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LARVICIDAL POTENTIAL OF *VETIVERIA ZIZANIOIDES* AGAINST DENGUE VIRUS SPREADING *AEDES AEGYPTI* MOSQUITOES

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ABSTRACT

The study on the larvicidal potential of different solvent crude (chloroform, water, acetone, hexane, ethanol, methanol) leaf extract of *Vetiveria zizanioides* against the dengue vector. The *Aedes aegypti* showed the root ethanolic extracts of vetiver contains alkaloids, flavanoides, saponins, tannins and steroids. The compounds are known to possess insecticidal and larvicidal activities. The chloroform extract shows the most effective larvicide among the various solvent extracts with the percentage mortality of 95% and 70% in 48 and 24hrs of exposure respectively. The high larvicidal activity is supported by the presence of phytochemicals such as alkkloides, saponins, tannins, flavanoides and steroids which showed combination effects interms of larvicidal action to mosquito larvae. The ethanolic extract of vetiver also shows good larvicidal activity 88% in 48hrs 68.42% in 24hrs. The water extract of vetiver shows no responds to mosquito larvae. The hexane extract and acetone extract having less effective against mosquito larvae. The LC - Mass spectra obtained determines the active fraction of chloroform had a major peak at retention time of 14.76 - 14.2 min with 621.31 peak area. These vetier extracts possessed different range of larvicidal property which may be used as a traditional mosquito control agent.

KEYWORDS

Aedes aegypti, Larvicidal and *Vetiveria zizanioides*.

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INTRODUCTION

Dengue is one of the most serious and fast emerging tropical diseases which in certain socio-ecological settings exacts disease burden that can be only be paralleled with that of malaria. Dengue, with its two severe clinical manifestation-dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), posses an increasingly perilous

situation due to lack of specific antiviral drugs or vaccins[1-2]. The dengue virus belongs to the family flaviviridae. It comprises four antigenetically distinguishable serotypes (DEN-1, DEN-2, DEN-3, DEN-4) transmitted genetically through the bite of various day-feeding mosquitoes; However, *Aedes aegypti* is the principal vector responsible for dengue transmission the world over[1]. The risk of dengue has increased in recent years due to rapid urbanization, and deficient water management including improper water storage practice in urban, and rural areas, leading to proliferation of mosquito breeding. The disease is spreading to newer geographical areas every year[2]. *Aedes aegypti* is supreme concern because of its wide distribution and close association with humans[3].

World-wide nearly 2.5-3 million people (40% of global population) continue to live at constant risk of contracting infection, while 50 million cases and 24,000 death are estimated to regularly occur annually in 100 endemic countries world-wide, including hospitalization of nearly 500,000 cases of which 90% are children. The southeast Asia region contributes 52% or 1.3 billion cases annually. India is one of the seven identified countries in the region regularly reporting incidence of DHF/DSS outbreaks and seems to be heading to transform into a major hyper-endemic niche for dengue infection in near future, with more and more never areas being struck by the epidemic dengue. The first confirmed report of dengue infection in India dates back to 1940s and thereafter several states began to report the disease which mostly struck in epidemic proportion often infecting heavy morbidity and mortality in rural environments[4]. In 2010 total 28292 cases and 110 deaths have been reported. Highest number of death were reported by Maharashtra (48) followed by Kerala (29) and Punjab (25). During 2014, 33320 cases and 86 deaths have been reported[2]. In Tamilnadu cases of dengue with same death were reported in 1996 for the first time, albeit detection of DEN-1, DEN-2, and DEN-4 viruses in the human sera in Tamilnadu[5,6].

Mosquito control includes targeting the adult mosquito through spraying chemical insecticides or by killing the mosquito larvae before they emerge into adult via using synthetic larvicides or botanical extract as an alternative larvicides[7]. Current strategies based on the elimination of breeding sites and applications of chemical insecticides for larval and adult mosquito control have resulted in development of resistance without eliminating the constant risk of dengue epidemics. Thus new approaches are urgently needed. Interest on possible use of environment friendly natural products such as extracts of plants or plant parts increased for vector control[8].

The vetiver is commonly known as resbira or sugandbmula, khas-khas, khus khus or khus grass is a fast growing perennial grass belonging to family Poaceae. It has thick fibers adventitious roots which are aromatic and highly valued. Vetiver is a tall, tufted, perennial, scented grass, with a straight stem, long narrow leaves and a lacework root system that is abundant, complex and extensive[9]. This grass grows throughout the plains of India ascending up to an elevation of 1200m. Through it originated in India, vetiver is widely cultivated in tropical regions of the world. The roots were down word 2-4 meters in depth. The most commonly used commercial genotypes of vetiver are sterile and this grass has rhizome buds for propagation. Pruning techniques are applied to promote root and leaf growth; this grass can grow vigorously on any kind of soil and climatic conditions[10,11]. Vetiver has been found to have better antimicrobial properties under laboratory conditions. It is well known as an eco-friendly plant that prevents soil erosion and rehabilitates metaliferous polluted land. It is cultivated for the production of the commercially important essential oils used in perfumery and aromatherapy[12,13].

Current research trends use plant extracts as alternative larvicides because they contain various phytochemicals that are specific in killing mosquito larvae without harming other organisms and the environment. Instead of using synthetic larvicides, the use of this plant derived products in controlling

mosquito larvae is inexpensive and environment friendly.

MATERIAL AND METHODS

Vetiveria zizanioides

Vetiveria zizanioides (Linn) Nash, a member of the family Poaceae commonly known as the *Khas-Khas*, *Khas* or *Khus* grass in India, is a perennial grass with thick fibrous adventitious roots which are aromatic and highly valued. This tufted grass grows throughout the plains of India ascending up to an elevation of 1200m. This grass is found occurring in wild state throughout tropical and subtropical plains, particularly along the riverbanks and over marshy land.

Taxonomic position of *V. zizanioides* is as under:

Kingdom	:	Plantae
Order	:	Poales
Family	:	Poaceaee
Genus	:	*Vetiveria*
Species	:	*zizanioides*

Collection of plant material and preparation of extract

The root of *Vetiveria zizanioides* were collected washed thoroughly, blotted and shade dried. The dried root were choked and converted into powder form by using automatic machine. This vetiver powder was converted into solution using solvents (sterile water, hexane, methanol, Chloroform, ethanol and acetone) by soxhelt apparatus.

Antibacterial activity assessment by agar well diffusion method

The antibacterial activity of *Vetiveria zizanioides* extract was evaluated using the agar well diffusion method[14]. 150ml of Muller Hinton agar (MHA) was prepared and allowed to sterilization at 121ºC for about 15 minutes. The petriplates were autoclaved in hot air oven at 121ºC for 45 minutes. The extract (water, hexane, methanol, ethanol, acetone, chloroform) has been made into 10, 20, 30, 40, 50, 60µl concentration. MHA of 20ml was dropped into the petriplates and was allowed to solidify. Then the different concentration of extracts was poured in the developed well and the plates were incubated for 24hrs at 37ºC. After 24hrs, antibacterial activity was assessed against the developed test organisms, namely *Staphylococcus aureus* and *Pseudomonas aeruginosa* by measuring the zone of inhibition.

Raring of *Aedes aegypti* larvae

The eggs of *A.aegypti* were produced from the Centre for research in Medical Entomology, ICMR, at Madurai, Tamilnadu, India. The eggs rafts of *A.aegypti* were kept in the tray containing tap water (culture medium) at laboratory condition (29+/-1ºC). After 24hrs incubation, the eggs were observed to batch out into first instar larvae. Appropriate amount of nutrients (sterilized yeast powder and dog biscuit in 1:1 ratio) were added to enhance the growth of larvae. The 4th instar larvae were used in the study.

Larvicidal bioassay

The plant extract were dissolved in 10mul of DMSO for solubility in water. Larvicidal activity was determined according to WHO protocol. The larvae were treated with the plant extract of 1000µg/ml concentration. A corresponding control was maintained. The larval mortality of fourth instars of *A.aegypti* was observed. The numbers of larvae surviving at the end of 48hrs were recorded and the percent mortality was calculated.

The % of mortality was calculated by,

(No. of larvae dead /No. of larvae) x 100

Corrected mortality was accounted for by. The extract which showed 80%-60% mortality were combined to check the larval activity by similar procedure as mentioned above.

Lethal concentration

The LC_{50} of the plant extract that showed 100% mortalit was determined by a similar procedure as mentioned above. 100, 250, 500 and 750µg/ml concentration used and the observation were recorded after 24hrs of incubation. The LC_{50} was determined by a probit analysis.

Phytochemical analysis

The qualitative phytochemical analysis method is used to identify the presence of active phytoconstituents in the vetiver extract such as carbohydrates, proteins, steroids, alkaloids, flavanoides, phenols, saponins and tannins. The

preliminary test was conducted as per standard test procedures.

Test for Carbohydrates

Fehling's Test

The plant extract of 2.0ml was mixed with 1.0ml of Fehling's solution. The mixed solutions were boiled for a few minutes. The formation of red of brick red precipitate indicates the presence of reducing sugar.

Benedict's Test

The 0.5ml of the extract was added to 5.0ml of Benedict's reagent and boiled in hot water bath for about 5 minutes. The appearance of red, yellow or green color precipitate showed the presence of reducing sugar.

Test for Protein

Millon's Test

Millon's reagent 1.0ml was added drop by drop with 3.0ml of water and 1.0ml of the extract and this mixture was heated, the white precipitate turns into the red which indicates the presence of proteins.

Biuret Test

The 1.0ml of the extract was added to 1.0ml of 10% sodium hydroxide and then heated. To this mixture, a drop of 0.7% copper sulphate solution was added drop by drop. Purplish violet color was formed and it indicates the presence of protein.

Ninhydrin Test

0.5ml of 2.0% ninhydrin reagent was added to 1.0ml of the extract and this was mixed thoroughly and heated for 2 minutes in the boiling water bath. The appearance of purple color indicates the presence of protein and free amino acid compounds.

Test for Steroids

Salkowki's Test

Concentrated sulphuric acid 1.0ml was added to 2.0ml of the extract carefully along the sides of the test tube, then the red color was formed in the chloroform layer which indicates the presence of steroids.

Test for Alkoloids

Wagner's Test

1.5% v/v of hydrochloric acid and a few drops of Wagner's reagent were added to acidify the plant extract of 1.0ml, then the formation of brown or reddish precipitate indicates the presence of alkaloids.

Meyer's Test

Few drops of Meyer's reagent were mixed with 1.0ml of the extract. A yellow creamy precipitate indicates the presence of alkoloids.

Test for Flavanoids

Alkaline Reagent Test

A few drops of the extract were added with 4 to 5 drops of sodium hydroxide solution. The intense yellow color was formed and turned into colorless on addition of a few drops of dilute hydrochloric acid indicates the presence of flavanoids.

Test for Phenols

Ferric Chloride Test

The 2.0ml of distilled water was added to 1.0ml of the extract followed by a few drops of 10% aqueous ferric chloride solution. Blue, green or violet color precipitate was appeared and it indicates the presence of phenols.

Lead Acetate Test

1.0ml of the plant extract was diluted with 3.0ml of distilled water to which a few drops of 1% aqueous lead acetate solution were added. The formation of yellow precipitate indicates the presence of phenols.

Test for Saponins

Foam Test

A few drops of sodium bicarbonate solution were added to 1.0ml of the vetiver extract and shaken it vigorously and kept this mixture for 3 minutes. A honey comb like froth was formed and it indicates the presence of saponins.

Test for Tannins

Ferric Chloride Test

Few drops of aqueous 5% Ferric chloride were mixed with 1.0ml of the extract. A bluish black color was formed and after sometime it gets disappeared in addition of a few drops of dilute sulphuric acid and a yellowish brown precipitate was formed which indicates the presence of tannins.

Lead Acetate Test

A few drops of 1% solution of lead acetate were added to 5.0ml of the extract, the formation of yellow[15, 16-21].

Column chromatography

Column chromatography involves the separation of compounds from a mixture by eluting the column with solvent of increasing polarity in a step wise manner and the collection of fractions according to the sequence regarding the eluted products being monitored by TLC. Column was packed with silica gel for column chromatography with 60-120 mesh size activated at 110ºC for 1 hr. The material to be chromatographed was adsorbed on silica gel for 5 min. Column was eluted with hexane as a solvent. The material to be chromatographed was dissolved in hexane and adsorbed on silica gel and the solution was allowed to flow down a vertical column. The extraction was carried out by eluting the column with solvents of increasing polarity and the various fractions were collected. For the recovery of the material the solvent was distilled on water bath or concentrated using rotary vacuum pump.

LC-MS analysis

The active fraction collected from column was dissolved in methanol (HPLC grade) followed by filtration using syringe filter and subjected to LC-MS analysis. The analysis was performed on a Waters LC-MS (Water Acquity System with Quatro Premier XE) consisting of a degasser, binary pump, auto sampler, and column heater. The column outlet was coupled to a Thermo fleet (LCQ-Fleet) Ion Trap mass spectrometer equipped with an ESI ion source. The sample was run on C18 reverse phase column with Mass Lynx 4.1 software and triple Quadrupole analyzer for 0-6 min. Gradient elution method was employed. LC conditions were as follows: solvent A: 0.1% Formic acid in water and solvent B: Acetonitrile; linear gradient, initial percentage of B (2%) to 6 minutes (98%); column temperature: 40ºC; flow rate: 0.4ml/min. MS parameters were as follows: ionization mode, positive; sheath gas, nitrogen; capillary temperature: 320ºC; capillary voltage: 5.0 kV; full scan acquisition, from 50 to 1000m/z at 2 scan/s.

RESULTS AND DISCUSSION

Antibacterial activity assessment by agar well diffusion method

The antibacterial activity test results of vetiver extract against gram positive bacterial pathogen namely *Staphylococcus aureus* and gram negative bacterial pathogens namely *Klebsiella pneumonia, Pseudomonas aeruginosa* by agar well diffusion method were shown in Table No.1 and the Figure No.1 shows the zone of inhibition.

The zone of inhibition against these bacterial pathogen were also shown in the Graph No.1. The zone of inhibition test results of vetiver chloroform extracts showed very good antibacterial activity of both gram positive and gram negative bacteria, *Staphylococcus aureus* (24mm), *Klebsiella pneumonia* (22mm) and *Pseudomonas aeruginosa* (22mm) in 60µl of concentration and 21mm, 19mm, 18mm in 50µl of concentration. The Vetiver ethanolic extracts also showed a good antibacterial against *Klebsiella pneumonia* (20mm) in 60µl than *Pseudomonas aeruginosa* (12mm) and gram positive *Staphylococcus aureus* (15mm). The vetiver acetone extract showed lower activity against those pathogenic microorganisms, *Staphylococcus aureus* (15mm), *Klebsiella pneumonia* (13mm) and *Pseudomonas aeruginosa* (14mm). Chloroform, ethanol and acetone compared with water hexane and methanolic extracts of vetiver resulted very less antibactertial activity both gram positive and gram negative bacteria. The chloroformic and ethanolic extracts proved that it has better controlled over the gram positive and gram negative microorganisms.

Larvicidal bioassay

Chloroform, water, acetone, hexane, ethanol and methanol extracts of *Vetiveria zizanioides* were screened for larvicidal activity. It was found that chloroform extract was highly toxic to the 4th in star larvae *A.aegypti.* Among the chloroform extract of vetiver shows 95% mortality after 48hrs of incubation. While in 24hrs incubation was 70%. The other vetiver extracts effect on larvae shown in Table No.2. The mosquito larvae exposed to vetiver extracts showed significant behavioral changes. The

most obvious sign of behavioral changes observed in *A.aegypti* was restlessness, loss of equilibrium which finally lead to death. Ethanol extract of vetiver exhibited 88% mortality in 48hrs. While in 24hrs, 68.40% mortality was exhibited. The methanol extract had 80% mortality after 48hrs of incubation and 53% mortality exhibited in 24 hrs incubation. All other vetiver extracts had less effect on the larvae. Because, the acetone exhibits 20% mortality in 24hrs and 34.63 % in 48hrs of incubation. And the hexane shows 15% and 16.12% mortality in 24hrs and 48hrs respectively. The water extract of vetiver was exhibits any mortality in the number of mosquito larvae. The % mortality and lethal concentration of different concentrations of vetiver solvent extracts were given in Table No.3. According to preliminary screening results 3 extracts were subjected to larvicidal bioassay which has above 80% larval mortality. Among them significant mortality rate was observed in chloroform extract of vetiver with LC_{50} value of 129. Likewise, Shivakumar, *et al*[7] reported that screening for larvicidal properties of different solvent leaf extracts of four plants among 20 extracts, 4 extracts gave high larvicidal potency with low concentration LC_{50} <197ppm. The findings of present study are quite compararble with previous report of Cavalcanti, *et al*[18]. Larvicidal activity of essential oils of Brazilian plant against *A.aegypti* and observed the LC_{50} to range from 60 to 553ppm. Similarly, Rahuman and Venkatesan[19] screened the petroleum ether extracts of *Citrullus colosynthis*; methanol extracts of *Cannabis indica* and *Momordica charantia*; and acetone extracts of *Trichosanthes anguina* against the larvae of the LC_{50} value are 74.57, 309.46, 492.73, 199.14 and 554.20ppm respectively.

Phytochemical analysis

Phytochemical screening tests were performed and the test results were presented in the Table No.4. The test revealed the presence of phytochemical constituents in extract such as carbohydrates, flavanoides, phenols, steroids, tannins and alkaloids, whereas saponins were absent in the vetiver extract.

LC - MS Analysis of Plant extracts

The LC- Mass spectra obtained determines the active fraction of chloroform had a major peak at retention time of 14.76 - 14.2 min with 621.31 peak area. Further, mass spectral analysis determined the molecular weight, which was found to be 365.28 i.e., m/z 316.2318 [M] + at positive mode. The LC-Mass spectra obtained determines the active fraction of ethanol had a major peak at retention time of 16.54 - 16.72 min with 606.40 peak area. Further, mass spectral analysis determined the molecular weight, which was found to be 336.28 i.e., m/z 316.2318 [M] + at positive mode. (Figure No.6) and (Figure No.7).

Table No.1: Antimicrobial activity of various vetiver solvent extracts against human pathogens

S.No	Vetiver Extracts	Concentration	Zone of inhibition (mm)		
			S. aureus	*K.pneumonia*	*P.aeruginosa*
1	Chloroform	10µl	12	-	-
		20µl	15	10	8
		30µl	17	12	12
		40µl	19	15	14
		50µl	21	19	18
		60µl	24	22	22
2	Water	10µl	-	-	-
		20µl	-	-	-
		30µl	-	-	-
		40µl	-	-	-
		50µl	-	-	-
		60µl	-	-	-
3	Acetone	10 µl	8	-	-
		20µl	9	-	-
		30µl	10	9	9
		40µl	11	10	12
		50µl	13	11	13
		60µl	15	13	14
4	Hexane	10µl	-	-	-
		20µl	-	-	-
		30µl	-	-	-
		40µl	-	-	-
		50µl	-	-	-
		60µl	9	-	-
5	Ethanol	10µl	-	-	-
		20µl	10	13	-
		30µl	11	14	-
		40µl	12	16	9
		50µl	14	17	11
		60µl	15	20	12
6	Methanol	10µl	-	-	-
		20µl	-	-	-
		30µl	-	-	-
		40µl	-	-	-
		50µl	-	10	-
		60µl	-	12	-

Table No.2: Larvicidal activity of various solvent extracts of *Vetiveria zizanioides*

S.No	Extract	% Mortality at 1000µg/ml concentration	
		24hrs	48hrs
1	Chloroform	70%	95%
2	Water	-	-
3	Acetone	20%	34.63%
4	Hexane	15.20%	16.12%
5	Ethanol	68.42%	88%
6	Methanol	53%	80%

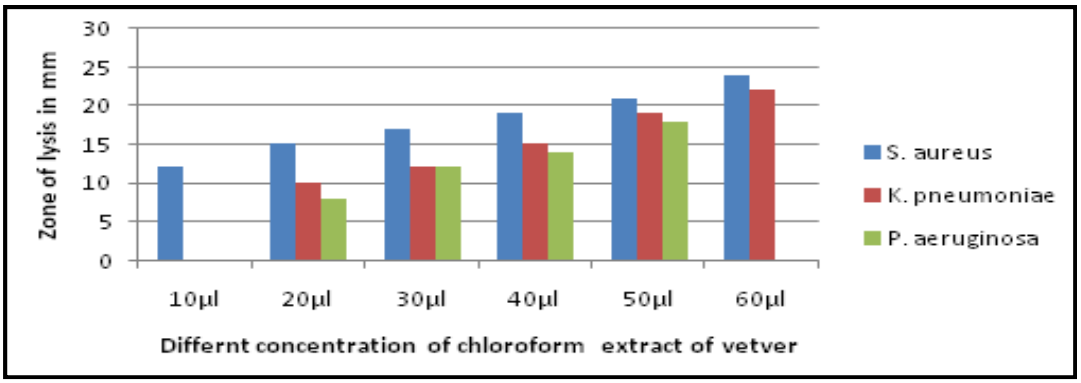
Lethal Concentration

Table No.3: Lethal concentration of plant extracts after 24hrs, According probit analysis

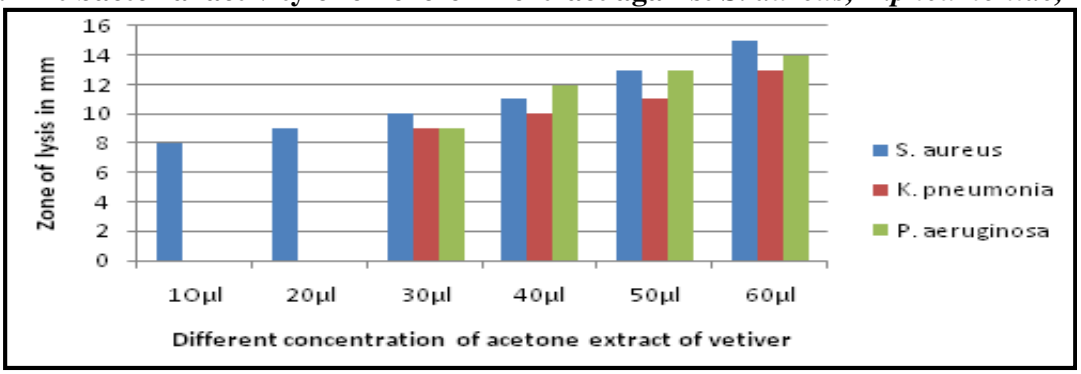
S.No	Vetiver extracts	Concentration mg/ml	% mortality	LC_{50} (mg/ml)
1	Chloroform	0.750	93 ± 2	128.83
		0.500	86 ± 5.22	
		0.250	69 ± 2.3	
		0.100	43 ± 2.4	
2	Ethanol	0.750	82.5 ± 2.2	106.54
		0.500	76 ± 3.2	
		0.250	55 ± 5.4	
		0.100	42 ± 7.2	
3	Methanol	0.750	75 ± 2	84.63
		0.500	59 ± 2.2	
		0.250	36 ± 4.2	
		0.100	30 ± 2.2	

Table No.4: Phytochemical analysis of *Vetiveria zizanioides*

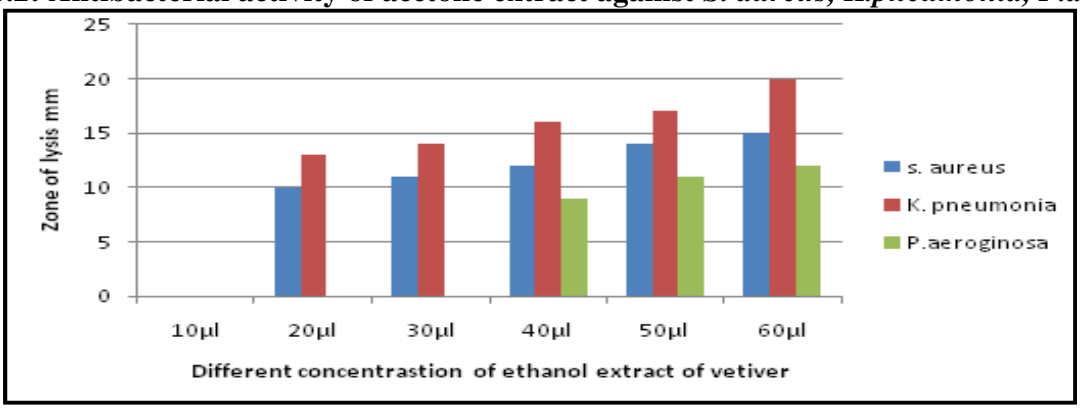
S.No	Plant constituents	Vetiver extract
	carbohydrates	
1	a) Fehling's test	+
2	b) Benedict	+
	Protein	
3	a) Millon's test	+
4	b) Biuret test test	+
5	c) Ninhydrin test	+
	Steroids	
6	a) Salkowki's test	+
7	Alkaloids	
8	a) Wagner's	+
9	b) Meyer's	+
	Flavanoids	
10	a) Alkaline reagent test	+
11	Phenol	
12	a) Ferric chloride test	+
13	b) Lead acetate test	+
	Saponins	
14	a) Foam test	+
15	Tannins	
16	a) Ferric chloride test	+
17	b) Lead acetate test	+



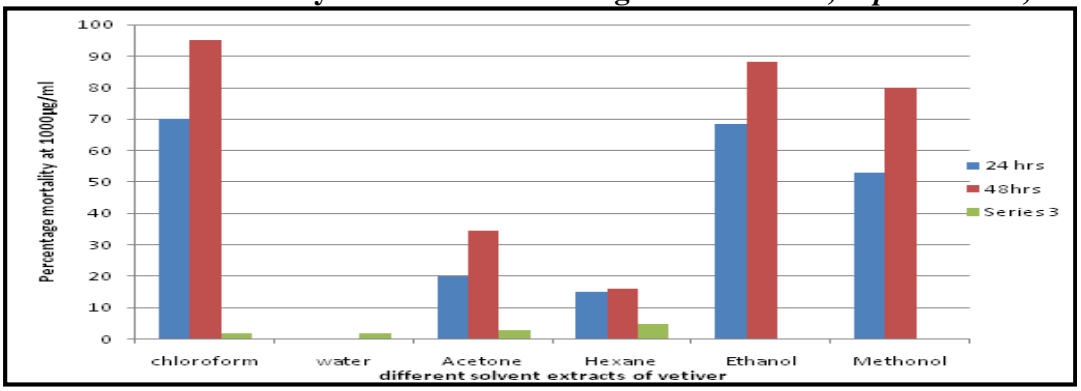
Graph No.1: Antibacterial activity of chloroform extract against *S. aureus, K.pneumoniae, P.aeruginosa*



Graph No.2: Antibacterial activity of acetone extract against *S. aureus, K.pneumonia, P.aeruginosa*



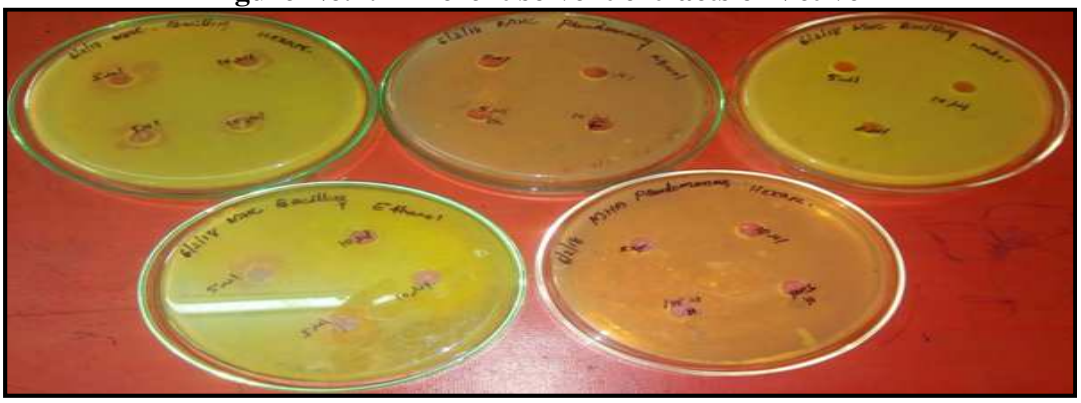
Graph No.3: Antibacterial activity of ethanol extracts against *S. aureus, K.pneumonia, P. aeruginosa*



Graph No.4: Larvicidal activity of various solvent extracts of Vetiver

Figure No.1: Vetiver grass and root

Figure No.2: Different solvent extracts of Vetiver



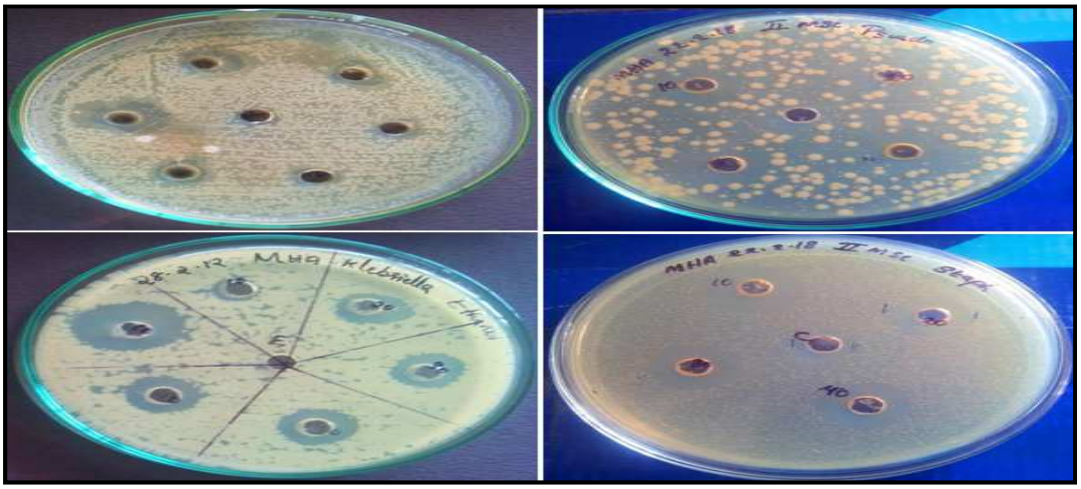


Figure No.3: Antibacterial activity of different extracts of *V.zizanioid against human pathogens*

Different stages of larval raring of *Aedes aegypti*

Figure No.4a: Eggs of *A.aegypti* on paper procured from ICMR, Madurai

Figure No.4b: Eggs were immersed in a tap water with feed

Figure No.4c: Hatch out of 1st instar Larvae

Microscopic view of different stage of larvae

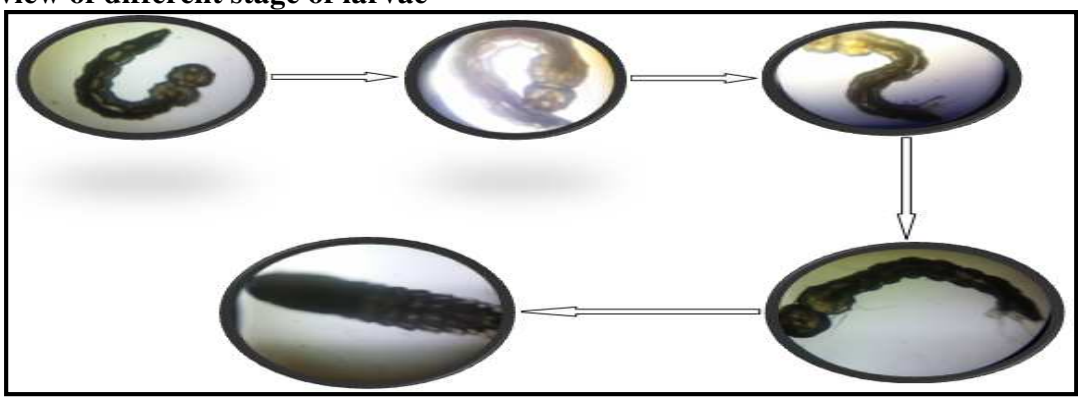


Figure No.5: Microscopic view of different stage of larvae

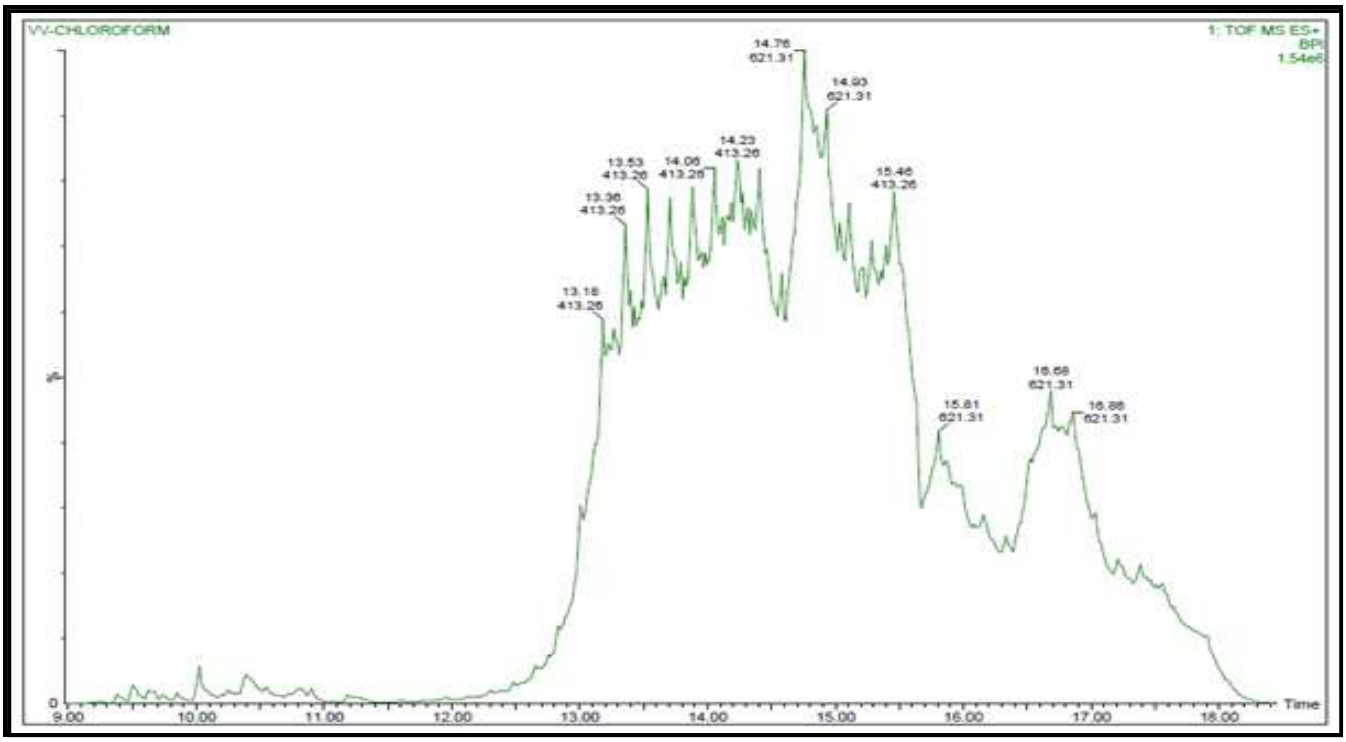


Figure No.6: LC-MS Spectra of the chloroform extract of *Vetiver*

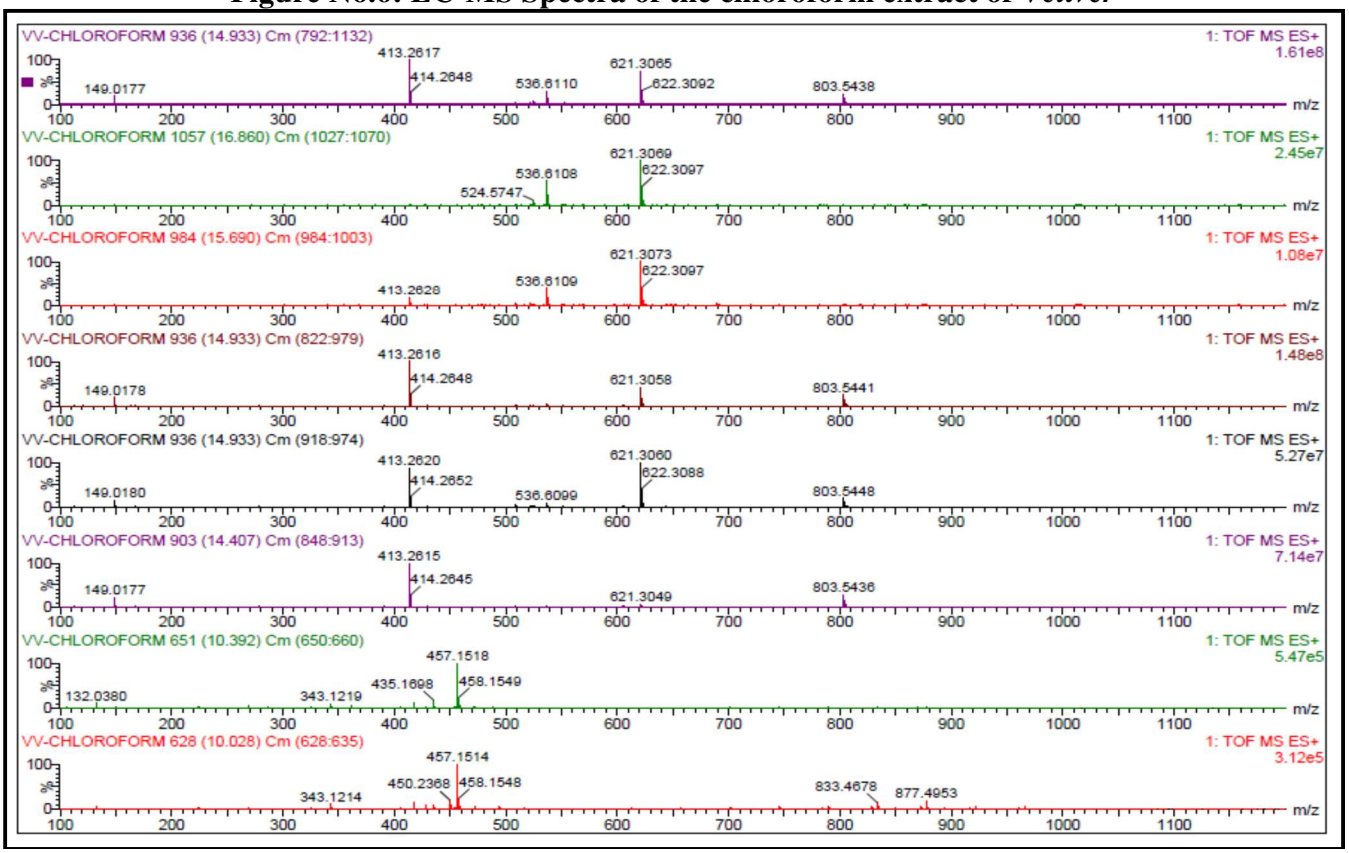


Figure No.7: LC-MS chromatograms of the chloroform extract of Vetiver

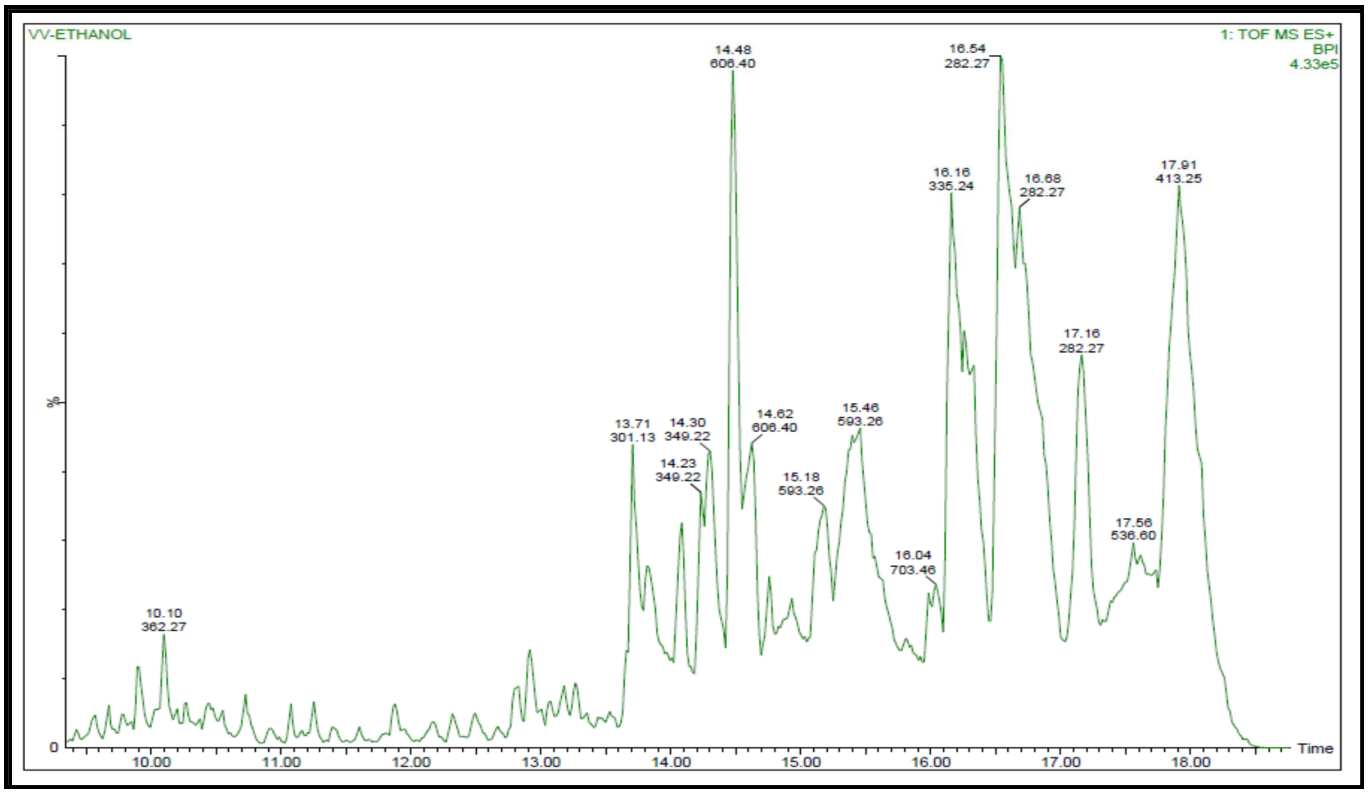


Figure No.8: LC-MS Spectra of the Ethanol extract of *Vetiver*

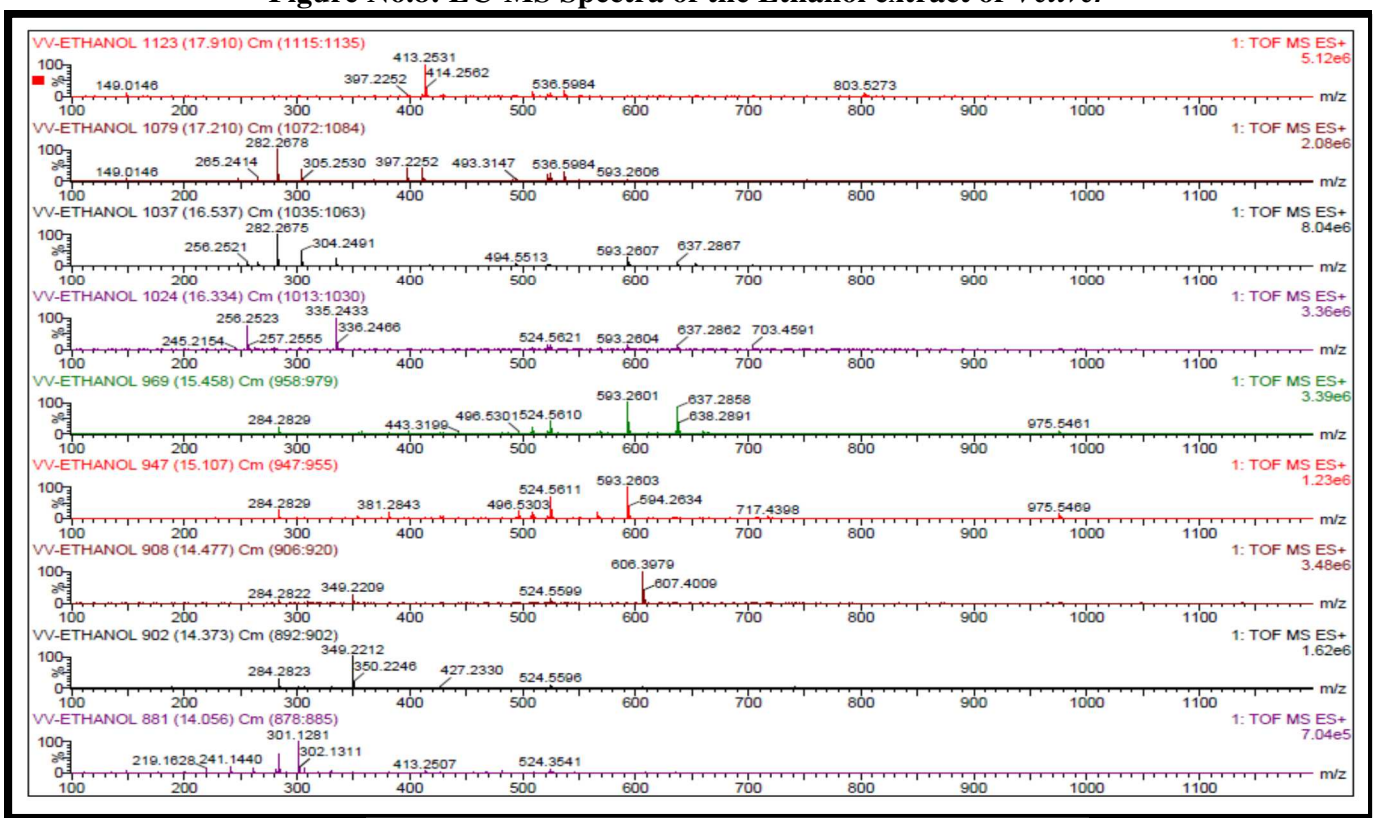


Figure No.9: LC-MS chromatograms of the chloroform extract of Vetiver

CONCLUSION

The study on the larvicidal potential of different solvent crude (chloroform, water, acetone, hexane, ethanol, methanol) leaf extract of *Vetiveria zizanioides* against the dengue vector. The *Aedes aegypti* showed the root ethanolic extracts of vetiver contains alkaloids, flavanoides, saponins, tannins and steroids. The compounds are known to possess insecticidal and larvicidal activities. The chloroform extract shows the most effective larvicide among the various solvent extracts with the percentage mortality of 95% and 70% in 24 and 48hrs of exposure respectively. The high larvicidal activity is supported by the presence of phytochemicals such as alkkloides, saponins, tannins, flavanoides and steroids which showed combination effects interms of larvicidal action to mosquito larvae. These vetier extracts possessed different range of larvicidal property which may be used as a traditional mosquito control agent.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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