Prospects of Ocimum Gratissimum Linn. (Lamiaceae) to Control Exorista Sorbillans Wiedemann (Diptera: Tachinidae) Menace of Silkworm in Serecosystem

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Research Article

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Abstract

Tachinid parasitoids are in focus mostly as biocontrol agents to be released against lepidopteran pest. But surprisingly certain tachinid parasitoids attack economically beneficial insects like silkworm and demands control measures against them. The uzi fly *Exoristasorbillans* (Diptera: Tachinidae) infests all commercially important silkworm species including *Antheraeassamensis* where it causes upto 80 percent crop loss. The control of such parasitoids is a difficult task as the larval stage is endoparasitic and chemical insecticides do not reach the targeted parasitoid without exposing the silkworm host. In the present study, we evaluated adulticidal activity of different solvent extracts and essential oil of *Ocimum gratissimum*. We found higher efficacy of essential oil in comparison to other solvent extracts. The effective fraction of oil was found to contain thymol or its isomer carvacrol as the major compound in GC-MS studies. Finally, *O. gratissimum* oil-based combination (MI) and carvacrol based combination of essential oil compounds (MII) were prepared, tested and found to be effective against the fly. In silico positive interaction of essential oil compounds with acetylcholinesterase enzyme model of the fly revealed that the said enzyme is one of the target proteins for these oil compounds to interrupt its function and subsequent lethal action.

Introduction

Tachinid flies have been gaining importance globally as promising biological control agent as many of them are natural predator of lepidopteran, coleopteran and some other insect pests. However, literature also reveals report on harmful effects of this group of insects on beneficial insects. The uzi fly *Exoristasorbillana* (Diptera: Tachinidae) is such a tachinid fly which is considered as one of the major constraints for the sericulture industry as they can parasitize all the commercial silkworm varieties along with some other lepidopteran caterpillars. The fly infestation has been reported from all the silk producing countries of the world\(^1\). In India the incidence of this fly infestation in mullberry silkworm *Bombyx mori*\(^2\), tasar silkworm *Antheraeamyliita* Drury\(^3\), eri silkworm *Samia Cynthia ricini* Boisduval\(^4\) and muga silkworm *Antheraeaassama* Westwood\(^5\) has been reported at different times and the fly menace still prevails. The overall damage caused by the fly may reach upto 40%. For outdoor and semidomesticated silkworm crop like *Antheraeaassamamensis* Helfer the damage even extends upto 80% in seed broods. The parasitoid spends entire larval stages inside the silkworm body and when mature, the maggots come out by making hole in the silkworm cocoon case to form pupae on soil. The infested silkworm dies in late larval stage or pre-pupal or pupal stage. Thus, the crop fails to propagate further. The silk cocoons commercially unreelable as the fly cut the cocoon case during emergence causing silk yield loss. To protect silkworm from *E. sorbillans* infestation, different methods viz. physical, mechanical, chemical and biological control have been suggested from time to time by earlier investigators. Use of chemical insecticides although give immediate and effective results but applications of chemical insecticides are not preferable at seri ecosystem as silkworms themselves are highly susceptible to the chemical insecticides and application of chemicals without exposing the silkworm is not feasible due to endoparasitic nature of the parasitoid. Similarly, microbial agents are also cannot be applied. Therefore, we hypothesize ecofriendly
products most particularly herbal products having selective toxicity as one of the rational alternatives to synthetic counterparts for controlling such pests of silkworm.

*Ocimum gratissimum* L. (Lamiales:Lamiaceae) is a perennial herb with a long history of traditional medicinal uses in countries across the world especially in the Africa and Asia. Extracts and essential oils of this plant have been reported to possess medicinal, bactericidal, fungicidal, nematicidal activities. Besides, the plant essential oil has been used safely in food, flavor and fragrance industry. It is a potential candidate of pharmaceutical industry with report of low mammalian toxicity. The plant has been already exploited for the control and management of many insect pests and parasitoids belonging to order Coleoptera, Lepidoptera, Isoptera, Diptera, Thysanoptera, Heteroptera etc but not found to be tested against uzi fly. Earlier we reported about the efficacy of ethanolic extract of this plant species. We have also observed lesser toxicity of *Ocimum* species against silkworm as compared to *Exoristas sorbillans*15,16. Therefore in the present study an attempt was undertaken to assess the adulticidal potentiality of different solvent extracts and essential oil of *Ocimum gratissimum* and its combination with other plant essential oil and constituent compounds against *E. sorbillans* so as to incorporate this plant product in uzi fly control program in future.

**Materials And Methods**

**Plant material**

Plant material was collected from Dibrugarh District of Assam and was identified by Botanical Survey of India, Shillong (Ref.No. BSI/ERC/2010/AKV/181).

**Preparation of crude plant extracts**

Fresh leaves of *Ocimum gratissimum* were washed, shade dried and ground to powder using electric grinder and preserved in refrigerator at 4°C for future use until extraction. Crude extract of leaf powder was prepared in absolute ethanol solvent. 150g of ground leaves were dissolved in 350ml of the solvent and kept for 72h. Extracts were filtered by using Whatman Filter Paper No 1 after 72 h and the solvents were removed under vacuum below 40°C.

**Fractionation of ethanol extract**

Ethanol extracts of the leaves of *Ocimum gratissimum* was further fractionated by using a series of solvents (Merck) on the basis of polarity namely petroleum ether, chloroform, butanol and water. Initially ethanol extract was prepared by dissolving ground leaf powder in absolute alcohol for 72h. The extract was then filtered and solvent was removed. The dried ethanolic extract was then weighed and taken in a separating funnel (2.5 liter) and petroleum ether was added and shaked for 30 minutes. It was then allowed to settle for 3h and the supernatant was decanted and filtered. The residue was extracted thrice adding same solvent and the filtrate was taken as petroleum ether extract. Sequentially the chloroform,
butanol and water were added and each solvent extract was prepared similarly as petroleum ether extract and stored in refrigerator for bioassay.

**Collection and preparation of plant essential oils**

Fresh leaves of the plant were collected, washed to remove dust, cut into small pieces and subjected for oil extraction by hydrodistillation method using Clevenger type apparatus. Two hundred grams (200g) of fresh leaves of the selected plant was put in 5 L round bottom flask per extraction of oil. The essential oil floating above water layer was collected after 4 hours of heating. Anhydrous sodium sulfate was added in the collecting vial to absorb traces of water. The oil was then stored in sealed screw cap vial in deep freezer (−20°C) for bioassay and analysis.

The percentage (v/w) of oil yield was calculated by using the following formula:

\[
\text{% of oil yield} = \frac{\text{Recovery of the Oil}}{\text{Weight of the plant}} \times 100
\]

**Culture of *Exorista sorbillans* Wiedemann**

Maggots of *Exorista sorbillans* (uzi flies) were brought from Govt. Sericulture Farm of Assam and kept in wire-netted wooden box (size: 30cm x 30cm) in insect culture room, Department of Life Sciences, Dibrugarh University. The newly emerged adult flies were provided with 20% honey solution soaked in cotton in petriplates for feeding in a well-ventilated insect culture room under natural light and temperature conditions (Max: 32°C; Min: 19°C).

**Bioassay with fractionated ethanol extract against *E. sorbillans***

Five percent concentration of petroleum ether extract, chloroform extract, butanol and water extracts derived from fractionation of ethanolic extracts were bioassayed against third day old adult uzi fly using contact residual film technique. 1.5ml of 5% concentration of each solution was applied into Whatman (110 mm) filter paper which was pasted in a petri dish and was allowed to dry in room temperature and then flies were released. The treated petri plates were covered by 110mm diameter glass funnel. The stem at terminal end of the funnel was covered with nylon net and thus aeration was facilitated to insects during treatment hours. For each experiment control was maintained where only the respective solvent was applied. Three replications were kept for each treatment and control set. Observation was taken at 1, 3, 6, 24, 30, 48 hours from the time of application. Abbot’s correction factor was applied in case of mortality of fly in control if occurred.

**Bioassay with effective solvent extract against *E. sorbillans***
The fractionated ethanolic extract which gave more than 50% death of *E. sorbillans* after 24h of treatment at 5% concentration was considered for further bioassay to get sub lethal concentration (LC50). For the purpose, different concentrations (0.01 to 15% at geometrically uniform interval) of the effective extract was prepared and subsequent bioassay against third day old adult uzi fly was carried out by contact residual film technique to determine LC50 value of the most effective solvent extract. Probit analysis was done for calculating LC50 using SPSS software.

**Bioassay with essential oil of *O. gratissimum***

Bioassay by using essential oil of *Ocimum gratissimum* against *E. sorbillans* was carried out by contact residual film technique as described above. Essential oil solutions of *O. gratissimum* were prepared in acetone solvent. For control, only acetone solvent at an amount of 1.5ml was applied to Whatman No.1 Filter paper against each test concentration. Third day old adult uzi flies were subjected to treatments. Three replications, each containing 10 insects were maintained for each treatment. Different concentrations of oil (0.01 to 0.5%) were prepared for determining LC50 value of essential oil of *O. gratissimum*. Lethal time for the essential oil against *E. sorbillans* was calculated by applying 1µl of crude essential oil topically, on thorax of the third day old uzi flies with the help of micropipette.

**Fourier Transfer Infrared Spectrograph (FTIR) analysis of active fraction and essential oil of the plant**

To identify the functional group of the constituent compounds, present in the bioassay guided active fractions of petroleum ether extract and essential oil of the *O. gratissimum*, FTIR analysis was performed. For this the fractionated active petroleum ether extract and essential oil was dissolved in chloroform. Spectra were taken in neat mode using Shimadzu Prestige-21 FTIR spectrophotometer.

**TLC (Thin layer chromatography) separation of essential oil of *Ocimum gratissimum*:**

Initially three solvent systems comprising of petroleum ether (boiling point 40-60°C) and ethyl acetate at the ratio of 10:1, 7:1 and 5:1 were prepared for separation of individual spots of the essential oils in aluminium TLC sheet (Merck). Finally, fractionation of oils was done using preparative TLC plate using the solvent system comprising of petroleum ether and ethyl acetate (5:1). Different fractions from the TLC plate were collected in the the solvent mixture and solvent was removed by distillation with temperature set at 40°C.

**Bioassay with different fractions of essential oil obtained from TLC and GC-MS analysis of active fraction:**

Bioassay of each individual fraction obtained from TLC was carried out by contact residual film technique using acetone (Merck) as solvent. Further GC-MS analysis of the best active fraction of oil was carried out to identify the compounds present in the active fraction of the oil.

For determining the compounds present in the active fraction of essential oil of *O. gratissimum* GC-MS analysis was done by using Jeol, Accu TOK GCV JMS-T100GCV MODEL. Column was HP5, 30-meter-
long, 0.25mm ID and 0.25nm film thickness. The conditions applied were: Injector temperature 250°C; Interface temperature 280°C. Column program was 80°C - 1 min, hold 5°C/Min- 250°C-10 Min, Hold-30°C/Min- 280°C. Carrier gas was Helium. Flow rate 1ml/min. Injection volume 0.2µl. Split ratio 1:100.

**Preparation of O.gratissimum essential oil based combination and bioassay against E.sorbillans.**

Based on essential oil of *O. gratissimum*, initially six combinations were prepared and tested on 3rd day old adult *E. sorbillans* using fumigant mode of application. Essential oil of some other locally available plants namely *Ocimum sanctum* (Lamiaceae), *Eucalyptus maculata* (Myrtaceae), *Callistemon linearis* (Myrtaceae), *Citrus sinensis* (Rutaceae) were considered to add as ingredient of combination on the basis of their efficacy obtained in our pilot studies. Topical application of each candidate essential oil at a dose 1µl per insect on thoracic region was done on third day old adult fly and lethal time was recorded. The prepared combinations for fumigant application were 5:0G, 4:1GOs, 4:1GEm, 4:1GCl, 4:1GCs and M. Synthetic gum (GripFix Adhesive Paste) was used as control release agent. The combination 5:0G comprised 5µl essential oil of *O. gratissimum* in total of 5ml synthetic gum. The combination 4:1GOs comprised 4µl essential oil of *O. gratissimum* and 1µl of *O. sanctum* in total of 5ml synthetic gum. The combination 4:1GEm comprised of 4µl essential oil of *O. gratissimum* and 1ul of *E. maculate* in total of 5ml synthetic gum. The combination 4:1GCl comprised of 4µl essential oil of *O. gratissimum* and 1µl of *C. linearis* in total of 5ml synthetic gum. The combination 4:1GCs comprised of 4µl essential oil of *O. gratissimum* and 1µl of *C. sinensis* in total of 5ml synthetic gum and the combination M comprised of equal amount of essential oil of *Ocimum gratissimum*(1µl), *Ocimum sanctum*(1µl), *Eucalyptus maculata*(1µl), *Callistemon linearis*(1µl), *Citrus sinensis*(1µl) in 5ml of synthetic gum. Fumigant mode of application was chosen for bioassay studies of each prepared combination against uzi fly. Freshly prepared combination was poured into plastic cap (cap of 15ml screw cap vial) and wrapped by muslin cloth from upper side and placed in conical flask of 500ml capacity. The muslin cloth prevented the flies to come in direct contact of the combination but allowed the vapor to come out and spread uniformly in the enclosed chamber (500ml volume). After keeping the formulation containing cap in the conical flask, 10 numbers of flies were released in each replication and the flask was sealed with the help of aluminium sheet and tightened by rubber band to keep the air blocked inside the flask. Response of the flies in terms of knock down and mortality was recorded from 1minute to 24hours at successive time interval.

**Preparation of combination from essential oil compounds and bioassay against E.sorbillans:**

Based on the efficiencies of combinations prepared from crude essential oil, further combinations were prepared by selecting essential oil compounds which have been reported and identified as major constituent compound of the concerned plant essential oils. These were Carvacrol, Thymol, Eugenol, Eucalyptol and Citral. Carvacrol and Thymol are the constituent compounds of *O.gratissimum*, Eugenol is the constituent compound of *O.sanctum*, eucalyptol is the constituent compound of both *E. maculata*, and *C.linearis*, citral is the constituent compound of *C. sinensis*. Two combinations were prepared taking these compounds. Fumigant mode of application was used for bioassay studies. Synthetic gum was
used as control release agent and plastic cap was used into which the prepared combination was poured. Combination marked as MII was prepared by mixing equal amount of Carvacrol (1ul), Thymol(1ul), Eugenol(1ul), Eucalyptol(1ul) and Citral (1ul) into a total of 5ml synthetic gum. Three replications were set for each combination. Another formulation C1 was prepared by mixing 5ul of carvacrol into 5ml of synthetic gum and the prepared formulation was kept in conical flask having 500ml capacity. Flies were then subjected to get exposure of the formulation and mortality data were recorded against time interval.

Docking performance of major essential oil compounds with acetylcholinesterase enzyme:

Ligand Preparation:

The 2D structure of three ligands were drawn using pubchem search tool in NCBI and converted in to 3D structure with the help of Chimera software and subsequently saved in pdb format.

Receptor Protein Preparation:

Acetylcholinesterase enzyme of *Exorista sorbillans* was selected as targeted protein for the ligand. The protein sequence (ACHE) was downloaded in FASTA format from protein database in NCBI and pasted in notepad. Protein BLAST was performed and based on blast result four pdb ids were selected as template protein (1dx4; 1qo9;5ydi;6arx;6ary) to construct model of ACHE protein of *E. sorbillans*. The selected template protein structures were downloaded from the PDB site (http://www.rcsb.org). For preparation of ACHE protein model, the Modeller 9.21 was used following the steps of basic modelling using the commands like build_profile.py, compare.py, align2d.py, evaluate_model.py. the generated models were uploaded in Procheck online service and Ramachandran plot for each model was obtained. Considering maximum percent value of amino acid residues present in most favored region and minimum in disallowed region, the most suitable model was selected for further docking with the selected ligands.

Docking:

Docking of the best model of ACHE protein was performed with essential oil compounds namely carvacrol, thymol, eugenol, eucalyptol, citral and the substrate acetylcholine in Python Molecular Viewer (PMV1.5.6) using AUTODOCK VINA in MGL tools. Best nine mode of interactions were generated for each docking with respective affinity values and rmsd values. The best mode for each docking having least rmsd value and high binding affinity were considered for analyzing the mode of binding interactions. Pictorial representation of binding interactions was analyzed in PMV and ligplus software.

Results

In the present study the ethanol extract of the plant was initially prepared and further fractionated by eleutropic series of solvents, namely petroleum ether, chloroform, butanol and water to identify the active solvent fraction against adult *E. sorbillans*. The result of the four successive solvent extracts showed that the petroleum ether extract of the plant had greater toxicity in comparison to the other three solvent
fractions at 5% concentration (Fig. 1). Onset of toxicity of O. gratissimum became apparent at 3 h post treatment and mortality increased up to 96.67 ± 3.34% at 48 h. Thus, mortality rate increased with increase in time. Chloroform extract caused 20 ± 0% and 20.83 ± 8.34% at 30 and 48 h after treatment respectively while butanol extract caused 14.50 ± 3.92 and 15.40 ± 4.82 respectively at 30 and 48 h after exposure and water extract caused no mortality of uzi fly. The calculated LC50 for petroleum ether extract was recorded as 5.38% (Y = 3.49 + 2.06X; R.Sq = 0.980) (Table 1, Supplementary Fig. 1).

Percentage of essential oil yield from fresh leaves of O. gratissimum was recorded as 0.63 ± 0.09 percent. A series of concentrations (0.01–0.5%) of the plant essential oil were applied to E. sorbillans by residual film technique in order to determine LC50 value which was calculated as 0.42% (Y = 6.07 + 2.81X; R.Sq = 0.941) (Table 1, Supplementary Fig. 2).

Table 1. Sublethal concentration (LC50) of petroleum ether extract and essential oil of Ocimum gratissimum against E. sorbillans (CRF)

| Sl No. | Extract      | Mode of application | LC50 (%) | Regression Equation | R² Value | X² Value | D.F. | Z-value | 95% CI   |
|--------|--------------|---------------------|----------|---------------------|----------|----------|------|---------|----------|
| 1      | Petroleum ether | Contact residual   | 5.38     | Y = 3.49 + 2.06X    | 0.980    | 553.53   | 37   | 30.74   | 2.058–2.338 |
| 2      | Essential oil   | Contact residual   | 0.42     | Y = 6.07 + 2.81X    | 0.941    | 185.26   | 16   | 17.20   | 2.672–3.325 |

FTIR analysis of petroleum ether extract of O. gratissimum showed absorption peak at 3471.87 indicating –NH stretching, at 2966.87 indicating –CH stretching, at 1734.01 indicating –C = O stretching and at 1462.04 indicating –CH asymmetric bending (Fig. 2, Table 2). Similarly, absorption peak of IR spectrum of essential oil of O. gratissimum at 3527.80 indicating presence of OH or NH stretching, 2962.66 indicating –CH stretching, 1618.28, 1581.53, 1510.26, 1450.26 indicating aromatic system and absorption peak at 1153.43 indicating presence of -C-O-C stretching (Fig. 2, Table 2).
Table 2
Infrared spectral data (cm$^{-1}$) and probable functional groups of petroleum ether extract and essential oil of *O.gratissimum*

| Name of extract     | Peak (cm$^{-1}$)   | Functional Group                  |
|---------------------|-------------------|-----------------------------------|
| Petroleum Ether     | 3471.87           | - NH stretching                   |
|                     | 2966.87           | - CH stretching                   |
|                     | 1734.01           | - C = O stretching                |
|                     | 1462.04           | - CH asymmetric bending           |
| Essential Oil       | 3527.80           | - OH or NH stretching (-OHgroup)  |
|                     | 2962.66           | - CH stretching                   |
|                     | 1618.28, 1581.53, 1510.26, 1450.26 | - Aromatic system |
|                     | 1153.43           | - C-O-C stretching                |

In the preparative thin layer chromatography, two fractions (F1, F2) were separated from the essential oil of *O.gratissimum* at solvent system consisting of petroleum ether and ethyl acetate (5:1). Effective fraction was determined by conducting bioassay of each fraction against 3rd day old uzi fly by using contact residual film method. F2 fraction of *O.gratissimum* was found effective against uzi fly. When the bioassay guided active TLC fraction (F2) of essential oil was subjected to GC/GC-MS analysis, three peaks were detected at retention time 10.3 and 16.2 and 21.7 m (Fig. 3) of which the major peak was observed at retention time of 10.3 minutes. The probable constituent compounds identified at this retention time from MS-analysis and library hit results was either thymol (Hit1) or phenol,2-methyl-5-(1-methylethyl)-(also known as carvacrol) (Hit2) having molecular weight 150 and molecular formula C10H14O (Fig. 5).

(RT: 10.3, 16.2, 21.7 M)

Fumigant toxicity of six formulation which were prepared based on essential oil of *O. gratissimum* and its combination with some other plant essential oil showed that the combination MI comprising equal amount of essential oil of *Ocimum gratissimum, Ocimum sanctum, Eucalyptus maculata, Callistemon linearis, Citrus sinensis* showed best result with sixty percent mortality at 24 h exposure period against 0–20 percent mortality caused by the rest of the prepared formulations (Table 3).

Table 3: Fumigant effect of different formulations of essential oil (500 ml air) against E. sorbillans
k-> knock down; d-> death; C-> Control (5 ml gum); MI (1:1:1:1:1)-> Formulation containing mixture of essential oil of *O. gratissimum, O sanctum, E. maculata, C. linearis, C. sinensis* at ratio of 1 µl each in total of 5 ml gum; 4:1GOs -> Formulation containing 4 µl essential oil of *O. gratissimum* and 1 µl of *O. sanctum* in total of 5 ml gum; 4:1GEm -> Formulation containing 4 µl essential oil of *O. gratissimum* and 1 µl of *E. maculata* in total of 5 ml gum; 4:1GCl -> Formulation containing 4 µl essential oil of *O. gratissimum* and 1 µl of *C. linearis* in total of 5 ml gum; 4:1GCs -> Formulation containing 4 µl essential oil of *O. gratissimum* and 1 µl of *C. sinensis* in total of 5 ml gum.

In our previous experiment we found higher toxicity of carvacrol than thymol\(^\text{17}\). Therefore, carvacrol (one of the major constituents of effective fraction of *O. gratissimum* oil) based formulations were prepared with synthetic gum and with other terpene compounds reported to present as dominant part in essential oil of *Ocimum sanctum, Eucalyptus maculata, Callistemon linearis, Citrus sinensis*. The result showed that the formulation MII consisting of carvacrol, citral, eugenol, eucalyptol showed higher mortality with 60% mortality of flies after 6 h of treatment and 100% mortality after 24 h of treatment at 500 ml of air volume in comparison to the formulation CI comprising of the carvacrol alone with maximum 30 percent mortality after 24 h of exposure (Fig. 6). The formulation of essential oil compounds (MII) was more effective than the formulation of the mixture of the crude essential oils (MI) (Fig. 6).
For studying the mode of action, using five template proteins (1dx4, 1qo9, 5ydi, 6arx, 6ary), a model of acetylcholinesterase enzyme of *E. sorbillans* having total amino acid residues 701 was constructed. The Ramachandran plot showed 90.5% amino acid residues of the model in most favored regions and with 0.3% residues in disallowed regions (Supplementary Fig. 3). While docking was performed using autodock software taking five essential oil compounds and the enzyme substrate acetylcholine as ligands with the modelled ACHE protein (Fig. 7), all the ligands were found to dock successfully to the protein molecule. Carvacrol with $-6.9$ kcal/mol binding affinity showed ten hydrophobic interactions with amino acid residues Gly 569, Ile 572, Glu 325, His 568, Tyr 458, Trp 173, Gly 238, Gly 239, Tyr 251, Ser 326 of the target protein. Thymol with $-5.1$ kcal/mol binding affinity interacted with ACHE via two hydrogen bonds with amino acids Asn 356 and Glu 534 with bond length 3.00 and 2.79 Å respectively and five hydrophobic interactions with amino acids His 526, Trp 651, Asn 652, Pro 531, Pro 358. Citral with $-4.1$ kcal/mol binding affinity showed eight hydrophobic interactions with Asn 490, Lys 491, Ile 488, Asn 487, Ile 415, Phe 527, Leu 416, Pro 358 residues of ACHE. Eugenol with binding affinity $-4.8$ kcal/mol interacted with ACHE via one hydrogen bond with amino acid Ala 194 having bond length 2.78 Å and four hydrophobic bonds with amino acids Leu 196, Trp 319, Lys 606, Asn 225. Eucalyptol with $-6.1$ kcal/mol binding affinity formed three hydrophobic bonds with Trp 173, Tyr 458 and His 568 residues of ACHE. Acetylcholine, the substrate of ACHE showed $-3.9$ kcal/mol binding affinity with two hydrogen bonds with amino acids Asn 533, Asn 652 with bond length of 3.05 Å and 2.81 Å respectively and also formed five hydrophobic bonds with Gln 537, Glu 534, Lys 622, Ala645, Ser 649 of ACHE protein (Fig. 7, Table 4).

Table 4: Binding affinity of selected ligand toward ACHE
Discussion

The solvent like ethanol can easily penetrate the cellular membrane to extract the intracellular ingredients from the plant material\textsuperscript{18}. In our earlier studies we found that the ethanolic extract of \textit{O. gratissimum} caused highest percent mortality over hot water, hydroalcohol and cold water extract\textsuperscript{14}. It is also reported that ethanol is a potent solvent to extract both polar and nonpolar compounds\textsuperscript{19}. Therefore, in the present investigation a stock of ethanolic extract was initially prepared to extract both polar and nonpolar compounds and further fractionated using eleutropic series of solvents to find out active solvent extract. The results suggested that the petroleum ether extract of the plant was the most effective among the four solvent extract tested indicating that the solvent extract might contain potential active ingredients against \textit{E. sorbillans} adult flies. Efficacy of nonpolar extract of plants including petroleum ether extract of \textit{O. gratissimum} and some other plants on dipteran insects was earlier reported by several workers\textsuperscript{20,21,22}. According to the principle of “like dissolves like”, solvents would only extract those compounds which have similar polarity with that of the solvents\textsuperscript{23,24,25,26}. Thus, the non-polar petroleum ether extract of the experimental plant under investigation extracted nonpolar compounds possibly phenylpropanoid, fatty

| Ligand/Substrate | Structure of ligand/substrate | Docking affinity (kcal/mol) | Distance from best mode Rmsd1.bRmsdu.b |
|------------------|-----------------------------|-----------------------------|---------------------------------------|
| Carvacrol        | ![Carvacrol Structure](image) | -6.9                        | 0.00                                  |
| Thymol           | ![Thymol Structure](image)   | -5.1                        | 0.00                                  |
| Citral           | ![Citral Structure](image)   | -4.1                        | 0.00                                  |
| Eugenol          | ![Eugenol Structure](image)  | -4.8                        | 0.00                                  |
| Eucalyptol       | ![Eucalyptol Structure](image)| -6.1                       | 0.00                                  |
| Acetylcholine    | ![Acetylcholine Structure](image)| -3.9                    | 0.00                                  |
acids, terpenes etc. which might be responsible for giving the efficacy of the solvent extract against *E. sorbillans*.

As the *O. gratissimum* plant is aromatic and contains good quantity of essential oil which has been reported effective against different insect pest including dipteran flies by many earlier investigators and as the nonpolar fraction of the ethanolic extract i.e. the petroleum ether fraction was found effective against uzi fly, we further focused on essential oil part of the plant for bioassay against *E. sorbillans*. Supporting our prediction, the result showed higher efficacy of *O. gratissimum* essential oil than the petroleum ether extract (Table 1) indicating nonpolar compounds as the responsible factors for the lethal toxicity of the plant against the fly. Although a considerable number of studies of earlier investigators highlighted effectiveness of this plant essential oil against many dipteran flies but the insecticidal potentiality of the plant oil was not found to be reported against *E. sorbillans*. We recorded 0.42% LC50 concentration of *O. gratissimum* oil which was quite higher than LC50 of petroleum ether extract (5.38%) against *E. sorbillans*. Similarly, efficacy of this oil against *Aedes aegypti* was earlier reported as 60 ppm. Fumigant action of *O. gratissimum* oil at a dose of 1µl·l−1·air reported to cause 98%, 99% and 100% mortality of *Rhyzoperthadominica, Oryzaephilussurinamensis* and *Callosobruchuschinensis* respectively at 24 h post treatment and eugenol i.e. the main constituent of the plant oil was reported to cause 79%, 61% and 100% mortality respectively of the same insects. In response to essential oil of *O. gratissimum*, the adult flies exhibited fast walking behaviour followed by sudden knock down. At higher dosage the knocked down insects could not recover and death ensued which might imply interference of the toxicant with the insect’s nervous system. Activity of the oil maybe attributed to action of a single major compound or synergistic actions of group of compounds. In order to know the nature of the functional groups present in the constituent compound(s) of the petroleum ether extract and essential oil of *O. gratissimum*, FTIR spectra analysis was done. The spectral peak revealed that the compounds present in the petroleum ether extract and the essential oil part of the plant comprised almost similar functional groups (Fig 2; Table 2).

In the preparative thin layer chromatography, two fractions (F1, F2) were separated from the essential oil of *O. gratissimum* at solvent system comprising of petroleum ether and ethyl acetate (5:1). Effective fraction was determined by conducting bioassay of each fraction against 3rd day old uzi fly by using contact residual film method. F2 fraction of *O. gratissimum* was found effective against uzi fly. When the bioassay guided active TLC fraction (F2) of essential oil was subjected to GC/GC-MS analysis three peaks were detected at retention time 10.3 and 16.2 and 21.7 min (Fig 3, Fig 4). The probable constituent compounds were identified as (i) thymol or phenol, 2-methyl-5-(1-methylethyl) - (RT=10.3 min; molecular weight 150, molecular formula C10H14O); (ii) oxamide, n-butyl-N’-(4-tert-butylphenyl) - (RT=21.7 min; molecular weight 276, molecular formula C16H24N2O2); (iii) oxamide,N-(4-tert-butylphenyl)-N’(1-methylpropyl) - (RT=21.7 min; molecular weight 276, molecular formula C16H24N2O2) (Fig 5). The major peak was at retention time 10.3 which was detected as either Thymol or phenol, 2-methyl-5-(1-methylethyl)-(also known as carvacrol) that might be the active compound of essential oil fraction (F2) of the plant showing adulticidal activity against uzi fly. Earlier thymol was recorded as major constituent
(48.1%) in essential oil of *O. gratissimum*(48.1%)\(^3^2\). On the contrary essential oil of *O. gratissimum* may comprise different constituent compounds at different percentages as shown by many authors\(^3^3,^3^4\). Phenol 2-methyl- 5-(1-methylethyl)- also known as Carvacrol is an isomer of thymol. Both thymol and carvacrol have antifungal and insecticidal potency\(^3^5,^3^6,^3^7\). We have reported potential activity of pure carvacrol compound (98-99% purity) against adult uzi fly with LC\(_{50}\) value of 194.5 ppm in contact residual mode of application\(^1^7\). The presence of carvacrol in the active oil fraction of the plant once again confirms its potential toxic affects against the fly. However biological interactions of terpene compounds with other compounds in mixture could not be predicted as some of the associations may show synergism, some other association show antagonism and sometimes with no apparent differences in total toxic effects.

While six *O. gratissimum* essential oil-based combinations were prepared and tested for assessing fumigant toxicity then the composition M comprising equal parts of *Ocimum gratissimum, Ocimum sanctum, Eucalyptus maculata, Callistemon linearis*, *Citrus sinensis* were found as the most effective one than the other combinations and *O. gratissimum* oil alone. This might be due to the synergistic effects of the constituents present in the essential oils of the selected plants. The finding is in conformity with the finding of Alves et al.\(^3^8\) who found mixture of essential oil of *Cymbopogon citratus, Cedrus atlantica* and *Corymbia citriodora* as potential adulticide against *Ceratitis capitata* (Diptera: Tephritidae). We have also recorded the combinations of essential oil of *Allium sativum* and *Ocimum sanctum* as potential larvicide against *Culex quinquefasciatus*\(^3^9\). In most of the earlier studies it is established that toxicity of a plant essential oil is mostly due to the activity of major constituent compounds. In the active fraction of *O. gratissimum* essential oil, the major compound detected was thymol or its isomer carvacrol. In our previous experiment as we found higher toxicity of carvacrol as compared to thymol against uzi fly\(^1^7\), therefore, carvacrol based formulations were prepared taking some other major constituents namely eugenol, eucalyptol, citral reported to be present in essential oil of *Ocimum sanctum, Eucalyptus maculata, Callistemon linearis* and *Citrus sinensis*. The result showed that the formulation MII comprising of carvacrol, citral, eugenol, eucalyptol showed higher mortality (60% mortality after 6h of treatment and 100% mortality after 24h of treatment) of flies at 500 ml of air volume in comparison to the formulation CI containing carvacrol alone with maximum 30 percent mortality after 24h of exposure in fumigant mode of application. The formulation of essential oil compounds (MII) was more effective than the formulation of the mixture of the crude essential oils (MI) (Fig6). Similar findings were disclosed by Dean et al\(^4^0\) where they described efficacy of aqueous formulation of nootkatone (5%) and carvacrol (5%) against *Ixodes scapularis* Say and *Amblyomma americanum* L. (Acari: Ixodidae). The insecticidal efficacy of the combinations of major essential oil compounds such as 1,8- cineole plus camphor, thymol plus \(\rho\)-cymene and citral plus limonene were reported against *Trichoplusiani*\(^4^1\). We have also reported some of the combinations of major essential oil compounds viz. eucalyptol plus eudesmol and carvone plus limonene as effective combinations than the individual compounds against adult *Aedes aegypti*\(^4^2\). But no such reports of formulation or combination of plant products are found to be reported in literature against
the silkworm parasitoid *E. sorbillans*. Therefore, the present finding can be very well explored further for developing commercial formulation to be used for the management of *E. sorbillans* in sericulture field.

We also studied the target site of action of the primary essential oil constituents of *O. gratissimum* and the others used in development of formulation. Findings of the previous researchers revealed that one of the primary targets of essential oils of plants and their terpene compounds as well as organophosphate and carbamate pesticides in insects are the acetylcholinesterase enzyme of nervous system\(^{43}\). The compounds inhibit AChE activity which subsequently lead to accumulation of the neurotransmitter acetylcholine at the neuronal synapse resulting in continuous stimulation and lack of coordination in the neuromuscular system and final death of the treated insect. While studying the affinities of the selected terpene compounds used in preparation of combination to be treated against uzi fly, we found positive interactions of all the selected compounds with the targeted enzyme. The highest affinity was recorded for the compound carvacrol (affinity -6.9 Kcal/mol) followed by eucalyptol (-6.1 kcal/mol), thymol (-5.1 kcal/mol), eugenol (-4.8 kcal/mol), citral (-4.1 kcal/mol). The lowest affinity was found for the enzyme substrate acetylcholine (-3.9 kcal/mol) among the all tested chemical compounds. The higher affinities of selected terpene compounds than the acetylcholine is a good indication that the compounds could effectively inhibit the enzyme. The result is consistent with our earlier findings of in silico interactions of terpene compounds with acetylcholinesterase enzyme of *Aedes aegypti* where we recorded higher binding affinities of eugenol, eucalyptol and carvacrol along with three other terpene compounds than acetylcholine\(^{44}\). Among the compounds thymol and acetylcholine showed both hydrogen and hydrophobic interactions while other compounds showed hydrophobic interactions with amino acid residues of the target protein. Recently Hussain et al\(^{45}\) reported positive binding interactions of twenty-eight essential oil constituents of the medicinal plant *Commiphoramyrrha* which included eugenol, β-eudesmol, caryophyllene, α-pinene, α-humulene etc. with more than -5 kcal/mol binding affinity with acetylcholinesterase enzyme. In another study Wang et al\(^{46}\) showed invitro acetylcholinesterase activity of honokiol and nine structurally related phenolic compounds against larvae of *Aedes aegypti*. From this finding it can be inferred that one of the reasons of the insecticidal activity of the combination might be due to the inhibitory effect of the constituents on AChE enzyme. However further study on extraction and isolation of the enzyme and activity test will be needed to confirm the same.

**Declarations**

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