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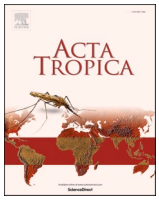

Insecticidal activity of phenylpropanoids from *Piper sarmentosum* and their interactions with glutathione *S*-transferase from adult mosquitoes

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ABSTRACT

This study investigated the insecticidal activity of three phenylpropanoids—asaricin (**1**), isoasarone (**2**), and trans-asarone (**3**)—isolated from the aerial parts of *Piper sarmentosum* against adult mosquito vectors: *Aedes aegypti, Aedes albopictus*, and *Culex quinquefasciatus*. Bioassay-guided fractionation of the plant's hexane extract led to the isolation of these phenylpropanoids. Compounds **1** and **2** exhibited strong adulticidal effects against *Aedes* species, with LD_{50} values $\leq$ 8.8 μg/mL and LT_{50} values $\leq$ 29 min. Meanwhile, *Culex quinquefasciatus* was less susceptible to these compounds, showing LT_{50} values $\leq$ 56 min. In contrast, compound **3** showed moderate toxicity across all species. To explore the potential resistance mechanism, glutathione *S*-transferase (GST) activity was measured and found to correlate positively with the LD_{95} values of the compounds. Mosquitoes exposed to all isolated phenylpropanoids at LD_{95} levels showed a significant increase in GST activity, suggesting its involvement in detoxification. Molecular docking studies further confirmed this interaction, revealing consistent binding of all three compounds to key residues (PRO11, GLU64, SER65, ARG66, and TYR105) within the GST active site. Despite increased GST activity—a known marker of metabolic resistance— compounds **1** and **2** remained highly effective, indicating that their mode of action may overcome or bypass common resistance pathways. These findings highlight the potential of *P. sarmentosum*-derived phenylpropanoids as promising botanical insecticides and underscore the importance of understanding enzyme-ligand interactions in developing effective mosquito control strategies.

1. Introduction

Aedes aegypti, Aedes albopictus and *Culex quinquefasciatus* are the main causes of several major transmitted epidemic diseases such as dengue fever, chikungunya, and yellow fever viruses (Li et al., 2015; Ravaomanarivo et al., 2014; Suman et al., 2011). These vector-borne diseases remain a global threat partly due to the emergence of resistant-strain mosquito species. Global estimates indicate that insecticide resistance has become so widespread that certain public health interventions are no longer effective (Tantely et al., 2010). One of the mechanisms of insecticide resistance is increased detoxification by having higher glutathione *S*-transferase (GST) activity, which catalyzes the binding of toxicants to glutathione (GSH), neutralizing its toxic activity while making it more water-soluble for fast excretion out of the cell (Bengtson Nash et al., 2014; Hemingway, 1983; Low et al., 2010). Reports have consistently found that high levels of GST facilitate insecticide detoxification through the catalysis of their conjugation (Grant, 1991; Grant et al., 1991), and this particular enzyme was found in several insects such as mosquitoes, house fly and Drosophila species (Clark et al., 1984, 1985; Taskin and Kence, 2004; Toung et al., 1990). Thus, GST was recognized for its importance in metabolizing toxic chemicals and developing insecticide resistance in insects (Hemingway, 2000). The rise in insecticide resistance has made vector control increasingly difficult, creating a pressing demand for alternative insecticides that are both selective and eco-friendly. (Villaverde et al., 2014). Plant-derived materials with a wide range of biochemical

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Table 1
Preliminary insecticidal activity of *P. sarmentosum* aerial part; hexane extracts (HE), dichloromethane extracts (DE), methanol extracts (ME) and hexane active fraction (H2) against adult *Ae. aegypti, Ae. albopictus and Cx. quinquefasciatus*.

Tested extracts and fractions*	**Tested adult mosquitoes** **								
	Ae. Aegypti			*Ae. albopictus*			*Cx. quinquefasciatus*		
	Slop ± SE	LD_{50} (μg/ml)[c] (95 % C.I.)	LD_{95} (μg/ml) [c] (95 % C.I.)	Slop ± SE	LD_{50} (μg/ml) [c] (95 % C.I.)	LD_{95} (μg/ml) [c] (95 % C.I.)	Slop ± SE	LD_{50} (μg/ml) [c] (95 % C.I.)	LD_{95} (μg/ml) [c] (95 % C.I.)
HE	3.047 ± 0.20	102.3[a] (73.3 to 156.4)	173.3[a] (162.1 to 232.8)	3.084 ± 0.212	97.1[a] (74.3 to 137.1)	235.5[a] (162.8 to 382.1)	3.372 ± 0.231	122.3[a] (95.0 to 170.8)	233.4[a] (168.2 to 495.9)
DE	5.7 ± 0.3	713.4[b] (647.9 to 780.3)	1378.2[b] (1190.1 to 1733.5)	5.342 ± 0.3	764.7[b] (731.1 to 999.1)	1553.8[b] (1428.3 to 1724.4)	2.4 ± 0.2	795.7[b] (731.1 to 1265.496	1595.7[b] (1376.4 to 1993.7)
ME	2.421 ± 0.149	302.5[c] (281.2 to 503.5)	1118.4[b] (696.5 to 2141.6)	2.404 ± 0.148	367.5[b] (274.1 to 782.2)	1219.3[b] (745.6 to 3418.2)	2.2 ± 0.4	553.2[b] (394.1 to 892.1)	958.4[b] (775.3 to 1671.4)
H2	2.406 ± 0.14	17.4[d] (7.8 to 33.7)	83.9[c] (40.2 to 1654.3)	2.528 ± 0.14	19.3[c] (13.2 to 27.2)	86.4[c] (52.7 to 242.7)	2.740 ± 0.15	20.1[c] (15.4 to 26.1)	80.2[c] (54.7 to 155.4)

* HE, DE and ME represent hexane, dichloromethane and methanol extracts of *P. sarmentosum* and H2 represent hexane active fraction.
** LD_{50} and LD $_{95}$ values followed by a common letter are not significantly different ($p < 0.05$) based on non-overlapping of the 95 % CI.

activities thus offer an alternative in minimizing these issues (Govindarajan et al., 2013; Neto Bandeira et al., 2013). In this context, *Piper sarmentosum* Roxb. (Piperaceae), known as "kadok" in Malaysia, was selected based on numerous reports of its high insecticidal potential (Satariah et al., 1999). This plant, which belongs under the *Piper* genus, is widely distributed in tropical and subtropical regions and has been used throughout history as traditional medicine, food spices and in recent years, more reports were published regarding its insecticidal activity (Hematpoor et al., 2016, 2017; Parmar et al., 1997; Qin et al., 2010; Sk et al., 2011). Phytochemical studies on *P. sarmentosum* species have led to the isolation of several biologically active secondary metabolites such as phenylpropanoids, alkaloids, pyrones and flavonoids (Parmar et al., 1997; Sk et al., 2011). Our previous investigation has led to the isolation and identification of three phenylpropanoids; asaricin (**1**), isoasarone (**2**) and *trans*-asarone (**3**) with huge insecticidal potential against different mosquito species and storage pests (Hematpoor et al., 2016, 2017). Compounds **1** and **2** were found to exhibit potent ovicidal and acetylcholinesterase (AChE) inhibition activities, evidenced by molecular docking studies. The compounds' ability to inhibit AChE and inflict neurotoxicity effects is believed to be their mode of action, which resulted in larval mortality (Hematpoor et al., 2016). In the present study, additional investigations were conducted to evaluate adulticidal activity, mosquito resistance, the correlation with GST activity levels, and the molecular interactions between the ligand (toxicant) and the GST enzyme.

2. Materials and methods

2.1. General experimental procedures

All solvents were of analytical grade and were distilled before use. Column chromatography was conducted using silica gel 60, 200 – 400 mesh ASTM (0.040 – 0.063 mm) (Merck, Germany). Aluminium-supported silica gel 60 F_{254} (20 × 20 cm) (Merck, Germany) was used for thin layer chromatography (TLC). Preparative thin layer chromatography (PTLC) silica gel 60 F_{254} glass plates (20 × 20 cm) (Merck, Germany) were also used for the separation of compounds besides using column chromatography. 1D-NMR and 2D-NMR spectra were recorded using a JEOL ECA 400 MHz NMR spectrometer with chloroform $CDCl_3$ (Merck, Germany) as the solvent. The LCMS-IT-TOF spectra were recorded on a UFLC Shimadzu Liquid Chromatography with *An spd*-M20A diode array detector coupled to an IT-TOF mass spectrometer. A Perkin-Elmer Spectrum 400 FT-IR Spectrometer was used to record the IR spectrum and spectroscopic grade chloroform was used as the solvent. UV spectra were recorded with spectroscopic grade methanol (CH_3OH) as solvent using a Shimadzu 1650 PC UV–Vis Spectrophotometer.

2.2. Plant material and extraction procedures

The aerial parts of *P. sarmentosum* were collected near Universiti Malaya. Diseased or damaged leaves were separated. The plants were first thoroughly rinsed and washed. Aerial parts were separated, followed by oven drying (60 °C) and milled in an electrical blender. Air-dried and powdered leaves material (2.3 kg) was extracted successively with hexane (3 L, 2x), dichloromethane (3 L, 2x), followed by methanol (3 L, 2x) at room temperature, which afforded 23.12 g, 21.02 g and 42.42 g of extracts, respectively. In a preliminary screening of the potential toxicity of the extracts towards *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes, the hexane extract showed the highest percentage of mortality (Table 1). This extract was then subjected to bioassay-guided fractionation and isolation.

2.3. Isolation and purification of compounds

The hexane extract was subjected to column chromatography using silica gel eluting with hexane and dichloromethane (CH_2Cl_2) (50:50; v/v). Nine fractions were obtained (H1-H9). Fraction H2 (0.87 g) exhibited the highest toxicity towards *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* adult mosquitoes (Table 1). This fraction was then purified via preparative TLC using hexane: CH_2Cl_2 (60:40; v/v), which resulted in the isolation of asaricin **1** (52 mg) ($R_f = 0.937$), isoasarone **2** (32 mg) ($R_f = 0.710$) and *trans*-asarone **3** (194 mg) ($R_f = 0.620$).

2.4. Characterization of isolated compounds

The structures of the isolated compounds were elucidated as asaricin (**1**), isoasarone (**2**) and *trans*-asarone (**3**) using various spectroscopic methods such as 1D-NMR, 2D-NMR, LCMS, UV, and IR as previously described (Hematpoor et al., 2016, 2017) and upon comparison with literature values (Patra and Mitra, 1981; Santos et al., 1998; Tanimori et al., 2009).

2.4.1. Asaricin 1

Yellow oil. LCMS-IT-TOF *m/z* 192.21118 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$, δ_H/ppm, *J*/Hz): 6.64 (1H, s, H-2), 6.51 (1H, s, H-5), 5.87–5.97 (1H, m, H-2′), 5.88 (2H, s, H-3a), 4.99–5.06 (2H, m, H-3′), 3.75 (3H, s, O<u>C</u>H$_3$–6), 3.28 (2H, d, $J = 6.8$ Hz, H-1′). ^{13}C NMR (100 MHz, $CDCl_3$, δ_C/ppm): 152.2 (C-6), 146.4 (C-3), 141.0 (C-4), 137.3 (C-2′),

120.8 (C-1), 115.3 (C-3′), 109.7 (C-2), 101.0 (C-3a), 95.0 (C-5), 56.6 (O$\underline{C}H_3$–6), 34.0 (C-1′).

2.4.2. Isoasarone **2**

Yellow oil. LCMS-IT-TOF *m/z* 231.0258 $[M+Na]^+$. ^1H NMR (400 MHz, $CDCl_3$, δ_H/ppm, *J*/Hz): 6.70 (1H, s, H-2), 6.54 (1H, s, H-5), 5.92–6.02 (1H, m, H-2′), 5.02–5.07 (2H, m, H-3′), 3.89 (3H, s, O$\underline{C}H_3$–4), 3.84 (3H, s, O$\underline{C}H_3$–3), 3.81 (3H, s, O$\underline{C}H_3$–6), 3.30 (2H, d, $J = 6.4$ Hz, H-1′). ^{13}C NMR (100 MHz, $CDCl_3$, δ_C/ppm): 151.5 (C-6), 148.1 (C-4), 143.2 (C-3), 137.5 (C-2′), 120.2 (C-1), 115.4 (C-3′), 114.1 (C-2), 98.2 (C-5), 56.8 (O$\underline{C}H_3$–3, O$\underline{C}H_3$–6), 56.4 (O$\underline{C}H_3$–4), 33.8 (C-1′).

2.4.3. Trans-asarone **3**

Yellow oil. LCMS-IT-TOF *m/z* 208.2625 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$, δ_H/ppm, *J*/Hz): 6.95 (1H, s, H-2), 6.66 (1H, dq, $J = 16.0$, 1.8 Hz, H-1′), 6.50 (1H, s, H-5), 6.11 (1H, dq, $J = 16.0$, 6.9 Hz, H-2′), 3.89 (3H, s, O$\underline{C}H_3$–4), 3.86 (3H, s, O$\underline{C}H_3$–3), 3.83 (3H, s, O$\underline{C}H_3$–6), 1.89 (3H, dd, $J = 6.9$, 1.8 Hz, H-3′). ^{13}C NMR (100 MHz, $CDCl_3$, δ_C/ppm): 150.8 (C-6), 148.9 (C-4), 143.5 (C-3), 125.2 (C-1′), 124.6 (C-2′), 119.2 (C-1), 109.9 (C-2), 98.1 (C-5), 56.9 (O$\underline{C}H_3$–3), 56.7 (O$\underline{C}H_3$–6), 56.3 (O$\underline{C}H_3$–4), 19.0 (C-3′).

2.5. Test mosquitoes

Adult mosquitoes were obtained from colonies cultured in the laboratory as described previously (Hematpoor et al., 2016). They were reared separately under laboratory conditions (27 ± 2 °C and 65 ± 5 % RH). Adult mosquitoes of *Ae. aegypti* and *Ae. albopictus* were fed with dried powder of cow liver while *Cx. quinquefasciatus* adult mosquitoes were fed with Brewer's yeast: mouse food (1:3). The 1-to-3-day-old adult mosquitoes were used for insecticidal assay.

2.6. Insecticidal activity

Insecticidal activity was analyzed by optimization of the standard procedures recommended by the World Health Organization (1981). Glucose solutions (10 % w/w) were prepared to feed mosquitoes in controlled environments. For a 10 % glucose solution, 100 *g* of glucose were dissolved in 900 mL of dechlorinated water (Haddad and Miller, 2019). This 10 % solution was used as the stock solution for further serial dilutions to obtain desirable concentrations of toxicants to feed the adult mosquitoes. These solutions were then administered to the mosquitoes during the testing phase. Compounds **1** and **2** were dissolved in 1 mL of acetone and 1 to 20 μg/mL concentrations were prepared with dechlorinated tap water inside 300 mL plastic cups. Since compound **3** was less effective, it was concentrated between 250 and 1000 μg/mL. Twenty adult mosquitoes were then introduced to each cup. Five replicates were maintained for each concentration and dead adult mosquitoes were counted after 24 h. Mosquitoes are considered dead when there are no signs of life and become immobile, such as lying on their back, with no leg or wing movements. The LD_{50} and LD_{95} values were calculated by probit analysis using Polo plus (LeOra Software LLC) ($p < 0.05$). Six concentrations of insecticides were used to determine the median lethal dose (LD) value. In all cases, the bioassay data were pooled and analyzed as described in WHO (1970). If the control showed more than 20 % mortality, the following formula was used to obtain the correct mortality percentage (Abbott, 1925; WHO, 1970).

$$X = (\%\,\text{test mortality} - \%\,\text{control mortality})/(100 - \%\,\text{control mortality}) \times 100$$

2.7. Median lethal time assay

Lethal time (LT) was used to determine adult mosquitoes' resistance to asaricin (**1**), isoasarone (**2**) and *trans*-asarone (**3**). LT_{50} and LT_{95} values are defined as the time to kill 50 % and 95 % of the mosquito's sample population, respectively. For time-mortality testing, based on the LD test, *Ae. albopictus, Ae. aegypti* and *Cx. quinquefasciatus* were individually exposed to compounds **1** (13 μg/mL), **2** (12 μg/mL) and **3** (1300 μg/mL). Batches of 25 mosquitoes were introduced into each cup. The number of dead adult mosquitoes was recorded at progressive time intervals until complete mortality or pupation was reached. Susceptibility was compared in terms of final mortality at 24-hour median lethal time (LT_{50}) calculated by log-probit regression using Polo plus (LeOra Software LLC) ($p < 0.05$).

2.8. Enzyme preparation

Enzyme preparations were carried out by homogenizing the adult mosquitoes at 0 °C (using crushed ice around the container to keep the temperature low) in 0.05 KH_2PO_4—NaOH (M-phosphate buffer, pH 7.5) using a glass homogenizer with a polytetrafluoroethylene pestle (PTFE) (5 %, w/v, homogenate). The mixture was centrifuged for 20 min at 5 °C and 16,000 rpm.

2.9. Determination of GST activity with biochemical assay (in vitro)

GST activity biochemical assay was conducted by using CDNB as substrate, in 96 well Microplate (4 mL) (Fisher Scientific) by sequential addition of 0.1 M phosphate buffer pH 6.5 (1.78 mL), enzyme preparation as mentioned above (0.1 mL), 50 mM of reduced GSH solution in buffer (0.1 mL) and 50 mM CDNB solution in acetonitrile (0.02 mL) (final volume of the routine incubation mixture is 2 mL). Enzyme activity was determined by continuously monitoring the change in absorbance at 340 nm for 3 min at 25 °C with a Synergy H1 Hybrid Multi-Mode Microplate Reader (Habig et al., 1974).

2.10. Treatment with GST-inducer candidates

Four replicates of 20 *Ae. Aegypti* adult mosquitoes were exposed to 10 %, 50 % and 95 % LD dosages of compounds **1, 2** and **3** separately using disposable plastic cups. Each replicate was kept in a separate cup for 24 h at 30 °C. One surviving adult mosquito from each replicate was homogenized (as described) in pools of 4 insects, where enzyme activity was determined from the average of 3 independent pools of 4 insects. Similar procedures were carried out for *Ae. albopictus* and *Cx. quinquefasciatus.*

2.11. Molecular docking study

The initial structure of *Drosophila melanogaster* for GST, the X-ray crystal structure from *Anopheles dirus* species (PDB ID: 1JLV) (Oakley et al., 2001) was used, and the molecular docking was performed where the binding site was defined from the center of the ligand found in the X-ray structure. The enzyme was then prepared under the protein preparation protocol implemented in Discovery Studio 2.5 (Accelrys Inc, 2.5.5). The missing residues from 103–135 and 574–585 were not included in this model since they were very far from the investigated binding sites. To prepare the protein, missing atoms were filled in, unnecessary forms were deleted, atom labels were corrected, and both water and X-ray blocking agents were removed.. Before minimization, molecular properties of the compound and the enzyme were described by CHARMM forcefield for the partial charge setting (Momany and Rone, 1992). The molecular docking was performed using Autodock/Vina (Trott and Olson, 2010), where the compound was flexible. The input site sphere was set at GSH-701 as its active site.

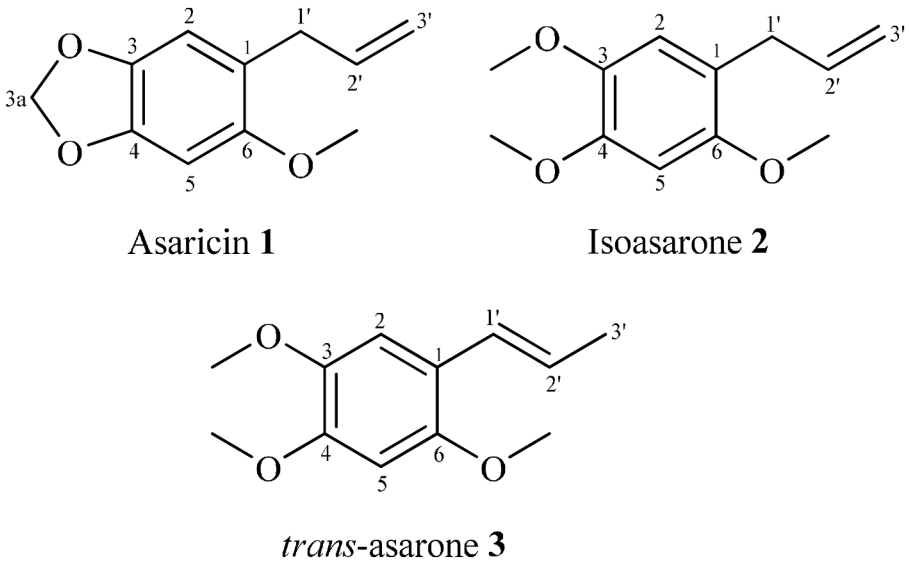


Fig. 1. Structures of asaricin **1**, isoasarone **2** and *trans*-asarone **3**.

Table 2
LD values of **1, 2** and **3** against adult mosquitoes of *Ae. aegypti, Ae. albopictus and Cx. Quinquefasciatus* with 95 % confidence index.

Compounds	**Tested adult mosquitoes***								
	Ae. aegypti			*Ae. albopictus*			*Cx. quinquefasciatus*		
	Slop ± SE	LD_{50} (μg/mL)[c] (95 % C.I.)	LD_{95} (μg/mL)[c] (95 % C.I.)	Slop ± SE	LD_{50} (μg/mL)[c] (95 % C.I.)	LD_{95} (μg/mL)[c] (95 % C.I.)	Slop ± SE	LD_{50} (μg/mL)[c] (95 % C.I.)	LD_{95} (μg/mL)[c] (95 % C.I.)
1	5.6 ± 0.70	4.3[a] (3.1 to 6.3)	7.3[a] (6.7 to 12.8)	4.3 ± 0.4	5.1[a] (4.1 to 8.1)	8.9[a] (6.0 to 14.2)	2.8 ± 0.31	7.3[a] (5.2 to 10.3)	13.2[a] (11.5to 19.6)
2	6.8 ± 0.9	4.1[a] (2.8 to 7.7)	7.1[a] (5.4 to 13.3)	4.3 ± 0.4	4.5[a] (3.6 to 8.7)	7.5[a] (5.6 to 11.3)	2.4 ± 0.2	8.8[a] (7.2 to 10.7)	12.4[a] (10.3 to 22.7)
3	8.8 ± 0.9	509.5[b] (391.2 to 643.5)	894.12[b] (692.4 to 1002.3)	5.3 ± 0.5	667.5[b] (584.3 to 803.1)	1345.2[b] (1283.4 to 1843.8)	3.4 ± 0.4	544.2[b] (474.6 to 902.2)	1304.46[b] (1120.1 to 1722.5)

* LD_{50} and LD_{95} values followed by a common letter are not significantly different ($p < 0.05$) based on non-overlapping of the 95 % CI.

3. Results

3.1. Preliminary tests

Preliminary toxicity testing was conducted using hexane (HE), dichloromethane (DE) and methanol (ME) extracts on *Ae. aegypti, Ae. albopictus and Cx. quinquefasciatus* adult mosquito (Table 1). As presented, the hexane extract exhibited the greatest insecticidal activity against all three mosquito species, showing LD_{50} of 102.3 μg/mL (*Ae. aegypti*), 97.1 μg/mL (*Ae. albopictus*) and 122.3 μg/mL (*Cx. quinquefasciatus*), in comparison to the DE and ME extracts (Table 1). Hence, HE was subjected to bioassay-guided fractionation where H2 was the most active fraction with LD_{50} and LD_{95} values of 17.4 and 83.9 μg/mL, respectively. Potent toxicity of H2 has resulted in the isolation and structural elucidation of asaricin (**1**), isoasarone (**2**) and *trans*-asarone (**3**) (Fig. 1).

3.2. Isolation and structural identification of isolated compounds

Three phenylpropanoids, asaricin (**1**), isoasarone (**2**) and *trans*-asarone (**3**) were isolated from the leaves of *P. sarmentosum*. Their structures were identified through the analysis of spectroscopic data as previously described (Hematpoor et al., 2016, 2017) together with comparison with the literature values (Patra and Mitra, 1981; Santos et al., 1998; Tanimori et al., 2009).

Table 3
LT_{50} and LT_{95} values of **1, 2** and **3** against adult mosquitoes of *Ae. aegypti, Ae. albopictus and Cx. Quinquefasciatus* with 95 % confidence index.

Compounds	**Tested adult mosquitoes***								
	Aedes aegypti			*Aedes albopictus*			*Culex quinquefasciatus*		
	Slop ± SE	LT_{50} (min) (95 % C.I.)	LT_{95} (min) (95 % C.I.)	Slop ± SE	LT_{50} (min) (95 % C.I.)	LT_{95} (min) (95 % C.I.)	Slop ± SE	LT_{50} (min) (95 % C.I.)	LT_{95} (min) (95 % C.I.)
1	5.6 ± 0.70	21[a] (15.3 to 32.3)	78[a] (46.7 to 82.8)	4.3 ± 0.4	29[a] (14.8 to 37.1)	82[a] (73.0 to 97.2)	2.8 ± 0.31	43[a] (39.3 to 64.3)	123[a] (102.5 to 139.6)
2	6.8 ± 0.9	17[a] (13.4 to 25.7)	56[a] (34.4 to 72.3)	4.3 ± 0.4	18[a] (14.6 to 28.3)	62 [b] (52.6 to 68.4)	2.4 ± 0.2	56[a] (44.2 to 79.2)	133[a] (86.3 to 142.7)
3		458[b] (353.4 to 565.6)	631[b] (593.4 to 677.2)		503 [b] (483.4 to 525.2)	732[c] (693.9 to 785.7)		781[b] (612.2 to 795.9)	1023[b] (983.4 to 1225.1)

* LD_{50} and LD_{95} values followed by a common letter are not significantly different ($p < 0.05$) based on non-overlapping of the 95 % CI.

Table 4
Specific GST enzyme activities of survived *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* adults after exposure to **1, 2** and **3** at LD_{10}, LD_{50} and LD_{95}.

Compounds	LD	GST specific activities (μmol/min/mg)*		
		Ae. aegypti	*Ae. albopictus*	*Cx. quinquefasciatus*
Asaricin **1**	10	0.178 ± 0.03	0.167 ± 0.03	0.216 ± 0.02
	50	0.238 ± 0.01	0.214 ± 0.02	0.299 ± 0.03
	95	0.358 ± 0.02	0.377 ± 0.05	0.424 ± 0.04
Isoasarone **2**	10	0.128 ± 0.01	0.141 ± 0.04	0.237 ± 0.03
	50	0.192 ± 0.01	0.232 ± 0.01	0.346 ± 0.01
	95	0.327 ± 0.03	0.353 ± 0.03	0.443 ± 0.01
Trans-asarone **3**	10	0.130 ± 0.01	0.120 ± 0.01	0.192 ± 0.01
	50	0.204 ± 0.03	0.197 ± 0.04	0.261 ± 0.01
	95	0.313 ± 0.03	0.281 ± 0.03	0.412 ± 0.04
Control		0.174 ± 0.01	0.157 ± 0.01	0.197 ± 0.02

* Values are expressed as mean ± standard deviation $p < 0.05$.

3.3. Insecticidal activity of isolated compounds

Isolated phenylpropanoids were tested against three species of mosquitoes in the search for potential bioinsecticides. The insecticidal activity of compounds **1, 2** and **3** at different concentrations was evaluated against the adult mosquitoes of *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus*. The LD_{50} and LD_{95} values of compounds **1, 2** and **3** against these insects were shown in Table 2, where they were found to be highly susceptible towards compounds **1** and **2** with LD_{50} values ranging between 4.3 – 7.3 μg/mL. Meanwhile, compound **3** showed the lowest toxicity against *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* with $LD_{50} > 500$ μg/mL and $LD_{95} > 800$ μg/mL.

3.4. Median lethal time assay

Exposure of *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* to asaricin **1** and isoasarone **2** for 120 min and *trans*-asarone **3** for 1200 min at respective LD_{95} concentrations resulted in LT_{50} values (Table 3). There were slightly higher tolerances of *Cx. quinquefasciatus* with LT_{50} of ≤ 56 min towards compounds **1** and **2** and LT_{50} of ≤ 781 min towards **3** compared to the *Ae. Aegypti* and *Ae. albopictus* mosquitoes. Meanwhile, *Ae. aegypti* and *Ae. albopictus* had similar relative tolerance towards compounds **1** and **2** (LT_{50} asaricin / LT_{50} isoasarone). Across all three species, compound **3** had the highest LT_{50} and LT_{95} values, signifying its moderate insecticidal activity.

3.5. GST activity

Biochemical assay revealed the differences in GST activities between all three adult mosquito species, as shown in Table 4. The specific GST activity of *Ae. aegypti* and *Ae. albopictus* in the control group were 0.174 and 0.157 μmol/min/mg respectively, significantly lower than that of *Cx. quinquefasciatus* (0.197 μmol/min/mg). The GST enzyme activity of each tested adult mosquito was positively correlated with their relative LD_{95} values of compounds **1, 2** and **3** (Fig. 2). The study showed that GST activity is linked to insecticide resistance, especially at LD_{95} levels. There was a clear positive correlation between GST activity and LD95, although it was not statistically significant ($p > 0.05$). These findings were then used to test whether GST activity increases when insects are exposed to GST-inducing compounds.

3.6. Treatment with GST-inducer candidates

There was no significant difference in GST activity between mosquitoes exposed to compounds **1- 3** at LD_{10} concentrations compared to that of the control group. Compound **1** increased GST activity by over 30 % in *Ae. aegypti* and *Ae. albopictus*, and by 51 % in *Cx. quinquefasciatus* at LD_{50} concentrations. At LD_{95} doses, compound **2** caused a 124.7 % GST rise in *Cx. quinquefasciatus*. Compounds **2** and **3** also led to elevated GST levels, whereas acetone exposure had no effect.

3.7. Binding affinity of phenylpropanoids towards active site of GST by molecular docking

The GST-ligand complex with the lowest calculated binding energy (Table 5) was selected from a series of poses in molecular docking for further analysis. Calculated binding energy would reveal the binding affinity of compounds at the binding site of GST, where lower binding energy suggests better binding affinity. Compounds **1, 2** and **3** were discovered to have similar binding affinities of −5 kcal/mol, −4.8 kcal/mol and −4.7 kcal/mol, respectively (Table 5). The superposition of these compounds oriented in the binding pocket of GST were shown in Fig. 3.

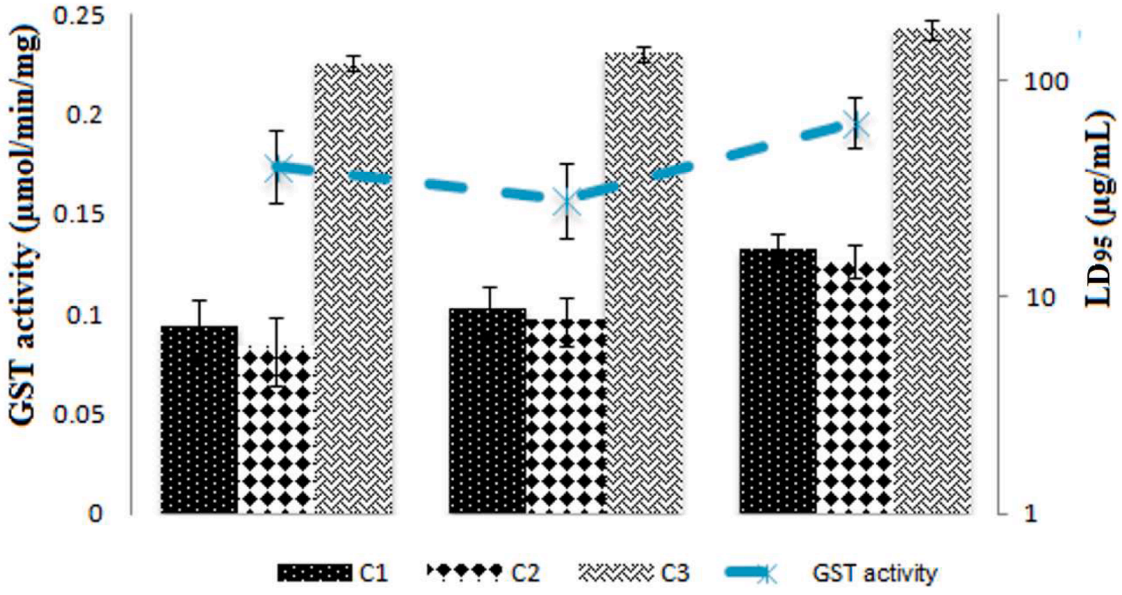
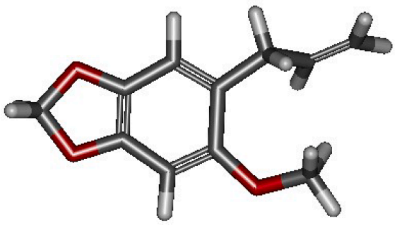
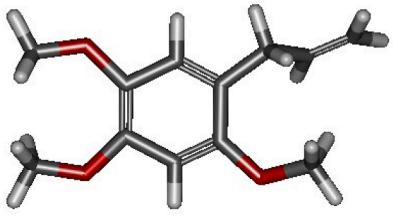


Fig. 2. Biochemical assay on GST activity and its correlation with LD_{50}. LD_{50} value of compound **3** was higher than 100 μg/mL to have better view on compounds **1** and **2** the column LD_{50} axis adjust to maximum 20 μg/mL.

Table 5
Binding interaction energy (kcal/mol) of **1**, 2 and **3** towards different binding sites in GST.

Inhibitors	Binding interaction energy (kcal/mol)
Asaricin **1** 	-5.0
Isoasarone **2** 	-4.8
Trans-asarone **3** 	-4.7

3.8. Molecular interaction of phenylpropanoid compounds toward GST

Insecticides might not work well if they easily and strongly bind to the GST enzyme, as the insect can detoxify and eliminate the toxicants before they reach their site of action. Measurements of interaction energy represented the binding strength between the insecticidal compound and GST. The interaction energy (IE) within 3 Å residues (as highlighted in Table 6) revealed that compound **1** has the highest interaction energy (−25.07 kcal/mol), followed by compound **3** (−32.81 kcal/mol) and compound **2** (−46.52 kcal/mol). Fig. 4 illustrated the detailed interactions, in particular, hydrogen bonds, denoted by a green dotted line, forming in the binding cavity. All compounds were found to form hydrogen bonds with the binding residues within the active site. Compound **2** formed four hydrogen bonds through SER65HG:O12, ARG66HH12:O14, ARG66HH12:O12 and ARG66H22:O14, (Fig. 4b), compound **3** formed two hydrogen bonds via ARG66HH12:O22 and ARG66HH22:O22 (Fig. 4c) whereas compound **1** formed only one hydrogen bond with the binding residues through SER65HG:O9 (Fig. 4a). The more hydrogen bonds a compound form, the more tightly it binds to the enzyme. Compound **2** formed three hydrogen bonds with ARG66, which explains its strong interaction at that site. Common binding residues for all three compounds—PRO11, GLU64, SER65, ARG66, and TYR105—were within 3 Å and had interaction energies below –2 kcal/mol. In compounds **2** and **3**, electrostatic forces contributed more to binding than van der Waals forces, unlike compound 1.

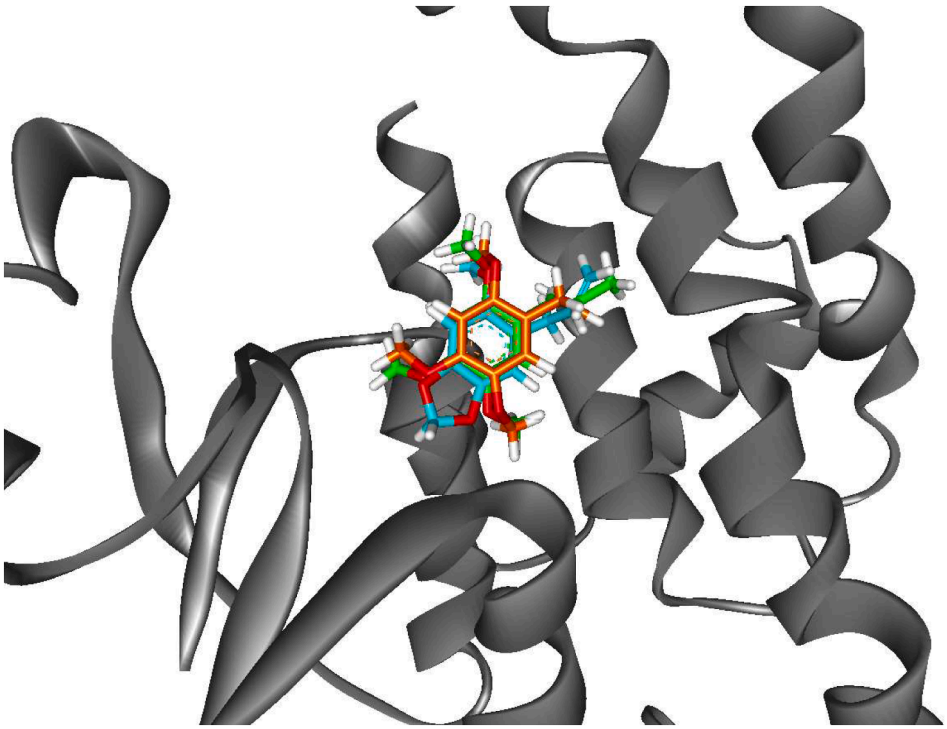


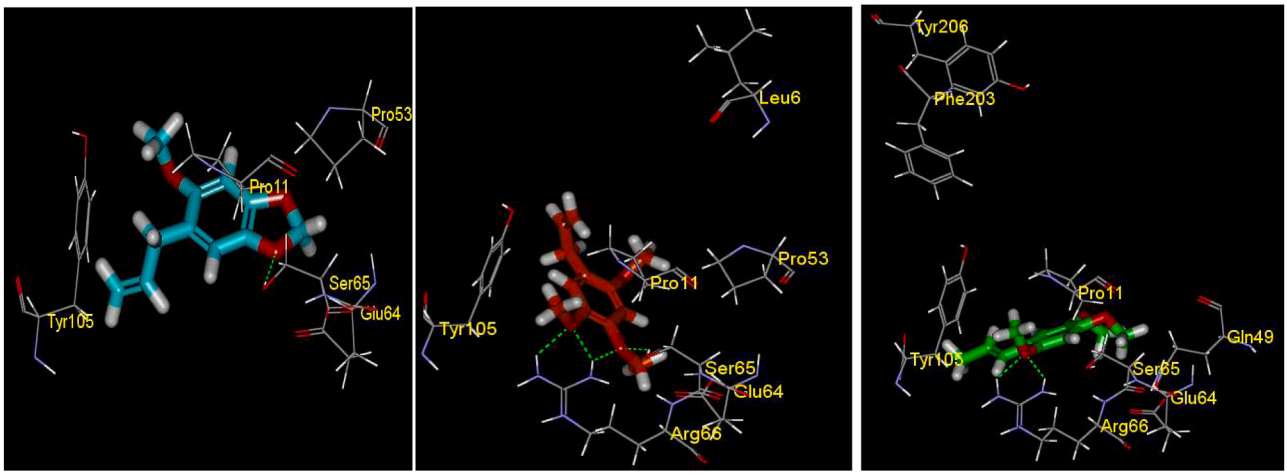
Fig. 3. Docking complexes of compounds **1** (blue), **2** (orange), and **3** (green) with GST.

Table 6
The interaction energy of compounds **1, 2**, and **3** toward GST. The highlighted GST residues are in the 3 Å binding with each compound.

Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)	Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)	Residue	IE (kcal/mol)	VDW (kcal/mol)	Electrostatics (kcal/mol)
GST Asaricin **1**				Isoasarone**2**				*Trans*-asarone **3**			
LEU 6	−1.29	−0.0074	−1.28	LEU 6	−3.68	−0.0091	−3.67	LEU 6	0.37	−0.0021	0.37
SER 9	0.46	−0.25	0.71	**SER 9**	0.19	−0.58	0.77	SER 9	−0.088	−0.17	0.081
ALA 10	−0.34	−0.23	−0.12	**ALA 10**	−0.74	−1.31	0.57	ALA 10	0.35	−0.75	1.1
PRO 11	−3.15	−3.44	0.28	**PRO 11**	−4.71	−4.77	0.057	**PRO 11**	−2.68	−2.32	−0.36
LEU 33	−0.01	−0.024	0.013	LEU 33	0.85	−0.026	0.88	LEU 33	0.67	−0.0095	0.68
GLN 49	−1.34	−0.43	−0.91	GLN 49	0.22	−0.38	0.59	**GLN 49**	−2.49	−1.15	−1.34
CYS 51	−0.1	−0.94	0.84	**CYS 51**	−1.71	−0.92	−0.79	CYS 51	−1.39	−0.93	−0.46
PRO 53	−2.38	−1.83	−0.55	PRO 53	−2.77	−0.56	−2.21	**PRO 53**	−1.94	−1.34	−0.61
GLU 64	−4.44	−2.79	−1.65	**GLU 64**	−6.49	−0.29	−6.19	**GLU 64**	−6	−1.91	−4.09
SER 65	−7.93	−2.14	−5.79	**SER 65**	−5.61	−1.45	−4.16	**SER 65**	−2.67	−0.87	−1.8
ARG 66	−1.41	−1.48	0.064	**ARG 66**	−21.36	0.68	−22.04	**ARG 66**	−7.6	−0.31	−7.29
MET 101	−0.12	−0.95	0.82	MET 101	2.06	−0.48	2.54	**MET 101**	0.33	−1.43	1.77
TYR 105	−5.54	−2.35	−3.19	**TYR 105**	−6.09	−3.26	−2.82	**TYR 105**	−7.64	−3.71	−3.94
GLN 106	−0.4	−0.47	0.066	GLN 106	−1.22	−0.16	−1.06	**GLN 106**	−1.56	−2.07	0.51
ALA 109	−0.77	−0.17	−0.6	ALA 109	−1.87	−0.17	−1.7	**ALA 109**	−0.56	−0.56	−0.00051
TYR 113	1.93	−0.23	2.17	TYR 113	0.57	−0.55	1.12	TYR 113	−1.72	−0.13	−1.59
PHE 117	0.019	−0.012	0.03	PHE 117	0.54	−0.024	0.57	PHE 117	−0.67	−0.0015	−0.67
PHE 203	−0.088	−0.045	−0.043	PHE 203	−1.62	−0.15	−1.47	PHE 203	−1.95	−0.03	−1.92
TYR 206	0.24	−0.0065	0.25	TYR 206	−0.18	−0.017	−0.16	TYR 206	0.38	−0.000084	0.34
3Å IE	−25.07	−15.92	−9.18	**3Å IE**	−46.52	−11.90	−34.60	**3Å IE**	−32.81	−15.67	−17.15
Total IE	−45.96	−20.43	−25.53	Total IE	−79.69	−17.19	−62.50	Total IE	−69.26	−20.45	−48.81

4. Discussion

Pesticides have a significant role in public health and agriculture. Conventional synthetic insecticides, while effective, have led to environmental pollution and selective pressure that accelerates the emergence of resistance in several insect species (Siddiqui et al., 2023). In this context, botanical insecticides derived from medicinal plants are gaining attention as promising alternatives due to their biodegradable nature, structural diversity, and lower toxicity to non-target organisms (Campbell et al., 1998; Gregorc and Poklukar, 2003). It has been proven that crude extracts of medicinal plants have toxic effects on different species of vectors, including mosquitoes (Wachira et al., 2014). Hexane extracts of *P. sarmentosum* demonstrated strong insecticidal activity against adult *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus*, with LD_{50} values $\leq$122 μg/mL. These values are indicative of a potent toxic effect, particularly when compared to other reported plant-based insecticides and essential oils reported in previous studies (Mullai et al., 2008; Nathan et al., 2007; Rajkumar and Jebanesan, 2009; Wilps, 1995). The local availability and edibility of *P. sarmentosum* further enhance its attractiveness as a source of natural insecticides, offering a cost-effective



(a) **(b)** **(c)**

Fig. 4. Docking structures of compound (a) **1** (b) **2** and (c) **3** toward GST and their closed contact residue interaction with the hydrogen bond interactions at SER65HG:O9 in (a), SER65HG:O12, ARG66HH12:O14, ARG66HH12:O12 and ARG66H22:O14 in (b) and ARG66HH12:O22 and ARG66HH22:O22 in (c). Residues interacted with compound with the interaction energy < -2 kcal/mol were shown.

solution that can reduce reliance on imported chemical products and support community-based vector control efforts (Stevenson et al., 2017). Following bioassay-guided fractionation, three phenylpropanoids—asaricin (**1**), isoasarone (**2**), and trans-asarone (**3**)—were isolated from the hexane extract. Among these, compounds **1** and **2** exhibited pronounced insecticidal activity, with LD_{50} values that varied between mosquito species.

Interestingly, both *Ae. aegypti* and *Ae. albopictus* displayed similar levels of susceptibility to compounds **1** and **2**, whereas Cx. quinquefasciatus required higher doses for comparable mortality, suggesting greater resistance. This species-specific variation in response may be attributed to physiological and biochemical differences, such as thicker cuticular barriers or enhanced detoxification pathways in *Cx. Quinquefasciatus* (Talipouo et al., 2021). Similar patterns of increased resistance in this species have been documented for other natural insecticides, reinforcing the notion that *Cx. quinquefasciatus* possesses more robust protective mechanisms (Chen et al., 2025; Lopes et al., 2019; Pridgeon et al., 2008). Moreover, the observed LT_{50} values were also higher for *Cx. quinquefasciatus*, further supporting its reduced sensitivity to phenylpropanoids.

Biochemical assays revealed that surviving adult mosquitoes exhibited elevated levels of glutathione *S*-transferase (GST), particularly in *Cx. quinquefasciatus*, establishing a clear relationship between GST activity and insecticide resistance (Luo et al., 2014). GST enzymes are known to play a critical role in detoxification by catalyzing the conjugation of glutathione to xenobiotic compounds, thereby facilitating their sequestration and excretion (Bagrij et al., 2001; Zhou et al., 2015). The positive correlation between GST activity and resistance to compounds **1**, **2**, and **3** suggests that GST-mediated detoxification is a primary mechanism enabling mosquitoes to withstand the toxic effects of these phenylpropanoids. Computational docking studies further confirmed this interaction, highlighting key residues—PRO11, GLU64, ARG66, and TYR105—within the GST binding pocket as important for the compounds' affinity. While compound **2** showed a stronger binding affinity to GST compared to compound **1**, both exhibited similar insecticidal activity, indicating that other detoxification pathways may also be contributing to overall resistance of the tested adult mosquitoes.

These findings suggest a multifaceted resistance mechanism, potentially involving other enzymatic systems such as cytochrome P450 monooxygenases and carboxylesterases (Hemingway et al., 2004; Nkya et al., 2013). The proposed detoxification mechanism involves the conjugation of the phenylpropanoid with GST, sequestration into intracellular compartments, and eventual transport out of the cell, which collectively mitigate the toxic effect of the compound. The ability of mosquitoes to upregulate GST in response to exposure, as demonstrated by increased enzyme levels in survivors of LD_{10} and LD_{95} treatments, underscores the adaptive nature of this resistance mechanism and emphasizes the need to monitor enzyme activity when deploying botanical insecticides.

The strong insecticidal activity of compounds **1** and **2** at low concentrations, combined with the safety profile of *P. sarmentosum*, suggests these compounds are promising candidates for further development. Their application could be tailored to target species with known susceptibility or incorporated into integrated vector management strategies to reduce selective pressure. However, before widespread implementation, additional studies are required to evaluate their environmental stability, formulation potential, and effects on non-target organisms. Incorporating GST inhibitors or designing analogs with reduced susceptibility to enzymatic detoxification may further enhance their efficacy. Ultimately, the results from this study contribute to the growing body of evidence supporting plant-based insecticides as sustainable alternatives and provide valuable insight into the molecular interactions driving mosquito resistance.

5. Conclusion

This study highlights the promising insecticidal potential of phenylpropanoids isolated from *Piper sarmentosum*—specifically asaricin (**1**) and isoasarone (**2**)—against key mosquito vectors, *Aedes aegypti, Aedes albopictus*, and *Culex quinquefasciatus*. The low LD_{50} values observed for compounds **1** and **2** confirm their strong toxicity at relatively low concentrations, with notable efficacy against *Ae. aegypti* and *Ae. albopictus*. Although *Cx. quinquefasciatus* exhibited higher resistance, likely due to elevated glutathione S-transferase (GST) activity, these mosquitoes remained susceptible to both compounds, indicating their potential utility even in resistant populations. Biochemical assays and molecular docking studies supported the role of GST as a key detoxification enzyme that interacts with all three compounds. The strong binding affinity of compounds **1** and **2** to critical residues within the GST active site highlights a plausible mechanism of detoxification, although other metabolic pathways may also be involved. Despite this, the insecticidal efficacy of both compounds remained substantial, reinforcing their value as bioactive agents in future vector control strategies. Given that *P. sarmentosum* is abundant, edible, and traditionally used in Southeast

Asia, the plant represents an environmentally safe and economically viable source for botanical insecticide development. Moreover, the moderate activity of trans-asarone (**3**), due to its amphiphilic structure, may still be valuable in multi-component formulations aimed at improving efficacy and stability. Therefore, asaricin and isoasarone are proposed as strong candidates for natural mosquito control agents, especially in Malaysia and neighbouring regions. Further research focusing on formulation development, in vivo toxicity assessments, and field trials will be essential to translate these findings into practical applications for integrated mosquito management.

CRediT authorship contribution statement

Arshia Hematpoor: Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Norhayu Asib:** Writing – review & editing, Validation, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sook Yee Liew:** Validation, Methodology, Investigation. **Vannajan Sanghiran Lee:** Validation, Software, Methodology, Investigation, Conceptualization. **Muhammad Afiq Ngadni:** Writing – review & editing, Validation, Funding acquisition.

Declaration of competing interest

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

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Data availability

No data was used for the research described in the article.

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