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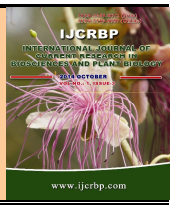

Original Research Article

Bio-Efficacy of *Terminalia chebula* Retz. (Combretaceae) against *Culex quinquefasciatus* Say (Diptera: Culicidae)

P.C.Sathya Narayanan[1], R. Kalpana Devi[2], M. Pushpalatha[3], R. Raveen[3] and S. Lingathurai[2]*

[1]PG Research Department of Zoology, Pachaiyappa's College for Men, Chennai-600 030, India
[2]PG Department of Zoology, Pachaiyappa's College for Men, Kanchipuram-631 501, India
[3]PG Research Department of Zoology, Madras Christian College, Chennai-600 059, India

**Corresponding author.*

A b s t r a c t

Hexane, chloroform and ethyl acetate extracts of *Terminalia chebula* leaves was tested under laboratory conditions against larvae and pupae of *Culex quinquefasciatus*. Among the four different concentrations (62.5, 125, 250 and 500ppm) tested, the dosage of 500ppm of chloroform extract had the most toxic effect (87.20% larval mortality and 56% pupal mortality). The LC_{50} values of chloroform extract of *T. chebula* leaves were 157.86 ppm and 386.16ppm against the larvae and pupae of *Cx. quinquefasciatus* respectively. Chloroform extract was more potent and showed a significant level at $p<0.05$. Larval and pupal toxicity of extracts were found to be dose dependent. The GC-MS analysis of effective extract was analyzed and described. A total of 23 compounds were identified. The major components in chloroform extract of *T. chebula* leaves were phytol isomer (17.43%), stigmast (11.40%), tetracosane (9.63%) and acetin, 1-mono (8.72%). In light of global efforts to find alternatives for currently used insecticides against *Cx. quinquefasciatus*, *T. chebula* and its constituent's advantage further research as potential mosquito larvicides and pupicides.

K e y w o r d s

Culex quinquefasciatus
GC-MS analysis
Larval mortality
Pupal mortality
Terminalia chebula

Introduction

Mosquitoes are one of the most medically significant transmitters as they extend variety of parasites and pathogens, which persist to have destructive effects on human beings. Mosquito-borne diseases such as malaria, filariasis, yellow fever and dengue cause widespread morbidity and mortality and are a major economic burden within disease-endemic countries (Sachs and Malaney, 2002; Boutayeb, 2006). Every year, about 300 million people are estimated to be affected by malaria, a major killer disease, which threatens 2,400 million (about 40%) of the world's population (Snow et al., 2005). The task of controlling mosquito populations is of the utmost importance in

the fields of public health because mosquitoes serve as the major vector for many diseases (James, 1992). At the present, this parasitic disease is one of the major health problems in India.

Recently mosquito control is based on the use of commercial synthetic insecticides which have potential toxic effect on public health and the environment. Chemical insecticides have been used during the past several decades to control varied dipteran pests (Poopathi and Archana, 2010). Use of synthetic insecticides is causing various problems such as environmental contamination, insecticide resistance and toxic hazards to humans and animals (Vatandoost et al. 2005, Davari et al. 2007). Increasing levels of resistance to commonly used insecticides have invariably led to multiple treatments and excessive doses, posing serious threats to both the environment and human health (WHO, 1985). Pesticides are indeed very effective in its use. But along with their useful effects, they also bring out serious harm to human health as well. Furthermore, these chemicals are expensive and are often toxic to both human and other animals and natural enemies.

The intensive use of chemical insecticides led to the development of resistant insect populations, resulting in a reduced control and often to a negative impact on various non-target organisms and on the environment in general (Charles and Nielsen-LeRoux, 2000). Previously Larvicidal and growth inhibitory activities of eight plant volatile oils of *Acorus calamus* (calamus oil), *Cinnamomum veerum* (cinnamon oil), *Cymbopogon nardus* (citronella oil), *Myrtus caryophyllus* (clove), *Eucalyptus globulus* (Eucalyptus oil), *Mentha piperita* (menthe oil), *Citrus limon* (lemon oil), *Citrus sinensis*, (orange oil) (Manimaran et al., 2013) have been reported against *Anopheles stephensi*, *Aedes aegypti* and *Cx. quinquefasciatus.*

Terminalia chebula is a moderate tree used in traditional medicines. It is belongs to the family combretaceae. It is commonly called as Black myrobalan, Ink tree (or) Chebulic myrobalan. It is extensively used in unani, ayurveda and homeopathic medicine (SuryaPrakash, 2012). It is used for the treatment of number of diseases like cancer, paralysis, cardio vascular diseases, ulcers, leprosy, arthritis, gout and epilepsy (SuryaPrakash, 2012). It has been reported as antioxidant (Suchalata and Devi, 2005), antidiabetic (Rao et al., 2006), antibacterial (Kannan et al., 2009), antiviral (Kim et al., 2001), antifungal, anticancerous, antiulcer, antimutagenic, wound healing activities etc. Fruits contain astringent substances - tannic acid, Chebulinic acid (Lee et al., 2010), gallic acid etc. Resin and a purgative principle of the nature of anthraquinone and sennoside are also present.

The toxicity of the plant *Moschosma polystachyum* was evaluated against early third instar larvae of *Culex quinquefasciatus* (Rajkumar and Jebanesan, 2004). Ilahi and Ullah (2013) studied the insecticidal activity of *Artemisia vulgaris* against fourth instar larvae of *Cx. quinquefasciatus.* In light of such trends around the globe, the urgent need for the development of selective mosquito control alternatives that can help to procure an effective resistance management strategy should be addressed adequately and promptly. This study was aimed at assessing the potential of *T. chebula* and their extracts for possible use as larvicide and pupicide against *Cx. quinquefasiatus* under laboratory conditions and to determine the chemical composition of the effective extract.

Materials and methods

Mosquito culture

Larvae of *Cx. quinquefasiatus* were collected from stagnant water areas of Kanchipuam Town, Tamil Nadu. The colony of larvae and pupae were separated and maintained the cultures. They were kept in plastic and enamel trays containing tap water. They were maintained and all the experiments were carried out at (27±2ºC) and 75–85% relative humidity under 14h/10h light and dark cycles. The larvae were fed on diet composed of Brewer's yeast, dog biscuits and algae collected from ponds in a ratio of 3:1:1. The feeding was continued until the larvae transformed into the pupal stage.

Larval mortality assay

Third instar larvae of *Cx. quinquefasiatus* collected from insect-rearing cage. Fifty mg of the plant extract was first dissolved in 100mL of respective solvent and distilled water (stock solution). From the stock solution, different concentrations were prepared with de-chlorinated tap water. Triton 80

(Qualigens, Mumbai, India) was added as an emulsified at a volume ratio of 0.05% in the test concentration solution. The larval mortality test was assessed by standard WHO procedure (WHO, 1992). For bioassay test, the larvae were taken in five batches of 25 in 100 mL test solution with de-chlorinated tap water. Four concentrations were prepared i.e 62.5, 125, 250 and 500 ppm. The control was set up with respective solvent, distilled water and Triton 80. The number of dead larvae was counted after 96hr of exposure and the percentage of mortality was reported from the average of five replicates. No food was offered during treatment. Dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region. Moribund larvae were those incapable of rising to the surface (within reasonable period of time) or showing the characteristic diving reaction when the water was disturbed.

$$\text{Mortality (\%)} = \frac{\text{Number of dead larvae}}{\text{Number of introduced larvae}} \times 100$$

$$\text{Corrected mortality} = [(\text{mortality in treatment} - \text{mortality in control}/100) - \text{Control mortality}] \times 100$$

All experiments were tested at room temperature (28 ± 2°C) for the three different solvent extracts.

Pupal mortality assay

A laboratory colony of the pupae was used to observe the pupicidal activity of plant extracts. Twenty five individuals of freshly emerged pupae were kept in 300mL plastic cups containing 100mL of de-chlorinated water with different concentrations. Five replicates were maintaining with each concentration of treatment. The control was maintained with distilled water and Triton 80. The numbers of dead pupae were counted after 96 h of treatment exposure and percentage of mortality was reported from the average of five replicates.

Gas Chromatography / Mass Spectrograph (GC/MS) analysis

The effective extract was analyzed by GC/MS to identify their phyto-compounds. GC/MS was performed using a Varian 3800 gas chromatograph directly coupled to a Varian Saturn 2000 Ion Trap (ITD) mass spectrometer with the whole system controlled by the Saturn GC/MS workstation (v5.2). The column used was a J and W DB-5 fused silica capillary column (30 m). GC/MS operating conditions followed those described by Adams (1995): injector temperature, 220ºC; transfer line, 240ºC; oven temperature, from 60 to 240ºC at 3ºC/min; carrier gas, Helium at 0.8 ml/min at 220ºC; sample injection volume, 0.2 ll; split ratio, 1:20. MS acquisition parameters were as follows: full scan with scan range 41–300 amu; scan time, 1.0 s; threshold, 1 count; AGC mode: on; microscans: 5; filament delay: 120 s; manifold – 60ºC.

Column head pressure was adjusted to 9.4 psi with extracts, which was used as an internal standard to set the flow rate at 0.8 ml/min, at which the retention times matched those reported by Adams (1995). A 10% (v/v) respective solvent as well as a 1% (v/v) solvent of extract was injected to allow a better identification of compounds. This was necessary to increase the sensitivity for minor compounds, especially for the extract of the plant which was similar in their major compounds.

The chromatograms obtained with the 10% (v/v) solution allowed the identification of some minor compounds while those obtained with the 1% (v/v) solution allowed a more accurate quantification of the major compounds, which were saturated at 10%. The analysis was repeated three times for each sample. Compounds were identified by comparing their GC retention times and mass spectra with Adams (1995, 2001), aided with authentic compounds and NIST mass spectra library. Quantification of extracts components (expressed as percentage of total peak area of chromatogram) was carried out by peak area normalization measurements.

Statistical analysis

The data of average larval and pupal mortality were subjected to Probit analysis for calculating lethal concentration 50% and 90% (LC_{50} and LC_{90}) and 95% fiducial limits of upper and lower confidence limit and chi-square values were calculated by using the SPSS 11. 5 and EPA 1.5 version program for rapid determination of lethal concentrations. LC_{50} and LC_{90} were calculated from toxicity data by using Probit analysis (Finney, 1971). Results with $p<0.05$ were considered to be statistically significant.

Results

The activity of *Terminalia chebula* crude extracts is often attributed to the complex mixture of active compounds. The preliminary screening is a good mean of evaluation of the potential larvicidal and pupicidal activity of plants popularly used for this purpose. The yield of the extracts were weighed and noted in Table 1. The extract showed a greater yield for chloroform (1.842g) followed by hexane (0.952g) and ethyl acetate extracts (1.205g).

Table 1. Yield of *Terminalia chebula* leaf-extract in three different solvents.

Solvent used for extraction	Extract yield (g)
Hexane	0.952
Chloroform	1.842
Ethyl acetate	1.205

The results of *Cx. quinquefasciatus* percent larval mortality are presented in Table 2. The results showed that all the solvent extracts recorded larval mortality. Among different solvent extracts, chloroform extracts was found to be more active followed by hexane and ethyl acetate extracts. At 500 ppm concentration of treatment chloroform exhibited 87.20% larval mortality towards *Cx. quinquefasciatus*. And remaining extract of 500 ppm concentration of hexane and ethyl acetate extracts were resulted 72.80 and 40.80% to *Cx. quinquefasciatus* at 96hr treatment. Other concentrations of chloroform extract of 62.5, 125 and 250 ppm showed 14.40, 45.60 and 67.20% mortality against *Cx. quinquefasciatus* respectively. Significant and dose dependent mortality ratio were observed all the treatment groups. No larval mortality was observed in ethyl acetate extract at 62.5 ppm treatment level. The mortality rate was higher in 96h bioassay and third-instar larvae are generally more susceptible than the other instars against most of the plant extract.

The results of the larvicidal activity of crude hexane, chloroform and ethyl acetate solvent extracts of *T. chebula* against the larvae, *Cx. quinquefasciatus* is presented in Table 3. Among the extracts tested, the highest larvicidal activity was observed in chloroform extract against *Cx. quinquefasciatus* followed by hexane extract with the LC_{50} and LC_{90} values were 157.86 and 227.20 ppm and 552.08 and 884.51 ppm, respectively. The lethal concentration data clearly showed that chloroform extract of *T. chebula* was the most lethal treatment since its LC_{50} and LC_{90} were significantly less (Chi-square was 1.700; significant at $p \leq 0.05$) than other treatments. At 500 ppm concentration of treatment chloroform exhibited 56.00% pupicidal activity towards *Cx. quinquefasciatus* (Table 4).

Table 2. Mortality (%) of *Culex quinquefasciatus* larvae in *Terminalia chebula* leaf extracts.

Extract tested	Concentration (ppm)			
	62.5	125	250	500
Hexane	4.00 ± 4.00^{b}	20.00 ± 4.89^{d}	47.20 ± 3.34^{d}	72.80 ± 1.78^{d}
Chloroform	14.40 ± 2.19^{c}	45.60 ± 3.57^{e}	67.20 ± 5.21^{e}	87.20 ± 3.34^{e}
Ethyl acetate	0.00 ± 0.00^{a}	7.20 ± 1.78^{c}	24.80 ± 5.21^{c}	40.80 ± 5.21^{c}
Control	0.00 ± 0.00^{a}			

* within columns, means (± SD) followed by same letter do not differ significantly (Turkey's test, $p<0.05$ level)

Table 3. Lethal concentrations of *Terminalia chebula* leaf extracts against the larvae of *Culex quinquefasciatus.*

Extract tested	LC_{50}	95% Fiducial limit		LC_{90}	95% Fiducial limit		Chi-square
		Lower	Upper		Lower	Upper	
Hexane	227.20	243.13	321.78	884.51	686.90	1263.38	0.514*
Chloroform	157.86	137.32	180.60	552.08	441.05	753.46	1.700*
Ethyl acetate	577.26	466.46	794.01	2105.28	1353.21	4368.12	3.212*

*$\chi 2$ values are significant at $p<0.05$ level.

The remaining extract of 500 ppm concentration of hexane and ethyl acetate extracts were resulted 42.40 and 39.20% to *Cx. quinquefasciatus.* Remaining concentrations of chloroform extract of 62.5, 125 and 250 ppm showed 4.80, 17.60 and 39.20% pupal mortality against *Cx. quinquefasciatus* respectively. Significant and dose dependent pupal mortality were observed all the

treatment groups. No mortality was observed in hexane and ethyl acetate extracts at 62.5 ppm concentrations. The results of the ANOVA carried out on mortality of mosquito larvae and pupae by different solvent extracts using different concentrations as variable revealed significant difference in larval and pupal mortality ($p \leq 0.05$ levels) (Table 4).

Table 4. Pupal mortality (%) of *Culex quinquefasciatus* in *Terminalia chebula* leaf extracts.

Extract tested	Concentrations (ppm)			
	62.5	125	250	500
Hexane	0.00±0.00^{b}	11.20±1.78^{c}	23.20±4.38^{b}	42.40±3.57^{b}
Chloroform	4.80±1.78^{c}	17.60±2.19^{d}	39.20±1.78^{c}	56.00±9.38^{c}
Ethyl acetate	0.00±0.00^{a}	4.80±1.78^{b}	24.80±1.78^{b}	39.20±1.78^{b}
Control	0.00±0.00^{a}			

* within columns, means (± SD) followed by same letter do not differ significantly (Turkey's test, $p<0.05$ level)

Among the extracts tested, the highest pupicidal activity was observed in chloroform extract against *Cx. quinquefasciatus* followed by hexane extract with the LC_{50} and LC_{90} values were 386.16 and 579.50 ppm and 1150.31 and 1452.83 ppm, respectively (Table 5). Ethyl acetate extract showed highest LC_{50} and LC_{90} values. This value indicates very lowest mortality towards *Cx. quinquefasciatus*. The lethal concentration data clearly showed that chloroform extract of *T. chebula* was the most lethal treatment since its LC_{50} and LC_{90} were significantly less (chi-square was 1.191; significant at $p \leq 0.05$) than other treatments.

Analysis of the chloroform extract from the *T. chebula* revealed a complex mixture of constituents (Table 6 and Fig. 1). Totally 23 phyto-compound groups were present in GC-MS analysis. The extracts represent mainly a mixture of phyto-compounds. The extract obtained from the seeds of *T. chebula* had the phytol isomer as the major component (17.43%), followed by the stigmasterol, which constituted 11.40%. This extract also showed the presence of tetracosane (9.63%) and tetracontane (2.42%). Remaining other compounds was present below level of 4.84%.

Table 5. Lethal concentrations of *Terminalia chebula* leaf extracts against the pupae of *Culex quinquefasciatus.*

Extract tested	LC_{50}	95% Fiducial limit		LC_{90}	95% Fiducial limit		Chi-square
		Lower	Upper		Lower	Upper	
Hexane	579.50	462.03	815.70	2341.70	1452.83	5179.22	3.802*
Chloroform	386.16	320.36	496.21	1748.16	1150.31	3370.34	1.191*
Ethyl acetate	587.18	476.70	803.16	2016.93	1314.65	4101.50	4.184*

*$\chi 2$ values are significant at $p<0.05$ level

Fig. 1: GC-MS chromotogram of chloroform extract of *Terminalia chebula* leaves.

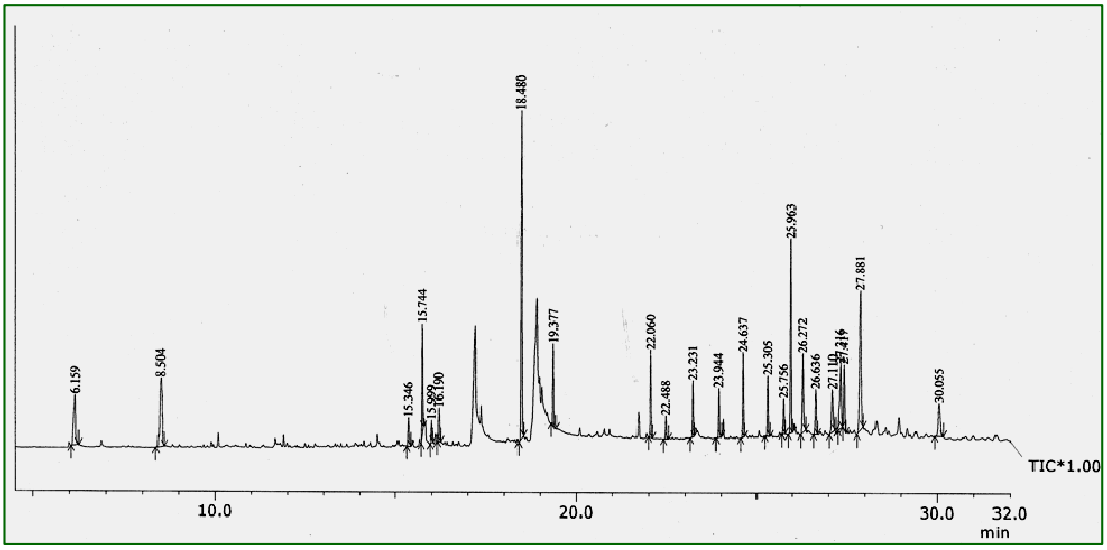

Table 6. GC-MS analysis of chloroform extract of *Terminalia chebula* leaves.

Peak No.	R. Time	Area	Area (%)	Name of phyto-constituent/group
1	6.159	44510225	4.72	1,2,3,-Propanetriol, monoacetate
2	8.504	63393938	6.72	Acetin, 1-mono-
3	15.346	15107323	1.60	(-)-loliolide
4	15.744	43583057	4.62	2,6,10-trimethyl,14-ethylene-14- pentadecne
5	15.999	8324771	0.88	3,7,11,15-tetramethylhexadec-2-en-1-ol
6	16.190	13245292	1.40	(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol
7	18.480	164493750	17.43	Phytol isomer
8	19.377	30135658	3.19	Neophytadiene
9	22.060	33185502	3.52	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester
10	22.488	9805942	1.04	Hexacosane
11	23.231	21591357	2.29	Hexatriacontane
12	23.944	19619908	2.08	n-Hexatriacontane
13	24.637	34835746	3.69	Tetratriacontane
14	25.305	25323268	2.68	N-tetratetracontane
15	25.756	15228137	1.61	Gamma.-Tocopherol
16	25.963	90885385	9.63	Tetracosane
17	26.272	41750609	4.42	Alpha.-Tocopherol-.beta.-D-mannosite
18	26.636	22866972	2.42	Tetracontane
19	27.110	28336978	3.00	Ergost-5-en-3-ol,(3.beta.)-
20	27.316	45714107	4.84	Stigmasterol
21	27.417	34175096	3.62	n-Hexatriacontane
22	27.881	107614909	11.40	Stigmast-5-en-3-ol,(3.beta.,24S)-
23	30.055	3020377	3.20	7,11,15-Tetramethyl-2-hexadecen-1-ol

Discussion

Mosquitoes are the most important insect groups that transmit many numbers of pathogens. This study confirmed the effects of mosquito species and extraction method on extract efficacy as reviewed by Mohan and Ramasamy (2007) and Sharma et al. (2006). In the present study the larvicidal and pupicidal potentials of chloroform extracts of seeds of the plant *T. chebula* was evaluated against *Cx. quinquefasciatus*. The extract of each part showed dose dependent increase in larvicidal and pupicidal activity. At higher doses, the extract caused a significantly higher mortality against the larvae of *Cx quinquefasciatus* vector species. Several investigations have mentioned that larvae of different mosquito species display different levels of susceptibility to the same extract to *Culex* larvae (Pizzaro et al., 1999; Rey et al.; 1999, 2001; Park et al., 2002). Ilahi et al. (2012) studied the larvicidal activity of aqueous extracts of bark, fruits and leaves of *Melia azedarach* (Linn) against *Cx. quinquefasciatus* at the concentrations of 50, 100, 500, 1000, 1500 and 2000 ppm. Among these extracts, the bark extract caused significantly higher mortality of 3rd and 4th instar larvae of *Cx. quinquefasciatus*.

The world health organization has estimated that globally the healths of at least 3 million people are affected by pesticides every year. Naturally occurring pesticides thus appear to have a prominent role in the development of future commercial pesticides, not only for agricultural crop productivity but also for the safety of environment and public health (Mandava, 1985).

The plants are considered as a rich source of bioactive molecules and they may be playing a vital role to control mosquitoes. Plant sources comprises enormously available group of phytochemicals and widely used to control mosquitoes (Jacobson, 1953). More than 2000 plant species have been reported to possess chemicals with pest control properties (Ahmed et al., 1984: Nataya et al., 2010; Pankaj and Anitha, 2010; Kamaraj et al., 2011) and among

them about 344 species of plants have been known to posses some degree of activity against the development of mosquitoes (Sukumar et al., 1991). Similarly, neem crude extract/ oil has specifically been reported by Kabaru and Gichia (2001) to inhibit mosquito development thereby disallowing pupation or adult emergence of the mosquito. Manimaran et al. (2013) have reported that eight plant volatile oils of *Acorus calamus* (calamus oil), *Cinnamomum verum* (cinnamon oil), *Cymbopogon nardus* (citronella oil), *Myrtus caryophyllus* (clove), *Eucalyptus globulus* (eucalyptus oil), *Mentha piperita* (menthe oil), *Citrus limon* (lemon oil), *Citrus sinensis*, (orange oil) and their oil formulation have larvicidal and growth inhibitory activity against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* at laboratory and field conditions.

A major drawback in the synthetic insecticide application is that they are non-selective and could be harmful to beneficial organisms, animals and human beings (Matsumara, 1975). Phytochemicals derived from various plants have proved as effective larvicidal and repelling compounds (Matsumara, 1975; Maradufa et al., 1978; Thangam and Kathiresan, 1993).

The use and practice of solvent extracts from certain plants for the control of mosquitoes have been studied by researchers since from longtime back. Botanical derivatives in mosquito control especially for mosquito larvae as alternative to synthetic insecticides offer a more environmentally friendly method of control (Irungu and Mwangi, 1995). Various plant species have been exploited to control the mosquito population throughout the world (Zebitz, 1984; Mwangi and Mukiama, 1988; Tare and Sharma, 1991; Sharma and Goel, 1994). The bioactive organic chemical contents may serve as insecticides, anti-feedants, oviposition deterrents, repellents, attractants etc. They are less toxic, easily biodegradable and do not have any adverse effects on the non target organisms.

In the present investigation the result of larval and pupal mortality of *T. chebula* against *Cx. quinquefasiatus* clearly showed that the potential effect at 500 ppm and the lowest mortality at 62.5 ppm, respectively. As the concentration of the plant extracts increases the total larval mortality and pupal were also found to be increased. The present results correlate with previous findings of Pushpanathan et al., (2006), when treated with *C. citrates* showed cent percent larvicidal activity on fourth instar larvae of *Cx. quinquefasciatus*. Govindarajan (2011) reported the larvicidal and ovicidal activity of *Cardiospermum halicacabum* leaf extracts against *Cx. tritaeniorhynchus* and *Ae. aegyptii*. Pushpanathan et al. (2006) reported the essential oils extracted from *Cymbopogan citratus* were evaluated for larvicidal and repellent activities against *Cx. quinquefasciatus*.

Conclusion

These results suggest that the chloroform extract of *T. chebula* leaves is promising as larvicides, pupicides against *Cx. quinquefasciatus.* Further studies are needed to determine the active ingredients in these phytochemicals and to know their mode of action, toxicity, and stability and to study their impacts on human health and non-target organisms in mosquito feeding habitats.

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