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EVALUATION OF THE INSECTICIDAL POTENTIAL OF THE LEAF EXTRACTS OF *PSIDIUM GUAJAVA* AND *PIPER BETLE* AGAINST *AEDES AEGYPTI* LARVAE

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ABSTRACT. Plant-based insecticides are getting attention as an alternative mosquito control strategy because of the emergence of insect resistance to currently used synthetic chemicals. Further, their high safety profile makes them ideal candidates for environmental applications. The current study evaluated the insecticidal potential of *Psidium guajava* and *Piper betle* leaf extracts against *Aedes aegypti* through in vitro and in silico approaches. In laboratory studies, the LC_{50} of n-hexane extract of *P. guajava* and ethyl acetate extract of *P. betle* were 95.21 ppm and 217.7 ppm after 24-h exposure, respectively. The gas chromatography-mass spectrometry analysis identified important bioactive compounds, including caryophyllene (21.2%), globulol (19.9%), squalene (8.3%), and γ-muurolene (6.6%) in *P. guajava* and hydroxychavicol (57%), 5-allyl-2-hydroxyphenyl acetate (5.6%), phytol (2.3%), and safrole (1.8%) in *P. betle* extract. In silico analysis of these compounds with target proteins acetylcholinesterase (AChE), S-adenosylhomocysteine hydrolase (SAHH), and sterol carrier protein-2 (SCP-2) in *Ae. aegypti* larvae showed that squalene from *P. guajava* had a higher binding affinity with AChE (–8.4 kcal/mol) compared to globulol (–7.3 kcal/mol). However, conventional hydrogen bonding, which is stronger and more stable, was observed in the globulol-AChE complex. The in silico analysis of *P. betle* phytochemicals demonstrated that hydroxychavicol, phytol, and safrole had binding affinities of –6.1 kcal/mol, –6.0 kcal/mol, and –6.0 kcal/mol with SAHH, respectively. A minor increase in binding affinity was observed in the safrole-SAHH complex (–6.1 kcal/mol), whereas no change was observed in the 5-allyl-2-hydroxyphenyl acetate-AChE complex (–5.9 kcal/mol) in 2-ligand binding mode. Since these bioactive compounds target the important proteins in the developmental processes of mosquito larvae, they can further be evaluated to design natural and organic insecticides against *Ae. aegypti.*

KEY WORDS *Aedes aegypti*, hydroxychavicol, larvicides, *Piper betle*, *Psidium guajava*, squalene

INTRODUCTION

Mosquitoes play a significant role in the ecosystem, but they also pose a significant threat to humans and animals worldwide, as many of them are vectors of various infectious agents. Vector-borne diseases like malaria, dengue, chikungunya, and Zika account for more than 17% of all infectious diseases, causing more than 700,000 deaths annually. The burden of these diseases is highest in tropical and subtropical areas, and they disproportionately affect the poorest populations. Among these, the *Aedes aegypti* (L.) mosquito is an important vector of many pathogenic viruses including yellow fever, dengue, Zika, and chikungunya viruses, thus contributing significantly to the global disease burden. Dengue is the most prevalent viral infection transmitted by *Aedes* species. More than 3.9 billion people in over 132 countries are at risk of contracting dengue, with an estimated 96 million symptomatic cases and an estimated 40,000 deaths every year (WHO 2024).

Controlling the mosquito population is crucial to restrict the spread of these diseases. Currently, this is done through the use of chemical insecticides targeting different developmental stages of mosquitos. However, the widespread use of these chemicals imparted damage to the environment, and nontarget organisms. Although synthetic pesticides like carbamates, pyrethroids, organochlorines, and organophosphates are frequently employed, they can cause resistance in mosquito populations and provide health and environmental problems, hence necessitating the development of alternative control strategies with a safer environmental profile and possibly a different mode of action than the existing chemical agents (Senthil-Nathan 2019, Alkuriji et al. 2020).

Since the larvae are restricted to aquatic environments and are simpler to eradicate, targeting the larval stage is the most localized and successful mosquito control strategy. Bioactive phytochemicals and essential oils found in plants have potent larvicidal properties with minimal effects on the environment and nontarget organisms. Therefore, biolarvicides can be an effective option in terms of relative protection, global availability, and low expense. It will be more cost-effective to search for locally grown medicinal plants than to purchase pricey imported ones for the purpose of controlling mosquitoes. These chemicals derived from natural sources, are mostly environmentally benign, mainly nontoxic to humans and other mammals, and biodegradable (Sengul Demirak and

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Canpolat 2022, Lima et al. 2024, Msugupakulya et al. 2024). Different parts of medicinal plants are a rich source of versatile metabolites with diverse biological activities, including insecticidal potential that may provide protection against pests and diseases (Flor-Weiler et al. 2023, Baz et al. 2024, Noorazlan et al. 2024).

The current study is focused on evaluating the larvicidal potential of *Psidium guajava* (L.) and *Piper betle* (Blanco). Guava leaf extract contains a high concentration of bioactive compounds such as quercetin, myricetin, hyperin, and apigenin (Pradhan et al. 2013, Aremu et al. 2024). The Piperaceae plant, *P. betle*, also locally known as paan, is an annual dioecious creeper that often grows in hotter and more humid areas. It grows in India, Pakistan, and other Southeast Asian countries including China and Vietnam and is often found in damp forests. *P. betle* leaf has piperol-A, piperol-B, sugars, starch, safrole, eugenol, terpinen-4-ol, allyl pyrocatechol monoacetate, eugenyl acetate, cadinene, hydroxychavicol, carvacrol, chavicol, caryophyllene, cineole, p-cymene, allyl catechol, chavibetol, and estragol as the main components (Martianasari and Hamid 2019, Lima et al. 2024). These plants have been used traditionally and contain numerous bioactive compounds, which could be promising candidates as insecticides. By investigating the larvicidal activity of these plants against *Ae. aegypti* and analyzing their bioactive compounds through *in vitro* and computational approaches, this study would eventually be helpful in developing safe and effective plant based natural larvicides. The development of organic larvicides is a multifaceted approach. It addresses the public health concern of mosquito-borne diseases, promotes environmentally friendly solutions, and leverages the power of natural resources. This research focuses on the in silico product designing using the n-hexane and ethyl acetate leaf extracts of *P. guajava* and *P. betle* respectively, as potential larvicides against *Ae. aegypti* under laboratory conditions.

MATERIALS AND METHODS

Plants used

The leaves of *P. guajava* and *P. betle* were collected from a local market. Only mature leaves were selected for consistency in phytochemical content. The plant specimens were deposited in the herbarium of Department of Botany, University of the Punjab, Pakistan, under the specimen numbers LAH30425A (*P. guajava*) and LAH30425 (*P. betle*).

Preparation of solvent extracts

The leaves of *P. guajava* and *P. betle* were washed with distilled water and shade dried. The leaves were then ground into a fine powder using an electrical blender. Sixty mg of *P. guajava* leaf powder was soaked in 300 ml of each solvent (n-hexane, acetone, and methanol) for 72 h at room temperature of 20°C. The mixture was then filtered using Whatman filter paper No. 41 (pore size 20 μm, 125mm diameter; Schleicher & Schuell, UK). The filtrate was air-dried at room temperature for a few days, until fully dried. Maceration of the *P. betle* leaves was carried out similarly to that of *P. guajava* leaves. Fifty g of *P. betle* leaf powder was packed by forming a thimble and dipped in 150 ml of each solvent (ethyl acetate and methanol) for 24 h. The extract was filtered out, and then 150 ml methanol was added to a beaker covered with foil; after 24 h, the ethyl acetate extract was filtered out. Both extracts were stored at 4°C till use (Yadav et al. 2020).

Concentration-response assays

Concentration-response assays were performed at the mosquito-rearing facility established at the National Institute of Health (NIH) Research Centre, King Edward Medical University, Lahore, Pakistan. Mosquitoes were reared according to the method developed by Raza (2020). Briefly, male and female mosquitoes were kept in $12'' \times 12'' \times 12''$ cages. Male mosquitoes were kept on a 10% sucrose solution, whereas female mosquitoes were blood-fed, using a stainless steel feeding device with chicken skin as a membrane. Plain filter paper strips lining the water containers were provided as oviposition sites. The strips containing mosquito eggs were collected, air-dried, and stored under laboratory conditions. For larval hatching, the filter paper strips containing mosquito eggs were immersed in boiled distilled water that had been cooled to room temperature. Larvae were kept in plastic trays containing 2 liters of water and pulverized fish as food for the larvae. The laboratory conditions were maintained at 28°C with 80% RH and 12:12 light/dark photo-/scoto- period.

A 30,000 ppm stock solution was prepared in dimethyl sulfoxide (DMSO) for the preparation of working concentrations. Experiments were performed in 5 batches using different concentrations. Each experiment was repeated at least 5 times. Distilled water alone or with the corresponding concentrations of DMSO was used as control. Assays were conducted in 20 ml glass tubes containing 5 ml of distilled water and corresponding extract concentration. A total of 10 fourth-stage larvae were added to each tube. Each treatment concentration and the control were replicated at least five times. The percentage mortality was recorded after 24 h and 48 h exposure from the mean of 5 replicates. The larvae were considered dead if they failed to rise to the surface of the water when they were probed (Supriya et al. 2019). Based on the data, percentage mortality was calculated for each concentration.

Statistical analysis

Probit regression analysis was applied to the mean larval mortality at each concentration. The median lethal concentrations (LC_{50} and LC_{90}) and 95% fiducial limits were determined by fitting a probit regression model. Additionally, the concentration-response curve was made, using the GraphPad Prism 10 software.

Gas chromatography-mass spectroscopy analysis

The separation of compounds was carried out using a gas chromatograph (TRACE-1300, Thermo Scientific) equipped with a capillary column (TR-35 MS), followed by identification of compounds using MS-ISQ mass spectrometer (Thermo Scientific). The dimensions of the column were 30 m × 0.25 mm internal diameter × 0.25 µm film thickness. Helium was used as the carrier gas. The temperature of the injection port was maintained at 250°C and the 1 µl sample was injected. The initial temperature of the column was maintained at 50°C for 1 min, and ramped at at 20°C/min to 300°C and held for 38 min. The temperature for mass spectrometer was maintained at 250°C. The identification of the compounds was done by comparing the spectra of the separated compounds with that of the spectra of the known compounds already available in NIST MS/MS library (mainlib).

In silico analysis

Screening of target proteins: In silico molecular docking was employed to predict potential target interactions and support hypotheses regarding the mode of action of the plant extract. The target proteins were screened based on the biochemical action of synthetic larvicides. The target proteins included acetylcholinesterase (AChE), S-adenosylhomocysteine hydrolase (SAHH), and sterol carrier protein-2 (SCP-2). The crystal structure of SCP-2 from *Ae. aegypti* (PDB ID: 2KSH) was retrieved from protein data bank (RCSB-PDB; https://www.rcsb.org/) (Burley et al. 2017). The 3D structures of AChE and SAHH were generated via I-TASSER (https://zhanggroup.org/I-TASSER/) (Yang and Zhang, 2015).

Screening of ligands: Phytochemical compounds present in high concentration were selected as potential therapeutic ligands for *in silico* analysis. The bioactive compounds identified in *P. guajava* included caryophyllene, globulol, squalene, and γ-muurolene. The bioactive compounds identified in *P. betle* included hydroxychavicol, phytol, safrole, and 5-allyl-2-hydroxyphenyl acetate. The 3D structures of these compounds were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) (Kim et al. 2019).

Molecular docking: The crystal structure of target protein SCP-2 was retrieved from the PDB. For the other target proteins, AChE and SAHH, whose structures were not available in PDB, ab initio modeling was employed. This process involved obtaining the complete amino acid sequences from the National Center for Biotechnology Information (NCBI) and then submitting them to I-TASSER for structure prediction. The model with the highest C-score was selected for each protein. Next, the protein structures were prepared using BIOVIA Discovery Studio. The 3D structures of the phytochemical compounds were retrieved from PubChem. Energy minimization of the ligands was performed using Vina Wizards to achieve stable conformations. These structures were then virtually screened using PyRx. Molecular docking was conducted with AutoDock Vina to predict the binding affinities of the ligands to the target proteins. Finally, the molecular interactions between the ligands and proteins were analyzed using BIOVIA Discovery Studio to determine binding affinities and stability (Rohs et al. 2005, Guedes et al. 2014).

RESULTS

Concentration-response assays of *P. guajava* and *P. betle* leaf extracts

The n-hexane extract of *P. guajava* leaves showed the highest larvicidal potential after 24 and 48 h as compared to acetone and methanol extracts. The LC_{50} value of n-hexane extract was 95.21 ppm after 24 h and 68.10 ppm after 48 h. The LC_{90} value of n-hexane extract was 175.93 ppm after 24 h and 135.04 ppm after 48 h. The LC_{50} and LC_{90} values of ethyl acetate extract of *P. betle* were 217.7 ppm and 315.7 ppm after 24 h, and 162.5 ppm and 232.2 ppm after 48 h, respectively. The LC_{50} and LC_{90} values of methanol extract were 239 ppm and 335.5 ppm after 24 h, and 218 ppm and 321.4 ppm after 48 h of exposure to *P. betle* extracts, respectively (Table 1). Figure 1 represents the concentration-response curve of extracts against *Ae. aegypti* after 24 h and 48 h exposure.

GC-MS analysis of *P. guajava* and *P. betle* extracts

This study investigated the chemical composition of n-hexane extract of *P. guajava* and ethyl acetate extract of *P. betle.* Results of GC-MS showed the presence of sesquiterpenes, steroids, and terpenoids in *P. guajava* and *P. betle* extracts. In addition, hydrocarbons, alkaloids, steroids, monoterpenes, triterpenes, carbohydrates, and volatile gasses were also detected. The major phytochemical compounds and their concentrations are summarized in Table 2.

Molecular docking analysis

The molecular docking of target proteins was performed through AutoDock Vina of PyRx (https://pyrx.sourceforge.io/downloads). Each protein was docked with a single ligand or combination of two or more ligands. The binding affinities of *P. guajava*

Table 1. Larvicidal activity of *Psidium guajava* and *Piper betle* extracts against fourth instar *Aedes aegypti*.

Plant	Exposure duration	Extract	LC_{50} (ppm)	95% fiducial limit (Lower–Upper)	LC_{90} (ppm)	95% fiducial limit (Lower–Upper)	Degree of freedom	Chi-square
Psidium guajava	24 h	n-Hexane	95	76–114	175.9	143–248	4	2.9
		Acetone	487.6	427–688	767	588–1826	3	3.4
		Methanol	383.7	357–409	512	474–571	6	3
	48 h	n-Hexane	68.1	48–84	135	107–202	4	1.4
		Acetone	385	349–439	597	503–866	3	0.1
		Methanol	316	291–338	422	389–475	6	1.7
Piper betle	24 h	Ethyl acetate	217.7	186.5–255.7	315.7	272.8–411	4	1.06
		Methanol	239	208–282.5	335.5	289.6–450	4	0.56
	48 h	Ethyl acetate	162.5	135.9–189.2	232.2	248.1–351.4	4	2.41
		Methanol	218	186.6–258	321.4	276.5–425.7	4	1.54
Water control*	24 h/48 h	—	0.0	—	0.0	—	—	—

* Water control resulted in 0% mortality after 24 h and 48 h exposure and was used as negative control.
Abbreviations: LC = lethal concentration; ppm = parts per million.

and *P. betle* phytochemicals with the target proteins are listed in Tables 3 and 4, respectively.

The molecular docking analysis of *P. guajava* phytochemicals revealed that squalene exhibited the highest binding affinity with AChE at –8.4 kcal/mol, indicating strong stability, whereas it has the lowest affinity with SCP-2 at –4.6 kcal/mol, suggesting weaker interactions. Caryophyllene, globulol, and γ-muurolene also show high affinity for AChE with binding affinities of –7.3 kcal/mol, –7.3 kcal/mol, and –7.8 kcal/mol, respectively, but lower affinities for SCP-2 (–5.6 kcal/mol, –5.4 kcal/-mol, and –5.2 kcal/mol) and SAHH (–6.8 kcal/mol, –6.2 kcal/mol, and –6.8 kcal/mol). When two ligands bound to SCP-2, their binding affinities changed, particularly with combinations involving squalene: caryophyllene and squalene show affinities of –5.5 kcal/mol and –5.3 kcal/mol, respectively, while γ-muurolene and squalene exhibit affinities of –5.6 kcal/mol and –5.3 kcal/mol. However, binding affinities remained unchanged for three or four ligands with any protein. Overall, squalene's interaction with AChE was the most notable, whereas its interaction with SCP-2 is the least stable.

The molecular docking analysis of *P. betle* phytochemicals in single ligand binding studies, phytol showed the highest binding affinity with AChE at −6.4 kcal/mol, but it did not form strong hydrogen bonds. Hydroxychavicol had a notable affinity with SAHH at –6.1 kcal/mol, forming strong bonds. Phytol and safrole also exhibited high affinities with SAHH (–6.0 kcal/mol), forming carbon-hydrogen and conventional hydrogen bonds. 5-Allyl-2-hydroxyphenyl acetate binds to AChE with –5.9 kcal/mol and hydroxychavicol with –5.7 kcal/mol, whereas the lowest binding energy was seen with 5-allyl-2-hydroxyphenyl acetate binding to SAHH at –5.0 kcal/mol. In 2 ligand binding studies, the binding affinity of 5-allyl-2-hydroxyphenyl acetate slightly increased against SCP-2 from –5.5 kcal/mol to –5.6 kcal/mol, and phytol's affinity increased from –5.5 kcal/mol to –5.7 kcal/mol against SCP-2. For AChE, the binding affinity of phytol decreased from –6.4 kcal/mol to –5.7 kcal/mol when combined with 5-allyl-2-hydroxyphenyl acetate. For SAHH, phytol's binding affinity decreased from –6.0 kcal/mol to –5.6 kcal/mol when combined with hydroxychavicol, safrole, and 5-allyl-2-hydroxyphenyl acetate. Additionally, safrole's binding energy improved to –6.1 kcal/mol when docked with SAHH, forming conventional hydrogen bonds. For combinations of three or four ligands, binding affinities remained consistent with those observed in double ligand binding interactions. In summary, the molecular docking analysis suggests that *P. guajava* phytochemicals, particularly squalene, have high potential as larvicides because of their strong AChE inhibition, whereas *P. betle* phytochemicals give moderate larvicidal activity through AChE and SAHH inhibition.

Molecular interaction analysis

The molecular interactions between ligand and target proteins was analyzed on BIOVIA Discovery

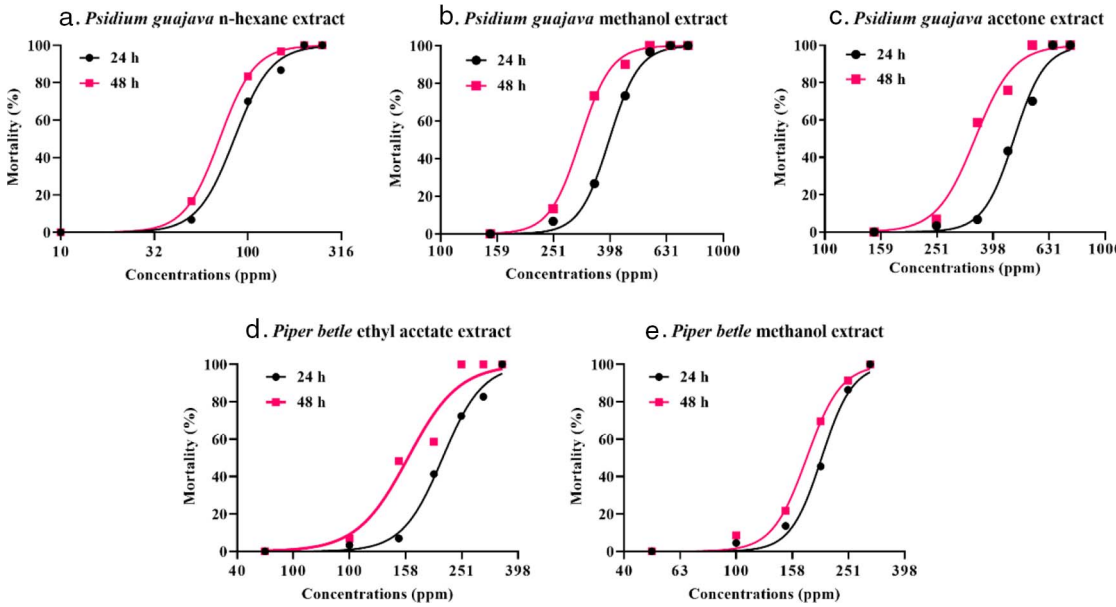

Fig. 1. Concentration-response curves of *Psidium guajava* extracts against fourth instar *Ae. aegypti* larvae: (a) n-hexane, (b) methanol, and (c) acetone. The concentration-response curve of *Piper betle* extracts against *Ae. aegypti* larvae: (d) ethyl acetate, and (e) methanol.

Studio. Globulol made stable interactions with AChE as compared to squalene. In case of *P. betle*, the binding affinity of ligand hydroxychavicol with protein SAHH was maximum when bound as a single ligand (–6.1 kcal/mol), but when bound in combination to other ligands, the binding affinity remained the same. Phytol showed binding affinity of (–6.0 kcal/mol) when it binds to SAHH.

DISCUSSION

Several studies have been conducted to overcome the burden of mosquito-borne diseases that have become a significant concern of public health. Multiple synthetic insecticides have been formulated and used commonly, but these synthetic products are harmful to human health. Therefore, researchers are putting efforts into designing such organic larvicidal products that have high efficiency against mosquitoes and are less harmful. Plant-based compounds have gained much attention in designing organic insecticides because of the presence of secondary metabolites that have insecticidal properties. The plants *P. guajava* and *P. betle* could be used to make natural larvicides since they are effective in killing *Ae. aegypti* larvae. Previous research has examined the larvicidal efficacy of several essential oil chemotypes; the results show that these oils are efficacious, with LC_{50} values ranging from 39.48 to 64.25 µg/ml (Dias et al. 2015). The present study evaluated the larvicidal activity of n-hexane and ethyl acetate extracts. Insecticidal activity against mosquitoes has been reported for the Indian *Piper* species such as *Piper cubeba* (Candolle), *Piper longum* (Miquel), and *P. betle* (Lee 2005). Bioactive phytochemicals from *P. betle* ethyl acetate extract were used in silico drug discovery, and two extracts of the plant demonstrated larvicidal efficacy against *Ae. aegypti*. Notably, Jayaraman and Venkatesalu (2015) reported that the ethyl acetate extract of this aromatic plant showed considerable larvicidal effectiveness with the LC_{50} and LC_{90} of 173.04 ppm and 442.73 ppm, respectively, against *Ae. aegypti*.

In the current study, 36 phytochemicals were identified by GC-MS studies in the n-hexane extract of *P. guajava* leaves, including high amounts of caryophyllene, globulol, squalene, and γ-muurolene. The key phytochemicals found in *P. betle* ethyl acetate extract that showed high larvicidal activity against *Ae. aegypti* were hydroxychavicol, 5-allyl-2-hydroxyphenyl acetate, phytol, and safrole. These bioactive substances, like synthetic larvicides that operate on the same targets to inhibit particular processes, were employed as ligands to interact with *Ae. aegypti* target proteins. Mendes et al. (2017) reported larvicidal activity of *P. guajava* essential oil against *Ae. aegypti*. Similarly, *P. betle* essential oil has demonstrated effectiveness as a bioinsecticide to control *Ae. aegypti* populations (Martianasari and Hamid 2019).

Literature indicates that phytochemicals from several plants have larvicidal potential against *Ae. aegypti* and these bioactive compounds could be developed as environmentally friendly organic larvicides (Larson et al. 2008). However, there is not enough literature available regarding in silico drug design against *Ae. aegypti* using *P. guajava* and *P.*

Table 2. Major phytochemical compounds of *Psidium gujava* and *Piper betle* identified through GC-MS analysis.

Plant Extract	Chemical name	Empirical formula	Molecular weight (g/mol)	Retention Time (min)	% Concentration	Nature of compound
P. gujava n-hexane extract	Caryophyllene	$C_{15}H_{24}$	204	8.59	21.21	Sesquiterpene
	Globulol	$C_{15}H_{26}O$	222	10.01	19.92	Sesquiterpene
	4-Isopropyl-1,6-dimethyl-1,2,3,4-tetrahydronaphthalene	$C_{15}H_{22}$	202	9.48	9.51	Sesquiterpenoid
	1H-Pyrrol-1-yloxy-2,5-dihydro-3-(methoxycarbonyl)-2,2,5,5-tetramethyl	$C_{10}H_{16}NO_3$	198	10.81	8.68	—
	Squalene	$C_{30}H_{50}$	410	24.49	8.33	Triterpene
	γ-Muurolene	$C_{15}H_{24}$	204	9.22	6.61	Benzoid
	α-Copaene	$C_{15}H_{24}$	204	8.17	4.81	Sesquiterpene
	Fenretinide	$C_{26}H_{33}NO_2$	391	12.09	1.88	Retinoid
P. betle ethyl acetate extract	Hydroxychavicol	$C_9H_{10}O_2$	150.17	10.407	57.40	Polyphenol
	5-Allyl-2-hydroxyphenyl acetate	$C_{11}H_{13}O_3$	193.46	20.50	5.63	—
	Benzaldehyde	C_6H_5CHO	106.12	6.82	3.59	Phenol
	8-Phytol	$C_{20}H_{40}O$	296.5	13.16	2.31	Terpenoid
	Neophytadiene	$C_{20}H_{38}$	278.5	19.76	2.01	Polyphenol
	Safrole	$C_{10}H_{10}O_2$	162.18	6.91	1.84	Polyphenol
	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	11.21	1.79	Fatty acids
	2-Hexadecen-1-ol	$C_6H_{32}O$	240.42	15.38	1.62	Fatty alcohol

Table 3. Binding affinities (kcal/mol) of *Psidium guajava* ligands with target proteins of *Aedes aegypti*.

	One ligand				Two ligands						Three ligands	Four ligands
Proteins	C	G	S	M	C+G	C+S	C+M	G+S	G+M	S+M	C+G+S	C+G+S+M
SCP-2	–5.6	–5.4	–4.6	–5.2	Same	–5.5, –5.3	–5.5, –5.6	–5.4, –5.3	Same	Same	Same	M= –5.6, S= –5.3
AChE	–7.3	–7.3	–8.4	–7.8	Same	Same	Same	Same	Same	Same	Same	Same
SAHH	–6.8	–6.2	–7.8	–6.8	Same	Same	Same	Same	Same	Same	Same	Same

Same: indicates that the values of binding affinities remain the same when ligands bind in combination of two, three or four ligands. Abbreviations: C: Caryophyllene, G: Globulol, S: Squalene, M: γ-Muurolene, SCP-2: Sterol carrier protein, AChE: Acetylcholinesterase, SAHH: S-adenosylhomocysteine hydrolase.

Table 4. Binding affinities (kcal/mol) of *Piper betle* ligands with target protein of *Aedes aegypti*.

Proteins	One ligand				Two ligands						Three ligands	Four ligands
	H	A	P	S	H+A	H+P	H+S	A+P	A+S	P+S	H+A+P	H+A+P+S
SCP-2	–5.5	–5.5	–5.5	–6.0	–5.3, same	–5.3, –5.7	–5.3, –5.9	Same, –5.7	Same, –5.9	–5.7, –5.9	–5.3, same, –5.7	H=–5.3, same, –5.7, –5.9
AChE	–5.7	–5.9	–6.4	–5.7	Same	Same, –5.7	Same	Same, –5.7	Same	Same	Same, same, –5.7	Same, same, –5.7, same
SAHH	–6.1	–5.0	–6.0	–6.0	Same, –5.2	Same, –5.6	Same, –6.1	–5.3, –5.6	–5.2, –6.1	–5.6, –6.1	Same, –5.2, –5.6	Same, –5.2, –5.6, –6.1

Same: indicates that the values of binding affinities remain the same when ligands bind in combination to other ligands.
Abbreviations: H: Hydroxychavicol, A: 5-allyl-2-hydroxyphenyl acetate, P: Phytol, S: Safrole, SCP-2: Sterol carrier protein-2, AChE: Acetylcholinesterase, SAHH: S-adenosyl homocysteine hydrolase.

betle bioactive phytochemicals. The current study explored this aspect by performing molecular docking between the *P. guajava* and *P. betle* bioactive compounds and the target proteins of *Ae. aegypti.* The bioactive compounds identified through GC-MS analysis were used as ligands for molecular docking with the target proteins of *Ae. aegypti*. The target proteins were selected on the basis of synthetic larvicides, which act on the same target proteins and kill *Ae. aegypti* by inhibiting their specific function. There are several databases used for the molecular docking of ligands with target proteins and their molecular interactions were analyzed by various tools such as PyMol and BIOVIA Discovery Studio. Sterol carrier protein (SCP-2), is a lipid binding protein, was docked with caryophyllene, globulol, squalene, and γ-muurolene. Among these four phytochemicals, caryophyllene exhibited the lowest docking energy (–5.6 kcal/mol); indicating its potential as an AeSCP-2 inhibitor. However, the phytochemicals from *P. betle* did not show strong binding interactions.

Squalene from *P. guajava* when docked with SAHH demonstrated the strongest binding affinity (−7.8 kcal/mol) among all phytochemicals through Van der Waals interactions with binding site residues. Hydroxychavicol, phytol, and safrole from *P. betle* have shown binding affinity of −6.1 kcal/mol, −6.0 kcal/mol, and −6.0 kcal/mol, respectively against SAHH, but these values changed in combination with other ligands. Notably, safrole-SAHH complex resulted in minor increase in binding affinity (−6.1 kcal/mol), whereas 5-allyl-2-hydroxyphenyl acetate-AChE complex showed no change in binding affinity (−5.9 kcal/mol) in two-ligand binding mode. The strong bonding interactions were because of the presence of C-H and conventional H bonds. These findings suggest that squalene, hydroxychavicol, phytol, and safrole can be organic inhibitors of AeSAHH, hence these can be promising candidates as plant-based larvicides. These function as inhibitors of SAHH, an enzyme in the activated methyl cycle that catalyzes the reversible hydrolysis of S-adenosylhomocysteine into homocysteine and adenosine. As it inhibits the SAHH, the hydrolysis of S-adenosylhomocysteine into adenosine and homocysteine is blocked. Resulting in accumulation of S-adenosylhomocysteine which inhibits Juvenile Hormone Acid Methyltransferase (JHAMT), preventing the synthesis of juvenile hormone. Thus interfering with the development of *Ae. aegypti* larvae, ultimately leading to larval death. Squalene from *P. guajava* and 5-allyl-2-hydroxyphenyl acetate from *P. betle* extract act as natural inhibitor of AChE which is essential to catalyze the breakdown of neurotransmitter acetylcholine into acetate and choline. This reaction is crucial in biochemical signaling in *Ae. aegypti*. By inhibiting AChE, 5-allyl-2-hydroxyphenyl acetate and squalene can halt the process, leading to the larval death.

Therefore, the present study explores that the ligands bind target proteins at different binding

affinities through different molecular interactions such as Van der Waals interactions, hydrogen bonding, alkyl, π-alkyl and σ-alkyl interactions. But in the binding pocket of protein only nonpolar amino acids make Van der Waals interactions through their nonpolar chains (Bissantz et al. 2010).

Before the formulation and development of any drug in pharmaceutical industries, in silico drug designing is performed to hypothesize inter- or intramolecular interactions between the target biomolecule (such as protein, DNA, or RNA) and potential ligands, as well as to determine the stability of the resulting complexes. The present study highlights the potential of natural phytochemicals, including squalene, globulol, hydroxychavicol, phytol, safrole, and 5-allyl-2-hydroxyphenyl acetate, as candidates for the development of botanical larvicides against *Ae. aegypti.*

In summary, the n-hexane extract of *P. guajava* and the ethyl acetate extract of *P. betle* demonstrated strong larvicidal potential against *Ae. aegypti* larvae under the laboratory settings. The important phytochemicals, including squalene, globulol, safrole, and 5-allyl-2-hydroxyphenyl acetate, identified in these plants effectively targeted the important proteins involved in the developmental processes of *Ae. aegypti* larvae. It has been shown through in silico analysis that these compounds interfere with biochemical signaling in *Ae. aegypti* larvae by blocking AChE and SAHH. These compounds can be subjected to experimental evaluation to design natural, organic and environmentally friendly insecticidal products to control *Ae. aegypti*.

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