



Original article

Larvicidal activity of plant extracts by inhibition of detoxification enzymes in *Culex pipiens*

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ABSTRACT

Objectives: Plant extracts have been a safe and eco-friendly substituent of chemical pesticides, used against mosquitoes to prevent vector-borne infections e.g., chikungunya, yellow fever, dengue, filariasis, dirofilariasis tularemia, malaria, and many other diseases.

Methods: The larvicidal activities of the acetone extracts of four plants e.g., *Lantana camara*, *Ruta chalepensis*, *Rhazya stricta*, and *Acalypha fruticosa* was evaluated against *Culex pipiens*. The composition secondary metabolites of the most effective extract (*L. camara*) was examined using gas chromatography-mass spectrometry (GC-MS). The expression level of Acetylcholinesterase (AChE) and Glutathione S-transferase (GST) genes were assessed through qRT-PCR.

Results: The results revealed that the extract of *L. camara* caused 98% mortality in *Cx. pipiens* followed by *R. stricta* (91%), *A. fruticosa* (79%), and *R. chalepensis* (69%) as compared to azadirachtin, used as a positive control. The results showed that LD₅₀ and LD₉₀ of the extract of *L. camara* were significantly higher as compared to *R. stricta*, *A. fruticosa*, and *R. chalepensis* extract. The extract of *L. camara* also significantly reduced the activities of AChE and GST as compared to the larvae, treated with the extracts of another plant extracts as well as a positive control. The chemical composition of *L. camara*, was determined through GC-MS.

Conclusions: The most important insecticidal compounds were including undecane, terephthalic acid, dimethyl-propane-thiosulfinate, fluorobenzoic acid octadecenoic acid. The insecticidal activity of the *L. camara* extracts against *Cx. pipiens* might be due to one or more of these compounds.

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1. Introduction

Mosquitoes are the most important pest, cause several infectious diseases e.g., dengue, chikungunya, malaria, filariasis, yellow fever, dirofilariasis tularemia, and many other diseases. *Culex pipiens* are common house mosquito which transmits several infections for example West Nile fever, Sindbis fever, lymphatic filariasis, and Japanese encephalitis (Brugman et al., 2018). *Cx. pipiens pipiens* and *Cx. pipiens pallens* are the two subspecies of *Cx. pipiens* (Yang et al., 2004). Female *Cx. pipiens* normally feed on the blood of vertebrae including human beings and birds and hence transmits infectious diseases from birds to humans and from

humans to humans. Eggs are laid in clumps on the surface of stagnant water bodies, such as pools, ditches, water tanks, and vases (Jang et al., 2020). The diseases transmitted by *Cx. pipiens* cause tens of thousands of deaths annually. Moreover, a majority of these diseases, for instance, West Nile fever, St Louis encephalitis, and lymphatic filariasis are not vaccine-preventable in humans (Rehman et al., 2019). Therefore, pest management remains an indispensable tool in the control and prevention of mosquito-borne infections.

Although *Lantana camara* is an ornamental plant, it is considered an invasive species in agricultural areas (Ghisalberti, 2000). *L. camara* is a valuable source of biologically active compounds, and phytochemical studies have shown the metabolites including terpenoids, phenylethanoid glycosides, flavonoids, and flavonoids are present in *L. camara*. Antifungal, antiprotozoal, antibacterial antiviral, allelopathic properties, antioxidant, activities of the metabolites extracted from *L. camara* has been detected (Elumalai et al., 2017). *Ruta chalepensis* is another small perennial shrub, used for the treatment of fever and joint pain in indigenous Arabic medicine (Günaydin and Savci, 2005). *R. chalepensis* is a

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flowering plant evergreen herb that grows up to 80 cm tall. Because of the variety of biological activities caused by their secondary metabolites, including antifungal, antioxidant, and anti-inflammatory properties, *Rutaceae* species have attracted a great deal of interest. In the Mediterranean region, one of the widely distributed species is *R. chalepensis*, usually referred to as fringed rue. The plant abounds in alkaloids and is well-known for its antioxidant properties (Elumalai., 2017). *Acalypha fruticosa* is also very well-known medicinal plant, belongs to the spurge family of Angiosperms. Dried leaves of *A. fruticosa* are used for making tea in Ethiopia. Furthermore, the leaf extracts of plants also show growth inhibition of agricultural pests (Lingathurai et al., 2011). *A. fruticosa* has been used extensively as a repellent or insecticide against ectoparasites and flies and also used as food, fodder. The powder of the dried leaves, soak in water is used heal the skin of animals. *Rhazya stricta* is known to possess some biological activity against insects and used in folk medicine. It was shown to be rich in alkaloids of different types, flavonoids, sterols and volatile oil. *R. stricta* possess a strong antimicrobial activity and also possess bioactive molecules such as alkaloids, terpenoids, antioxidants.

The insects manage the toxicity through their antioxidant defense mechanism, which consists of both enzymatic and non-enzymatic antioxidant components (Turhan et al., 2020; Türkan et al., 2020). Acetylcholinesterase (AChE) and Glutathione S-transferases (GSTs) are multifunctional enzymes that play a central role in the detoxification of both endogenous and xenobiotic compounds and protection against the toxicity (Taslimi and Gülçin, 2018; Gülçin et al., 2020b).

In the present study, the pesticidal activity of acetone extracts from *L. camara*, *R. chalepensis*, *R. stricta*, and *A. fruticosa* leaves was tested against *Cx. pipiens*. The basic aim of the study was to compare the insecticidal activities of the four plants and to identify the most significant plant against the *Cx. pipiens*. moreover, the study was also aimed to determine the capability to overcome the antioxidant enzymes such as AChE and GST in larvae which are produced and withstand the toxicity.

2. Materials and Methods

2.1. Reproduction of *Cx. pipiens*

The *Cx. pipiens* larvae have been collected from the laboratory for mosquito studies from the Mosquito Research Laboratory, King Abdulaziz University Saudi Arabia. The larvae were reared on artificial food, added in containers (25 × 35 × 6 cm) filled water. The colony was maintained under the following conditions: 27 °C ± 2 °C, 70% ± 5% R.H.

2.2. Collection and processing of plant material

The plants used in the study, i.e., *L. camara*, *R. chalepensis*, *R. stricta*, and *A. fruticosa*, were collected from different places in Saudi Arabia. The leaves were plucked, washed with tap water, and shade dried for a few days. Finally, the dried leaves were ground to a fine powder with the help of a laboratory grinder (Phillips, Germany).

2.3. Preparation of extract of different plants

Exactly 40–60 g of leaf powder was added to a Soxhlet apparatus, along with 200 ml of absolute acetone. The extraction continued for six hours. The extracts were then concentrated in a rotary evaporator until they became considerably viscous. To prepare the stock solutions, 1 ml of acetone extracts were mixed with 99 ml of 0.3% dimethyl-sulphoxide (prepared in distilled water). Finally,

100, 300, 500, 700, and 1000 mg/l of working solutions were made in distilled water.

2.4. Larvicidal bioassay of different plants against *Cx. pipiens*

After preparing the aforementioned concentrations of the plant extract, each preparation was added to 250 ml glass beakers. Then 25 early fourth instar larvae were introduced in each assembly. Experiments were run in quadruplicates. The larvae were given standard larval food and the temperature was kept 28 ± 2 °C. After 24 h of exposure, percentage mortality was noted. Thenceforth, cumulative mortalities of larvae and pupae were documented daily. Live pupae were transferred to new beakers containing fresh water. Partially emerged adults or adults who failed to leave the water surface were not considered viable (Maheswaran et al., 2008). Negative and positive control reactions were conducted. In the positive control, 1% of Azadirachtin was used. LC₅₀ and LC₉₀ of the plant extracts were also calculated using probit analysis.

2.5. Assessment of AChE in larvae

Acetylthiocholine was used to evaluate the AChE activity as described by Ellman et al. (1961). Five heads of the mosquito larvae were homogenized in a solution containing 1 ml Triton X-100, 38.03 mg ethylene glycol tetra-acetic acid (EGTA), 5.845 g NaCl, and 80 ml Tris buffer (10 mM, pH 7). At 5000 rpm, the homogenate was centrifuged for 5 min and 100 µl of supernatant was added to 1 ml of Tris (0.1 M, pH 8), 100 µl of 5–5'-dithiobis (2-nitrobenzoic acid) (DNTB) in Tris (0.01 M, pH 8) buffer. 100 µl acetylthiocholine was added after 5 min. The activity of AChE was measured by a spectrophotometer (Shimadzu, Japan) at 412 nm (Topal et al., 2017; Cakmak and Gülçin, 2019; Aras et al., 2019; Gülçin et al., 2020a).

2.6. Analysis of GST activity in larvae

The decapitated larvae were homogenized in 0.1 M sodium phosphate buffer at pH 6, centrifuged for 30 min at 10,000 rpm and activity the GST was assessed as described by Gülçin et al., (2016a) Absorption changes were recorded at 340 nm by means of spectrophotometers (Shimadzu, Japan). According to Bradford (1976), the protein content was evaluated using bovine serum albumin as the standard (BSA, Sigma).

2.7. Identification of compounds through GC–MS analysis

The plant selected has been dried and crushed into a fine powder using mortar and pestle. During the drying and extraction of plant metabolites, proper action was taken to ensure that bioactive constituents were not lost or destroyed. Using acetone, the extraction was carried out and the extract was filtered through a 0.45 µm filter. At a split ratio of 10:1, exactly 2 µl of sample volume was injected into GC–MS (Agilent Technologies, USA). The helium gas (He) was used at a flow rate of 1 ml/min as a carrier gas. The column was set at 60 °C for 2 min and then increased to 160 °C for 5 min at a rate of 5 °C/min. In the electron ionization system, in electron impact mode, the ionization energy to detect the ions was 70 eV. Compared with the library present in the NIST library was the spectrum of detected compounds in the extract.

2.8. Statistical analysis

The results were presented as mean ± SD, and to perform statistical analyses, MS office Excel 2016 was used. DMRT was performed using the Statistic Analysis Method (SAS 9.1, USA) to detect major variations between treatments and controls.

3. Results and discussion

3.1. Larvicidal, pupicidal and emergence inhibition bioassay of different plant extract

The compounds are often inexpensive, biodegradable, and highly effective, therefore they are used frequently against the pest (Ahmed et al., 2016). The results of our study showed that the larvicidal activity of *L. camara* extract was 14% when 100 mg/l concentration was applied. However, the mortality was increased up to 98% when 1000 mg/l concentration was applied. Interestingly, fewer adults emerged from the surviving pupae, as we increased the concentration of the plant extract. This indicated that the plant extract is highly toxic for the pupae, caused incomplete emergence of an adult from a pupa (Fig. 1A and 1B). The extract of the *L. camara* caused inhibition of pupa production from larvae (Fig. 1C and 1D) and also caused larvae deformities (Fig. 1E and 1F). The total percentage inhibition, observed at 1000 mg/l was up to 98% (Fig. 2A).

Previous studies reported very strong larvicidal activities of acetone, ethanolic and methanolic extracts of the *L. camara* against *Anopheles stephensi*, *Aedes aegypti* and *Cx. quinquefasciatus*, larvae (Abutaha et al., 2018a). The larvicidal activity of *L. camara* was evaluated in previous studies and the results revealed a very high level of insecticidal activities against mosquitos (Fatope et al.,

2002). The methanol and ethanol extract were prepared and their larvicidal activity was observed against *Cx. pipiens* and *Aedes aegypti*. The extracts exhibited a profound larvicidal activity.

The *R. stricta* plant extract was also used to determine the larvicidal activity against the *Cx. pipiens*. The percentage of larvae mortality was 6, and 71% when 100 and 1000 mg/l of the extract *R. stricta* was applied respectively. Similarly, only 9 pupae out of 29 were transformed in to adults, showed 91% inhibition at the concentration of 1000 mg/l (Fig. 2B). Commercially available larvicidal chemical azadirachtin was used as a positive control.

The rate of larval mortality of *A. fruticosa* extract was 5 and 64% at 100 and at 1000 mg/l respectively. Moreover, at the concentration of 1000 mg/ml, 21 out of 36 larvae were transformed in to adults and the percent inhibition at 100 and 1000 mg/l was 16 and 79% respectively (Fig. 3A). Commercially available larvicidal chemical azadirachtin was used as a positive control. The chloroform extracts of the leaves of *A. fruticosa* showed the best activity against the cabbage moth. The leaves were found to have terpenoids, saponins, and other pesticidal compounds. Previous study showed the potential of leaf extracts of *Acalypha indica* against *Anopheles stephensi* (Lingathurai et al., 2011).

The rate of mortality of larvae after the application of 1000 mg/l of the extract of *R. chalepensis* was 49%. The number of adults emerging from the surviving pupae were decreased and percent inhibition at the concentration of 1000 mg/l was increase up to

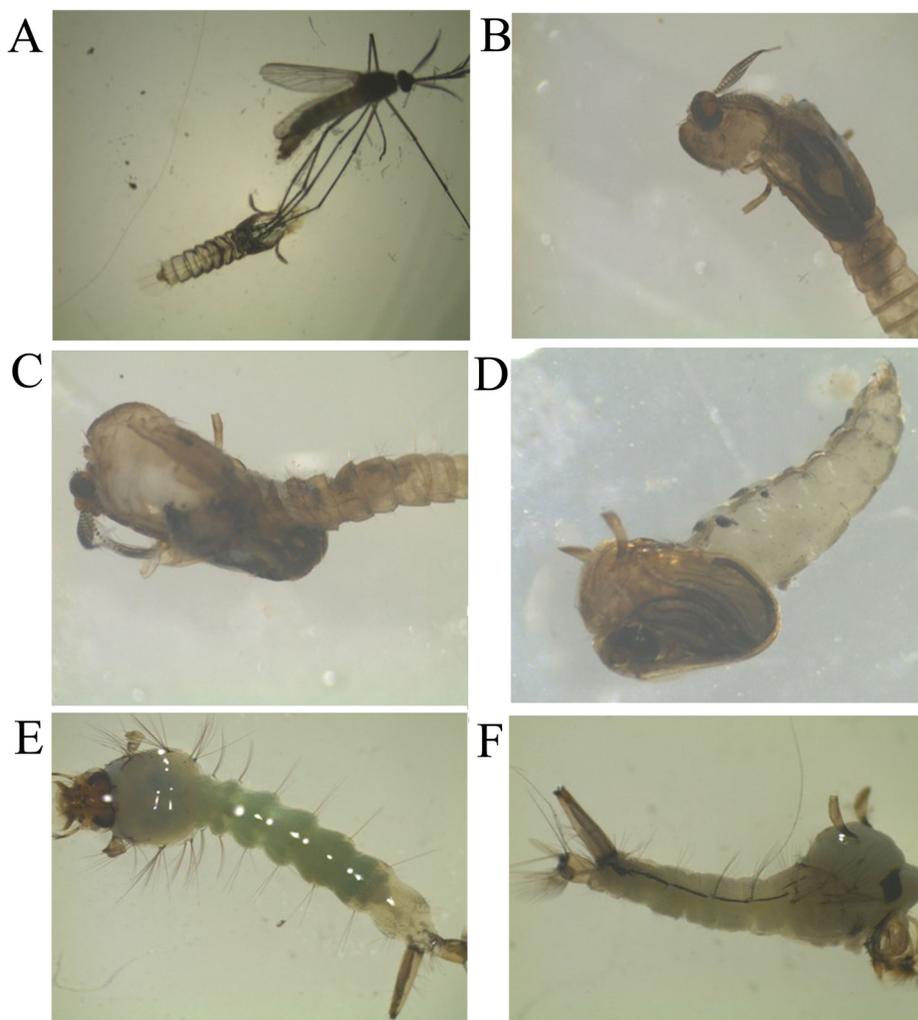


Fig. 1. (A) Incomplete emergence of the adult mosquito from pupa (B) the antennae, mouth parts, and legs of the mosquito are still attached in the pupal exuvia. (C) Emergence of adults from pupa (D) Intermediate stage between adult and pupa (E) Generation of pupa from larvae (F) Intermediate stage between larva and pupa.

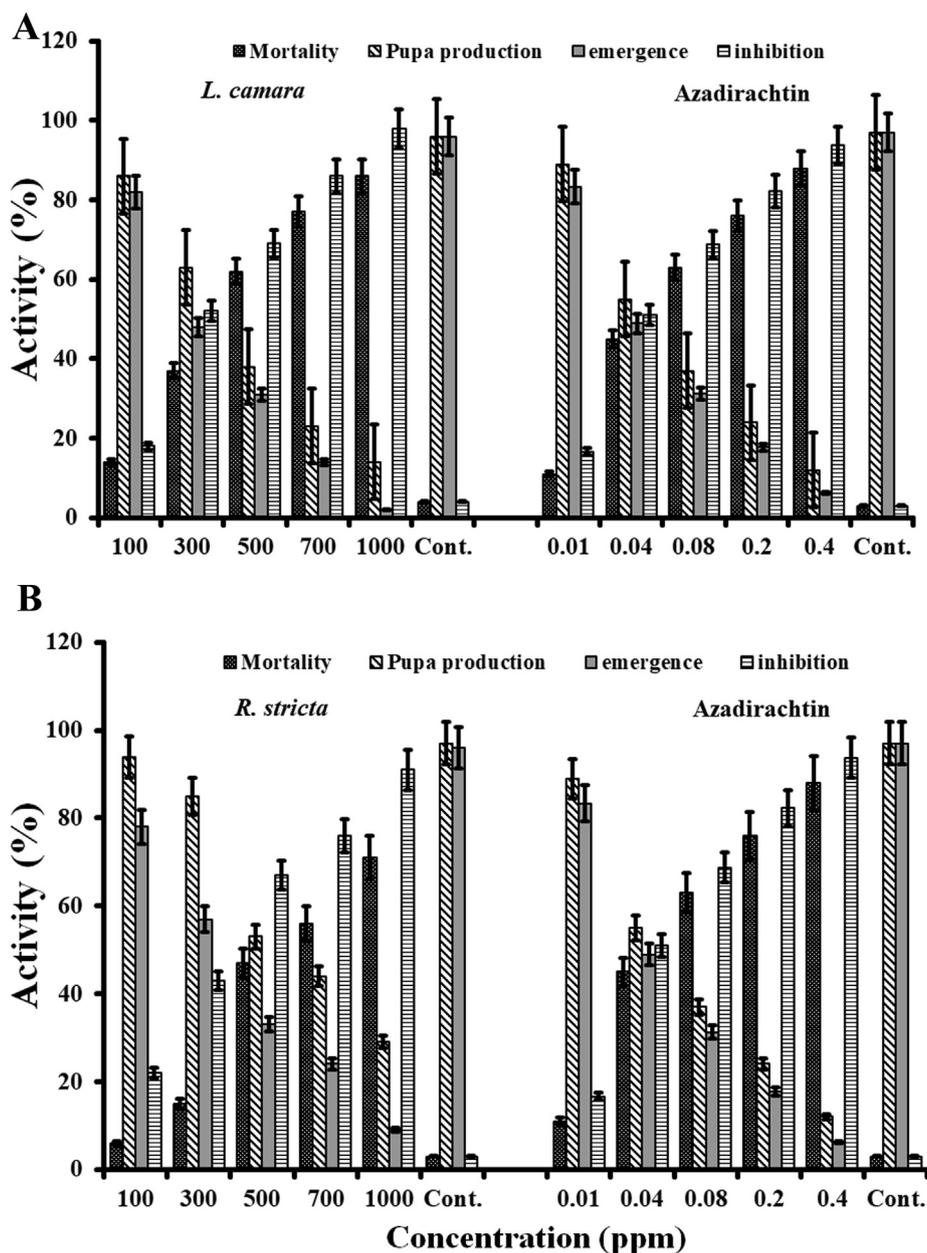


Fig. 2. (A) Larvicidal activity of the acetone extract of *Lantana camara* against *Cx. pipiens*. The different concentrations of the extract were used to assess the larval mortality, rate of pupa production, rate of emergence, and rate of inhibition. (B) Different concentrations of extracts of *Rhazya stricta* was assessed against *Cx. pipiens* estimate it's larvicidal activity. The assessment was carried out to determine the larval mortality, rate of pupa production, rate of emergence, and rate of inhibition. Different concentrations of azadirachtin were used as positive control.

69% (Fig. 3B). Commercially available larvicidal chemical azadirachtin was used as a positive control. The studies focused on *Ae. albopictus* (Conti et al., 2013), *Ae. Aegypti* (Ali et al., 2013), *Cx. pipiens* and barber's pole worm (Brugman et al., 2018).

The overall results revealed that the extract of *L. camara* caused 98% larval mortality showing a significantly higher rate of mortality as compared to activities of all the other plant extracts (Fig. 4). Initial studies indicated that the leaves of *L. camara* is a rich source of bioactive molecules (Fatope et al., 2020). In addition, the larvicidal activity of the *L. camara* was even slightly higher than the azadirachtin used as a positive control. The extract of *R. stricta* caused 91%, *A. fruticosa* and *R. chalepensis* caused 79% and 69% mortality in mosquito larvae respectively. Previous reports also revealed that *L. camara* was successfully used against insects and caused 100% mortality even after 24 h (Keziah et al., 2015). However, the extract

of *Nepeta cataria* showed 70% mortality as compared to *L. camara* when used against insects in equal concentration and the mortality of *S. oryzae* was up to 74% (Xu et al., 2016).

3.2. Estimation of lethal concentrations of different plant extracts

The value of lethal concentration (LD_{50} and LC_{90}) of a chemical means an amount needed to kill 50% and 90% of the members of a tested population respectively. In the present study, the extract of *L. camara* was evaluated and the results revealed that (Table 1) the LC_{50} for the plant extract was found to be 264 mg/l and the LC_{90} was 844.8 mg/l. The Chi value calculated was 8.178. Similarly, the LC_{50} and LC_{90} for *R. stricta* were 293.4 and 1294 mg/l, respectively (Table 1). The value of the calculated Chi was 6.1123. The LC_{50} and LC_{90} of the extract of *A. fruticosa* were 435.6 and

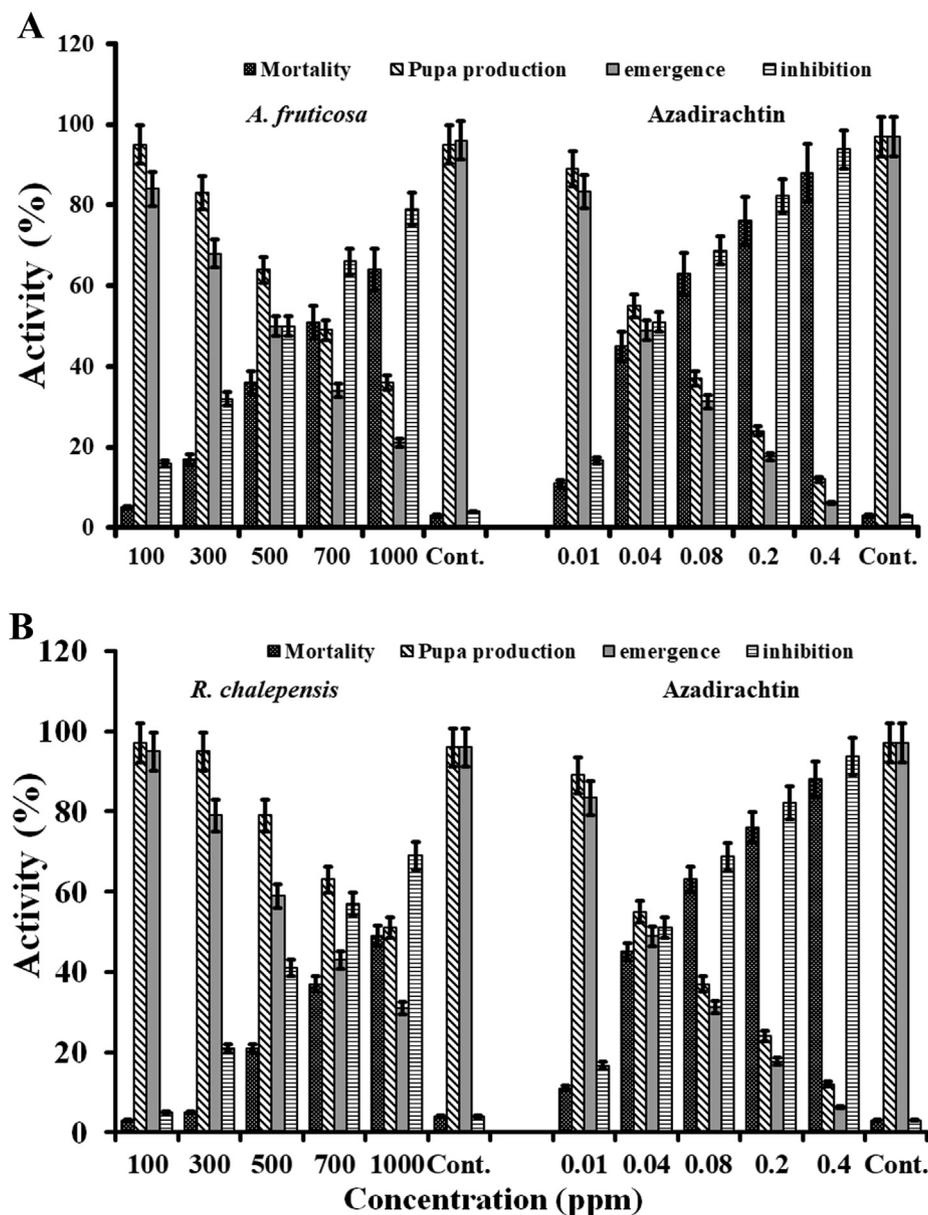


Fig. 3. (A) Larval mortality, rate of pupa production, rate of emergence, and rate of inhibition of *Cx. pipiens* were determined after treatment of the extract (different concentrations) of *Acalypha fruticosa*. (B) Toxicity of the extract of *Ruta chalepensis* was determined against larvae of *Cx. pipiens*. the larval mortality, rate of pupa production, rate of emergence, and rate of inhibition were assessed after treatment of different concentrations of the extract. Azadirachtin were used as positive control in different concentrations.

2167 mg/l, respectively (Table 1). The value of calculated Chi was 4.9. The LC_{50} and LC_{90} of the leaf extract of *R. chalepensis* were 611.9 and 2284 mg/l, respectively (Table 1). The value of calculated Chi was 1.3368. The LC_{50} dose of *O. basilicum* was applied against the *A. stephensi* larvae different stages with mortalities ranged from 0.276% to 0.305%, (Dris et al., 2017). The toxic effect of plants e.g., *Cymbopogon citrates* *Acorus calamus*, *Ocimum basilicum*, *Mentha arvensis*, and *Saussurea lappa* were assessed against larvae of *Cx. quinquefasciatus* and *Ae. Aegypti*, the results revealed the highest larvicidal activity in the volatile oil of *O. basilicum* with LC_{50} of 75.35 and 92.30 mg/l respectively (Enan, 2005). The overall larvicidal activity of extract of *L. camara* was higher as compared to azadirachtin (commercially available larvicidal chemical), used as a reference, and positive control and was also higher than the *R. stricta*, *A. fruticosa*, and *R. chalepensis*. Based on our results and

results of the other studies enticed us to investigate the chemical composition of *L. camara* through (GC–MS).

3.3. Assessment of chemical insecticidal compounds of *L. Camara* through GC–MS

The extract of *L. camara* showed the highest insecticidal activity against *Cx. pipiens* as compared to other plant extracts e.g., *R. stricta*, *A. fruticosa*, and *R. chalepensis* used in this study. Therefore, *L. camara* was subjected to GC–MS analysis to determine the chemical constituents of the extract of *L. camara*. The GC–MS profile of the compounds showed some very important biological active metabolites that have been reported with very strong insecticidal activities (Fig. 5). A wide array of a chemical compound of *L. Camara* has been reported, which showed the importance of the

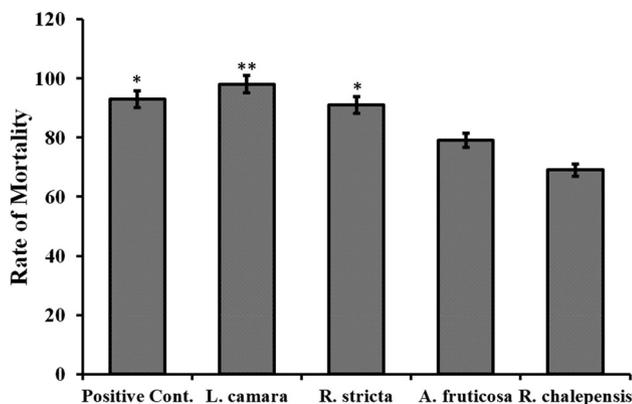


Fig. 4. A comparison of larvicidal activities caused by different plant extracts against *Cx. Pipiens*.

plant extracts (Table 2). The wide array of the metabolites can be attributed to the influence of gene pool which is translated into a variety of metabolites against a wide range of insects (Khan et al., 2017; Ullah et al., 2020).

The compounds such as Cyclopentasiloxane were found in *Cornus officinalis*, where 5-hydroxymethyl furfural was the most important metabolite, showed significant insecticidal activity against mosquito which showed strong larvicidal agent against different insects including mosquitos (Ullah, 2019). Ullah et al. (2015) detected secondary metabolites i.e., benzylideneacetone, acetylated phenylalanine-glycine-valine indole, proline-tyrosine, oxindole, *p*- and hydroxyphenyl propionic acid which showed very

strong insecticidal activities against different insects such as *Plutella xylostella*, *Manduca sexta*, *Salix exigua*. Naphthalene was also detected in the extract of *L. camara* in the present study which has been used as an insecticidal compound. *Tineola bisselliella* and *Tinea pellionella* demonstrated that Naphthalene showed insecticidal activity against a wide range of insects such as moth, mosquitos, and other insects (Supriya et al., 2010).

3.4. Assessment of AChE activity in larvae

The activity of AChE was determined in the larvae of *Cx. pipiens* treated at the concentration of 1000 mg/l of the extract of *L. camara*. The results (Fig. 6A) showed that the extract significantly ($P < 0.05$) inhibited the specific activity of AChE in larvae as compared to the other plant extract as well as azadirachtin, used as a positive control. Results showed that the extract of *L. camara* significantly restricted the AchE activity in larvae as compared to the larvae treated with other plant extract and/or positive control. Due to these results it has been suggested that the extract of *L. camara* significantly reduced the production of antioxidants as compared to other plant extracts which might be the reason to cause a higher rate of mortality in mosquitos (Abutaha et al., 2018; Rehman et al., 2020).

Chemicals including camphor and fenchone have been reported as AChE inhibitors in several insects such as *Tribolium castaneum* and *Sitophilus oryzae* (Farnesi et al., 2012). GC-MS profiling revealed the presence of 5-Hydroxymethylfurfural, naphthalene, Cyclopentasiloxane, benzene dicarboxylic acid, tridecanoic acid. Most of these chemicals have been reported as a strong pesticide. These compounds inhibit the AChE, which is used to protect the

Table 1
LC₅₀, LC₉₀, and Probit analysis of the plant extracts and Azadirachtin.

Plant/ Pesticide	LC ₅₀ (ppm)			LC ₉₀ (ppm)	Slope	Chi	
	Conc.	Lower Limit	Upper Limit			Calculated	Tabulated
<i>Lantana camara</i>	264	168.9	352.3	844.8	2.537	8.178	7.8
<i>Rhazya stricta</i>	293.4	248.7	338.6	1294	1.988	6.1123	7.8
<i>Acalypha fruticosa</i>	435.6	374.4	506.3	2167	1.834	4.9543	7.8
<i>Ruta chalepensis</i>	611.9	539.3	705.5	2284	2.240	1.3368	7.8
Azadirachtin	0.044	0.0359	0.0536	0.294	1.558	1.2958	7.8

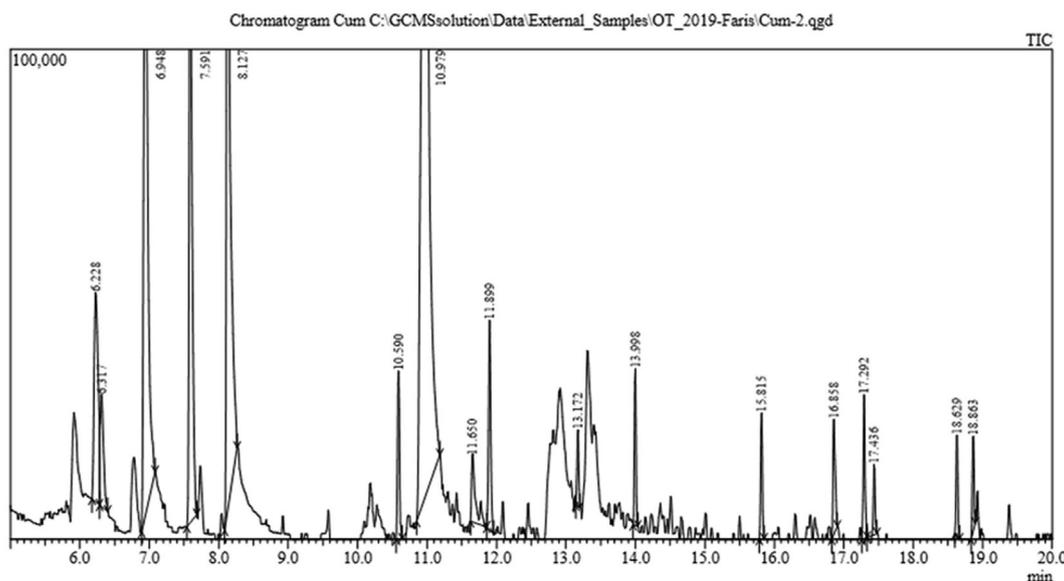


Fig. 5. GC-MS spectra of the extract of *Lantana camara* shows the peaks of different compounds.

Table 2list of the chemical compounds detected from the acetone extract of *L. camara* through GC-MS.

Peak#	RT	Area	Height	A/H	Name
1	6.228	171,231	42,738	4.01	Undecane, 3,7-dimethyl-
2	6.317	73,832	22,839	3.23	3-Nonen-1-ol, (Z)-
3	6.948	531,544	123,380	4.31	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
4	7.591	397,235	143,486	2.77	Naphthalene
5	8.127	629,514	163,043	3.86	5-Hydroxymethylfurfural
6	10.59	66,344	34,354	1.93	Hexane, 3,3-dimethyl-
7	10.979	1,330,639	176,843	7.52	Sucrose
8	11.65	54,738	13,431	4.08	Butyl(dimethyl)silyloxycyclopentane
9	11.899	90,674	42,854	2.12	Tetrasiloxane, 3,5-diethoxy-1,1,1,7,7,7-hexamethyl-3,5-bis(trimethylsiloxy)-
10	13.172	26,305	15,605	1.69	Hexane, 3,3-dimethyl-
11	13.998	52,812	31,936	1.65	N-(Trifluoroacetyl)-N,O,O'-tetrakis(trimethylsilyl)norepinephrine
12	15.815	44,140	25,810	1.71	Cyclohexasiloxane, dodecamethyl-
13	16.858	54,228	23,471	2.31	Methyl valerate
14	17.292	51,583	29,348	1.76	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
15	17.436	22,593	14,381	1.57	1-(2-Methoxyethoxy)-2-methyl-2-propanol, methyl ether

RT, retention time; A/H, ration of area and height.

insects against the insecticidal compounds, and hence the extract was highly toxic to the insects due to these metabolites.

3.5. Analysis of the GST activity in larvae

The GST activity was measured in larvae treated with different plant extracts and results revealed that the GST activity was significantly reduced in larvae treated with extract of *L. camara* as compared to other plants extract as well as a positive control (Fig. 6B). The GST has been reported to play an important role in stress physiology and are implicated in intracellular transport and various

biosynthetic pathways (Rehman and Rather, 2019). The extract of *L. camara* showed the most significant larvicidal effects because it was succeeded to reduce the GST level and hence the immune level of the larvae was overcome by the toxicity of *L. camara* earlier as compared to other plants (Gülçin et al., 2016b; Gülçin et al., 2018; Khan et al., 2020).

4. Conclusion

To sum up, all the plants included in the study showed an appreciable larvicidal activity and the highest activity was exhibited by *L. camara*. The study further necessitates the complete phytochemical analysis of the leaf extracts. The extract of *L. camara* significantly reduced the activities of AChE and GST enzymes in larvae as compared to other plant extract. The phytochemical analysis will indicate clearly that what compounds present in the leaves bear larvicidal activity. Furthermore, the toxic effect of the plants on animals should also be checked, only then they can be deemed safe for human use.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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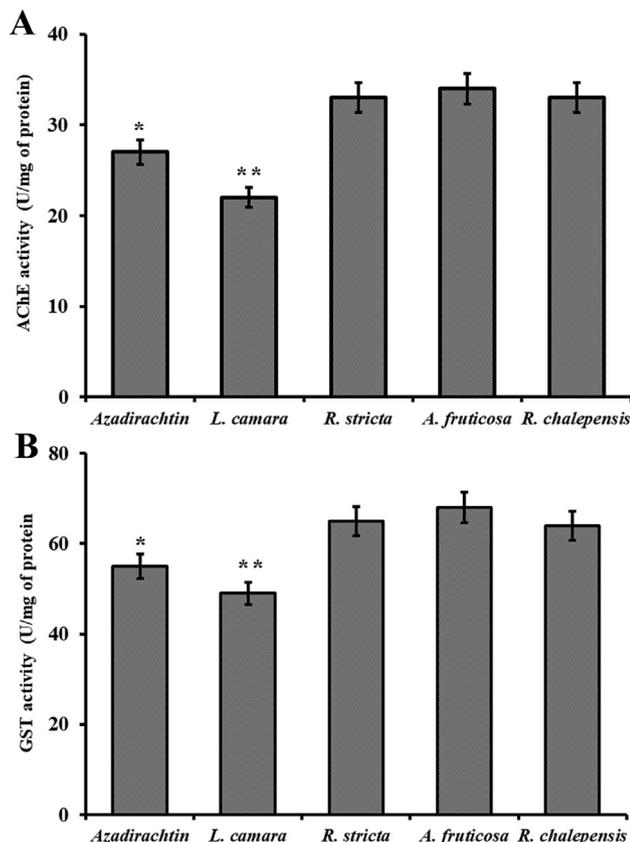


Fig. 6. Assessment of antioxidant enzymes in larvae. (A) Analysis of AChE in the larvae of *Cx. pipiens* treated with different plant extract. (B) Analysis of GST in the larvae of *Cx. pipiens* treated with different plant extract. The (*) represents positive control and (**) represents significant difference from control.

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