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Larvicidal activity and GC-MS analysis of *Piper longum* L. leaf extract fraction against human vector mosquitoes (Diptera: Culicidae)

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Abstract
Mosquitoes are the vectors for transmitting major public health diseases like dengue, filariasis, and malaria. Among the various mosquito larval control agents, plant extracts and isolated phytocompounds are good alternatives to control vector mosquitoes. The objective of this study is to test the efficacy of leaf extract fraction prepared from the locally available plant, *Piper longum* L in decimating the larvae of *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus*. *Piper longum* leaf extract fraction was isolated through column chromatographic separation and phytochemical analysis was carried out by standard procedures. The larvicidal assay was carried out following WHO methods, and the *P. longum* fraction was tested at various concentrations (10-80µg/dl) on the third instar larvae of *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus*. The 24h LC_{50}, LC_{90} values were as determined using probit analysis. GC-MS analysis was done for the identification of bio-active compounds present in the specific *P. longum* leaf fraction. The fraction of *P. longum* leaf extract contains secondary metabolites such as alkaloids, glycosides, saponins, phytosterols, diterpenes, triterpenes, phenols, tannins, steroids, and terpenoids. The LC_{50} and LC_{90} values were 38.96 and 41.53; 45.06, and 71.16; 72.02 and 76.80 µg/dl against *Ae. Aegypti*, *An. stephensi*, and, *Cx. quinquefasciatus* respectively. Twenty-nine phytocompounds were identified by GC-MS. The present results suggested that the fraction of *P. longum* leaf extract checked for mosquito larvicidal activity had an excellent potential for controlling selected human vector mosquitoes.

Keywords: column chromatography, extraction, medicinal plant, phytochemical compounds, soxhlet apparatus

1. Introduction
Vector-borne diseases account for more than 17% of infections, causing more than one million deaths annually [1]. Pathogens for diseases such as dengue fever, malaria, Japanese encephalitis, and filariasis are transmitted by the three genera of mosquitoes namely *Aedes*, *Anopheles*, and, *Culex* [2, 3]. More than 3000 mosquito species belonging to 34 genera in the world, among them only about 300 species are capable of transmitting diseases to humans and other vertebrates [4]. Approximately 40 million people in India suffer from mosquito-borne diseases annually and contribute significantly to disease burden, death, poverty, and social debility all over the world, particularly in tropical countries [5]. *Aedesaegypti* is the principal vector of dengue fever which is one of the most important vector-borne viral diseases worldwide [6, 7]. Dengue fever can debilitate the patient for a week or more, or as the hemorrhagic form which may lead to death [8]. *Anopheles stephensi* is the major vector of malaria in the Indian subcontinent and some West Asian countries. Malaria remains the most serious vector-borne disease affecting about 300-500 million people and 1.4 to 2.6 million deaths annually throughout the world. More than 40% of the world's population lives in areas prone to malaria [9]. *Culex quinquefasciatus* is the vector of *Wuchereria bancrofti* and causes lymphatic filariasis and is possibly the most abundant house mosquito in towns and cities of tropical countries [10]. According to WHO [11], about 90 million people worldwide are infected with *W. bancrofti*. There are approximately 40 million people who experience severe disability due to lymphatic filariasis [12].

One of the approaches to combat mosquito-borne diseases relies largely on the interruption of the aquatic stages or by killing the adult mosquitoes using chemical insecticides. The drastic effects of chemical insecticides for controlling mosquitoes have received wide public apprehension [13]. To overcome these problems associated with conventional mosquito control, great efforts are required to develop innovative or complementary control techniques for mosquito species, which has resulted in the search for eco-friendly, cost-effective, and target specific insecticides against mosquito species [14]. Phytochemicals have been reported to be alternatives to synthetic pesticides and many of them are effective in mosquito control [15]. Plants or parts of plants possess a consortium of chemicals with unique biological activity [16]. Over 2000 plant species contain chemicals with pest control properties [17], and among them, several species of plants have been shown to have some degree of activity against mosquitoes [18].

Plant kingdom encompasses the thousands of medicinal plants, and they have been tested for their biological properties to develop medicines, pesticides, cosmetics, and food ingredients, mainly due to the presence of phytochemical constituents in these medicinal plants. Chemical compounds produced by plants known as phytochemicals are either primary metabolites, such as proteins, amino acids, common sugars, purines, and pyrimidines of nucleic acids, chlorophyll, secondary metabolites like alkaloids, flavonoids, terpenes, lignans, steroids, saponins, phenolics, and glycosides [19]. Which carry important beneficial properties like anti-inflammatory, antidiabetic, antiaging, antimicrobial, antiparasitic, anticancer, antioxidant, and also mosquito larvicidal properties [20]. *Piper longum* L. (Pippali) is a slender, aromatic, creeping, perennial herb occurring in the hotter parts of India, from Central Himalayas to Assam and evergreen forests of the Western Ghats from Konkan to Kerala [21]. Common usage of *P. longum*is for stomach aches, cough, tumors, and similar ailments. Piperine, piperlongumine, pipernonaline, and piperidine are the important compounds derived from *P. longum* [22, 24]. The various bioactive phytochemicals are characterized using Gas Chromatography-Mass Spectrometry (GC-MS), which is a very compatible technique, commonly used for the identification and quantification of the required bio-active compounds [25, 27]. This study was designed to assess the larvicidal potential of the column chromatographic fraction 1 (9:1, i.e. Chloroform: Ethanol) of *P. longum* leaf extract on the larvae of *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus*. GC-MS analysis of *P.longum* leaf extract fraction was done to identify the major phyto compounds available in the fraction.

2. Materials and Methods

2.1. Collection of *P. longum* leaves

Leaves were plucked from botanically authenticated *P. longum* plants, available in certain private gardens. The plucked leaves were cleaned thoroughly with tap water and dried at room temperature for 7-10 days in the shade. The dried samples were powdered using an electric blender and the sifted fine powder was transferred to airtight containers for further use.

2.2. Extract Preparation

P. longum leaf powder samples were extracted in a soxhlet apparatus using a highly polar solvent, ethanol. The extraction process was continued for about 12 hours during which time about 15 cycles of extract movement were noticed between the middle chamber and lower flask. The extraction was stopped when the solvent in the middle chamber was totally colourless. The extract was then concentrated in a Rotary Vacuum Evaporator at 40 °C. Once the raw extract was totally dry, the powder was scrapped out, weighed, and placed inside a screw-capped glass bottle for further use

2.2.1 Column Chromatographic Characterization

About 50 mg of the crude *P. longum* extract was dissolved in 10 ml ethanol. The chromatographic column consisted of 60-120 mesh silica gel packed inside a glass column. The compact column was initially packed and compacted using petroleum ether. The compact column was thoroughly checked for close-packing silica gel. The extract dissolved in ethanol was mixed with non-polar solvent, chloroform in 9:1 proportion. The fractions were collected separately in numbered 50 ml beakers. The fraction was checked for the presence of active ingredients using Thin-Layer Chromatography (TLC). TLC plates were prepared using 40-micron size silica gel slurry spread on glass plates and activating in a hot air oven at 110 ºC for about 1 hour. The plates were cooled and about 1µl of each chromatographic fraction were loaded at the base of the plate using a micropipette. The plates were placed inside a chamber containing about 50 ml of a solvent mixture containing 5:5 ethanol and chloroform. The chamber was closed with a glass lid and the TLC plate was taken out when the solvent reached the top portion of the plate. The movement of the solute was followed by developing the plate inside another chamber concentrated with iodine vapours.

2.3. Phytochemical Analysis

The presence of different phytochemical constituents with significant mosquito larvicidal activity in the chromatographic fraction was established using standard qualitative procedures [28, 30].

2.4. Mosquito Rearing

Egg cards of *Ae. Aegypti*and eggs of *An. Stephens*i, and *Cx. quinquefasciatus* were procured from the Centre for Research in Medical Entomology (CRME), ICMR, Madurai. The eggs were incubated in the laboratory in three separate trays containing tap water. The larvae that hatched out were fed with powdered dog biscuits and yeast in the ratio of 3:1.

2.5. Larvicidal Activity

The larvicidal activity of *P. longum* leaf extract fraction on mosquito larvae was assessed by using the standard method prescribed by WHO [31]. The third instar *Ae. aegypti*, *An. Stephensi*, and *Cx. quinquefasciatus* were raised in the laboratory and removed for the experiments at the appropriate time. The larvae were exposed to the toxicants in clean 100ml glass beakers. The concentrations ranging from 10-80 (µg/dl) were tested against *Ae. aegypti*, *An. Stephensi*, and *Cx. quinquefasciatus* larvae. Four replicates were maintained for each concentration. The exposed larvae were continuously monitored and the mortality was recorded after 24hr.

2.6. GC-MS analysis

The fraction was further characterized using Gas

Chromatography and Mass-Spectral analysis. GC-MS analysis was carried out in South Indian Textile Research Association (SITRA), Coimbatore. Fraction dissolved in ethanol was analyzed using gas chromatography (THERMO GC- TRACE ULTRA VER: 5.0, THERMO MS DSQ-II), and the spectra pertaining to each RT values was further characterized using mass- spectral analysis. CAS library reference was used to elucidate the structure of compounds available in a particular fraction.

2.7 Statistical Analysis

The larval mortality data were subjected to probit analysis for calculating LC_{50} and LC_{90} values and their 95% Upper (UCL) and Lower Confidence Limits (LCL), were calculated using the dose effect probit Analysis [32].

3. Results

3.1. Screening of phytochemicals

The preliminary phytochemical screening of *P. longum* leaf extract fraction revealed the presence of alkaloids, saponins, phytosterols, diterpenes, triterpenes, phenols, tannins, steroids, and terpenoids (Table 1).

Table 1: Phytochemical analysis of column fraction (9:1) of *P.longum* leaf extract

Sl. No	Phytochemicals	Fraction 1
1.	Alkaloid	+
2.	Glycoside	-
3.	Saponin	+
4.	Phytosterols	+
5.	Diterpenes	+
6.	Triterpenes	+
7.	Phenol	+
8.	Tannins	+
9.	Flavonoids	-
10.	Steriods	+
11.	Terpenoids	+

+Signs denotes the presence
- Signs denotes the absence

3.2. Larvicidal efficacy

The larvicidal activity of leaf extract fraction of *P. longum* was evaluated under laboratory condition. *P. longum* was an effective larvicide of the third instar larvae of *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus*. The larvicidal efficacy was expressed by LC_{50} and LC_{90} values at 24h exposure time. The LC_{50} and LC_{90} values against early third instar larvae of *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus* were 38.96 and41.53; 45.06 and 71.16; 72.02 and 76.80(µg/dl),respectively(Table 2). The results indicate that the leaf extract fraction of *P. longum* possesses the potential for controlling mosquito populations.

Table 2: Probit analysis of the mortality response of third instar mosquito larvae to chromatographic fraction (9:1) of *P. longum* leaf extract after exposure for 24h.

Conc. (µg/dl)	Mosquitoes					
	Ae. aegypti		*An.stephensi*		*Cx. quinquefasciatus*	
	LC_{50}(µg/dl)	LC_{90}(µg/dl)	LC_{50}(µg/dl)	LC_{90}(µg/dl)	LC_{50}(µg/dl)	LC_{90}(µg/dl)
	LCL-UCL	LCL-UCL	LCL-UCL	LCL-UCL	LCL-UCL	LCL-UCL
10						
20						
30						
40	38.96	71.16	41.53	72.02	45.06	76.80
50	14.47-63.45	46.67-95.65	17.04-66.02	47.53-96.51	20.57-69.55	52.31-101.29
60						
70						
80						

Conc. – concentration. LC_{50}– lethal concentration that kills 50% of the exposed larvae, LC_{90} – lethal concentration that kills 90% of the exposed larvae, LCL- lower confidence limit(95%),UCL-upper confidence limit (95%).

3.3. Identification of phyto compounds

Total of twenty-nine main bio-active compounds present in the leaf extract fraction of *P. longum* were identified by using GC-MS (Table 3 and Fig 1). The results indicate that the leaf extract fraction of *P. longum* possesses the potential for controlling mosquito populations.

Table 3: GC-MS Characterization of *P.longum* leaf extract fraction (9:1)

RT	Name of the compound	Molecular formula	Molecular Weight
3.37	Benzene, methyl- (CAS)	C7H8	92
3.61	Ethynylcyclopentene-(1)	C7H8	92
5.18	Benzene, 1, 3, 5-trimethyl- (CAS)	C9H12	120
8.20	Dodecane, 5, 8-diethyl- (CAS)	C16H34	226
9.22	Memantine	C12H21N	179
10.42	Naphtho [1, 2-b] furan-2, 8(3H, 4H)- dione, 3a, 5, 5a, 9b-tetrahydro-3,5a,9-trimethyl-,[3S-(3à, 3aà, 5aá, 9bá)]- (CAS)	C15H18O3	246
11.31	Tetradecane (CAS)	C14H30	198
11.31	Tetradecane (CAS)	C14H30	198
13.76	Allyl-5-t-butylhydroquinone	C13H18O2	206
15.53	Hexadecane (CAS)	C16H34	226
15.80	Indan, 1-(2-methylpropenyl)-2-thiocyanato-	C14H15NS	229
17.35	(5, 8-Dihydro-6-methyl-5, 8-etheno-4H-3a-azaazulen-4-y liden) acetonitrile	C14H12N2	208
19.75	1-Cyano-1, 1-Dideuterio hexadecane	C17H31D2N	251
20.30	5-(Hydroxymethyl)-2-(1-methyl-2-imidazolyl)-1H-benz imidazole	C12H12N4O	228
21.22	Acetic acid, 2-acetoxymethyl-4-acetylamino-4-cyano-butyl ester	C12H18N2O5	270
22.28	Pentadecanoic acid, 14-methyl-, methyl ester (CAS)	C17H34O2	270
23.07	3, 7, 8-Trimethylpyrido [2, 3-d] pyrimidine-2(3H)-, 4(8H)-dione	C10H11N3O2	205
23.60	Hexadecanoic acid, ethyl ester	C18H36O2	284
25.62	10-Octadecenoic acid, methyl ester (CAS)	C19H36O2	296
26.13	Heptadecanoic acid, 9-methyl-, methyl ester (CAS)	C19H38O2	298
26.93	Docosanoic acid, 8, 9, 13-trihydroxy-, methyl ester (CAS)	C23H46O5	402
28.93	á-d-Mannofuranose, 2, 3:5, 6- di-O-Ethylborandiyl-1-O-(10-undecen-1-yl) -	C21H38B2O6	408
30.55	Heptacosane (CAS)	C27H56	380
31.16	6"-(2-Hydroxy-3-methyl-3-butenyl)-Amentoflavone	C35H26O11	622
31.44	2, 12-Dibromo-7-phenyl-5, 6, 8, 9-tetrahydrobenz[a,j]anthracene-14-carboxylate	C33H28Br2O4	646
31.86	2, 11, 13, 22, 23, 25-Hexaoxa-1, 12(1, 3, 2)-Dibenza-24(2, 9)-1, 10-phenanthrolinabicyclo [10.10.3] pentacosaphane	C40H44N2O6	648
32.17	1, 2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	C16H22O4	278
32.97	Docosane (CAS)	C22H46	310
34.98	Heptacosane (CAS)	C27H56	380
35.57	1, 4-Dioxaspiro [4.5] decane-7-butanoic acid, 6-methyl-, 2-(methylsulfonyloxy) ethyl ester	C16H28O7S	364
36.60	7á, 9á:8à, 10à-Bis (Dimethylmethylenedioxy) 7, 8, 9, 10- tetrahydrobenzo [a] pyrene	C26H24O4	400

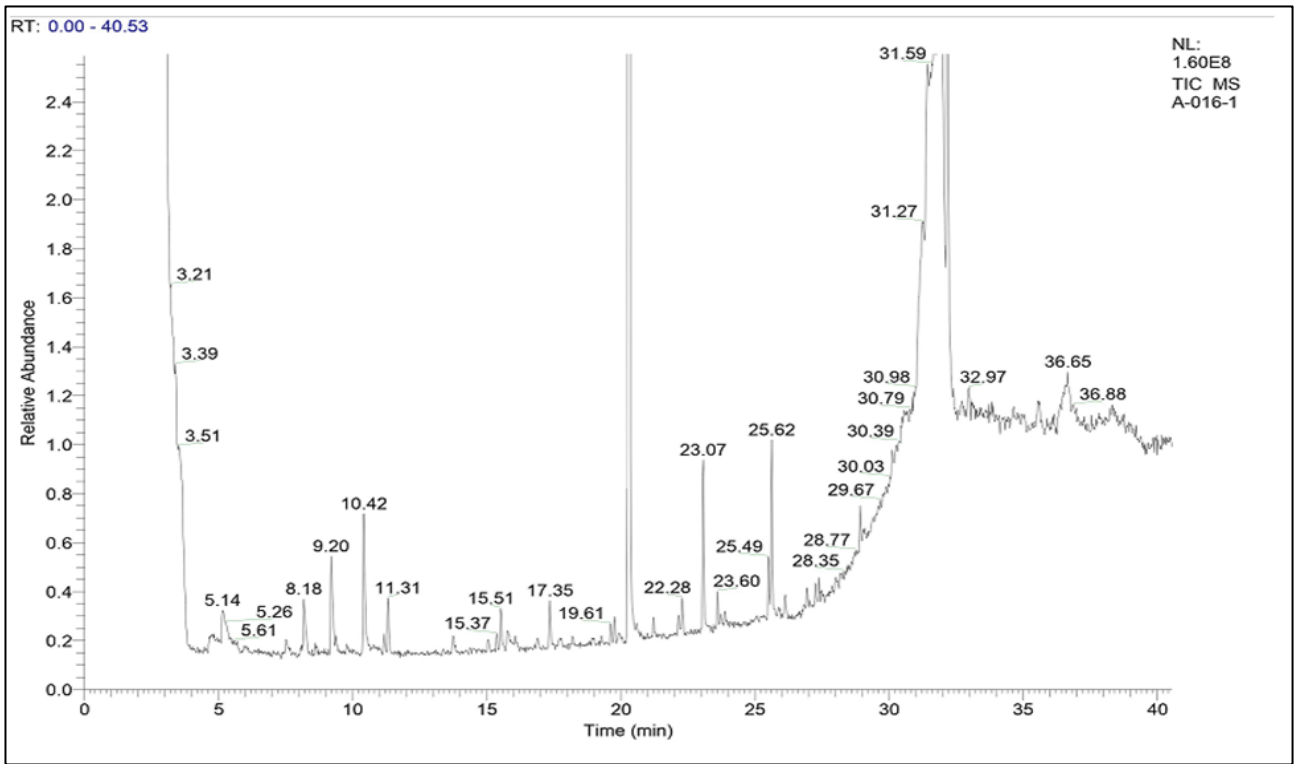

Fig 1: GC-MS Chromatogram of *P. longum* leaf extract fraction (9:1)

4. Discussion

The biocidal activity of botanicals is better understood when column fractions are used. The column fractions of *Acacia nilotica* leaves studied by Ukwuani Kwaja *et al.* [33]. Phytochemical screening of *A. nilotica* revealed the leaves contain terpenoids, saponins, tannins, steroids, and phenols. The phytochemical analysis of column fractions of *Tagetes erecta* L. reported by Devika and Justin Koilpillai [34]. Mosquitoes are the most dangerous insects since they transmit several pathogens to humans. Howard *et al*. demonstrated the larval control can be an effective control tool due to the low mobility of larval mosquitoes, especially where the principal breeding habitats are man-made and can be easily identified [35]. Gleiser and Zygaldo discussed that the vector control program with plant extracts focused more on the elimination of mosquitoes in the larval stage. The advantage of targeting larvae is that they cannot escape from their breeding sites until the adult stage and also reduce overall pesticide use in the control of adult mosquitoes by aerial application of adulticidal chemicals [36]. Cetin *et al*. suggested that the plant extracts and isolated compounds would be good alternatives to control vector mosquitoes [37]. Bilal *et al*. reported that the plants produce many compounds naturally for defense against their pathogens and other plant-eating insects. Hence plants having different kinds of compounds and many of them possess varied levels of activity against insect pests. These plant isolated compounds could be utilized for the control of mosquitoes as they are very effective and biodegradable and not dangerous to human beings and to the environment [38]. Venkaetachalam and Jebasan identified that the phytochemicals derived from plant sources act as larvicides, insect growth regulators, repellents, and oviposition attractants and have different activities [39]. Several investigators have shown phytochemicals exhibited medicinal as well as insecticidal activities [40-45]. It was concluded from this study the presence of these phytochemicals in *P. longum* leaf extract fraction might be the reason for its larvicidal activity. In the present study, the *P. longum* leaf extract fraction showed enhanced larvicidal activity against all three mosquito species studied. GC-MS analysis of *P. longum* showed twenty-nine phytocompounds, among which compounds such as hexadecanoic acid andmethyl ester showed insecticidal, nematicidal, and pesticidal activities [46]. Hexadecanoic acid ethyl ester is responsible for larvicidal activity [47-49]. Thus the compounds identified in *P. longum* by GC-MS presumably led to the mortality of the larval forms of the three common mosquitoes tested.

5. Conclusion

The bio-active compounds in the leaves of *P. longum* show mosquito larvicidal action. It is interesting to note that the same extract exercises a killing effect on the larvae of three different species of mosquitoes. One of the fractions of *P. longum* leaf extract showed effective larvicidal properties against *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus*. Since *P. longum* is freely available in the different tracts along the Western Ghats, there is every possibility to utilize the mosquito larvicidal compounds in the leaves of this plant in controlling the larval forms of vectors. The bio-active compounds responsible for the larvicidal action may be characterized and chemical analogues may be formulated for commercial applications

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7. Conflict of Interest: The authors declare that there is no conflict of interest.

8. Source of Funding: No funding was received for conducting this study.

9. Ethical Approval: This study does not involve the use of animals or human subjects

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