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Development of Bio-larvicide for *Anopheles stephensi* through Selected Phytoligands from the Leaf of *Eucalyptus grandis* Against Mosquito Acetylcholinesterase: An *In Silico* Approach

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***Abstract*:** The mosquito, *Anopheles* sp. causes mosquito-transmitted diseases to human. The present study was determined percentage mortality of larvae (*Anopheles stephensi*) by leaf extract (*Eucalyptus grandis*) in aqueous medium and the inhibitory effects of selected bioactive compounds present in the leaf against the mosquito acetylcholinesterase through an *in silic*o approach. The acetylcholinesterase (receptor) was obtained (PDB ID: 2AZG) from the Protein Data Bank (PDB) and the canonical SMILES of selected eleven phytoligands were obtained from PubChem database. The three-dimensional (3D) structure of phytoligands were procured from online CORINA software. The softwares, PyRx (Version 0.8) for receptor-ligand binding and T.E.S.T. (Version 4.1) for QSAR modelling to know predictive toxicity were used in the present study. The present results indicated that the percentage mortality was observed to the larvae (*A. stephensi*) at higher concentration ranges 70–100% during 48h exposure by the aqueous leaf extract of *E. grandis*. The binding interactions resulted six compounds may be reversible inhibitors find the binding just opening of the active site while five compounds may be irreversible inhibitors to obtain binding opposite side of the active site. The rat oral LD_{50} values range between 1383.11 to 4286.15 mg/kg, which may be indicated low toxic compounds with biodegradable capacity. It is concluded that experimental bioassay and in silico study, individual and/or combinations of phytoligands of *E. grandis* might be used as bio-larvicide to develop *A. stephensi* immobility. It is suggested experimental bioassay with each bioactive compound and molecular dynamics study for the validation of the present data.

Keywords: eucalyptus leaf extract; bioassay; mosquito bio-larvicide; mosquito acetylcholinesterase; molecular docking; QSAR modeling; phytocompounds

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I. INTRODUCTION

The mosquito-transmitted diseases are global threat and it has been reported also in many parts of India [1-3]. Among other mosquito species, *Anopheles stephensi* is a suitable vector for malaria and contains various species of *Plasmodium* [1-8]. From past to recent studies, several insecticides have been discovered to eradicate larvae of mosquito, but a critical issue has evolved due to the resistance of the synthetic larvicides and found unable effort for mosquito control as immobility to larvae [9-15]. On the other hand, these synthetic chemicals have a potent toxicological impact to human through the food chain [16].

According to researchers, in present scenario, several phytochemicals are potential for mosquito (*Anopheles* sp.) larvicides as their origins are from plants [17-19]. Many of these phytochemicals are suitable non-toxic agents for mammals, including human and also biodegradable environmental safe compounds [17-22]. Besides these, the leaf extracts of *Eucalyptus* sp. (family Myrataceae) have already been showed larvicidal activity to the mosquito larvae (*Anopheles* sp., etc.) in the laboratory study [23-25], but the detection of exact compound for inhibition of the acetylcholinesterase activity by an *in silico* approach and the cause of immobility to the larvae has not been attempted earlier.

In general, in silico means bioinformatics in which computational prediction study viz. molecular docking to know receptor-ligand binding can easily be achieved [26-27], QSAR (Quantitative Structure Activity Relationship) modelling to know predictive toxicity, mutagenicity, developmental toxicity, etc. [28]. The *in silico* study is related to design bio-larvicides from bioactive compounds of plants, which is well known for mosquito prevention [29]. Several researchers have mentioned that the immobility or mortality of larvae by using insecticides due to acetylcholinesterase enzyme (AChE) inhibition, which affects the neurotransmission [30]. It is noteworthy, synthetic insecticides showed resistance to the mosquito gene of AChE and the target site is an insect-specific cysteine residue, found peripheral site of acetylcholinesterase [31]. According to researchers, new insecticides can only be suitable when low or non-toxic

effect without resistance to insects and particular inhibition target site is achieved [30-36].

Generally, QSAR (Quantitative Structure Activity Relationship) is done for faster screening of toxicity evaluation through mathematical modelling, which relates the structural features or molecular descriptors and the biological properties of any compound [37-39]. This study helps to predict toxicity such as LC_{50} (median lethal concentration) or LD_{50} (median lethal dose), etc. in several biota viz. daphnids, fish, rats, etc. [40-41]. Another part of *in silico* study, is also based on quantitative structure–activity relationship (QSAR) models, which can be used to formulate new compounds or drugs and screen chemical libraries [37-45].

The present study was determined percentage mortality of the larvae of *Anopheles stephensi* (Liston, 1901) by aqueous extract of leaf and to predict mosquito acetylcholinesterase inhibition by the established bioactive compounds in the leaf of *Eucalyptus grandis* through an *in silico* approach with special reference to molecular docking and QSAR modeling.

II. MATERIALS AND METHODS

Collection of leaf sample and preparation of extract

The eucalyptus leaf sample was collected nearby college campus, Serampore, West Bengal, India. The aqueous leaf extract of *Eucalyptus grandis* was prepared by using fresh leaves. The extraction was done by the methods of Zareen et al. [25] with some modifications. All the leaves were washed thoroughly in the running tap water, followed by distilled water, then kept on the blotting paper to soak the excess water. The leaves of 20 nos. were kept for drying in the shed at room temperature and made it in powder form. Finally, the powder was dissolved in dechlorinated tap water. The solution was filtered and taken in a clean glass bottle as a stock solution (100%).

Bioassay of *Anopheles stephensi* larvae

From this stock solution, different dilutions were prepared as 70%, 40%, 20% and 10%. The supplied larvae (*Anopheles stephensi*) were kept in the aerated water prior to toxicity test and 10 nos. were used in each petri dish as per serial dilutions (100% - 10%). This test was done in duplicate as replica. In each dilution and for 0hr, 24hr and 48hr exposure, the percentage mortality was observed.

Protein retrieve

The crystal structure of mosquito protein acetylcholinesterase (PDB ID: 2AZG) was selected (Figure 1) and retrieved from protein data bank (http://www.rcsb.org/) because this protein is susceptible in the larvae of mosquito due to inhibition by compound(s) [46].

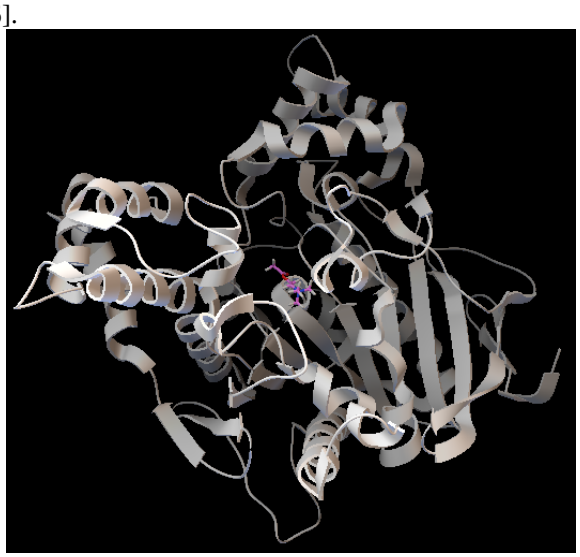


Figure 1. Ribbon representation of crystal structure of acetylcholinestarase protein of mosquito. Ball and stick structure is acetylcholine located at catalytic site

Phytoligands selection

Established 11 phytocompounds viz. aromadendrene, terpineol, eucamalol, alloocimene, eucalyptol, isopulegol, p-cymene, limonene, linalool, citronellal and citronellol were selected as per literature on *Eucalyptus* sp. reported by Hardel and Sahoo, [47] and Nair et al. [48]. The SMILES (simplified molecular-input line-entry system) string for each compound were retrieved from the NCBI PubChem database (http://www.ncbi.nlm.nih.gov/pccompound/), all the phytoligand molecules were converted into 3D structure by using the CORINA online software (http://www.mol-net.de) after inserting the canonical SMILES string for each chemical and all the 3D structure of each ligand are depicted in Figure 2.

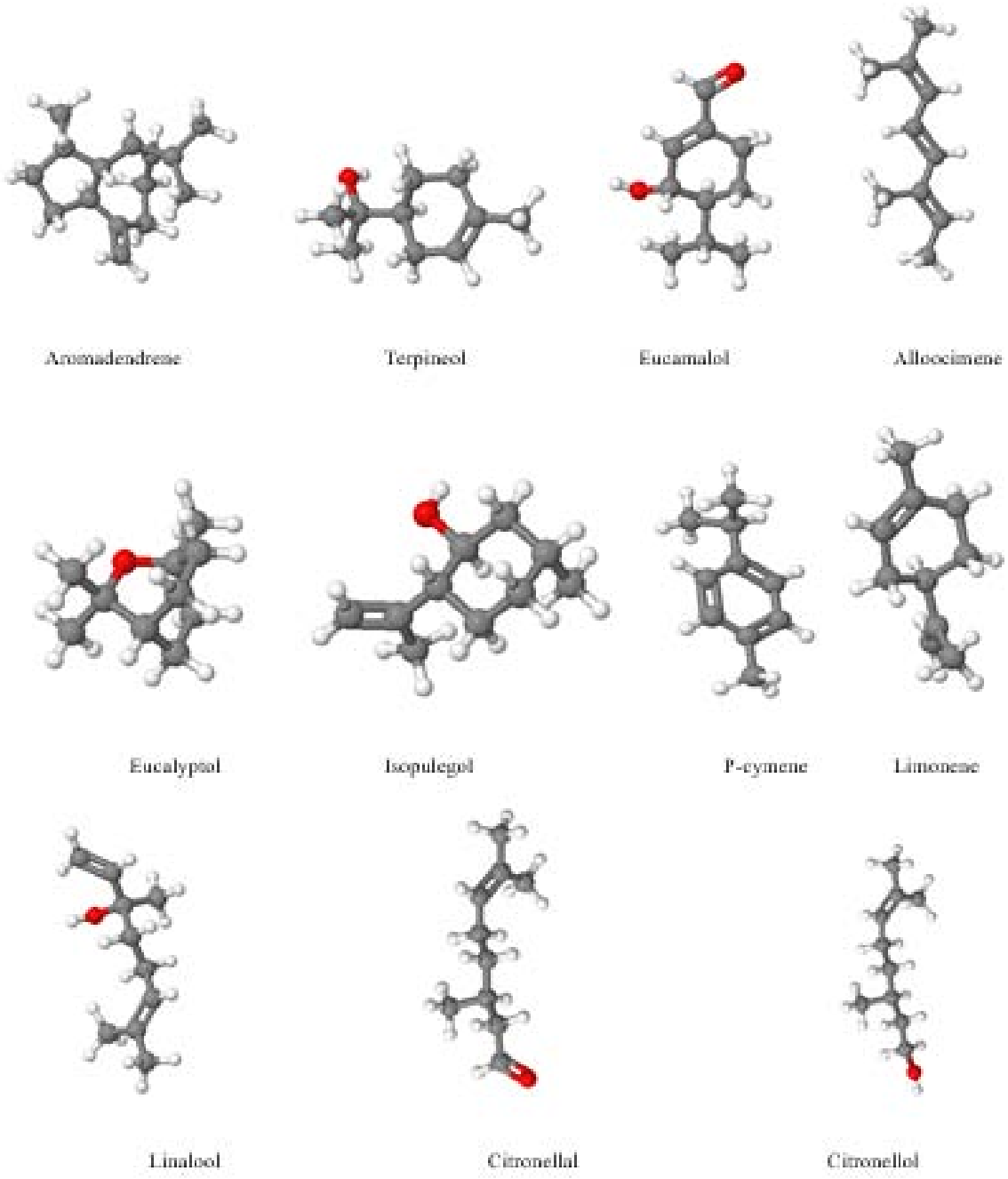


Figure 2. 3D structure of phytochemicals found in the leaf of *E. grandis*

Molecular docking study

The molecular docking was done through PyRx software (Version 0.8) developed by Trott and Olson [49]. The molecular docking result for each receptor-ligand binding was visualized through pdbqt output by using this tool. The docking site on this target protein was inserted within a grid box with the dimension values for X: 63.3502 Y: 74.6010 Z: 72.2942 Å, with a grid spacing value of 0.375 Å, values from centred X: 116.14 Y: 103.95 Z: -142.83 Å were noted. This tool helps to predict the energy value as well as receptor-ligand binding site for each phytoligand [50-51].

Predictive toxicity study by QSAR modelling

The QSAR modeling software package was used to estimate the LC_{50} of *Daphnia magna* and *P. promelas* and rat oral LD_{50} values of established phytochemicals of *E. grandis* (Figure 2). In the present study, Toxicity Estimation Software Tool (T.E.S.T.), Version 4.1 was used [28]. The predicted values for LC_{50} and LD_{50} were obtained after operating the above mentioned software.

III. RESULTS AND DISCUSSION

Percentage mortality for larvae of *Anopheles stephensi*

In Table I, different dilutions of leaf extract such as 10%, 20%, 40%, 70% and 100% within 24hr exposure 40%, 40%, 60%, 80% and 90% mortality were recorded while 10, 20, 40, 70 and 100 dilutions of leaf extract within the 48hr duration 70%, 80%, 90%, 100% and 100% mortality recorded. The present bioassay results for larvae of *Anopheles stephensi* indicated highest percentage mortality

(90%) in 100% leaf extract within 24hr duration while 100% mortality was obtained in 70% - 100% dilution of leaf extract of *E. grandis* within 48hr exposure. It was already known that the aqueous extracts of different dilutions of *Eucalyptus* sp. caused mortality to the larvae of *Anopheles* sp. mosquito, which is supporting the present data of larval mortality in both the durations [23-25]. The R^2 values were observed 96.70% and 85.03% for 24hr and 48h duration respectively. The regression curve for each duration is depicted in Figure. 3. The present study revealed that the phytochemicals present in the leaf of *E. grandis* potential to immobile mosquito larvae of *A. stephensi* and leaf extract can be used as larvicide for mosquito control [24].

Table I. Percentage dilution of neem leaf extract versus percentage mortality of larvae

Extract concentrations (% dilution)	***Species used (in nos.)***	***Duration of exposure (in hr)***		
		0	***24***	***48***
		% mortality	***% mortality***	***% mortality***
Control (0)	10	0	0	0
10	10	0	40	70
20	10	0	40	80
40	10	0	60	90
70	10	0	80	100
100	10	0	90	100

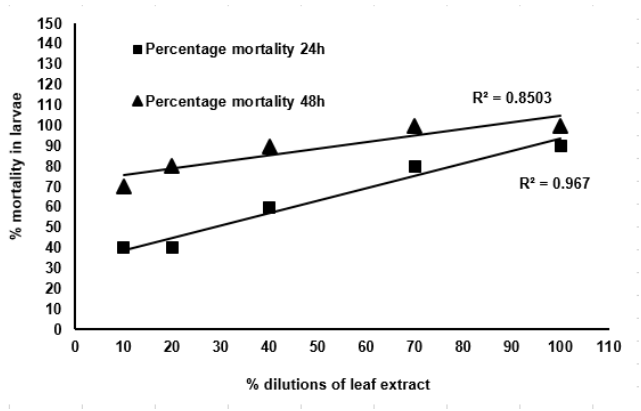


Figure 3. Regression curve of % mortality for larvae of *A. stephensi* versus % dilution of leaf extract of *E. grandis*

Receptor-ligand binding through molecular docking

It was observed from Table II that the binding energy value (kcal/mol) in sequence as aromadendrene (-7.4), terpineol (-6.3), eucamalol (-5.7), alloocimene (-5.5), eucalyptol (-5.5), isopulegol (-5.5), p-cymene (-5.4), limonene (-5.2), linalool (-5.1), citronellal (-5.0) and citronellol (-5.0) were observed. The researchers have already been studied reversible and irreversible inhibition in the acetylcholinesterase protein [30-36; 52] and earlier, there have been found compounds that developed resistance in the genes of AChE [31; 52]. The isolation of this particular phytochemical can be suitable from eucalyptus leaves for mosquito larvae, especially *A. stephensi* control through bio-larvicide development [24].

3D ribbon representation for binding position for all above phytoligands was obtained through PyRx software and the 3D structure of binding interaction studied. According to Djogbenou et al. [52], compound(s) binding with the residues viz. Asn, Asp and Gly at 140, 189, 24 and 119 position as insecticides resistance while catalytic triad at position of Ser199, Glu325 and His439 for AChE protein have been investigated (Figure 4A).

It was observed that near mouth of the catalytic site of AChE, the ligands such as aromadendrene (connected with hydrophobic residues Asp72, Phe75, Asn85, Tyr332 and Trp84 without hydrogen bonding), citronellal (connected with hydrophobic residues Phe76, Tyr332 and Arg339 without hydrogen bonding), limonene (connected with hydrophobic residues Trp280, Asp72, Phe75 and Tyr332 without hydrogen bonding), alloocimene (connected with hydrophobic residues Trp280, Tyr121, Phe288, Tyr332 and Phe329 without hydrogen bonding), p-cymene (connected with hydrophobic residues Trp280 and Tyr332 without hydrogen bonding) and eucamalol (connected with hydrophobic residues Tyr121, Asp72 and Trp84 without hydrogen bonding) while opposite to the catalytic site the ligands viz. terpineol (connected with hydrophobic residues Leu131, Ser145, Val100, His132, Val19 and Val29 with one hydrogen bonding at Asn98), citronellol (connected with hydrophobic residues Leu31, Asn98, Val29 and Val100 with two hydrogen bonding at Ser145 and Ala125), eucalyptol (connected with hydrophobic residues Pro63, Thr126, Leu31 and Ser145 without hydrogen bonding) and isopulegol (connected with hydrophobic residues Pro63, Thr126, Ala125, Leu31, Asn98, Tyr130, ASP131 and His132 without hydrogen bonding) were observed (Table II and Figure 4B).

In Figure 4C, the binding interactions revealed that aromadendrene, eucamalol, limonene, alloocimene, p-cymene and citronellal may be reversible inhibitors to show binding just the mouth of the active site, which supported by other studies for inhibitory effect of compounds [30-31; 53-54] while terpineol, eucalyptol, isopulegol, linalool and citronellol may be irreversible inhibitors to obtain binding opposite side of the active site [30]. In the other words, reversible inhibitors are commonly used in the treatment of neurodegenerative diseases while the mechanism of irreversible inhibition found for compounds as insecticides and nerve agents [30].

Table II. Molecular docking for leaf phytochemicals of *E. grandis* against acetylcholinesterase protein of mosquito

Sl. No.	***Ligands***	***Binding energy (Kcal/mol)***	***Hydrophobic residues***	***H-bond residues***
1.	Aromadendrene	-7.4	Asp72, Phe75, Asn85, Tyr332 & Trp84	---
2.	Terpineol	-6.3	Leu131, Ser145, Val100, His132, Val19 & Val29	Asn98
3.	Eucamalol	-5.7	Tyr121, Asp72 & Trp84	---
4.	Alloocimene	-5.5	Trp280, Tyr121, Phe288, Tyr332 & Phe329	---
5.	Eucalyptol	-5.5	Pro63, Thr126, Leu31 & Ser145	---

6.	Isopulegol	-5.5	Pro63, Thr126, Ala125, Leu31, Asn98, Tyr130, ASP131 & His132	---
7.	P-cymene	-5.4	Trp280 & Tyr332	---
8.	Limonene	-5.2	Trp280, Asp72, Phe75 & Tyr332	---
9.	Linalool	-5.1	Arg133, Asp131, His132, Val29 & Leu31	---
10.	Citronellal	-5.0	Phe76, Tyr332 & Arg339	---
11.	Citronellol	-5.0	Leu31, Asn98, Val29 & Val100	Ser145 & Ala125

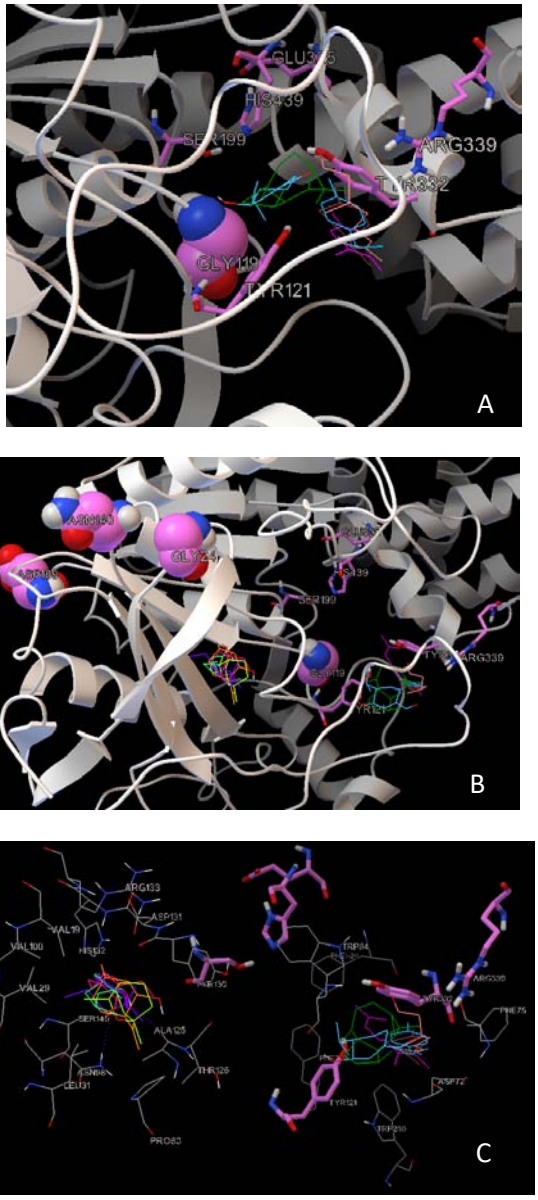


Figure 4. Pictorial representation of receptor-ligand binding interaction. A = Ribbon structure of protein, line structure of ligands and ball structure of residue Gly119; B = Ribbon structure of protein, line structure of ligands and ball structure of resistance residues Asn140, Asp189, Gly24 & 119, catalytic triad Ser199, Glu325 & His439 as ball & stick structure; C = Binding interactions through line structures of tested compounds, blue dotted lines represent the hydrogen bonding only in two compounds

QSAR modeling for predictive toxicity

The present predictive toxicity study was done on 11 types of common phytochemicals, which have already been reported in the leaf of *Eucalyptus* sp. [47-48]. The CAS (Chemical Abstracts Services) no. for each phytocompound along with the acute toxicity (LC_{50}) prediction data in *D. magna* and *Pimephales promelas* and rat oral LD_{50} value were tabulated in Table III. Out of the 11 common phytochemicals, only in 8 compounds were obtained predictive toxicity data by using T.E.S.T. For *D. magna*, it was observed the predicted LC_{50} data (mg/l), for terpineol (4.68), eucalyptol (44.96), isopulegol (2.19), p-cymene (5.86), limonene (2.17), linalool (1.77), citronellal (4.01) and citronellol (11.50) while in *P. promelas*, terpineol (29.47), eucalyptol (91.83), isopulegol (8.47), p-cymene (6.04), limonene (1.34), linalool (9.24), citronellal (4.93) and citronellol (6.24) respectively. In case of rat oral LD_{50} data (mg/kg), the values were obtained for compounds viz. terpineol (2755.15), eucalyptol (1383.11), isopulegol (2785.28), p-cymene (3265.39), limonene (4286.15), linalool (2054.27), citronellal (4065.79), and citronellol (3118.25) respectively (Table III).

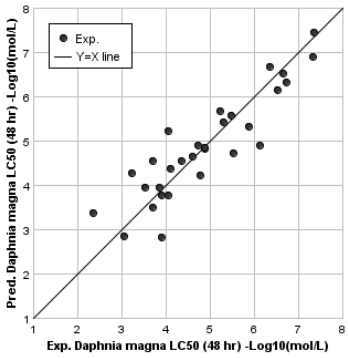
The predictive toxicity study through QSAR modelling is helpful prior to experimental bioassay, which can easily be determined the exact suitable chemical compound [54-56]. The present results indicated few compounds were toxic to *D. magna* as well as *P. promelas* but low toxic to the mammal especially rat oral exposure. These compounds are easily biodegradable and may not be persistent in the aquatic bodies like other synthetic larvicides [57]. The predicted results with the statistical interpretation for each phytochemical to *D. magna*, *P. promelas* (LC_{50} data) as well as rat oral exposure (LD_{50} data) were obtained through T.E.S.T. software. Only 3 compounds namely aromadendrene, eucamalol and alloocimene unable to predict the LC_{50} and LD_{50} values due to CAS number was not identified by the present tool (Table III).

The statistically significant value through the regression curve (R^2 value) for individual chemical was obtained by T.E.S.T. and is also tabulated in Table III and individual value of phytochemical for FDA model fit result is depicted (Figure 5 A-H, a-h and i-viii). According to Golbraikh and Tropsha [59] and Golbraikh et al. [58], if R^2 value beyond 0.6 or 60%, then a QSAR prediction model is acceptable. The statistical interpretations are very important and validation of predictive data through QSAR modelling is well-established [58].

Table III. QSAR-prediction data for phytochemicals of *E. grandis* leaf

Sl. No.	***Phytochemicals***	***CAS no.****	***Predicted LC_{50} values of Daphnia magna (mg/l)***	***R^2 values (in %)***	***Predicted LC_{50} values of P. promelas (mg/l)***	***R^2 values (in %)***	***Predicted Rat oral LD_{50} values (mg/kg)***	***R^2 values (in %)***
1.	Aromadendrene	72747-25-2	N.F.	---	N.F.	---	N.F.	---
2.	Terpineol	8000-41-7	4.68	81.0	29.47	98.0	2755.15	78.3
3.	Eucamalol	152246-70-3	N.F.	---	N.F.	---	N.F.	---
4.	Alloocimene	673-84-7	N.F.	---	N.F.	---	N.F.	---
5.	Eucalyptol	470-82-6	44.96	86.0	91.83	93.1	1383.11	77.0
6.	Isopulegol	89-79-2	2.19	89.0	8.47	90.3	2785.28	82.0
7.	P-cymene	99-87-6	5.86	93.0	6.04	82.3	3265.39	76.1
8.	Limonene	138-86-3	2.17	95.5	1.34	89.4	4286.15	72.0
19.	Linalool	78-70-6	1.77	89.3	9.24	87.0	2054.27	77.0
10.	Citronellal	106-23-0	4.01	80.2	4.93	89.1	4065.79	84.0
11.	Citronellol	106-22-9	11.50	77.0	6.24	82.0	3118.25	72.1

* Data obtained from ChemIDPlus (https://chem.nlm.nih.gov/chemidplus/name/); N.F. = Not found CAS no in T.E.S.T. database



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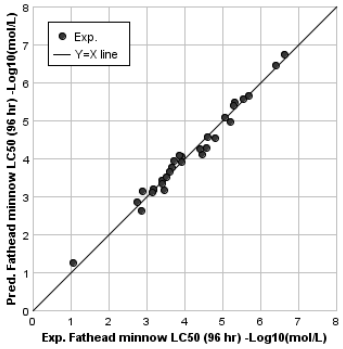

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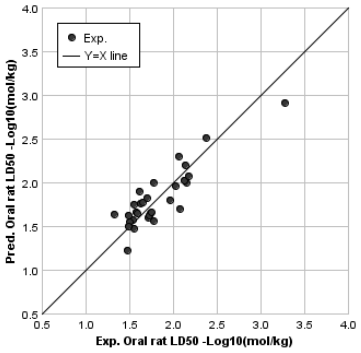

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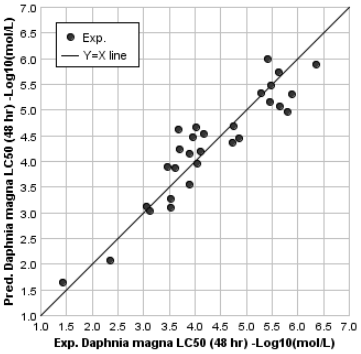

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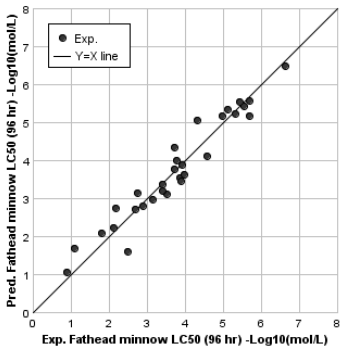

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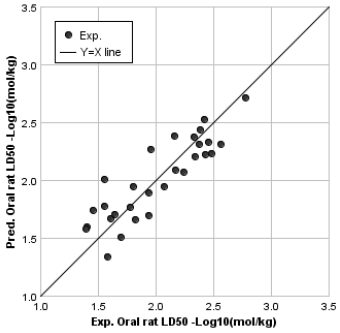

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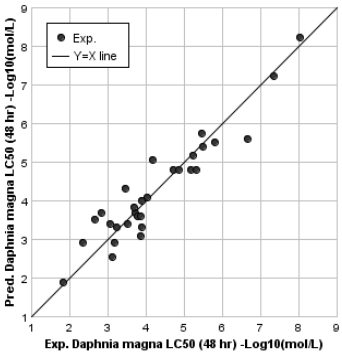

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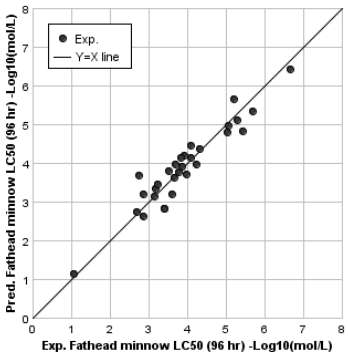

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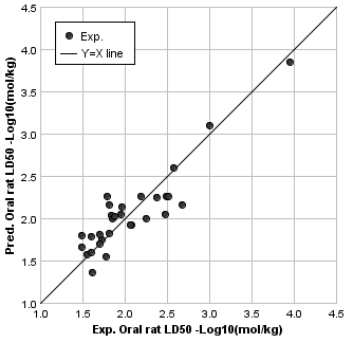

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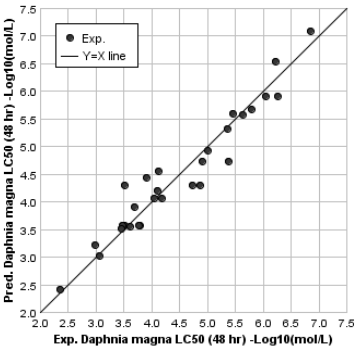

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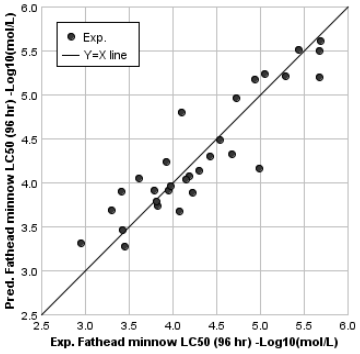

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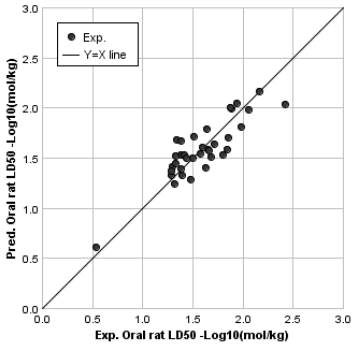

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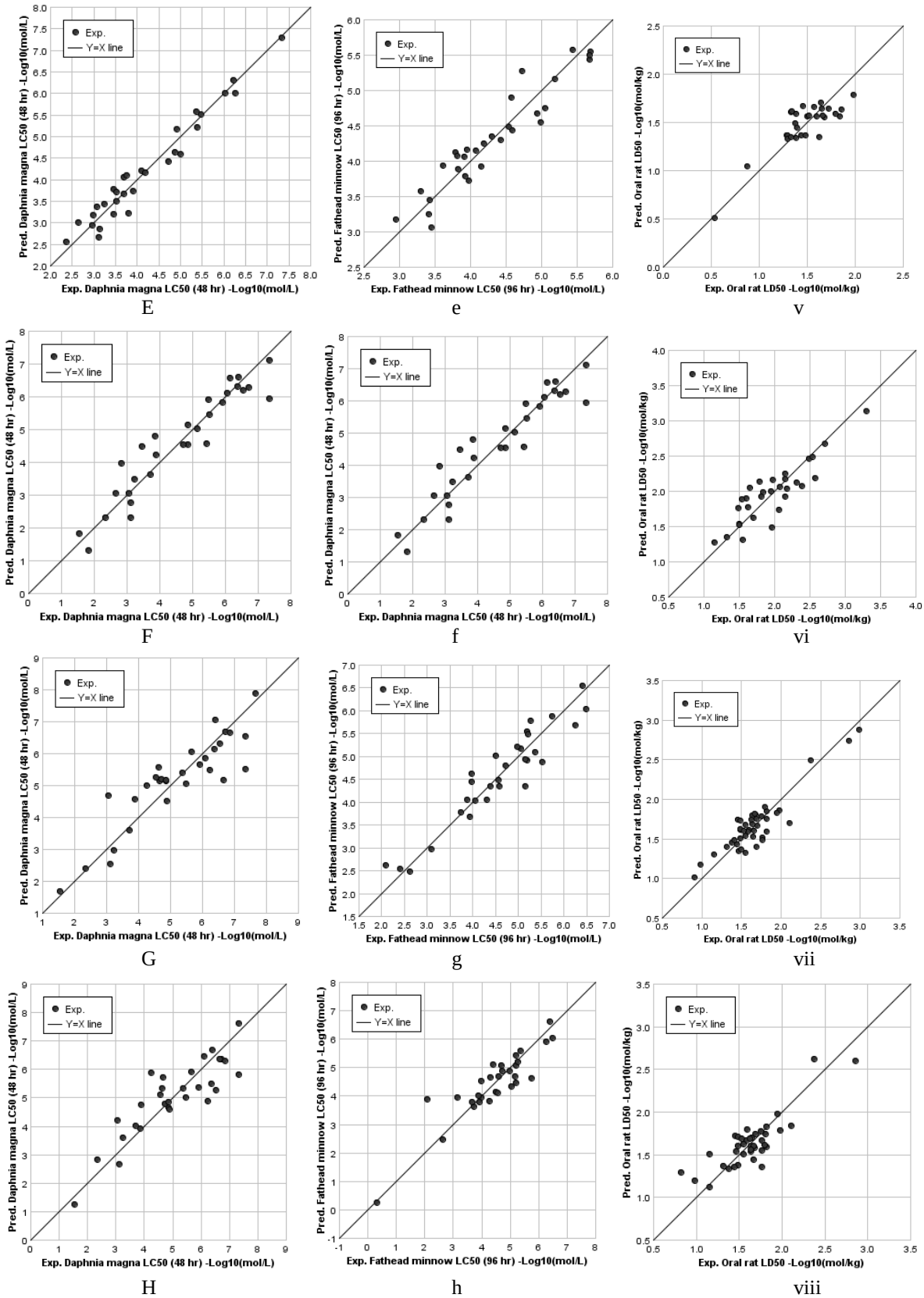

Figure 5. Statistical analysis and regression curve for toxicity prediction of phytochemicals (terpineol, eucalyptol, isopulegol, p-cymene, limonene, linalool, citronellal and citronellol). A-H and a-h = LC_{50} prediction curve for *D. magna* and *P. promelas*; i-viii = rat oral LD_{50} prediction curve

IV. CONCLUSION

In the present study, mosquito AChE inhibitory activities through *E. grandis* leaf extracts of bioassay on larvae of *A. stephensi*, followed by molecular docking and QSAR modelling by selecting bioactive compounds of leaf were investigated. It is concluded that leaf extracts of *E. grandis* in 70% - 100% dilution showed 100% mortality during 48hr duration as like synthetic pesticides [57]. In docking results against receptor mosquito AChE (PDB ID: 2AZG), among

11 compounds, 6 phytoligands viz. aromadendrene, eucamalol, limonene, alloocimene, p-cymene and citronellal may be reversible inhibitors to show binding just the mouth of the active site while others 5 phytoligands viz. terpineol, eucalyptol, isopulegol, linalool and citronellol may be irreversible inhibitors to obtain binding opposite side of the active site were obtained. In QSAR modelling, mammalian toxicity with special reference to rat oral LD_{50} value ranges 1383.11 to 4286.15 mg/kg, which indicated low toxic compounds and may have a biodegradable capacity in water. It is suggested *in vitro* and *in vivo* study to know resistance genes of mosquito (*A. stephensi*) AChE as reported for synthetic insecticides prior to developing larvicide [60].

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest for the present study.

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