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In silico and *In vitro* anti-mosquito analysis of some secondary metabolites from *Citrus* species on *Aedes aegypti* and *Culex quinquefasciatus*

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Abstract
The plant-based insecticides are a reliable and eco-friendly alternative to synthetic chemicals. Therefore, the aim of the present investigation is to identify the best insecticidal activity of secondary metabolites from *Citrus* species peel, such as naringenin, naringin, hesperidin and diosmin, by *in silico* analysis. Further, the same was analyzed for larvicidal, pupicidal and adultcidal activities, and histopathological changes in the midgut of *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes. Thus, based on the *in silico* analysis, naringenin showed the highest binding affinity towards the target protein AeSCP-2 with least binding energy value of -9. 75511 kcal/mol. Moreover, naringenin showed larvicidal and pupicidal activities after 24 h of exposure of naringenin at a different concentration like 2, 4, 6, 8 and 10 ppm. The larval mortality of LC_{50} and LC_{90} values of naringenin were 3. 537 and 9. 940 ppm against *Ae. aegypti*, meanwhile 3. 537 and 9. 940 ppm for *Cu. quinquefasciatus.* The pupal mortality at LC_{50} and LC_{90} were 3. 418 and 9. 848 ppm for *Ae. aegypti*; 3. 700 and 10. 009 ppm of naringenin for *Cu. quinquefasciatus*, respectively. The adutcidal effect was observed for naringenin at 3, 6, 9, 12 and 15 ppm against the mosquitoes. The adult mortality value of LC_{50} and LC_{90} were 5. 390 and 14. 701 for *Ae. aegypti* while 5. 719 and 15. 138 ppm for *Cu. quinquefasciatus*, respectively. Furthermore, histopathological examination also clearly shows destructive midgut regions in *Ae. aegypti* and *Cu. quinquefasciatus.* Thus, the present results demonstrated that naringenin is as good candidate for natural insecticide production that can help control the mosquito proliferation.

Keywords: *Aedes aegypti*, *Culex quinquefasciatus*, sterol carrier protein-2 and naringenin

Introduction
Vector-borne diseases, for example, malaria, dengue, yellow fever and filariasis, are considered to be serious health issues. Mosquito species belonging to the genera *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* play significant roles in the transmission of such vector-borne diseases [1]. The *Aedes aegypti* vector transmits several yellow fevers like dengue, chikungunya, viruses and other diseases [2]. *Culex quinquefasciatus* is an important vector of lymphatic filariasis [3]. According to the World health organization, the prevalence of dengue had been estimated to be around 3. 9 billion people (128 countries), who are at risk of getting infected by dengue viruses [4]. Based on [5] report, each year nearly 120 million people get infected by filariasis, over 40 million get severely disfigured and disabled, and almost 76 million have hidden damage to the lymphatic and renal system while remaining are symptomless.
Aedes aegypti and *Culex quinquefasciatus* are found abundantly in many tropical areas of the world and breed very fast in drains and stagnant water reservoirs; their development involves the holometabolous life cycle, which includes four life stages: eggs, larvae, pupae and adults [4]. Although the use of synthetic insecticides can interrupt these disease transmissions, the repeated use of insecticides can effectively destroy the ecosystem and lead to a resurgence of the mosquito population [6].
Various plants and/or plant-based products have been proven to be effective in controlling mosquitoes, are safer for the environment and are mostly target specific [7][8]. Citrus species have been reported as a source of botanical insecticides as a variety of these plants contains secondary metabolites that show insecticidal activity against several coleopterans and dipterans. Orange peel extract as pest management has many advantages over synthetic pesticides [9]. Previously, [10]. reported that *Citrus sinensis* orange peel extract had larvicidal, pupicidal, repellent and adulticidal activity against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*. The major secondary metabolites of *Citrus* species peel include naringenin, naringin, hesperidin, diosmin, didymin, poncirin, eriocitrin, narirutin, luteolin, etc. [11]. Among them, there is no proper evidence about the mosquitocidal activity of naringenin, naringin, hesperidin and diosmin. Hence, the main goal of this study is to analyse anti-mosquito activity for these phytocompounds by *in silico* study. Furthermore, the best phytocompound would be utilized for larvicidal and pupicidal, effects against *Aedes aegypti and Culex quinquefasciatus*.

Materials and Methods
In silico study
The three-dimensional crystal structure of the sterol carrier protein of *Aedes aegypti* (AeSCP-2) (PDB ID: 1PZ4) was retrieved from Protein data bank (PDB) (www. rcsb. org/pdb/). The coordinate file for AeSCP-2 was obtained by using the molecular visualization viewer, i. e., the SPDB (www.expasy.org/spdbv/) viewer. Amino acids in active site

of AeSCP-2 were SER-18 to HIS-28, and it was also confirmed by using binding pocket detection server tools (pocket finder and Q-site finder) (www. modelling. leeds. ac. uk/qsitefinder). The predicted binding sites were based on the binding energy that contained 17 amino acids making up this binding cavity. All the predicted ligand binding site residues are listed in the

Table 1: Binding site amino acids and its structural topology of AeSCP-2 (PDB: 1PZ4)

Amino acid in the binding pocket		Binding site amino acids in structural unit of AeSCP2
VAL-8, PHE-9, ARG15 & LEU-16	:	Alpha – Helix
SER-18, ILE-19, ASP-20, ARG-24, GLN-25 &VAL-26	:	Ist Loop
TYR-30 & PHE-32	:	B-Sheet-I
MET-46 & LEU48	:	B-Sheet-II
LEU-64 & MET-66	:	B-Sheet-III

Selected compounds

The PubChem database was utilized for retrieving the selected four phytocompounds (Figure 1). All the selected chemical structures were generated (from SMILES notation) using Chem Sketch Software (www.acdlabs.com). After successfully building the structures and geometry optimization, it was followed by energy minimization. The energy minimization procedure was done for 100 cycles, using the Chimera software.

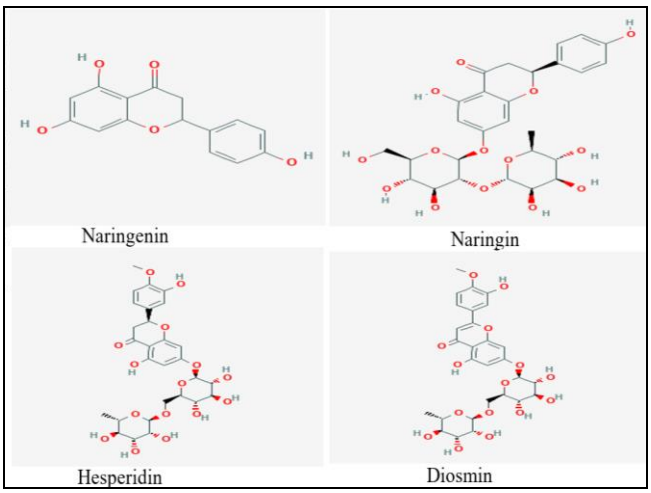

Fig 1: Molecular structures of selected phytocompounds

Molecular docking using auto dock 4. 0

Molecules were taken from the top-ranked ligand and interaction studies were carried out using Auto Dock 4. 0

Protein-Ligand Docking

Protein preparation

The Auto Dock 4. 0 was used for performing the docking process. The initial steps for protein preparation were adding of polar hydrogens to the target protein AeSCP-2. Next the appropriate partial atomic charges were assigned. The charged protein was converted to the 'PDBQ' format to read the Auto Grid. However, in most modeling systems, polar hydrogens are added in a default orientation, assuming that each new torsion angle would be around 0° or 180°. Additionally, without some form of refinement, it would lead to spurious locations of the hydrogen-bonds. One option was that the hydrogens were relaxed and a molecular mechanics minimization was performed on these structures. Another program used was "pol_h", where the default-added polar hydrogen structure was taken as the input. Moreover, favorable locations for each of the movable protons were sampled and only the best position of each was selected. This type of 'intelligent' placement of the movable polar hydrogen is particularly important for amino acids serine, tyrosine, and threonine.

Ligand preparation

Firstly, the hydrogens were added to all the atoms in the ligands and was ensured that all their valences were properly completed. This was carried out using ADT (molecular docking package). Further, it was ensured that the atom types were corrected before addition of the hydrogens. Based on the preference, i. e. whether charged and/or neutral carboxylates and/or amides are required, the pH was specified (automatically). Furthermore, the partial atomic charges were assigned to the ligand molecules. These charges were written in 'PDBQ' format that had columns similar to a Brookhaven PDB format, but also has an added column for the partial atomic charges.

The setting and running of the auto grid

All the pre-calculated grid maps, one for each atom type present in the ligands being docked, which were required for Auto Dock to make the docking calculations very fast. These maps were calculated by using the Auto Grid. A grid map was produced with a three-dimensional lattice of the regularly spaced points, that surround (either entirely or partially), and the centered on the active sites of the macromolecules/ligands, i. e., 17 amino acids of AeSCP-2 protein structure. A typical grid point spacing varied between 0. 2Å to 1. 0Å, although the default was approximately 0. 375Å. Potential energy of a "probe" atom or the functional groups was due to all the atoms in the macromolecules being stored in each point within the grid maps.

An input grid parameter file that usually carries an extension of "gpf", was also required for Auto Grid. The maximum as well as the minimum energies found during the grid calculations process for AeSCP-2 were stored in log file. With all these important features of the Auto Grid, it was set exactly on the active site of the AeSCP-2 (1PZ4) as well as the grid parameter file was written.

Running of the auto dock

The molecular docking was carried out using a Genetic Algorithm, i. e., the Least Square (GA-LS) algorithm, using Auto Dock 4. 0. After the grid maps were prepared by the Auto Gird as wewll as the docking parameter file (dpf) was ready, the Auto Dock job was run. The docking results were viewed using 'getdocked'. It was called as "lig. macro. dlg. ", and all the docked conformation outputs were visualized as well as analyzed. From the various poses of docking, the complex formed with the least energy and with a very stable conformation was selected as the final result.

In vitro analysis

Chemicals

Naringenin was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals as well as the reagents used were of analytical grade and were purchased from Merck Sigma-Aldrich, Himedia, Mumbai, India.

Collection of eggs and maintenance of larvae

The eggs of *Ae. aegypti* and *Cu. quinquefasciatus* were collected from VCRC (Vector Control Research Centre), Puducherry, a unit of ICMR, using an "O"-type brush. These eggs were then brought to the laboratory and transferred to 18×13×4-cm enamel trays that contained 500 mL of water for the hatching process. All the mosquito larvae were fed with pedigree dog biscuits and yeast at as ratio of 3:1. The feeding was continued till all the larvae transformed into pupal stage.

The maintenance of the pupae and adults

All the pupae collected from the culture trays were transferred to plastic containers (12×12 cm) that contained 500 mL of water, by using a dipper. Further, the plastic jars were maintained in a 90×90×90-cm mosquito cage for the emergence of adults. The mosquito larvae were all maintained at 27+2°C, relative humidity of 75–85 %, under a photoperiod of 14:10 (light/dark). Additionally, a 10 % sugar solution was provided for a period of 3 days before the blood feeding stage.

The adult mosquito vectors

All the adult female mosquitoes were allowed to feed on the blood of a rabbit (a rabbit/day, exposed on the dorsal side) for 5 days to ensure adequate blood feeding for 5 days. After blood feeding, enamel trays with water from the culture trays were placed in the cage as oviposition substrates.

Larval and pupal toxicity test

Laboratory colonies of mosquito larvae/pupae were utilized for the larvicidal and pupicidal activities. A total of twenty-five numbers of first to fourth instars larvae and pupae were introduce into 500 mL of the test medium containing a particular concentration of the 2, 4, 6, 8 and 10 ppm of naringenin in plastic cups in six replications. In the control, the same number of larvae and pupae were maintained in 500 mL of dechlorinated water containing an appropriate volume of DMSO. All the containers were maintained in the laboratory at the room temperature (28±2°C) with naturally prevailing photoperiod (12 hours of light & 12 hours of dark). If any larvae/pupae were considered to be dead if it was not moving when prodded repeatedly with a soft brush. The mortality of each pupa was recorded after 24 h of exposure period to the compound (naringenin) by following the Abbott formula [12].

Adulticidal bioassay

The adulticidal bioassay was performed by the [13]. Appropriate concentrations of naringenin were diluted with ethanol to achieved different concentrations and applied on the Whatman no. 1 filter papers (size 12× 15 cm). The control papers were treated with ethanol under similar conditions. The adulticidal activity of the naringenin was evaluated at various concentrations (3, 6, 9, 12 and 15 ppm). Twenty female mosquitoes were collected and gently transferred into a plastic holding tube. The mosquitoes were first allowed to acclimatize in holding tubes for 1 hour and then exposed to test paper for 1 hour. Then, at the end of the exposure duration, the mosquitoes were transferred back to the holding tube and kept 24 h for the recovery period. A pad of cotton soaked with 10 % glucose solution was placed on the mesh screen. Each of the experiments was replicated six times along with the appropriate control. The mortality of mosquitoes was determined at the end of the 24 hour recovery period. LD_{50} and LD_{90} with their 95 % confidence limits were determined using Log probit analysis test [14].

Histopathological studies

After the exposure of 24 hours naringenin treated, the alive larvae were then collected for final examination. All the larvae were rinsed with distilled water before the fixation with the Bouin's solution, followed by dehydration using graded ethanol and toluene series. Then, the larvae were embedded in paraffin, sectioned and stained with Haematoxylin and Eosin before the examination using compound microscope [15].

Statistical analysis

Data were arranged in an Excel sheet. The statistical analysis for the experimental data was performed using the computer software Stat Plus 2009 (Analyst Soft, Canada) to find the lethal concentration against larvae (LC_{50} and LC_{90}) out in 24 hours by probit analysis with a reliability interval of 95%. Additionally, to determine if there was a significant statistically difference among different doses of naringenin against mosquito larvae, student's t-test was used to analyze the difference of the percentage of mortality. The results with different superscripts (a, b, c) in each experimental group were significantly different at $p<0.05$.

Results

In silico study

The results obtained in the *in silico* study revealed the possible binding orientation of the selected four phytocompounds, i. e., naringenin, naringin, hesperidin and diosmin, against the mosquito sterol carrier protein (AeSCP-2) marker. Table 2 enlists the binding free energy values that were obtained from docking each of the four selected phytocompounds (ligands). Figures 2 illustrate the type of interactions that were observed between the ligands and the AeSCP-2 protein.

Table 2: Binding energies obtained for the selected phytocompounds aginst AeSCP2

S. No	Name of the phytocompound	Docking energy/ binding free energy (BFE) kcal/mol
1	Naringenin	-9. 75511
2	Naringin	-9. 29459
3	Hesperidin	-9. 39762
4	Diosmin	-9. 02305

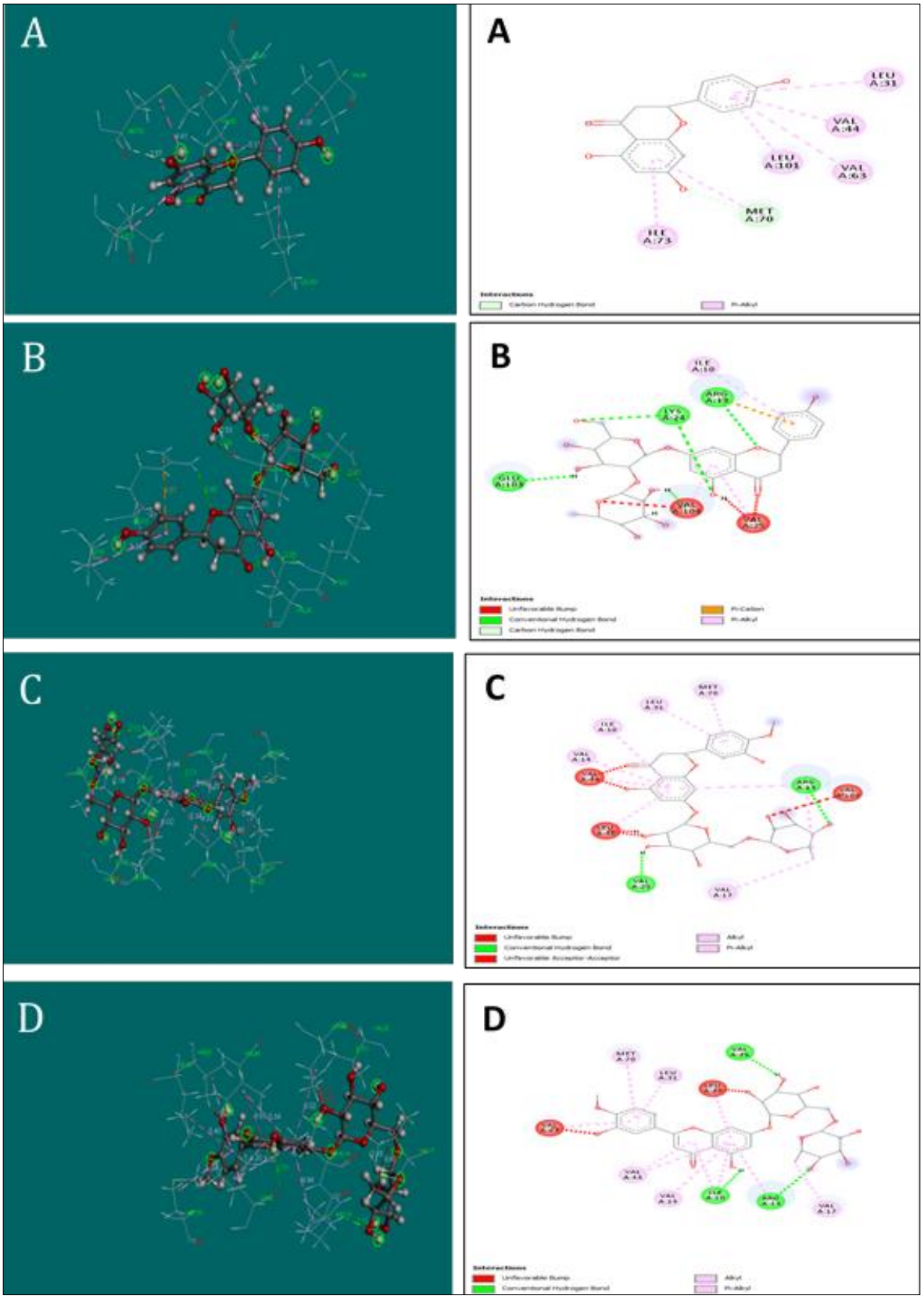

Fig 2: Docking results obtained (3D and 2D structural interactions) for AeSCP2 protein with selected phytocompounds.

(A1 & A2: Naringenin; B1 & B2: Naringin; C1 & C2: Hesperidin; D1 & D2: Diosmin)

The docking of target protein AeSCP-2 with naringenin revealed the presence of one carbon-hydrogen bond with MET A: 70 residue, and six Pi-Alkyl interactions with ILE A:73, LEU A:101, VAL A:63, VAL A:44 & LEU A:31 residues (Figure 3 A1 & A2). The docking energy or binding free energy obtained for naringenin was -9. 75511 kcal/mol.

The naringin docking with AeSCP-2 protein showed three unfavourable bumps with VAL A:104 & VAL A:25 residues, four conventional hydrogen bond with GLU A:103, LYS A:24 & ARG A:13 residues, one carbon-hydrogen bond with VAL A:104 residue, one Pi-Cation interaction with ARG A:13 residue, and three Pi-Alkyl interactions with ILE A:10, VAL A:104 & VAL A:25

residues (Figure 3 B1 & B2). The docking energy or binding free energy obtained for naringin was -9. 29459 kcal/mol.
The docking between hesperidin and the target protein AeSCP-2 revealed the presence of unfavourable bumps and unfavourable acceptor-acceptor interactions with VAL A:104, LEU A:46 & VAL A:44 residues, two conventional hydrogen bonds with VAL A:25 & ARG A:13, and nine Alkyl & Pi-Alkyl interactions with VAL A: 14, ILE A:10, LEU A:31, MET A:70, VAL A:44, LEU A:46, ARG A:13 & VAL A:17 residues (Figure 3. 2 C1 & C2). The docking energy or binding free energy obtained for hesperidin was -9. 39762 kcal/mol.
The diosmin docking against the AeSCP-2 protein showed the presence of two unfavourable bumps with the residues VAL A:63 & LEU A:46, three conventional hydrogen bonds with VAL A:25, ILE A:10 & ARG A:13 residues, and Alkyl & Pi-Alkyl interactions with MET A:70, LEU A:31, VAL A:63, VAL A:44, VAL A:14, ILE A:10, ARG A:13, VAL A:17 & LEU A:46 residues (Figure 3. D1 & D2). The docking energy or binding free energy obtained for diosmin was -9. 02305 kcal/mol.
Thus, among all the four selected phytocompounds, naringenin showed the highest binding affinity towards the target protein AeSCP-2 with a least binding energy value of -9. 75511 kcal/mol.

Larvicidal and pupicidal activities of naringenin

The different concentrations (2, 4, 6, 8 and 10 ppm) of naringenin were used to observe the larvicidal and the pupicidal activity against *Ae. aegypti* and *Cx. quinquefasciatus* (Table 3 & 4). The highest larval mortality of 98. 9 per cent was noticed at 10 ppm of naringenin, whereas the lowest larval mortality of 36. 2 per cent was observed at 2 ppm concentration for *Ae. aegypti*. The larval mortality was significantly increased when the concentration of naringenin was increased. The LC_{50} and LC_{90} values of naringenin were 3. 23 and 9. 321 ppm, respectively. The chi-square value was 0. 733, which showed that the larvicidal activity was significant at p<0. 05 level (Table 3). At the concentration of 10 ppm, naringenin showed the highest mortality for *Ae. aegypti* pupae and was found to be 95. 9 per cent, while the lowest mortality for *Ae. aegypti* pupae were 33. 2 per cent at a concentration of 2 ppm naringenin. The values of LC_{50} and LC_{90} naringenin were 3. 418 and 9. 848 ppm, respectively. The chi-square value was 0. 459, that demonstrated pupicidal activity at a significant level of p<0. 05 (Table 3).
The highest larval mortality of 90. 1 per cent was noticed at 10 ppm of naringenin for *Cx. quinquefasciatus*, whereas the lowest larval mortality of 33. 1 per cent was observed at 2 ppm concentration. The larval mortality was significantly increased when the concentration of naringenin increased. The LC_{50} and LC_{90} values of naringenin were 3. 537 and 9. 940 ppm, respectively. The chi-square value was 0. 432, which showed that the larvicidal activity was significant at p<0. 05 level (Table 4).
At the concentration of 10 ppm, naringenin showed the highest mortality for *Cx. quinquefasciatus* pupae and was found to be 90. 1 per cent, while the lowest mortality for *Cx. quinquefasciatus* pupae were 30. 00 per cent at a concentration of 2 ppm naringenin. The values of LC_{50} and LC_{90} naringenin were 3. 700 and 10. 009 ppm, respectively. The chi-square value was 0. 374, that demonstrated pupicidal activity at a significant level of p<0. 05 (Table 4).

Adulticidal activity of naringenin

Table 5 shows the adulticidal activity of naringenin against *Ae. aegypti* and *Cx. quinquefasciatus*. In *Ae. aegypti*, the mortality rate of 34. 2, 46. 2, 62. 4, 78. 4, and 92. 8 per cent were observed at 3, 6, 9, 12 and 15 ppm naringenin concentration, respectively. The highest mortality rate of 92. 7 per cent was present at a concentration of 15 ppm naringenin, whereas the lowest mortality of 34. 2 per cent was observed at 5 ppm naringenin concentration. The LC_{50} and LC_{90} values of naringenin against *Ae. aegypti* were 5. 390 and 14. 701 ppm, respectively. The chi-square value was 0. 488, which showed that the adulticidal activity was significant at p<0. 05 level (Table 5).
The mortality rate of *Cx. quinquefasciatus* of 32. 5, 45. 3, 63. 9, 76. 8 and 89. 8 per cent were observed at 3, 6, 9, 12 and 15 ppm naringenin concentration, respectively. The highest mortality rate of 89. 8 per cent was present at a concentration of 15 ppm naringenin, whereas the lowest mortality of 32. 5 per cent was observed at 5 ppm naringenin concentration. The LC_{50} and LC_{90} values of naringenin against *Cx. quinquefasciatus* were 5. 719 and 15. 138 ppm, respectively. The chi-square value was 0. 591, which showed that the adulticidal activity was significant at p<0. 05 level (Table *5).*

Effect of Naringenine on the midgut histology

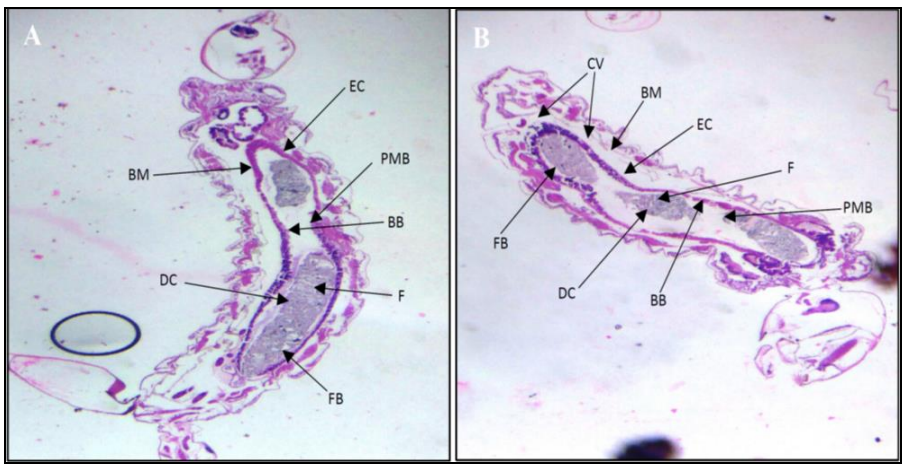

Fig 3: Longitudinal cross section through the anterior midgut of 3rd instar larvae of *Aedes aegypti*

A. Control: Larval midgut of the control group showing well developed brush border (BB), basal membrane (BM), digestive cells (DC), food bolus (F), intestinal epithelial (EC), fat body (FB) and peritrophic membrane (PMB); and (B) Treated with LC50 of naringenin, showing the effect after 24 h of exposure: Midgut of larvae exposed to 3. 23 ppm naringenin, showing destructive brush border (BB), degenerative digestive cells (DC), degenerative basal membrane (BM) and degenerated digestive cells (DC), cellular vacuolization (CV), degeneration in peritrophic membrane (PMB), distribution of food bolus (FB), vacuolated intestinal epithelial (EC), smaller fat bodies (FB), etc. (40X).

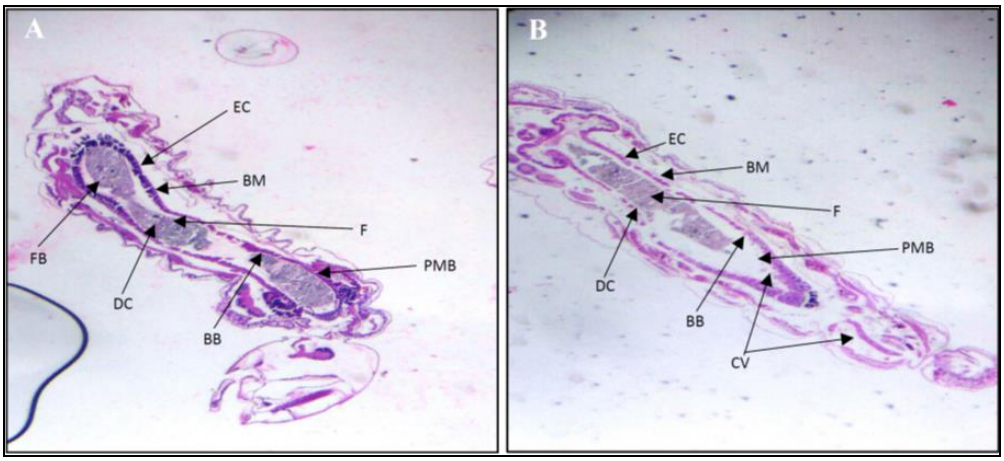

Fig 4: Longitudinal cross section through the anterior midgut of 3rd instar larvae of *Cx. Quinquefasciatu*

B. (A) Control: Larval midgut of the control group showing well developed brush border (BB), basal membrane (BM, digestive cells (DC), food bolus (F), intestinal epithelial (EC), fat body (FB) and peritrophic membrane (PMB); and (B) Treated with LC_{50} of naringenin, showing the effect after 24 h of exposure: Midgut of larvae exposed to 3. 23 ppm naringenin, showing destructive brush border (BB), degenerative digestive cells (DC), degenerative basal membrane (BM) and degenerated digestive cells (DC), cellular vacuolization (CV), degeneration in peritrophic membrane (PMB), distribution of food bolus (FB), vacuolated intestinal epithelial (EC), smaller fat bodies (FB), etc. (40X).

Figure 3 & 4 shows the longitudinal cross-section (40X) of the anterior midgut of 3rd instar larvae of control and naringenin treated Ae. aegypti and Cx. quinquefasciatus, respectively. The control group showed well-developed brush border, basal membrane, digestive cells, food bolus, intestinal epithelial, fat body and peritrophic membrane. The naringenin treated larvae at 3. 23 ppm concentration, showed destructive brush border, degenerated digestive cells, basal membrane and digestive cells, cellular vacuolization, degeneration in the peritrophic membrane, distribution of food bolus, vacuolated intestinal epithelial, smaller fat bodies, etc.

Table 3: Larvicidal and pupicidal activities of naringenin against *Ae. Aegypti*

Stages	Concentration (ppm)	Morality (%)	LC_{50} (ppm) (LCL-UCL)	Regression Equation	LC_{90} (ppm) (LCL-UCL)	x^2 df=3
Larva	2	36.2 ± 2.76^a				
	4	55.4 ± 4.22^b				
	6	68.1 ± 5.19^c	3.23 (2.098-4.981)	Y=1.9886x + 3.9871+	9.321(9.294-12.968)	0.733
	8	81.1 ± 6.18^d				
	10	98.9 ± 7.52^e				
Pupa	2	33.2 ± 2.53^a				
	4	49.6 ± 3.78^b				
	6	67.2 ± 4.41^c	3.418 (2.485-4.700)	Y=2.8672x + 3.4709	9.848(7.306-10.998)	0.459
	8	80.1 ± 6.10^d				
	10	96.5 ± 7.35^d				

Significant at $p<0.05$; Control: nil mortality; LC_{50}: lethal concentration that kills 50% of the exposed larvae or pupa; LC_{90} : that kills 90% of the exposed larvae or pupa; LFL lower fiducidal limit; UFL upper fiducidal limit; x^2 : Chi-square value; df: degrees of freedom.

Table 4: Larvicidal and pupicidal activities of naringenin against *Cx. Quinquefasciatus*

Stages	Concentration (ppm)	Morality (%)	LC_{50} (ppm) (LCL-UCL)	Regression Equation	LC_{90} (ppm) (LCL-UCL)	x^2 df=3
Larva	2	33.1 ± 2.52^a				
	4	48.5 ± 3.68^b				
	6	63.0 ± 4.84^c	3.537 (2.54-4.924)	Y=2.7073x+3.5178	9.940(7.966-11.044)	0.432
	8	79.4 ± 6.05^d				
	10	95.9 ± 7.29^e				
Pupa	2	30.0 ± 2.30^a				
	4	45.6 ± 3.47^b				

	6	61.2 ± 4.66[c]	3.700(2.699-5.072)	Y=2.8511x-3.3828	10.009(7.92-11.982)	0.374
	8	73.8 ± 5.62[d]				
	10	90.1 ± 6.86[e]				

Significant at p<0.05; Control: nil mortality; LC_{50}: lethal concentration that kills 50% of the exposed larvae or pupa; LC_{90}: that kills 90% of the exposed larvae or pupa; LFL lower fiducidal limit; UFL upper fiducidal limit; x^2: Chi-square value; df: degrees of freedom.

Table 5: Adulticidal activity of naringenin against *Ae. aegypti* and *Cx. Quinquefasciatus*

Concentration (ppm)	Morality (%)	LC_{50} (ppm) (LCL-UCL)	Regression Equation	LC_{90} (ppm) (LCL-UCL)	x^2 df=3
Ae. aegypti					
3	34.2 ± 2.60[a]	5.390(3.764-7.718)	Y=2.4398x+3.2154	14.701(13.082-16.987)	0.488
6	46.2 ± 3.54[b]				
9	62.4 ± 4.75.[c]				
12	78.4 ± 5.97[d]				
15	92.7 ± 7.07[e]				
Cx. quinquefasciatus					
3	32.5 ± 2.48[a]	5.719(3.934-8.313)	Y=2.3033x+3.2556	15.138(14.541-17.728)	0.591
6	45.4 ± 3.46[b]				
9	63.9 ± 4.87[c]				
12	76.8 ± 5.85[d]				
15	89.8 ± 6.84[e]				

Significant at p<0.05; Control: nil mortality; LC_{50}: lethal concentration that kills 50% of the exposed larvae or pupa; LC_{90}: that kills 90% of the exposed larvae or pupa; LFL lower fiducidal limit; UFL upper fiducidal limit; x^2: Chi-square value; df: degrees of freedom

Discussion

Typically, mosquito control is carried out by targeting either adults or immature larvae. Use of synthetic insecticides has been the primary strategy in controlling the larval population of mosquitoes. However, continuous use of these chemicals for killing larval is increasing environmental contamination. This has led to the need for alternative eco-friendly products for controlling the mosquito larvae [16].

Sterol is a vital compound required for most insects and mosquitoes to complete their life cycle. Unfortunately, mosquitoes cannot synthesize the sterol, and it depends on the mammals for ingesting the same. Mosquitoes take up/ingest the sterol from the decaying plant materials in their larval stage in the form of phytosterol, which is then converted further to cholesterol for utilizing it for growth and reproduction [17]. This conversion occurs with the help of carrier protein AeSCP-2 in *Aedes aegypti* [18]. AeSCP-2 protein is expressed in the midgut, and it is the main region for cholesterol absorption. AeSCP-2 has been proposed to be associated with the uptake as well as the delivery of cholesterol, an essential nutrient for the insects, across the cellular barriers, between the midgut and the hemocoel, where it is then moved to different sites for storage and/or utilization [19].

To block the carrier protein AeSCP-2, several compounds have been screened by various researchers [20, 21]. In the present study, the phytochemicals, naringenin, naringin, hesperidin and diosmin were utilized to dock (*in silico*) with the mosquito cholesterol carrier protein AeSCP-2. Among the four selected phytocompounds, naringenin showed the highest binding affinity towards the target protein AeSCP-2 with a docking energy, i. e., least binding energy value, of -9. 75511 kcal/mol. The docking energy (binding free energy) obtained for naringin, hesperidin, and diosmin are -9. 29459, -9. 39762 and -9. 02305 kcal/mol, respectively.

The above results revealed that Citrus species have insecticidal activity due to the presence of these phytocompounds, which can inhibit the cholesterol metabolism in mosquitoes leading to its death. Moreover, these phytocompounds have known pharmacological activities [22]. and is widely found in *Citrus* species, and is thus both non-toxic to humans and can be assumed to be ecofriendly. Therefore, based on the present results, further study (*In vitro*) will be carried out on naringenin.

The larvicides play a vital role in controlling mosquitoes in their breeding sites. These also show a negative impact on areas of beneficial and non-target organisms. Once the adult mosquito is developed and flies in the air, it is very difficult to go after and destroy them. The best way of mosquito control is to destroy it in the larval stage and also at the immature (larvae and pupae) stages of mosquitoes that are most susceptible to controlling agents [23].

Secondary metabolites like phenolics, terpenoids and alkaloids in plants, may act as larvicides, antifeedants, moulting hormones, oviposition deterrents, repellents, juvenile hormone mimics, growth inhibitors, antimoulting hormones as well as attractants against target pests [24]. It has been previously reported that some types of flavonoids have had an effect on agricultural pests with ovicidal effect, oviposition, fecundity, mortality, weight reduction, and the emergence of adults [25]. Earlier, [26]. reported that flavonoids like quercetin, rutin, and naringin showed positive effects for controlling both nymphs and adults of the aphid. Present results, the larvicidal activity of LC_{50} and LC_{90} values of naringenin were 3. 23 and 9. 321 in *Ae. aegypti* and 3. 537 and 9. 940 ppm in *Cx. quinquefasciatus*, respectively. Furthermore, pupicidal activity at LC_{50} and LC_{90} naringenin were 3. 537 and 9. 940 against *Ae. aegypti* and 3. 700 and 10. 009 ppm against *Cx. quinquefasciatus*, respectively. These results indicate that naringenin showed larvicidal and pupicidal activity against *Ae. aegypti* and *Cx. quinquefasciatus*. This effect may be because naringenin blocks sterol carrier protein, which was confirmed in phase I *in silico* study. The larvicidal and pupicidal activity of the naringenin was supported by the findings of [27]., who reported that rutin showed larvicidal LC_{50} and LC_{90} at 75. 53 and 206. 06 ppm against *Cx. quinquefasciatus.*

To avert the proliferation of the mosquito-borne diseases as well as to enhance the quality of the environment as well as the public health, mosquito control is an essential activity [28]. In order to avoid the resistance, alternative methods in vector control are necessary. Plant secondary metabolites are an excellent source for controlling mosquitoes due to their efficiency, easy biodegradability, development of less

to non-toxic products, and may be applied to mosquito breeding places [29, 30]. The current study showed the adulticidal LC_{50} and LC_{90} of naringenin were 5. 390 and 14. 701 ppm against *Ae. aegypti*, while 5. 719 and 15. 138 ppm against *Cx. quinquefasciatus.* Naringenin may kill the adult mosquitoes by either inhibit digestion or alter the behaviour, growth, and development. The above findings were similar with the reports by [31]., who reported the bioactivity of 4 flavonoid compounds, i. e., poncirin, rhoifolin, naringin and marmesin expressed potent adulticidal activity against *Ae. aegypti*, and the lethal concentration LC_{50} and LC_{90} values ranged between 0. 082 to 0. 122 mg/l and 0. 152 to 0. 223 mg/l, respectively.

Many plant insecticides primarily target the midgut of the mosquito larvae, and some are able to interfere with the larval development into the adult stage, even at sub-lethal concentrations [32]. The metamorphosis of *Ae. aegypti* larvae comprise comprehensive transformations of the insect body, including a remodelling of the midgut, where larval digestive cells are completely replaced [33, 34]. The current study, naringenin treated larvae of *Ae. aegypti*, *Cx. quinquefasciatus* at 3. 23 ppm concentration, showed destructive brush border, degenerative digestive cells, degenerative basal membrane, degenerated digestive cells, cellular vacuolization, degeneration in the peritrophic membrane, distribution of food bolus, vacuolated intestinal epithelial and smaller fat bodies compare to control. This result corroborates previous results demonstrating a reduction in lipid size in fat body cells of *Cx. quinquefasciatus* larvae after exposure to 1. 25 ppm of ivermectin [35]. This method of vacuolation may indicate that these cells are in a death process, possibly due to the presence of toxic substances that may alter microvilli size in the midgut of insecticide-exposed larvae, as described by [36].

Conclusion

The present study provides significant evidence that naringenin, a plant-derived phytocompound, have larvicidal, pupicidal and adulticidal activities against both *Ae. aegypti* and *Cx. quinquefasciatus*. Hence, it can be an excellent candidate to control the mosquito population. However, further examinations are warranted to prove their long-term effects as potent phyto-insecticides in the field conditions as well as on the ecosystem.

Conflict of Interest

The authors declare no conflict of interest

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