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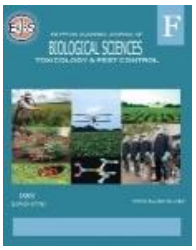

Efficacy of the Green Tea, *Camellia sinensis* Leaves Extract on Some Biological Activities of *Culex pipiens* and the Detection of Its Phytochemical Constituents

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ABSTRACT

The mosquitocidal and repellent activity of the green tea, *Camellia sinensis* leaves extract against *Culex pipiens* was evaluated. Toxicity of this extract increased with increasing the concentration, the recorded LC_{50} was 540 and 600 ppm for larvae and pupae, respectively. The extract showed a significant prolongation in both larval and pupal duration. In addition, it caused a great effect in pupation and adult emergence percent. The green tea, *Camellia sinensis* leaves extract was found to have a remarkable malformative effect on both larvae and pupae. The green tea leaves extract was effective in exhibiting antifeedant and repellent activity against *Culex pipiens* mosquitoes; it gave the highest protection (100%) from the bites of starved females at the dose of 6 mg/cm^2. The major bioactive chemical constituents in green tea leaves extract were identified using HPLC and FT-IR analysis. These results suggest that the leaves extract of green tea, *Camellia sinensis* can be considered as alternative mosquitocide and repellent agent.

INTRODUCTION

Culex pipiens is one of the most widely distributed species around the world. *Culex pipiens* is the primary vector of several arboviruses' diseases, such as West Nile virus, Japanese encephalitis virus, and Rift Valley fever virus, as well as worms responsible for lymphatic filariasis (Lerdthusnee *et al*., 1995; Jang *et al*., 2002; Nauen 2007 and Diaz-Badillo *et al*., 2009).

Mosquito control, in view of their medical importance, assumes global importance. Synthetic insecticides are quick, effective and most popular methods of pest control. However, repeated and injudicious application of synthetic insecticides has often resulted in the development of resistance insects strains (Liu *et al*., 2005). In addition to resistance, some mosquitocides may be associated with diverse environmental hazards, such as accumulation of non-biodegradable residue, biomagnification in the food chain, and toxicity to human and animal health (Rawani *et al*., 2009). Therefore, there is a growing need to find new, safe and effective alternatives.

The botanical insecticides are generally pest-specific and are relatively harmless to non-target organisms including man. As well as they are biodegradable and harmless to

the environment. Also, plants comprise a rich pool of phytochemical compounds that can be used to replace synthetic chemical insecticides (Ghosh *et al*., 2012).

Extracts from leaves, flowers, and roots of plants and oils were found to have mosquito larvicidal activity. Alaoui (2009) tested aqueous extracts of several medicinal plants, sage (*Salvia officinalis*), marjoram (*Origanum majorana*) and rosemary (*Rosmarinus officinalis*) on *Culex pipiens*; the mortality rate was very interesting. Furthermore, studies by Tchoumbougnang *et al*. (2009) have shown that *Thymus vulgaris* also has insecticidal activity against *Anopheles* and *Aedes*. The results found by Sayah *et al*. (2014) confirm the larvicidal activity of the essential oils of *Citrus aurantium*, *Citrus sinensis*, and *Pistacia lentiscus* which have been tested on *Culex pipiens* larvae.

Keeping in view the importance of plant extracts as a safe and major alternative to synthetic insecticides the present study was aimed to test larvicidal, pupicidal and repellent efficacy of green tea (*Camellia sinensis*) leaves extract against *Culex pipiens* mosquito under laboratory conditions.

MATERIALS AND METHODS

Mosquito Rearing:

Culex pipiens mosquitoes were used in this study; they were reared and maintained for several generations at the Medical Entomology Research Laboratory at Zoology and Entomology Department, Faculty of Science, Al-Azhar University, Cairo, under controlled conditions using the standard procedures (Kasap and Demirhan, 1992).

Plant Extraction:

The dried leaves of green tea, *Camellia sinensis* were pulverized into powder using a grinding machine. 10 g of powder was weighed and added to 100 ml of prepared solution, consisting of 70% ethanol + 8% ethyl acetate + 22% distilled water in a glass container. The mixture was immersed in a water-bath set at 60^{o} C for 5 hours and stirring of the mixture was performed at intervals. The mixture was then removed from the bath and allowed to cool at room temperature for 72 h; after that, it was filtered. The combined solvent extract was concentrated under vacuum at 40^{o} C until solvents were completely removed. This dried extract was used for the biological activity tests.

Extract Analysis:

The major compounds in the plant extract were identified using High-Performance Liquid Chromatography HPLC (Sykam ROUTINE HPLC System S 500) at EPRI institute.

The characterized function groups and the confirming of the constituents of the extract were analysed by FT-IR spectroscopy (Model 960 Moog, ATI Mattson Infinity Series, USA) at EPRI institute.

Bioassays:

Different concentrations of the plant extract were prepared by dissolving the required amount of the plant extract in distilled water, 1 ppm of PEG 40 Hydrogenated Castor Oil was added as an emulsifier.

Bioassays were conducted in plastic cups containing the needed amount of the plant extract dissolved in 250 ml of distilled water. Twenty-five third instar larvae of the mosquito were then transferred to the test medium; the experiment was performed in three replicates for each concentration. Control larvae received 1 ppm of (PEG40) in 250 ml of distilled water. All larvae were exposed until pupation, and mortality was recorded at 24-h intervals. Dead larvae and pupae were counted and removed immediately.

The morphological aberrations of the dead larvae and pupae were determined and counted; also, the larval and pupal durations were calculated.

The growth index was estimated by using the following equation of Saxena and Sumithra, (1985):

Growth index = A / B (where: A = % of adult emergence and B = mean developmental period in days).

Repellent Activity:

Evaluation of the repellent activity of extract of *Camellia sinensis* leaves was carried out against the adult females of *Culex pipiens*. Three to four days old blood starved female *Culex pipiens* mosquitoes were kept in a net cage (20 cm x 20 cm x 20 cm). One ml from each concentration was directly applied on 30 cm^2 of the ventral surface of a pigeon after removal of feathers, and then the pigeon was placed in the cage containing the starved mosquitoes. After two hours the fed and unfed females were counted. The experiment was conducted three times; fifteen females were used in each experiment. The negative control test was carried out using water and PEG40, and a positive control test was carried out using commercial repellent (DEET) from Jonson Wax Egypt.

The repellency percent was calculated by using the following equation (Abbott, 1925):

Repellency % = [(%A -%B) / (100- %B)] x 100 (where: A = percent of unfed females in treatment and B = percent of unfed females in control).

Statistical Analysis:

Statistical analysis of the data was carried out according to the method of Lentner *et al*., (1982). LC_{50} was calculated using multiple linear regression (Finney, 1971).

RESULTS

Larval mortality of *Culex pipiens* after the treatment by the extract of *Camellia sinensis* leaves was observed. Data presented in Table (1) showed that *Camellia sinensis* leaves extract had no effect on the third instar larvae of *Culex pipiens* after 24h, while after 48h only the two highest concentrations 800 and 1000 ppm showed 1.3% of larval mortality. The concentration of 1000 ppm caused 4% of mortality after 72h.

By the end of the larval stage, the complete larval mortality (100%) attained at the highest concentration (1000 ppm). Meanwhile, the larval mortality was 13.3 % at the lowest concentration (200 ppm) compared to 0 % for untreated larvae.

Table (1): Effect of *Camellia sinensis* leaves extract on the mortality, malformation duration of larvae and pupation percent of *Culex pipiens*.

Conc.	Larval mortality %				Larval	Larval dur.	Pupation
(ppm)	24h	48h	72h	total (at the end of l.d.)	mal. %	mean ± SD.	%
Control	_	_	_	_	_	4.6 ± 1.2	100
200	_	_	_	13.3	5.3	7.8 ± 2.4*	86.7
400	_	_	_	28	16	8.9 ± 1.3*	72
600	_	_	1.3	49.3	42.7	10.5 ± 2.6**	50.7
800	_	1.3	_	78.7	69.3	13 ± 2.5**	21.3
1000	_	1.3	4	100	96	_	_

Where: Conc = concentration, l.d. = larval duration, mal. = malformation, dur. = duration, SD = Standard Deviation, * = Significant ($P < 0.05$) and ** = highly Significant ($P < 0.01$).

The *Camellia sinensis* leaves extract induced remarkable malformation effects on the treated larvae. At the lowest concentration (200 ppm) the malformation percent was 5.3%, this percentage increased to 96% at the highest concentration (1000 ppm). There were no malformed larvae in the control group.

The mean larval duration was significantly affected by all used concentrations of *Camellia sinensis* leaves extract; it reached to 13 ± 2.5 days at the concentration of 800 ppm compared to 4.6 ± 1.2 days for the control group.

Pupation percent of treated larvae was decreased as the concentration increased. At the highest concentration (1000) ppm no pupae were produced compared by 100% of the untreated ones.

Data given in Table (2) indicated that the lethal effect of *Camellia sinensis* leaves extract was extended to the pupal stage, the pupal mortality percent reached to 68.8% at the concentration of 800 ppm compared by 1.3% of pupal mortality at the control group. On the other hand, the malformation percent was increased by increasing the concentration; it recorded 68.8% at the concentration of 800 ppm, while at the control group no malformed pupae were observed.

The mean pupal duration was 1.56 ± 0.52 days for the control group; this duration was significantly prolonged by all used concentrations, it reached to 2.6 ± 0.55 days at the concentration of 800 ppm.

The total larval and pupal mortality was increased to 100% at the highest concentration (1000 ppm), while it was 25.3% at the lowest concentration (200 ppm) compared by 1.3% at the control group.

A negative correlation between the adult emergence percent and the concentration was observed, where it recorded 0.0% at the highest concentration (1000 ppm) and 86.2% at the lowest concentration (200 ppm). The adult emergence percent at the control group was 98.7%.

Table 2: Effect of *Camellia sinensis* leaves extract on mortality, malformation, duration of pupae, adult emergence percent, sex ratio and growth index of *Culex pipiens.*

Conc. (ppm)	Pupal mort. %	Pupal mal. %	Pupal dur. mean ± SD.	larval and pupal mort. %	Adult emerg. %	Sex ratio (male: female)	Mean development period (days) ± SD.	Growth index
Control	1.3	_	1.56 ± 0.52	1.3	98.7	(0.9 : 1)	6.16 ± 1.72	16
200	13.8	3.1	2.7 ± 0.74*	25.3	86.2	(0.8 : 1)	10.5 ± 3.14*	8.2
400	24.1	13	2.6 ± 0.63*	45.3	75.9	(0.9 : 1)	11.5 ± 1.93**	6.6
600	55.3	36.8	2.53 ± 0.52*	77.3	44.7	(1.1 : 1)	13.03 ± 3.12**	3.43
800	68.8	68.8	2.6 ± 0.55*	93.3	31.3	(1.5 : 1)	15.6 ± 3.05**	2.01
1000	_	_	_	100	_	_	_	_

Where: Conc. = concentration, mort. = mortality, mal. = malformation, dur. = duration, emerg. = emergence SD = Standard Deviation, * = Significant (P < 0.05) and ** = highly Significant (P < 0.01).

The results in Table (2) showed also that the sex ratio of adults emerged from pupae developed from untreated larvae (male: female) was (0.9: 1), while the larvae treated with different concentrations of *Camellia sinensis* leaves extract revealed more or less ratio of emerged males to females. The calculated sex ratio was (0.8: 1) at the lowest concentration (200 ppm) and (1.5: 1) at the highest concentration (800 ppm).

The mean developmental period was significantly prolonged by increasing the concentration; it recorded 15.6 ± 3.05 days at the concentration of 800 ppm compared by 6.16 ± 1.72 days for the untreated group.

A very retarded effect on the growth of larvae, pupae, and adults *Culex pipiens* was

observed especially at the highest concentration (800 ppm), the growth index recorded 2.01 compared by 16 for the untreated group.

The data in Table (3) showed the LC_{50}, the Slope (b) and the Correlation coefficient (r^2) for both larval and pupal mortalities. As shown from the results, the calculated LC_{50} was 540 ppm for the larval mortality, and 600 ppm for the pupal mortality.

Table 3: Relative efficiency of *Camellia sinensis* leaves extract against *Culex pipiens* larvae and pupae

Stage	LC_{50} (PPM)	Slope (b)	Correlation coefficient (r^2)
Larvae	540	0.1025	0.9808
Pupae	600	0.0883	0.9641

The repellent activity of *Camellia sinensis* leaves extract against starved *Culex pipiens* females was recorded in Table (4). At the highest dose (6 mg/cm^2), the extract induced 100% repellency, while it induced 25.6% repellency at the lowest dose (0.75 mg/cm^2) compared to 100% repellency for DEET.

Table 4: Repellency effect of *Camellia sinensis* leaves extract on *Culex pipiens* females.

Dose mg/cm^2	No. of tested females	Fed females		Unfed females		Repellency %
		No.	%	No.	%	
0.75	45	32	71.1	13	28.9	25.6
1.5	45	24	53.3	21	46.7	44.2
3	45	12	26.7	33	73.3	72.1
6	45	0	0	45	100	100
DEET	45	0	0	45	100	100
Control	45	43	95.6	2	4.4	_

Figure (1) showed the chromatogram of *Camellia sinensis* leaves extract by HPLC. The eleven major compounds were identified with different retention periods, as shown in Table (5). The results demonstrated that peaks numbers 1, 2, 6 and 11 are qualitatively important components in *Camellia sinensis* leaves extract. They were identified as theanine, theobromine, caffeine, and catechin, respectively. Peaks numbers 3, 4, 5, 7, 8, 9 and 10 were identified as gallocatechin, epigallocatechin, epicatechin, epigallocatechin gallate, catechin gallate, gallocatechin gallate, and epicatechin gallate, respectively. The molecular structures of these compounds are shown in Figure (2).

The FT-IR analysis was taken for the characterization of *Camellia sinensis* leaves extract constituents and is given in Figure (3). FT-IR spectra showed peaks which may be assignable to O–H and/or N–H stretching (3386.43 cm^{-1}), symmetric and asymmetric of methyl groups (2941.56 and 2887.77 cm^{-1}), C = C stretching (2090.75 cm^{-1}), C = O stretching (1644.61 cm^{-1}), benzene ring (1410.85 cm^{-1}), C–N stretching (1232.59 cm^{-1}),

C–O–C stretching (1110.13 and 1043.98 cm^{-1}), secondary cyclic alcohols (993.17 cm^{-1}), aromatic C–H bending (923.65, 852.49 and 674.86 cm^{-1}).

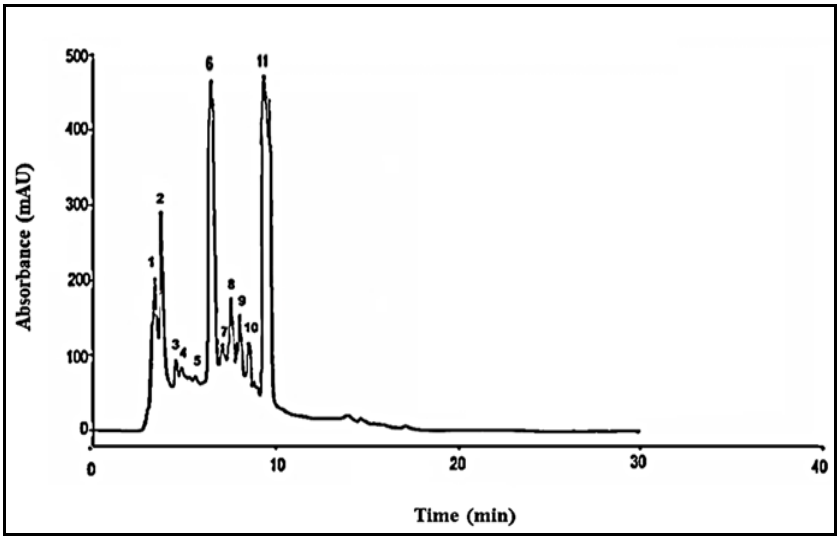

Fig. 1: HPLC chromatogram of *Camellia sinensis* leaves extract

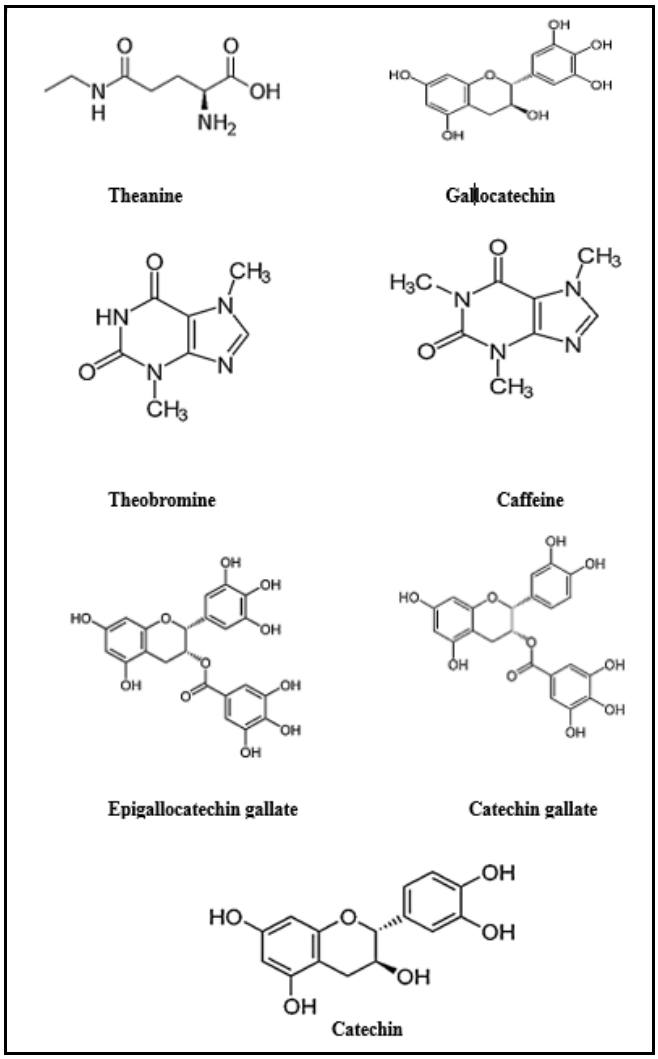

Fig. (2): The molecular structures of *Camellia sinensis* leaves extract components

Table 5: The main chemical components of *Camellia sinensis* leaves extract.

Peak no.	Ret. time (min.)	Chemical formula	Compound name
1	3.4	$C_7H_{14}N_2O_3$	Theanine
2	3.7	$C_7H_8N_4O_2$	Theobromine
3	4.5	$C_{15}H_{14}O_7$	Gallocatechin
4	4.8	$C_{15}H_{14}O_7$	Epigallocatechin
5	5.5	$C_{15}H_{14}O_6$	Epicatechin
6	6.4	$C_8H_{10}N_4O_2$	Caffeine
7	7	$C_{22}H_{18}O_{11}$	Epigallocatechin gallate
8	7.5	$C_{22}H_{18}O_{10}$	Catechin gallate
9	7.9	$C_{22}H_{18}O_{11}$	Gallocatechin gallate
10	8.4	$C_{22}H_{18}O_{10}$	Epicatechin gallate
11	9.4	$C_{15}H_{14}O_6$	Catechin

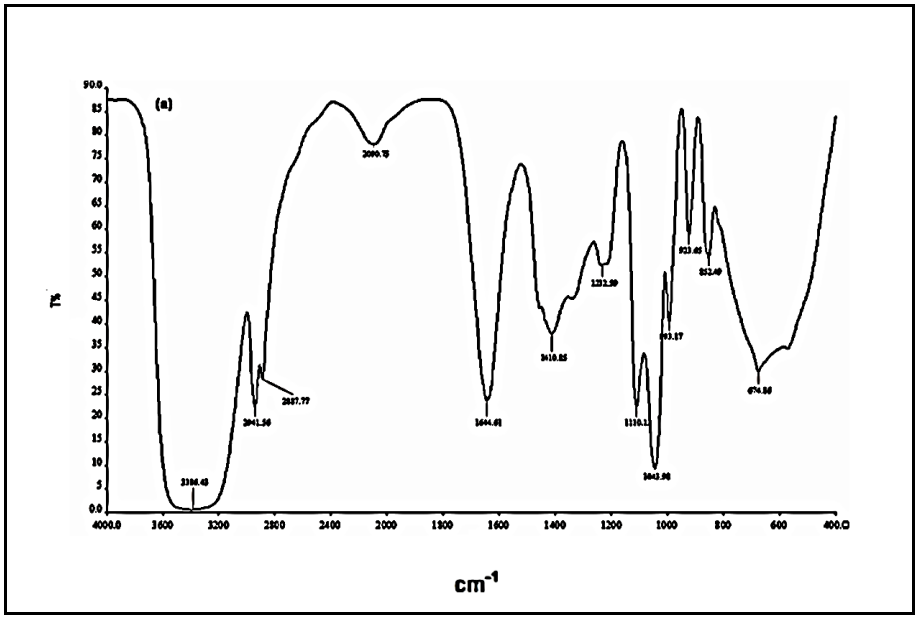

Fig. 3: FT-IR spectrum of *Camellia sinensis* leaves extract.

DISCUSSION

Mosquito-borne diseases are one of the most public health problems in developing countries. It can be controlled by preventing mosquito bite using repellent, causing larval mortality and killing mosquitos. Eliminating the larval stage is advantageous because the mosquitoes cannot disperse or acquire human pathogens (Hardin and Jackson, 2009).

Due to the numerous problems associated with the use of synthetic insecticides, there are extreme concerns in plant extracts as potential sources of natural insect control agents.

In this study, we evaluate the insecticidal and repellent potency of *Camellia sinensis* leaves extract as a promising insecticide and repellent agent against *Culex pipiens* mosquito.

Our results reveal a direct relationship between the larval mortality and the exposure time and also between the mortality rate of larvae and the concentration, to which they were exposed. The LC_{50} was found to be 540 and 600 ppm for larvae and

pupae, respectively. Moreover, there was a remarkable reduction in pupation and adult emergence percent. These results are in agreement to some extent with those of Jackson *et al*. (2016), they reported that larval exposure to methanolic crude extract of *Camellia sinensis* leaves extract at 250 ppm and 500 ppm for 24h resulted in larval mortality rate of over 90 % in *Anopheles gambiae* and 75 % in *Anopheles arabiensis*. A relatively lower concentration of 100 ppm resulted in moderate mortality rates of < 50% in both species, but induced growth disruption effects evident as abnormal larval-pupal intermediates and disrupted adult emergence.

Exposure of immature larval and pupal stages to *Camellia sinensis* leaves extract induces significant effects on the developmental timing of both larvae and pupae. These results agree with Dieng *et al*., (2016) report where dengue vectors exposed to sublethal doses of *Camellia sinensis* leaves extracts were found to exhibit an extended larval developmental time, reduced pupation and adult emergence rates. It was, therefore, speculated that this extract could have induced impairment of these important hormone-regulated developmental steps and processes. Also, a similar phenomenon was reported by Lopez *et al*. (2015), when *Drosophila melanogaster* larvae were treated with green tea polyphenolic extracts.

The *Camellia sinensis* leaves extract induced several morphological aberrations in the larvae, pupae, and adults of *Culex pipiens*. The percent of morphological aberrations in *Culex pipiens* increased with greater concentrations of the extract. Abnormalities commonly observed in this work were deformed larvae or larvae with dark colour. Dead larval-pupal intermediate stages mostly with the head of a pupa and the abdomen of a larva had been observed. Also deformed, pupal-adult intermediates and incompletely emerged adults had been observed. These results indicate a metamorphosis-inhibiting effect of the *Camellia sinensis* leaves extract, which could be caused by a disturbance of hormonal control or interference in chitin synthesis during the molting process (Saxena and Yadav, 1983; Mwangi and Rembold, 1988 and Al-Sharook *et al*., 1991). Several previous studies have described morphological aberrations induced by plant extracts on mosquito larvae. Karmegam *et al*. (1997) observed that indigenous plant extracts affect *Culex quinquefasciatus* larval morphology such as larval-pupal intermediates. Similar observations were made by Khater and Shalaby (2008) with abnormalities of *Culex pipiens* larval morphology such as pigmented and twisted larvae after exposure to some plant oils. The malformed larvae, pupae, and adults were not able to develop normally and died.

The growth index was considerably reduced; it decreased as the concentration of the *Camellia sinensis* leaf extract increased. These results are in line with Saxena *et al*. (1993) and Shaalan *et al.* (2005).

The *Camellia sinensis* leaves extract possesses effective skin repellent activity against *Culex pipiens*, it induced 100% repellency at the dose of 6 mg/cm^2. The present results are in agreement with results obtained by Tawatsin *et al*. (2001) who reported repellent activity against *Aedes aegypti*, *Anopheles dirus*, and *Culex quinquefasciatus*, which is due to 5% vanillin, which has been added to the essential oil of *Curcuma longa*. Venketachalam and Jebanesan (2001) have also reported that the repellent activity of methanol extract of *Ferronia elephantum* leaves against *Aedes aegypti* activity at 1.0 and 2.5 mg/cm^2 concentrations gave 100% protection up to 2.14±0.16 h and 4.00±0.24 h, respectively, and the total percentage protection was 45.8% at 1.0 mg/cm^2 and 59.0% at 2.5 mg/cm^2 for 10 h.

Phytochemicals derived from plant sources act as larvicides, insect growth regulators, repellent, and ovipositor attractant and have different activities which have been observed by many researchers (Venketachalam and Jebasan, 2010). Our results

demonstrated that the *Camellia sinensis* leaves extract contains eleven major compounds identified by HPLC and confirmed by FT-IR analysis.

Caffeine is a xanthine alkaloid, and an important component in green tea extract because of its stimulant properties. Caffeine may play an important role against some pathogens (Van Breda *et al*., 2012) and also, it was reported to interfere with mosquito larval development (Laranja *et al*., 2003). Itoyama and Bicudo (1992) showed the harmful effects of caffeine on *Drosophila prosaltans*, related to fecundity, egg-laying capacity and longevity which is consistent with our results.

A series of polyphenolic compounds such as catechins (e.g. gallocatechin, epigallocatechin, epicatechin, epigallocatechin gallate, catechin gallate, gallocatechin gallate, epicatechin gallate, and catechin) have been identified from *Camellia sinensis* leaves extract. Catechins are reported to have an antioxidant effect (Zhang *et al*., 1997, Hodgson, 2008). Ray *et al*. (1999) reported that the toxicity of polyphenols is exerted on the midgut epithelium of larvae; this observation supports the toxicity of the phenolic derivative, catechins. Also, Catechin, a phenolic derivative, has been reported previously from *Casuarina equisetifolia* and other plants like *Ricinus communis* and *Ulmus davidiana* to possess insecticidal properties (Jang *et al*., 2002). Elumalai *et al*. (2015) found that the isolated catechin from *Leucas aspera* showed pronounced larvicidal activity at very low concentrations. The LC_{50} and LC_{90} values of catechin were 3.05 and 8.25 ppm against *Ae. aegypti*, 3.44 and 8.89 ppm against *An. stephensi*, and 3.76 and 9.79 ppm against *C. quinquefasciatus*, respectively. Catechin in roots of *Podocarpus nagri* showed growth inhibitory effects on *Heluothis virescens* larvae (Zhang *et al*., 1992).

Conclusion

In this study, we assayed some biological activities and repellent effects of green tea, *Camellia sinensis* leaves extract, as it is one of the most consumed beverages in the world, against *Culex pipiens* mosquito. We found that it exhibits toxic effects on *Culex pipiens* larvae and pupae. The time of development for both larvae and pupae was prolonged. This is probably due to the high concentration of active compounds present in green tea such as caffeine and catechins. Also, these results indicated that green tea has a remarkable repellent effect against *Culex pipiens* females. These results demonstrate the potential of this plant species to be included as an alternative to control and repellent for *Culex pipiens*.

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