dried in vacuo and weighed, however, both were mixed to perform the larvicidal assay. The extracts were stored at 4°C. Stock solution (10% w/v) was prepared by dissolving the plant extract in acetone. Tween-80 was also added as emulsifying agent at the concentration of 0.02% (v/v) in the final test solution. Different concentrations were prepared by adding required volume of stock solution in beakers containing 100 ml of water.

Mosquito species

Laboratory colonies of *Ae. aegypti* and *An. stephensi* were maintained at 26 ± 2°C, 70 ± 10% relative humidity and a photoperiod of 12 : 12, Light : Dark at the Ecotoxicological Laboratory, Department of Zoology, University of Rajasthan, Jaipur, India.

Larvicidal bioassay

Standard methods for testing the susceptibility of mosquito larvae to insecticides[24] were followed in all the experiments with slight modifications. The flavonoid extracts of different parts of both the selected plants were used at 300, 400, 500 and 600 ppm dilutions and 50, 75, 100 and 200 ppm dilutions in bioassay against late III or early IV instar larval stages of *Ae. aegypti* and *An. stephensi* respectively. In all, 20 larvae were exposed to the extracts at each concentration, in final volume of 100 ml formulation taken in 250 ml glass beaker. Three replicates for each concentration and the control (with acetone and emulsifier) were tested for larval bioefficacy. The larval mortality at different concentrations and in control was recorded after 24 h continuous exposure. The corrected mortality was analyzed using Abbott's formula[25] wherever required. The mortality data were analyzed by

log-probit method[26] and lethal concentration (LC) values (50 and 90) were calculated. Data from larval mortality were subjected to analysis of variance (ANOVA). Statistical software SPSS was used for data analysis.

GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) analysis of the most active flavonoid extract from flower-buds of *V. negundo* was carried out by GC-MS Shimadzu Model QP-2010 mass spectrometer under the following conditions: DB-Polyethylene glycol coated fuse silica capillary column (30 m length × 0.25 mm ID × 0.25 µm film thickness): Helium carrier gas (1.34 ml/min); 250°C injector temperature; 240°C interface temperature; and 200°C on source temperature. Column temperature programmed at 60°C with 10°C/min rise to 230°C. For GC-MS detection ionization energy of 70ev was used. The components were identified based on National Institute of Standards Technology (NIST) Library.

RESULTS

The percent mortality LC_{50} and LC_{90} values and their 95% lower and upper confidence limits for the late III or early IV instar larvae of *Ae. aegypti* and *An. stephensi*, (treated with various concentrations of flavonoid extracts of different parts of the selected plants) have been recorded and tabulated in Tables 1 & 2 respectively. From the results it is evident that flavonoid extracts of different parts of both the plants showed differential efficacy against the selected larvae of *Ae. agypti* and *An. stephensi*. Flavonoid extracts of whole aerial part of *A. paniculata* was found to be inactive against the selected larvae of *Ae. aegypti* even at the concentration of 600 ppm, whereas it had shown 70% mortality for *An. stephensi* at the concentration of 200 ppm (LC_{50} - 107.15 and LC_{90} - 363.07). Among different flavonoid extracts (leaf, stem, root and flower) of *V. negundo* flavonoid extract of flower-buds had shown highest mortality (100%) at the concentration of 600 ppm (LC_{50} - 323.59 and LC_{90} - 478.63) for the late III or early IV instar larvae of *Ae. aegypti* (Fig. 1) and at the concentration of 200 ppm (LC_{50} - 58.88 and LC_{90} - 120.22) for the late III or early IV instar larvae of *An. stephensi* (Fig. 2). Flavonoid extracts of other parts of *V. negundo* (leaf, stem and root) were found comparatively less active for larvae of *Ae. aegypti* as compared to

Table 1. Toxicity of flavonoid extracts of different parts of *V. negundo* and *A. panicualta* against IV instar larvae of *Ae. aegypti*

Plant parts	Doses in ppm	Percent kill against late III or early IV instar larvae of *Ae. aegypti*	LC_{50} in ppm (LCL–UCL)	LC_{90} in ppm (LCL–UCL)	ANOVA *p*-value
A. paniculata whole aerial part	300	0	ND	ND	ND
	400	0			
	500	0			
	600	0			
Vitex leaf	300	0	794.32	1995	0
	400	18.33 ± 2.88	(2327.35–271.09)	(680.88–5845.35)	
	500	26.66 ± 2.88			
	600	36.66 ± 5.77			
Vitex stem	300	0	1412.53	4467	0
	400	10 ± 0	(1130.02–1765.66)	(3573.60–5583.75)	
	500	16.66 ± 2.88			
	600	28.33 ± 2.88			
Vitex root	300	6.66 ± 2.88	602.55	1174.89	0
	400	20 ± 0	(547.77–662.80)	(1067.27–1291.40)	
	500	36.66 ± 2.88			
	600	45 ± 5			
Vitex flower	300	48.33 ± 2.88	323.59	478.63	0
	400	68.33 ± 2.88	(302.42–346.24)	(447.31–512.13)	
	500	85 ± 0			
	600	100 ± 0			

Sixty larvae (3 replicates of 20 each) were treated at each dose level; ND–Not done; LCL–Lower confidence limit; UCL–Upper confidence limit; ppm–Parts per million; The data are indicated as the mean ± SEM; (n = 3). One way ANOVA was used which shows significant difference with respect to control ($p \leq 0.05$).

Table 2. Toxicity of flavonoid extracts of different parts of *V. negundo* and *A. panicualta* against IV instar larvae of *An. stephensi*

Plant parts	Doses in ppm	Percent kill against late III or early IV instar larvae of *An. stephensi*	LC_{50} in ppm (LCL–UCL)	LC_{90} in ppm (LCL–UCL)	ANOVA *p*-value
A. paniculata whole aerial part	50	18.33 ± 2.88	107.15 (90.80–126.43)	363.07 (307.68–428.42)	0
	75	35 ± 8.66			
	100	58.33 ± 2.88			
	200	70 ± 0			
Vitex leaf	50	8.33 ± 2.88	131.82 (101.4–171.36)	346.73 (260.71–450.74)	0
	75	25 ± 5			
	100	35 ± 5			
	200	68.33 ± 2.88			
Vitex stem	50	0	223.90 (183.52–273.15)	707.90 (580.24–863.63)	0
	75	10 ± 0			
	100	25 ± 5			
	200	48.33 ± 2.88			
Vitex root	50	25 ± 5	83.17 (68.73–100.63)	275.42 (227.61–333.25)	0
	75	46.66 ± 5.77			
	100	65 ± 5			
	200	80 ± 5			
Vitex flower	50	46.66 ± 5.77	58.88 (53.52–64.76)	120.22 (92.92–112.44)	0
	75	56.66 ± 5.77			
	100	80 ± 5			
	200	100 ± 0			

Sixty larvae (3 replicates of 20 each) were treated at each dose level; ND–Not done; LCL–Lower confidence limit; UCL–Upper confidence limit; ppm–Parts per million; The data are indicated as the mean ± SEM; (n = 3). One way ANOVA was used which shows significant difference with respect to control ($p \leq 0.05$).

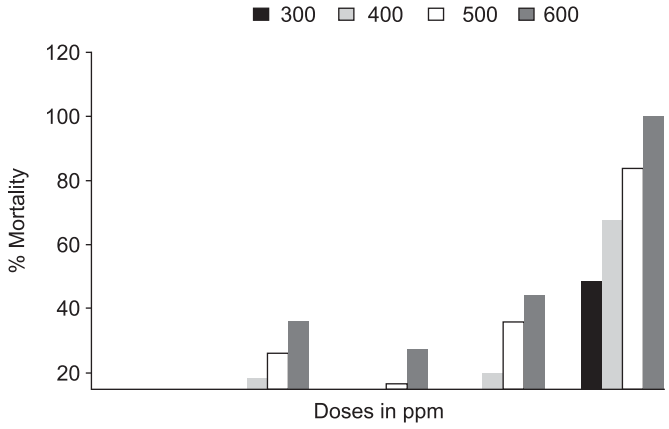

Fig. 1: Percentage mortality of flavonoid extracts of different parts of *Vitex negundo* and *Andrographis paniculata* against late III or early IV instar larvae of *Aedes aegypti* Linnaeus.

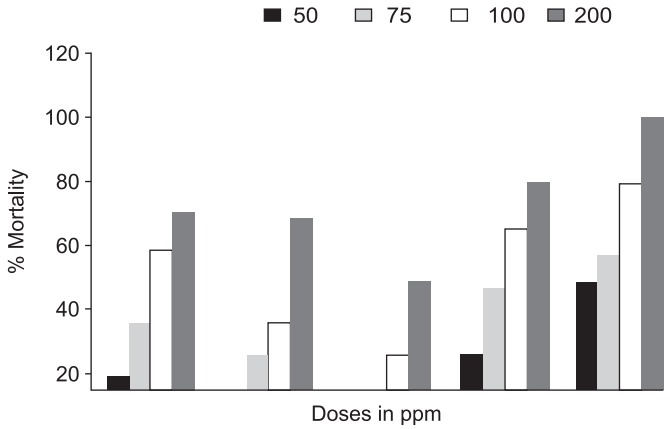

Fig. 2: Percentage mortality of flavonoid extracts of different parts of *Vitex negundo* and *Andrographis paniculata* against late III or early IV instar larvae of *Anopheles stephensi* Liston.

larvae of *An. stephensi*. Thus, the susceptibility of two mosquito species to the different extracts was observed to be more in *An. stephensi* when compared to *Ae. aegypti.*

Further, effect of different flavonoid extracts was observed to be dose dependent as evident by an increase in percent mortality with increasing concentrations. These results get substantial confirmation from the findings of other workers[27–29], who had also reported the dose-dependency of plant extracts against mosquito larvae. A general behavioural change in the larvae of both the mosquito species was observed and it was seen that larvae slowly became inactive within few hours of

treatment with flavonoid extracts of flower-buds extract of *V. negundo*. The microscopic examination of dead larvae showed disintegration of integument probably due to removal of chitin and abnormal stretching of body specially the neck region. These symptoms suggest growth regulating and probably neurotoxic action of the flavonoid extracts of flower-buds of *V. negundo*, However, further study is needed to substantiate this view.

Since flavonoid extract from flower-buds of *V. negundo* showed promising larvicidal activity, it intended us to explore the chemical constituents by GC-MS analysis. The spectral data of GC-MS analysis of the extract are shown in Fig. 3. In all, samples of 80 compounds were identified. The retention time, name, molecular weight and the structure of some of the components of the test extract were ascertained (Table 3, Fig. 3).

The major compounds identified in flavonoid extract of flower-buds of *Vitex* were Phenol (26.83%), Naphthalene (4.95%), 2,3-dihydrobenzofuran (6.79%), Phenol-2,4-Bis (1,1-dimethyl) (4.49%), flavones 4'-OH,5-OH,7-di-O-glucoside (0.25%) [Fig. 4], 5-hydroxy-3, 6, 7, 3',4'-pentamethoxy flavones (0.80%) [Fig. 5] and many more.

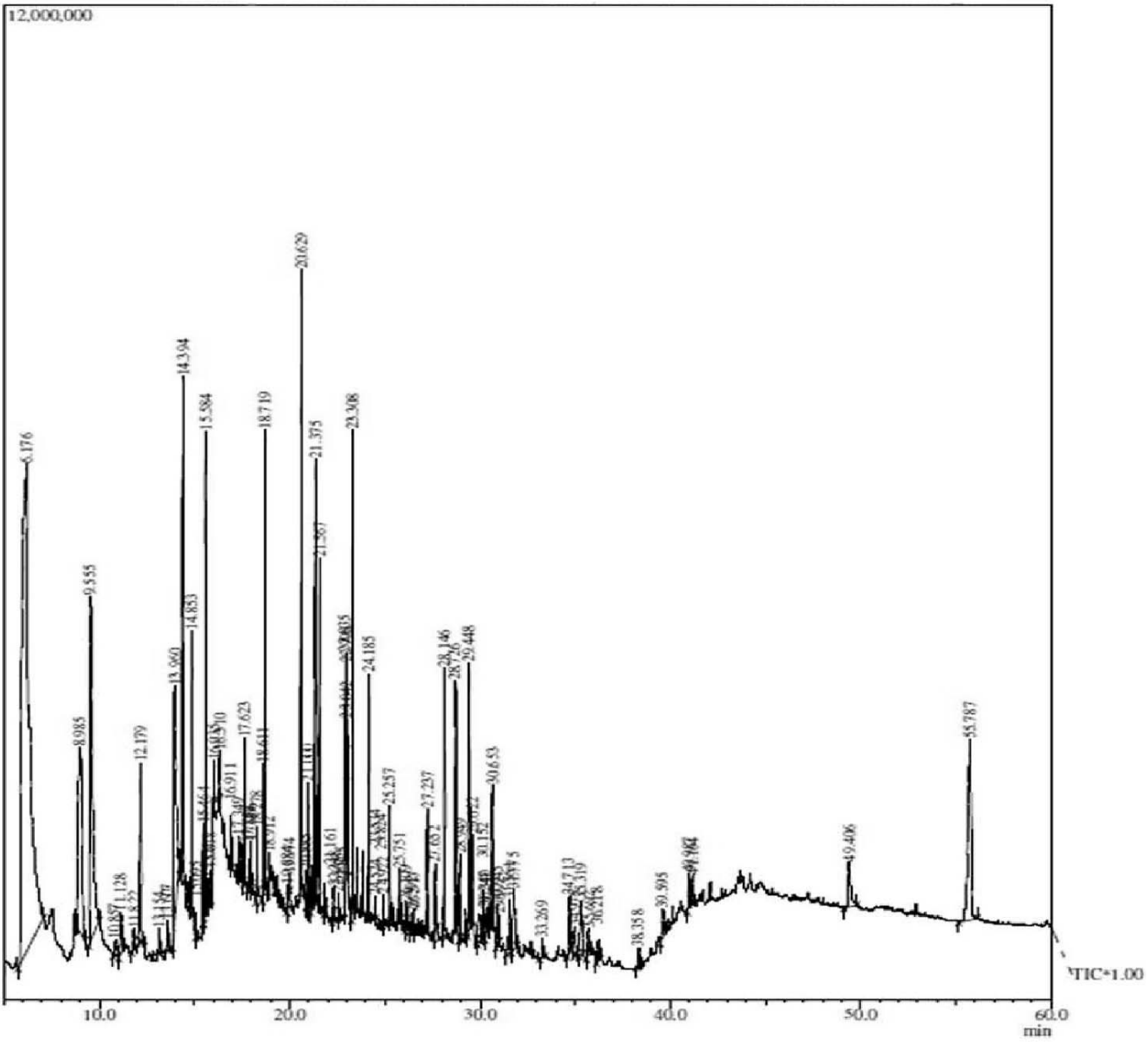

Fig. 3: GC-MS analysis of bound flavonoid extract of flower-buds of *Vitex.*

Table 3. Important compounds identified in the GC-MS analysis of bound flavonoid extract of flower–buds extract of *V. negundo*

Peak	Retention time	Area (%)	Chemical formula	Compound name	Molecular weight
1	6.176	26.83	C_6H_6O	Phenol	94
2	8.983	4.95	$C_{10}H_8$	Naphthalene	128
3	9.558	6.79	C_8H_8O	2,3-dihydrobenzofuran	120
4	12.175	1.89	$C_{14}H_{30}$	Tetradecane	198
5	13.958	3.33	$C_8H_8O_3$	4-hydroxybenzoic acidmethylester	152
6	14.392	4.49	$C_{14}H_{22}O$	Phenol-2,4-Bis(1,1-dimethyl)	206
7	14.850	1.75	$C_{11}H_{20}O_4$	Azelacicacid, dimethylester	216
8	20.625	4.49	$C_{17}H_{34}O_2$	Palmiticacid methylester	270
9	15.583	2.71	$C_{17}H_{34}$	Octadecane(n-7)	254
10	24.925	0.13	$C_{12}H_{20}O$	5,5,8a-trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	180
11	30.342	0.25	$C_{27}H_{30}O_{15}$	Flavones 4'-OH,5-OH,7-di-O-glucoside OR Kaempferol-3-O-rutinoside	594
12	30.650	1.21	$C_{16}H_{22}O_4$	1,2-benzenedicarboxilic acid, mono(2-ethylhexaylester)	278
13	34.975	0.34	$C_{15}H_{24}O_3$	Ovidin A	252
14	49.408	0.80	$C_{20}H_{20}O_8$	5-hydroxy-3,6,7,3',4'-pentamethoxy flavones	388
15	55.783	4.30	$C_{30}H_{50}O_7Si_5$	3,5,7-tris (trimethylsiloxy)-2[3,4-di (trimethylsiloxyphenyl] 4H-a-bezopyran-4-one	662

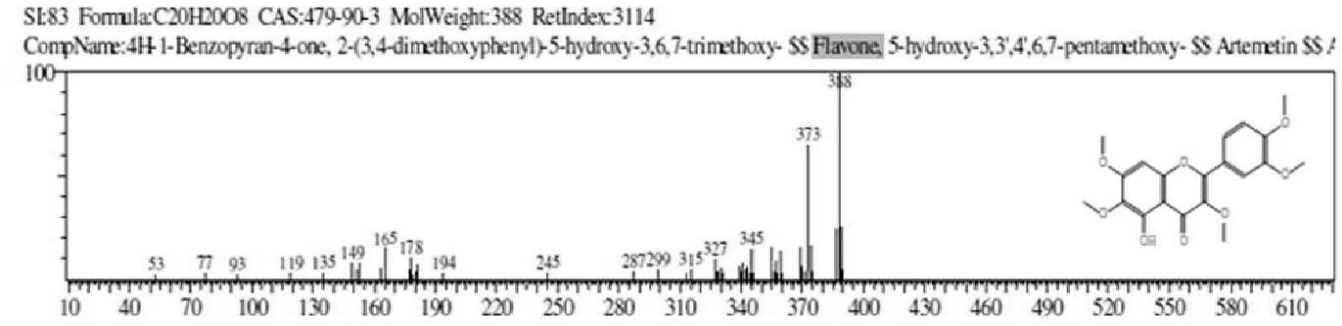

Fig. 4: Mass spectrum of 5-hydroxy-3,6,7,3',4'-pentamethoxy flavones.

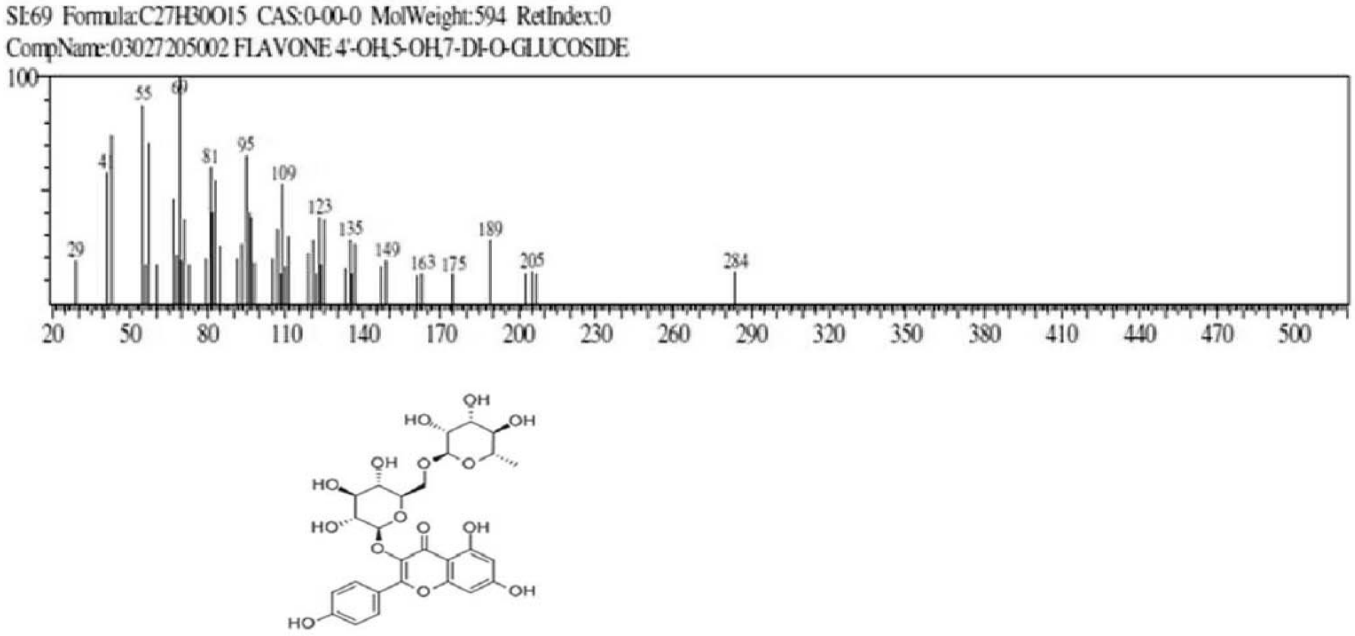

Fig. 5: Mass spectrum and structure of Kampferol-3-O-rutinoside.

DISCUSSION

Today, the environmental safety is considered to be of paramount importance. Hence an insecticide should be eco-friendly, which is generally not observed in chemical or synthetic pesticides. This safety could only be ascertained through plant-based insecticides. Phytochemicals may serve as suitable alternatives to synthetic insecticides in future as these are relatively safe, inexpensive and readily available in most parts of the world. In the present study, various flavonoid extracts were studied in a dose dependent manner. Flavonoid extract from flower-buds of *Vitex* particularly was found to have higher rate of larvicidal activity against *An. stephensi* and *Ae. aegypti*, whereas in the case of other extracts (obtained from different parts), the concentrations had to be increased for better larvicidal effect.

Larvicidal activity of partially purified extracts of leaves of *V. negundo, Nerrium oleander* and seeds of *Syzygium jambolanum* on different instars of *Culex quinquefasciatus* and *An. stephensi* had been reported by Pushpalatha & Muthukrishnan[30]. Larvicidal activity of fatty acid methyl esters of different species of *Vitex* against *Culex* had also been reported by Kannathasan *et al*[31]. Differential larvicidal efficacy of four species of *Vitex* against *Cx. quinquefasciatus* had been reported by Kannathasan *et al*[32]. Whole plant ethanolic extract of *A. paniculata* had been studied for larvicidal, pupicidal, adulticidal and ovicidal properties against the malaria vector by Kuppusamy and Murugan[33]. Synergistic activity of *A. paniculata* with *Bacillus thuringiensis* against malaria vector *An. stephensi* was also tested by Kuppusamy *et al*[34]. Larvicidal and ovicidal efficacy of different extracts of *A. paniculata* was tested against *Cx. quinquefasciatus* and *Ae. aegypti* by Govindarajan[35]. Bioactivity of four flavonoid compounds from *Poncirus trifoliate* L. was tested against the dengue fever vector by Rajkumar and Jebanesn[36].

The results of the present study revealed that most of the compounds, identified in the GC-MS spectral studies belong to phenolic group and other compounds identified in GC-MS are mostly hydrocarbons which have so far not been reported to have any bioactivity. As the flavonoids which have been screened in the present study belong to phenolic group, and the larvicidal activity can be assigned to the synergistic effects of phenolic compounds (that are present in substantial amount) which also include flavonoids. However, more studies are required to be done before the flavonoid extract of flower-buds of *V. negundo* could be exploited at commercial scale.

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