Larvicidal activity and histopathological alterations effected by *Kyllinga nemoralis* grass weed extracts on the mosquito vectors

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Abstract

Vector-borne diseases are one of the utmost contributors to human mortality and morbidity in the tropical and subtropical regions. Vector control remains the most effective measure and is often the only way to prevent disease outbreaks as there are no vaccines for many vector-borne diseases. Owing to their quick action, chemically synthetic insecticides are the first line of action, but their constant use has led to the development of resistance and permanent residual effect on the surroundings which can be harmful to animals including human. Therefore, biologically active weeds have attracted considerable interest in mosquito control programs in the recent time. In the present study, the crude hexane, chloroform, ethanol, methanol and aqueous whole grass extracts of *Kyllinga nemoralis* were tested for the larvicidal efficacy against the early fourth instar larvae of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*. Mortality was recorded after 24 hours of treatment. Preliminary phytochemical analysis revealed the strong presence of various phytocompounds responsible for the mortality of the mosquito larvae. TLC and GC-GC-MS analysis of the ethanol extract of *Kyllinga nemoralis* revealed the presence of phytocompounds with insecticidal properties.

***Keywords*:** *Kyllinga nemoralis*, phytochemical analysis, Histopathology, *Anopheles stephensi*, *Culex quinquefasciatu*

Introduction

Mosquitoes spread more diseases than any other group of arthropods and present an immense threat to millions of people, since they act as vectors for important parasites and pathogens, causing millions of death annually [1,2]. 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 [3,4,5]. These diseases are also responsible for the mortality, morbidity, economic loss and social disruption [6]. Approximately 2 millions of people die globally and atleast 300 million cases of malaria has near reported each year [7]. Mosquitoes also cause allergic responses in humans that include local skin and systemic reactions, such as angioedema [8].

India reports 1.48 million malaria cases and about 173 deaths: 1.4 million suspected and 11,985 confirmed chikungunya cases; 5,000 *Japanese encephalitis* (JE) cases and approximately 1,000 deaths; 383 dengue cases and 6 deaths during 2006 and 2007 [9]. So, control of mosquito larvae is a public health importance in preventing emerging adults and their emerging pathogens [10].

Mosquito control is essential for the proliferation of mosquito borne diseases, environmental quality and public health. [11]. Vector control remains the most effective measure and is often the only way to prevent disease outbreaks because there are no vaccines for many vector-borne diseases and drug resistance is an increasing threat. One of the methods to manage these diseases is to control the vectors for bringing about interruption in disease transmission. The control of mosquitoes at larval stage is considered as an efficient way in the integrated vector management [12].

Worldwide all water sources are common habitats for the immature stages of vector mosquito species and reducing mosquito-diseases morbidity in both urban and rural areas where a sufficient proportion of larval habitats can be targeted [13].

Insect repellent is known to play an important role in preventing vector-borne diseases by reducing man-vector contact. Currently a variety of repellents are marketed to control mosquito-human contact in the form of mats, coils, lotions and vaporizers. There also are mosquito repellent products available based on sound productions, particularly inaudible high frequency ultrasounds [14].

Synthetic insecticides have been not very successful due to human, technical, operational, ecological and economic factors. This may resulted to the development of resistant strains of mosquitoes. One of the major drawbacks of chemical insecticides is that they are non-selective and could be harmful to other organisms in the environment [11].

During the last decade, various studies on natural plant products against mosquito vectors indicate them as possible alternatives to synthetic chemical insecticides. Mosquito control programmes largely target the larval stage at their breeding sites with larvicides [15]. Biologically active plant materials have attracted considerable interest in mosquito control programs in the recent times. Biopesticides provide an alternative to synthetic pesticides because of their generally low environmental pollution, low toxicity to humans, and other advantages [16].

Natural products, especially those derived from plants, have been used to help mankind sustain its health since the dawn of medicine. Over the past century, the phytochemicals in plants have been a pivotal pipeline for pharmaceutical discovery. The importance of the active ingredients of plants in agriculture and medicine has stimulated significant scientific interest in the biological activities of these substances [17]. Plants produce a broad range of bioactive chemical compounds. Several plants have been screened due to their insecticidal properties and crude extracts of many plant species have shown promising mosquitocidal activities. Some phytochemical insecticides are even more effective than synthetic ones [18].

Kishore *et al.,* [19] 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 such as alkanes, alkenes and simple aromatics, lactones, essential oils and fatty acids, terpenes, alkaloids, steroids, isoflavonoids, pterocarpans and lignans. Researchers have also documented the isolation of several bioactive toxic principles from various plants and reported their toxicity against different mosquito species.

In spite of the enormous economic importance of the grasses, this family has rarely been exhaustively surveyed for its chemical constituents. Major problems in surveys are the sheer size of the family (6000-9000 species) and the difficulties of taxonomic verification. Our knowledge of the flavonoid and related phenolic constituents in the *Gramineae* is particularly scanty since only some 100 species from 70 genera have been examined previously [20,21,22].

In India and South East Asian countries plants are known for their medicinal uses therefore, it appears more feasible to use these species for economical purpose such as source of medicines and insecticides

for the management of these plants in place where they have attained the status of weeds. However, knowledge on insecticidal property of *Cyperaceae* is much limited [23,24].
Nut grass has been used by the people who live in few areas of Indonesia as a home remedy against stomach disorders especially dysentery. In addition, nut grass has possible antimicrobial properties due to the presence of polyphenols in the plant [25].

The phytochemical screening revealed that *Pennisetum purpureum* is rich in tannins, alkaloids, flavonoids, saponins, cyanogenic glycosides and oxalates, but with very little phytate. Alkaloids, flavonoids, saponins, and tannins are known to have antimicrobial activity, as well as other physiological activities [26,27]. In fact, flavonoids have a wide range of biochemical and pharmacological activities in mammalian and other biological systems. They possess anti inflammatory, antioxidant, anti allergic, hepatoprotective, anti thrombic, antiviral and anti carcinogenic activities [28].

Kyllinga nemoralis leaves contain many biologically active chemicals like essential oils (terpenes α-cyperone, β-selinene, and α- humulene),6 terpenoids, saponins and phenolic compounds8. The rhizomes possess flavonoids, triterpenoids and glycosides [29].

Hexane extract of tuber of plant *Cyperus rotundus* was tested for repellent activity against mosquito vector *Anopheles culicifacies*, *Anopheles stephensi* and *Culex quinquefasciatus*. Results showed that the tuber extracts were effective for repellency of the entire mosquito vector even at a low dose [30].
The present study focuses on the larvicidal activity of *Kyllinga nemoralis* grass weed extracts against the fourth instar larvae of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus.*

Materials and Methods

Selection of Plant

The grass *Kyllinga nemoralis* (Fig. 1) belonging to the family *Poaceae* was collected from the natural population in the Presidency College campus, Chennai, Tamilnadu, India. The plant was identified and authenticated by Prof. P. Jayaraman and deposited at the plant anatomy research center (PARC) West Tambaram, Chennai-45, Tamil Nadu, India.The collected plants were washed with tap water and distilled and then it was dried for 14 days.

Plant extract preparation

The dried plants were sieved and powdered using electric blender. The grass powder was extracted successively using solvents respectively aqueous, chloroform, ethanol, hexane, methanol. 50gm powder was soaked in 500ml of each solvent. The extracted solvents were concentrated using Rotary evaporator and stored it under 4^{o}C in air tight bottles for further use.

Selection of mosquito species

The fourth instar larvae of *Aedes aegypti*, *Anopheles stephensi* and *Culex quiquefasciatus* were selected for this present study. The larvae were procurred from the Entomology Research Institute (ERI), Loyola College, Chennai, Tamilnadu, India.

Phytochemical screening

Phytochemical screening of the samples was carried out as described by [31,32]. The plant extract samples were screened for secondary metabolites like carbohydrates, saponins, alkaloids, flavonoids, glycosides and proteins *etc*.

Separation of bioactive compounds using TLC

Preparation of plant extract

10 mg / ml of the plant extract in ethanol solvent was used for TLC examination.

TLC Plate preparation

The silica gel 60º F 254 coated aluminum sheets were slice in size 1.5 x 6.5cm and the prepared ethanol plant extract was loaded on silica plate and air dried.

Mobile phase preparation

The ethanol extracts were standardized in ethyl acetate with acetone and finally in hexane: ethyl acetate (2:1) ratio [33].

GC-MS analysis

The sample was prepared in the concentration range of 0.2 - 0.5 mg/ml and injected by flow analysis at a flow rate of 10 µl min-1. The recorded mass was in the range 100-500 m/z using the mass spectrum was obtained using an Agilent technologies (6890 N) instrument (JEOL GCMATE II) fitted with an electron spray ionization source. Software version 4.0 was used for data acquisition. The positive ion mode, using a spray voltage at 3.5 kV, at a source temperature of 80oC, was employed for recording the spectra. Mass spectra were recorded under electron impact ionization at 70 eV energy.

Mosquito Larvicidal Bioassay

The larval susceptibility tests were done according to standard procedures [34]. A laboratory reared vector mosquitoes viz., *Aedes ageypti Anopheles stephensi* and *Culex quinquefasciatus* free of exposure to insecticides and pathogens. Cyclic generations of vector mosquitoes were maintained at 25-29°C insectariums. Larvae were fed on powdered dog biscuit and yeast in the ratio of 3:1 and adult mosquitoes on 10 per cent sucrose solution.

Larval suscepectibility test was carried out as described by [35]. A total of three trials with five replicates per trial for each concentration was done against mosquito vectors. The crude plant extracts were separately tested for its larvicidal potential against fourth instar larvae of *A. ageypti, A. stephensi* and *C. quinquefasciatus.* The Stock solution (1000 ppm) of the extract was prepared by dissolving 100 mg of crude extract in 1 ml acetone and volume raised to 100 ml with distilled water. From the stock solution different dilution of 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm were prepared in 200 ml distilled water and 20 fourth instar larvae were released and mortality was recorded after 24 h. The beakers were kept in a temperature controlled room at 28°C ± 2°C and the larvae were exposed to 200 ml water containing 0.1 ml of acetone which served as control.

Larval susceptibility tests

The different concentrated solutions of extracts was prepared and tested against the three mosquito larvae *A. aegypti, A. stephensi* and *C. quinquefasciatus* were placed in each test solutions to observe the larvicidal properties as per the following procedure.

Groups of 20 larvae were placed in glass beakers containing 200 ml of the grass extract solution. Control experiments without extract were run in parallel. The larvae in each solution were then left for 24 h and the numbers of dead larvae were counted after 24 h of exposure, and the percentage mortality was calculated from the average of five replicates and mortality in the control was corrected by Abbott's [36] formula.

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

Dose-response bioassay

From the stock solution, different concentrations ranging from 25 to 150 ppm were prepared. Based on the preliminary screening results, the prepared grass extracts were subjected to dose-response bioassay for larvicidal activity against *A. aegypti, A. stephensi* and *C. quinquefasciatus*. The numbers of dead larvae were counted after 24 hours of exposure, and the percentage mortality was reported from the average of five replicates.

Statistical analysis

The average larval mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90} [37] as described by [38] and other statistics at 95% fiducial limits of upper confidence limit and lower confidence limit and chi-square values were calculated using the software (SPSS, 11.5).

Results and Discussion

Phytochemical Screening

The phytochemical screening of whole plant extracts of *Kyllinga nemoralis* revealed the strong presence of carbohydrates, steroids, saponins, flavonoids, alkaloids, anthocyanin, quinines steroids and coumarins, phenols, cardiac glycosides, acids, terpenoids and triterpenoids were present [39] in the ethanol extract of the *Kyllinga nemoralis.* (Table 1). As ethanol extract showed the strong presence of various phytocompounds hence it was further subjected to larvicidal assay**.**

Larvicidal Bioassay

The ethanol extract of *Kyllinga nemoralis,* exhibits effective larvicidal activity at very low concentrations against the fourth instar larva of all three mosquito species tested. Ethanol extracts of *Kyllinga nemoralis* showed 100% mortality at 200 ppm against the fourth instar larvae of *A. stephensi* , *C. quinquefasciatus* and 250 ppm against *A. aegypti*. Ethanol extract at low concentration of 100 ppm showed 50 to 60% mortality, when exposed for 24 h. Ethanol extracts were also found to be equally effective showing 80 to 95% activity against the fourth instar larvae of the three mosquito species tested (Tables 1.3, 1.4 and 1.5). Based on the probit analysis between the concentrations of plant extract against the fourth instar larvae of *A. aegypti, A. stephensi* and *C. quinquefasciatus* after 24 h exposure (Fig.3.a, b and c), the results were presented in Tables 2,3 and 4 The ethanol extract of *Kyllinga nemoralis* was found to be more toxic against *A. stephensi* and *C. quinquefasciatus* with LC50 value of 72.077 ppm and 79.266 ppm respectively and LC90 value of 139.843 ppm and 161.294 ppm respectively, when compared to its toxicity against *A. aegypti* with LC50 value of 92.234 ppm and LC90 value of 209.768 ppm respectively (Fig. 1.0). Methanol extracts of *Kyllinga nemoralis* showed LC50 and LC90 values of 90.635 ppm and 191.748 ppm against *C. quinquefasciatus* when compared to *A. stephensi* (93.918 ppm and 205.217 ppm) and *A. aegypti* (95.853 ppm and 218.108 ppm) respectively. All the extracts (aqueous, hexane and chloroform) also showed good larvicidal activity at a relatively high concentration when compared to ethanol and methanol grass extracts. Since the ethanol extract showed potent larvicidal activity, when compared to the other extracts, it was takenup for further studies.

Thin Layer Chromatography analysis

The phytocompounds present in the ethanol extracts were further analysed by TLC (Fig.2). Ethanol extract of *Kyllinga nemoralis* showed 10 bands with R_f values of 0.34, 0.41, 0.45, 0.53, 0.56, 0.61, 0.63, 0.67, 0.71 and 0.76.

GC-MS analysis

The spectral data of GC-MS analysis of the extract is shown in Fig. 4. The retention time names, molecular weight of the some of the componenets of the test extract were ascertained. Ten compounds were identified by GC-MS. Some of the compounds observed were 1. Hexadecanoic acid, methyl ester , 2. 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, 3. Methyl 9-cis,11-trans-octadecadienoate, 4. Ethyl 9,12,15-octadecatrienoate, 5. Ethyl 9,12-hexadecadienoate, 6. 2H-1-Benzopyran, 3,4-dihydro-7-methoxy-3, 7. 5-Heptadecene, 1-bromo-, 8. 17-(1,5-Dimethylhexyl)-10,13-dimethyl-2, 9. 8-[2-(2-Amino-phenyl)-2-oxo-ethyl]-3-(1-, 10. Campesterol, 11. A-Neo-18.alpha.-oleana-3,12-dien-30-oic, 12. 7-Heptadecyne, 17-chloro-, 13. 4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a, 14. Lup-20(29)-en-28-oic acid, 3-hydroxy-, (Table. 5).

Histopathological studies

The first aspect investigated in this work was the histological structure of the midgut epithelial cells of the untreated 4th instar larvae of *Anopheles stephensi*(. The midgut of the dipteran larvae has been subdivided into two different regions, each including one characteristic cell type. Since the midgut of the

larvae is the principle region of absorption and digestion, its histoarchitecture was studied. The midgut is lined with nucleated columnar cells with granulated cytoplasm [40]. The posterior midgut is characterized by tall epithelial cells (dark cells: [41]. Dark cells show normal intercellular contacts along the whole lateral plasma membranes, normal nuclei, a well developed brush border, and a normal adhesive basement membrane, as observed in control sections [42]. The alimentary canal is associated with circular and longitudinal muscles. The cuticle was found to be intact and gastric caeca showed distinct layer of epithelial cells in the control larvae. The histopathological changes were observed in the treated 4th instar *Anopheles stephensi* larvae. All the treated larvae developed distinct lesions and damages affecting mainly the midgut epithelium and secondarily the caeca, muscles and fat tissues. The epithelial cells of the gastric ceacae start to burst and cytoplasmic rejection of cell material mixed with food column was observed. Some of the cells degenerated and showed lysis. The treated larvae showed degeneration of muscles and shrinkage of fat tissue and the wall of the cytoplasm appeared irregular and were completely separated from each other. The gut contents were also found to be mixed with haemolymph. Previous histological studies have revealed that midgut region of the treated larvae is considered as the primary site for cellular alterations [43]. The above observations recorded could be responsible in causing larval mortality. These results are in line with the finding reported by [44,45].

Besides, many researchers have suggested the synergistic effect of plant materials could represents an important tool to replace the chemical repellents used today [46].

Conclusion

This study aims to evaluate the efficacy of *Kyllinga nemoralis* grass extracts against the four instar larvae of *A. aegypti*, *A. stephensi* and *C. quinquefasciatus*. It was observed that these extracts exhibited insecticidal activity against all the three mosquito larvae.

Table 1. Phytochemical screening of grass extracts of *Kyllinga nemoralis*

S. No	Secondary metabolites	Aqueous	Chloroform	Ethanol	Methanol	Hexane
1	Carbohydrate	+++	+++	+++	+++	+++
2	Tannins	+	-	+	++	-
3	Saponins	+++	+	+++	+++	+
4	Flavonoids	++	+++	+++	++	+
5	Alkaloids	++	++	+++	+++	+
6	Anthocyanin	+++	-	+++	+++	+
7	Quinones	-	-	+++	+	+
8	Glycosides	-	-	-	-	-
9	Cardiac glycosides	++	-	+++	+	-
10	Terpenoids	-	-	++	+	-
11	Triterpenoids	+	-	++	+	-

12	Phenols	++	+	+	+++	-
13	Coumarins	+++	++	+++	++	+
14	Acids	++	-	+	++	+
15	Protein	++	--	-	-	-
16	Steroids	++	+	+++	+++	+

Table 2. Larvicidal activity of *Kyllinga nemoralis* grass extracts against fourth instar larvae of *A. aegypti*

Extracts	Concentration (ppm)	24hr % Mortality	LC_{50} (LCL–UCL) (ppm)	LC_{90} (LCL–UCL) (ppm)	Chi-Sq
Aqueous	50	14	172.743 153.454 ± 198.589	623.061 464.004 ± 985.830	22.860
	100	26			
	150	39			
	200	54			
	250	71			
Ethanol	50	23	92.234 54.525 ± 124.185	209.768 151.396 ± 487.103	10.984
	100	52			
	150	71			
	200	89			
	250	100			
Chloroform	50	16	134.614 89.946 ±195.771	395.198 247.384 ± 2090.861	14.058
	100	33			
	150	48			
	200	63			
	250	87			
Hexane	50	9	244.218 206.785 ± 313.217	1073.168 687.528 ± 2318.385	0.204
	100	21			
	150	34			
	200	42			
	250	52			
Methanol	50	22	95.853 49.745 ± 134.803	218.108 151.657 ± 693.013	18.177
	100	48			
	150	66			
	200	85			
	250	100			

Table 3. Larvicidal activity of *Kyllinga nemoralis* grass extracts against fourth instar larvae of *A. stephensi*

Extracts	Concentration (ppm)	24hr % Mortality	LC_{50} (LCL–UCL) (ppm)	LC_{90} (LCL–UCL) (ppm)	Chi-Sq
Aqueous	50	16	146.187 130.826 ±164.104	488.712 383.649 ± 701.156	4.710
	100	33			
	150	51			
	200	63			
	250	78			
Chloroform	50	17	140.553 125.744 ± 157.364	466.605 369.245 ± 659.908	5.357
	100	36			
	150	53			
	200	64			
	250	80			
Ethanol	50	29	72.077 49.631 ± 91.080	139.843 109.604 ± 219.459	9.507
	100	64			
	150	92			
	200	100			
	250	100			
Hexane	50	10	232.323 198.832 ± 291.013	977.361 645.016 ± 1978.267	1.829
	100	22			
	150	31			
	200	43			
	250	57			
Methanol	50	22	93.918 47.051± 132.960	205.217 143.088 ± 651.891	19.944
	100	48			
	150	66			
	200	89			
	250	100			

Table 4. Larvicidal activity of *Kyllinga nemoralis* grass extracts against fourth Instar larvae of *C. quinquefasciatus*

Extracts	Concentration (ppm)	24hr % Mortality	LC_{50} (LCL–UCL) (ppm)	LC_{90} (LCL–UCL) (ppm)	Chi-Sq
Aqueous	50	14	148.371 133.539 ± 165.614	463.287 370.175 ± 643.848	3.136
	100	30			
	150	49			
	200	64			
	250	76			
Chloroform	50	11	210.768 182.951 ± 255.620	855.360 586.029 ± 1598.790	1.112
	100	23			
	150	35			
	200	48			
	250	59			
Ethanol	50	27	79.266 42.700 ± 108.646	161.294 116.904 ± 362.965	17.231
	100	55			
	150	83			
	200	100			
	250	100			
Hexane	50	13	186.295 164.214 ± 218.083	707.457 510.060 ± 1192.200	2.607
	100	24			
	150	38			
	200	53			
	250	65			
Methanol	50	23	90.635 47.180 ± 126.591	191.748 135.855 ± 526.812	19.355
	100	52			
	150	71			
	200	96			
	250	100			

Table 5. GC-MS analysis of Ethanol extract of *Kyllinga nemoralis*

S. No	RT	Name of the compound	Peak Area (%)	Amount
1.	29.884	Hexadecanoic acid, methyl ester	8.141e+6	1.442
2.	34.122	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	2.983e+7	5.284
3.	34.558	Methyl 9-cis,11-trans-octadecadienoate	1.650e+7	2.923
4.	35.040	Ethyl 9,12,15-octadecatrienoate	1.169e+7	2.071
5.	35.865	Ethyl 9,12-hexadecadienoate	7.003e+6	1.240
6.	51.502	2H-1-Benzopyran, 3,4-dihydro-7-methoxy-3	1.718e+6	0.304
7.	53.104	5-Heptadecene, 1-bromo-	983238	0.174
8.	55.126	17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,	2.622e+6	0.464
9	57.898	8-[2-(2-Amino-phenyl)-2-oxo-ethyl]-3-(1-	2.670e+6	0.473
10	56.904	Campesterol	7.726e+7	13.685
11	58.973	A-Neo-18.alpha.-oleana-3,12-dien-30-oic	8.074e+6	1.430
12	59.381	7-Heptadecyne, 17-chloro-	1.255e+7	2.223
13	59.716	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a	6.678e+6	1.183
14	60.271	Lup-20(29)-en-28-oic acid, 3-hydroxy-,	6.613e+6	1.171

Fig. 1. *Kyllinga nemoralis* grass selected for the study

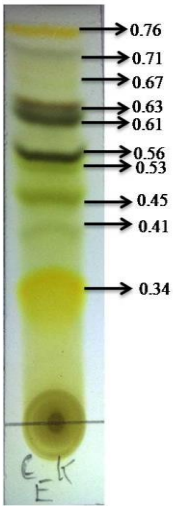


Fig.2. Thin layer chromatography analysis of Ethanol extract of *Kyllinga nemoralis*

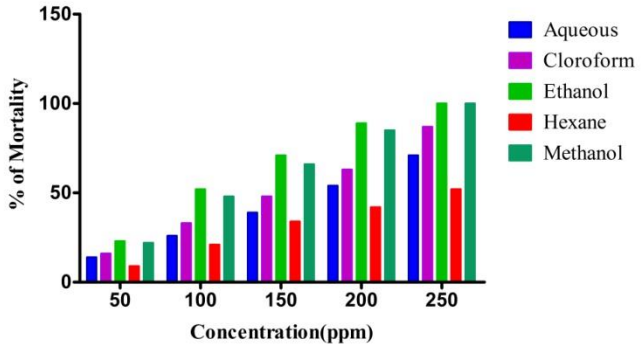


Fig 3a. ***Aedes aegypti***

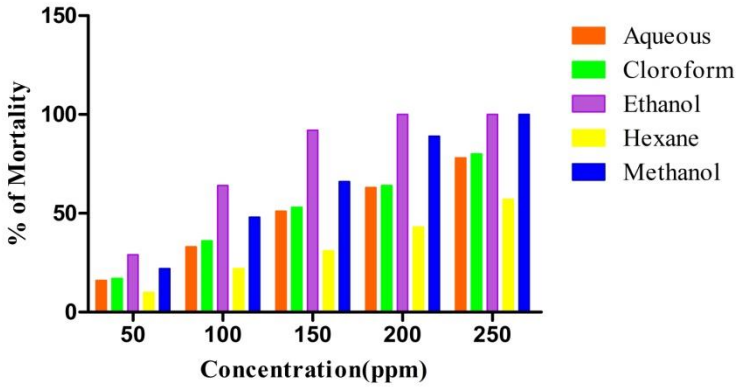


Fig 3 b. ***Anopheles stephensi***

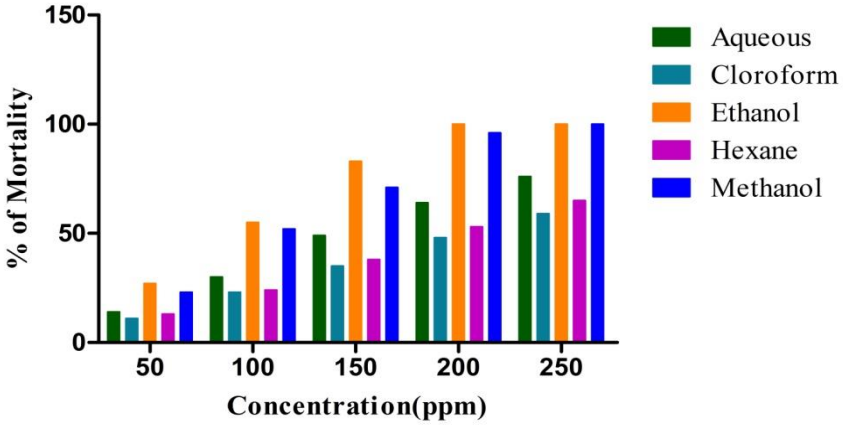


Fig 3c. ***Culexquinquefasciatus***

Fig 3 (a,b and c). Larvicidal activity of ***Kyllinga nemoralis*** **grass extracts against fourth Instar larvae of** ***Aedes aegypti, Anopheles stephensi*** **and** ***C. quinquefasciatus***

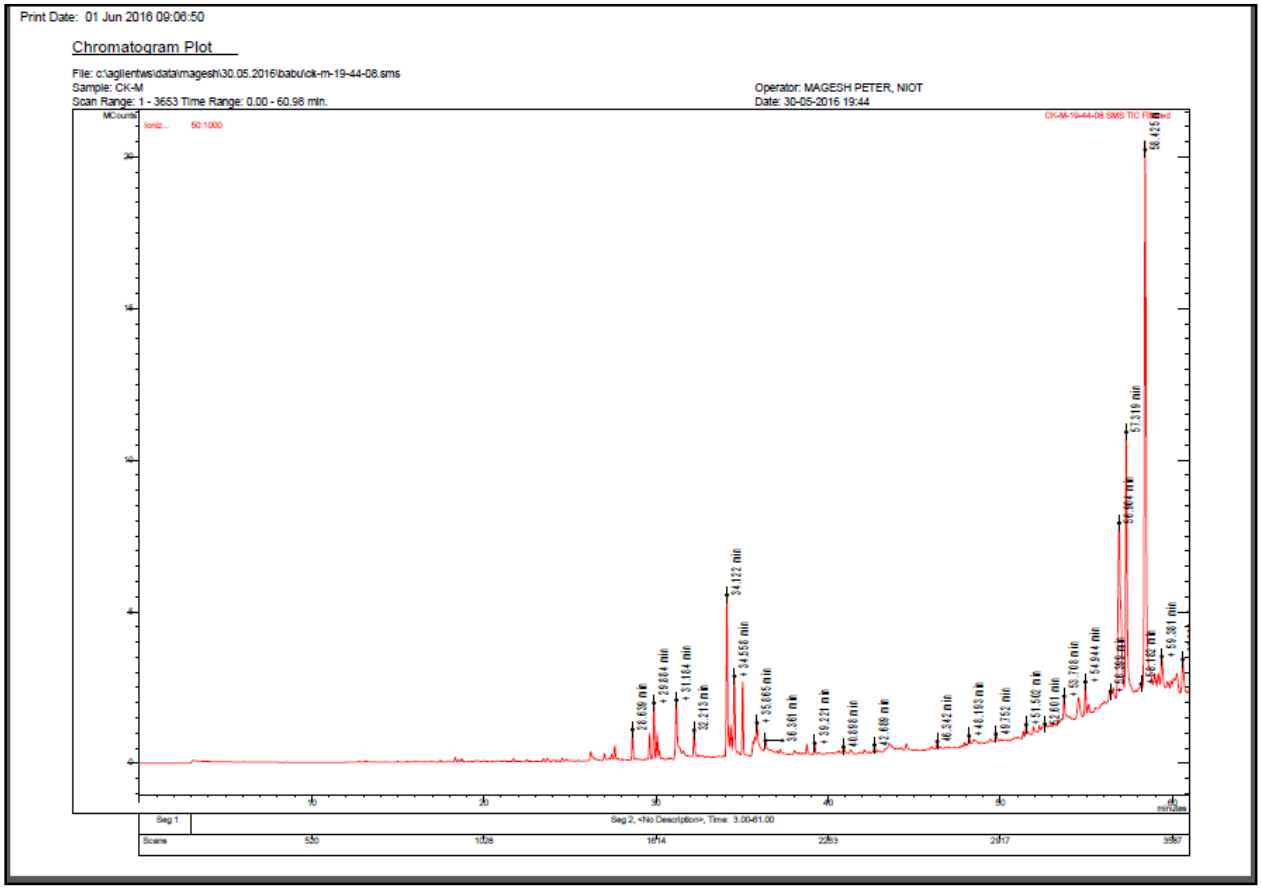
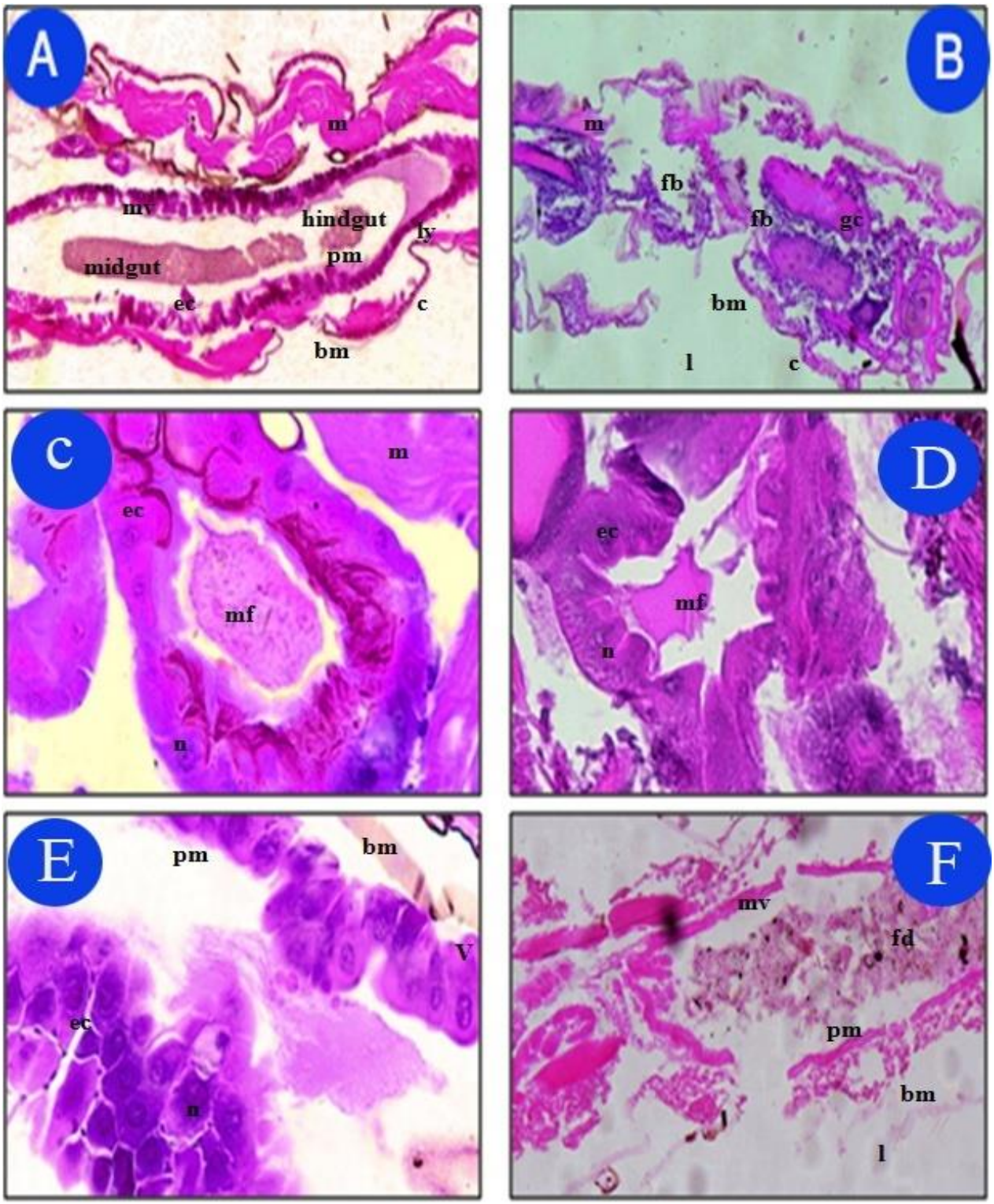


Fig 4. GC-MS chromatogram of ethanol extract of *Kyllinga nemoralis*



m- muscle, ec- epithelial cells, pm-peritrophic membrane, bm- basement membrane,v- vesicle, n- nucleus, l- lumen, fb- food bolus, mv- micro villi, mf- muscle fibrelae, c- cuticle, gc- gastric caeca.

Fig. 5 Histopathological alterations of control (a,c,e) and treated (b,d,f) 4th instar larvae of *Anopheles stephensi*(400X).

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