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

NR Padma Priya and RD Stevens Jones

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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 phytochemicals 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 (10-80 µg/dl) on the third instar larvae of *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus*. The 24h LC50, LC90 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 LC50 and LC90 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 phytochemicals 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]. *Aedes aegypti* 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, anti-dietetic, 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. longumis 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 °C 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. Stephensii, 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 insecticides 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 LC50 and LC90 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).

| Sl. No | Phytochemicals       | Fraction 1 |
|--------|----------------------|------------|
| 1.     | Alkaloid             | +          |
| 2.     | Glycoside            | -          |
| 3.     | Saponin              | +          |
| 4.     | Phytosterols         | +          |
| 5.     | Diterpenes           | +          |
| 6.     | Triterpenes          | +          |
| 7.     | Phenol               | +          |
| 8.     | Tannins              | +          |
| 9.     | Flavonoids           | -          |
| 10.    | Steroids             | +          |
| 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 LC50 and LC90 values at 24h exposure time. The LC50 and LC90 values against early third instar larvae of *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus* were 38.96 and 71.16; 45.06 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.

| Conc. (µg/dl) | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 |
|---------------|----|----|----|----|----|----|----|----|
| *Ae. aegypti* | 38.96 | 71.16 | 41.53 | 72.02 | 45.06 | 76.80 |
| *An. stephensi* | LCL-UCL | LCL-UCL | LCL-UCL | LCL-UCL | LCL-UCL | LCL-UCL |
| *Cx. quinquefasciatus* | LCL-UCL | LCL-UCL | LCL-UCL | LCL-UCL | LCL-UCL | LCL-UCL |

Conc. – concentration. LC50 – lethal concentration that kills 50% of the exposed larvae, LC90 – 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 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.    | Steroids             | +          |
| 11.    | Terpenoids           | +          |
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-(3a, 3a, 5a, 9b)]- (CAS) | C15H18O3         | 246              |
| 11.31| Tetradecane (CAS)                                        | C14H30            | 198              |
| 11.31| Tetradecane (CAS)                                        | C14H30            | 198              |
| 13.76| Allyl-5-t-butylhydroquinone                              | C13H20            | 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-ethano-4H-3a-azaazulen-4-y liden) acetonitrile | C14H12N          | 208              |
| 19.75| 1-Cyano-1, 1-Dideuterio hexadecane                       | C17H31D2N         | 251              |
| 20.30| 5-(Hydroxymethyl)-2-(1-methyl-2-imidazolyl)-IH-benz imidazole | C12H12N2O      | 228              |
| 21.22| Acetic acid, 2-acetoxymethyl-4-acetylamino-4-cyano-butyl ester | C12H18N2O2      | 270              |
| 22.28| Pentadecanoic acid, 14-methyl-, methyl ester (CAS)       | C17H34O2          | 270              |
| 23.07| 3, 7, 8-Trimethylpyrido [2, 3-d] pyrimidin-2(3H)-, 4(8H)-dione | C10H11N2O2     | 205              |
| 23.60| Heptadecanoic acid, 9-methyl-, methyl ester (CAS)        | C19H36O2          | 284              |
| 24.13| Docosanoic acid, 8, 9, 13-trihydroxy-, methyl ester (CAS) | C23H46O3         | 402              |
| 28.93| 4-d-Mannofuranose, 2, 3, 5, 6-di-O-Ethyl(hexydiyl)-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]anthracene-14-carboxylate | C33H28Br2O4   | 646              |
| 31.86| 2, 11, 13, 22, 23, 25-Hexaaxa-1, 12[1, 3, 2]-Dibenzen-24[2, 9]-1, 10-phenanthrinabicyclo[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-butanolic acid, 6-methyl-, 2-(methylessulfonyloxy) ethyl ester | C16H28O7S       | 364              |
| 36.60| 7a, 9a:8a, 10a-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 bio-activity of compounds is better understood when column fractions of Phytolacca dodecandra leaves are used. The column fractions of A. nilotica revealed the leaves contain terpenoids, saponins, tannins, steroids, and phenols. The phytochemical analysis of column fractions of Tabernaemontana dodecandra 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]. Venkaatachalam 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 [40-41]. 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 phytochemicals, among which compounds such as hexadecanoic acid and methyl ester showed insecticidal, nematicidal, and pesticidal activities [42]. Hexadecanoic acid ethyl ester is responsible for larvicidal activity [43-45]. 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 Aedes 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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9. Ethical Approval: This study does not involve the use of animals or human subjects

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