Effect of (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone from Blumea axillaris (Lam.) DC. plant against An. stephensi and Cx. quinquefasciatus vector mosquitoes (Diptera: Culicidae) and expression of different functional genes

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Research Article

Keywords: Blumea axillaris, Mosquito, Larvicidal, Ovicidal, Histopathology, qRT-PCR

Posted Date: January 17th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2476047/v1

Abstract

The present study was conducted to evaluate the mosquitocidal efficiency using a compound isolated from *Blumea axillaris (Lam.) DC.* against *Culex quinquefasciatus* and *Anopheles stephensi*. The compound isolated from leaves of *B. axillaris* is (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone. This isolated compound was exposed to eggs and larvae of *An. stephensi* and *Cx. quinquefasciatus* and with different concentrations of 0.5, 1.0, 1.5, and 2.0 ppm. Primarily, the hexane extract of an isolated compound exhibited promising larvicidal activities of LC^{50} and LC^{90} values of 155.811 and 566.763 ppm against *An. stephensi* and 77.215 and 99.653 against *Cx. quinquefasciatus* at 24 hours. Secondly, the compound isolated from *B. axillaris* at 2-ppm concentration exhibited LC^{50} and LC^{90} values of 0.85 and 3.59 and 1.19 and 3.678 ppm against *Cx. quinquefasciatus* and *An. stephensi* larvae, respectively. Furthermore, histopathological studies revealed serious damage to the larval midgut cells in both *Cx. quinquefasciatus* and *An. stephensi* (treated with compound). Additionally, it showed 81.0% and 84.2% ovicidal activity against both *Cx. quinquefasciatus* and *An. stephensi* eggs at 120 h post-treatment. In addition, we also analyzed the influence of isolated compound on the expression of different functional genes of *An. stephensi* and *Cx. quinquefasciatus* namely (1) *White* (*Wh*) gene, which is responsible for eye pigmentation; (2) the *Glutathione transferase* (*GST*) gene that helps for survival when exposed to any insecticides or pesticides; (3) *Cytochrome P450 monooxygenase* (*CYP450*) gene that enhances resistance in mosquitoes to digest pesticides and (4) *Esterase* (*Est*) gene, which helps in resistance to the variety of insecticides. These overall studies and results clearly suggest that compounds from *B. axillaris* could act as good mosquitocidal agents against both *Cx. quinquefasciatus* and *An. stephensi* and the compound were also first time reported as mosquitocidal activity.

Introduction

Mosquitoes are the major transporter of viruses and are responsible for severe health problems in humans and domestic animals all over the world (Medlock et al. 2012; Organization 2013; Hillary et al. 2020). Globally, every year, more than 1 billion cases and 1 million deaths are recorded due to these mosquito-borne infectious diseases. Mosquitoes belonging to the genera *Aedes*, *Anopheles*, and *Culex* transmit dengue, chikungunya, yellow fever, zika, malaria, and filariasis to humans (Hillary and Ceasar 2021). Bite of these mosquitoes causes allergic responses with high fever, rash, nausea, limb swelling, and sometimes bleeding and death. Therefore, real vector control approaches are needed to avoid mosquito-borne diseases and improve community health (Hillary and Ceasar 2021).

In recent years, the most commonly used methods to control vector mosquitoes depend on chemical pesticides such as organophosphates, organochlorides, carbamates, methoprene, pyriproxyfen, and N, N-Diethyl-meta-toluamide (DEET) (Liu et al. 2005; Amer and Mehlhorn 2006). However, these chemicals, being pesticides, risk human life and the environment (Wang and Jacobs-Lorena 2013). Therefore, researchers focus on environment-friendly alternatives like plant extract, bacteria, and fungi instead of using chemical insecticides (Khan et al. 2012). Among these, the literature reveals a sufficient amount of work on mosquito control using plant extracts and plant compounds (Govindarajan et al. 2008; Anstrom et al. 2012). So far, more than 300 plants (including *Azadirachta indica*, *Cassis tora*, *Cestrum nocturnum*, *Delonix elata*, *Carapa guinesis*, *Solanum xanthocarpum*, *Orthosiphon thymiflorus*, etc.) have been identified, which have insecticidal activity against vector-borne mosquitoes (Schmutterer 1990; Shaalan et al. 2005; Kumar et al. 2012). Several phytocompounds (like nilocotin, 3, 5-dimethoxy-3', 4', 6, 7- bismethylendioxyflavone, N-methyl-N-cis-styrylcinnamamide, saponin, ecbolin A, ecbolin B, etc.) isolated from various plants are also been utilized for controlling the vector-borne mosquitoes (Cecilia, 2014; Reegan et al., 2014).

Therefore, in the present study, we selected medicinal plant *Blumea axillaris* and isolated compounds and used against *An. stephensi* and *Cx. quinquefasciatus* mosquitoes. *B. axillaris* (Lam.) DC is a Synonyms of *Blumea wightiana* (DC), *Blumea mollis* (D. Don) Merr. It's belongs to the Asteraceae family (Tribe Inuleae). It's an aromatic annual herb that is native to Southeast Asia, south of Sahara, and South America (Sreelekha et al. 2017). The *B. axillaris* having many medicinal properties and used for treating diarrhoea, asthma, dropsy, skin diseases, wounds, and external parasites (Sreelekha et al. 2017). This plant also reported to be an anti-inflammatory, anticancer, antibacterial, anti-phytofungal and antioxidant activities (Sreelekha et al. 2017; Sivanandhan et al. 2018). Isolated essential oil and alcoholic extract of this plant also showed promising mosquito larvicidal activities (Senthilkumar et al. 2008). Previously, few compounds isolated from *B. axillaris*, such as 4-hydroxy-7-ethoxycarvotanacetone (ethanol extract of the whole plant) (Bohlmann et al. 1979), 4-hydroxy-7-tigloyloxycarvotanacetone (ethanol extract of the whole plant) (Bohlmann et al. 1979), 4*R*, 5*S*)-4-hydroxy-7-tigloyloxy carvotan acetone and its acetate derivative 4*R*, 5*S*)-4-acetoxy-7-tigloyloxy carvotan acetone, which showed good mosquitocidal activities (Sivanandhan et al. 2018, 2021; Subramaniyan et al. 2021).

Therefore, in this present study we isolated an active compound from *B. axillar* leaves (n-hexane extract) and checked mosquitocidal activities, particularly *An. stephensi* and *Cx. quinquefasciatus* (omitted *Aedes aegypti*, since mortality could not be observed). The

isolated compound was screened for larvicidal, ovicidal and histopathological effects against *An. stephensi* and *Cx. quinquefasciatus*. In addition, we also studied the influence of phyto-compound on the expression of different functional genes of *An. stephensi* and *Cx. quinquefasciatus* namely (1) *White* (*Wh*) gene, which is responsible for the eye pigmentation; (2) *Glutathione transferase* (*GST*) gene that helps for the survival when exposed to any insecticides or pesticides; (3) *Cytochrome P450 monooxygenase* (*CYP450*) gene that enhances resistance in mosquitoes to digest pesticides and (4) *Esterase* (*Est*) gene, which helps in resistance to the variety of insecticides.

Materials And Methods

Plant collection and identification

B. axillaris plant was collected from Cuddalore District, Tamil Nadu, India. The plant was collected during the flowering stage in the month of March to May 2018. Taxonomist Dr. Pandikumar from Entomology Research Institute (ERI), Loyola College, Chennai, performed the taxonomical identification of the plant species. Voucher specimen (ERISS-02) of *B. axillaris* plant was deposited in the herbarium at ERI.

Preparation Of Crude Extract

The collected plant materials were shade-dried at room temperature and coarsely powdered using an electric blender. The powdered material (100 g) of the *B. axillaris* plant was sequentially extracted using hexane, chloroform, and methanol solvents. After 72 h of soaking, the extract was filtered through Whatman No.1 filter paper and dried in a vacuum. Solvents in the extract were removed using a rotary vacuum evaporator at 45–55°C and the crude extracts were stored at 4°C in the refrigerator for further bioassay.

Mosquito Larvae Rearing

An. stephensi and *Cx. quinquefasciatus* mosquitoes' larvae were collected from stagnant water in the Coovam River, Chennai, during August 2018. The collected larvae were maintained in the Entomology Research Institute, Loyola College, Chennai at a temperature of 28 ± 2 ˚C and relative humidity (RH) of 75 ± 5% with a photoperiod of 12 ± 0.5 hours (hrs). Larvae were kept in plastic containers at a density of 75 larvae per container containing 100 ml of water (pH-7.0) to which 3:2 g of dog biscuits and brewer's yeast were added and were kept free from pathogens, repellents, and insecticides. The plastic containers were covered with netting material. The first (F1) generation larvae were used for the experiment (Cecilia et al., 2014).

Extraction and Column chromatography for B. axillaris **hexane extract**

The *B. mollis* hexane extract was subjected to column chromatography. The dried crude extract (25 gm) was blended with silica gel (Acmae's 60–120 mesh) and loaded on a glass column, which was packed with silica gel (Acmae's 100–200 mesh). The silica column was initially washed with hexane and the extract was eluted with solvents of increasing polarity starting in the following order: (1) hexane (100%); (2) hexane: ethyl acetate (9:1); (3) hexane: ethyl acetate (8:2); (4) hexane: ethyl acetate (6:4); (5) hexane: ethyl acetate (1:1); (6) ethyl acetate (100%); and (7) methanol (100%). All fractions were collected in 250 ml conical flasks separately. The collected fractions were subjected to TLC and their R_f values were calculated. In total, 150 fractions were obtained and all the fractions were subjected to TLC analysis. Based on the TLC profile, similar fractions were pooled together and finally, seven fractions were obtained. The seven fractions were screened for larvicidal activity, which was reported by Sivanandhan et al. (2020). The fractions, which showed good larvicidal activities, were selected to identify new active compounds.

Chemicals And Instruments

Acmes's silico gel (100–200 mesh) was used for column chromatography. Silico gel F_{254} pre-coated aluminium plates (Merck, 0.2 mm thickness) were used for TLC. 10% alcoholic H_2SO_4 was used for visualization (heated at 110 degrees for 5 min until colours appeared).

FT-IR spectrum was taken on a Perkin-Elmer grating spectrophotometer in a KBr disc. ^{1}H-NMR (700 MHz) and ^{13}C-NMR (175 MHz) spectra were recorded on a Bruker instrument in $CDCl_3$. Chemical shift values are given in δ scale with TMS (Tetramethylsilane) as the internal standard.

2D NMR (COSY, HSQC, HMBC, and NOESY) were also recorded. HRESI-MS was recorded on the LC-MS TOF Shimadzu instrument. Preparatory HPLC was run on a JASCO instrument. Analytical grade chemicals and solvents used were for this study.

Isolation Of The Active Constituent

The active hexane extract was subjected to column chromatography over silica gel (Acmes's 100–200 mesh) packed in hexane. The column was eluted with hexane and ethyl acetate mixtures with increasing amounts of ethyl acetate resulting in 7 fractions. Fraction 4 eluted with hexane–ethyl acetate 6:4 showed good antifungal activity reported by Sivanandhan et al. 2020.

Purification Of Active Principle By Preparatory Hplc

Fraction 4 eluted with hexane–ethyl acetate 3:2 and was washed with little methanol. Further purification of the compound was achieved by preparative HPLC (Column COSMOSIL 5C18 MS 11, JASCO Gulliver System, 250 × 20 mm, 5 µm, gradient elution acetonitrile - H_2O 40–60%, 2 ml/min UV 215 nm). The same active fraction, the compound (4*R*, 5*S*) – 4 – hydroxyl – 7 - tigloyloxy carvotan acetone already been reported from the plant by Sivanandhan et al. (2020). The active compound identified was (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone.

Larvicidal Assay Using (4r, 5s) 4-hydroxy-7-angeloyloxycarvatoneacetone

Larvicidal assay was done via a previously used method of WHO (2005) with small modifications (Organization 2005). Twenty (20) F1 generation larvae each of *An. stephensi* and *Cx. quinquefasciatus* were introduced to the test concentrations of 0.5, 1, 1.5, and 2.0 ppm for the isolated compound. The isolated compound was prepared using DMSO (249 ml of water and 1 ml of DMSO); five replications were maintained for each concentration. The azadirachtin (Sigma Aldrich) and temephos (Sigma Aldrich) were used as positive controls at the same test concentrations of 0.5, 1, 1.5, and 2.0 ppm, with five replicates. The water was served as a negative control. *An. stephensi* and *Cx. quinquefasciatus* larvae mortality rate was observed after 24 hrs of exposure. Then mortality rate for each assay of dead larvae was done using the following formula (a). The corrections for mortality were done using Abbot's formula when control mortality was below 5% (b) (Abbott 1925).

(a) Percentage of mortality: $\frac{\mathrm{No.ofdeadlarvae}}{\mathrm{No.oflarvaeintroduced}}$

(b) Corrected percentage of mortality: $\frac{1-n\mathrm{in}T\mathrm{aftertreatment}}{n\mathrm{in}C\mathrm{aftertreatment}}$

Where *n* is the number of larvae, *T* is the number of treated larvae, and *C* is the number of larvae in control. The corrected percentage mortality value for each concentration was consider to estimate LC_{50} and LC_{90} values by using US EPA probit analysis software (version 1.5).

Ovicidal Assay Using (4r, 5s) 4-hydroxy-7-angeloyloxycarvatoneacetone

The ovicidal activity was assessed using the prescribed method of Elango et al. (2009) with slight modifications. Twenty (20) freshly laid eggs of *An. stephensi* and *Cx. quinquefasciatus* were exposed to the test concentrations of 0.5, 1, 1.5, and 2.0 ppm for the compound. The isolated compound was prepared using DMSO (249 ml of water and 1 ml of DMSO); five replications were maintained for each concentration. The azadirachtin (Sigma Aldrich) and temephos (Sigma Aldrich) were used as positive controls at the same test concentrations of 0.5, 1, 1.5, and 2.0 ppm with five replicates. The water was served as a negative control. The ovicidal activity was evaluated up to 120 hrs post-treatment. After 120 hrs post-treatment, the control, and treated eggs were observed under the microscope and photographed using a stereo zooming microscope (Wild M7S TYP 308700, Switzerland). The unhatched eggs with unopened opercula were counted in each experiment; hatchability of the egg was observed using a compound microscope at 120 hrs post-treatment, and the ovicidal treatment was assessed in percentage by using the following formula.

Percentage of egg mortality: $\frac{\mathrm{Numberofunhatchedeggs}}{\mathrm{Totalnumberofeggs}} \times 100$

Effect Of Isolated Compounds On Non-target Organisms

The effect of active compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone was tested based on the Maheswaran and Ignacimuthu (2012) method against non-target organisms such as *Poecilia reticulata* (predatory fish) collected from a pond of Fishery Research Institute, Chetpet, Chennai, India. The collected predator was exposed to different test concentrations of 0.5, 1, 1.5, and 2.0 ppm for the active compound. Temephos and azadirachtin were used as a positive control with 5 replicates. The mortality rate was observed after a 24 hrs exposure. After the treatment, the predators were free from the isolated compound and left free to normal water. The LC_{50} and LC_{90} values were obtained using probit analysis (version 1.5).

Percentage of mortality: $\frac{\text{No.ofdeadlarvae}}{\text{No.oflarvaeintroduced}}$

Histopathology

The treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone) and control larvae of *An. stephensi* and *Cx. quinquefasciatus* were performed according to the previously described method (Raymond et al., 2007) with slight modifications. The tissues were fixed in Carnoy 2 (Sigma-Aldrich, India) for 72 hrs. Sequential dehydration was done in the tissue using alcohol viz., 50, 60, 70, 80, 90, and 100% for every 2 hrs. After sequential dehydration, the samples were placed in xylene for 6 hrs and then transferred to a hot air oven with wax for embedding for 2 hrs. After 2 hrs, de-waxing was done with xylene for 5 minutes. Then the tissues were stained with Ehrlich's hematoxylin and eosin. Finally, the tissues were washed with 100% alcohol. Then the observation was made with the microscope (Motic images plus 2.0 ML, China) connected to a computer and treated and untreated larvae of *An. stephensi* and *Cx. quinquefasciatus* were photographed. The larval midgut cells of treated larvae were observed and compared with the control.

Gene Identification And Primer Design

Nucleotide sequences of *Wh*, *GST*, *CYP50, Est*, and *Actin* genes of *An. stephensi* and *Cx. quinquefasciatus* were retrieved from the NCBI database. The primers were designed in the conserved regions of the mosquito nucleotide sequence using the online Primer3 software tool (https://primer3.ut.ee/) (Tables 1, 2).

Table 1
Details of An. stephensi **gene specific primers used for qRT-PCR**. The details such as name of the gene, gene ID, forward and reverse primer, and product length with their temperature are provided.

Name of the gene	Forward primer (5' to 3')	Reverse Primer (5' to 3')	Product length	Tm (˚C)
Wh	CATCAACGGAGCGCTTTTC	GGATAGGTGATCGAGGTGAAC	210	59
CYP 450	ATGCCGAAGGATACCGCCAA	CGTGCGCAGATTGTTCACCA	198	59
EST	CGGGCCGGATTTCTTGGTTC	TTCGGGTCCCTCCAAAGGC	186	59.1
GST	CCAACGCCGACAACGAGAAG	CTTCTTGCACCGCTCCAACC	200	59
ACTIN	ATCGACAATGGGTCGGGCAT	GTCCTTCTGGCCCATTCCGA	126	59

Abbreviations: *Wh*, *white*; *CYP450, Cytochrome P450 monooxygenase; Est, Esterase; GST, Glutathione transferase.*

Table 2

Details of Cx. quinquefasciatus **gene specific primers used for qRT-PCR**. The details such as name of the gene, gene ID, forward and reverse primer, and product length with their temperature are provided.

Name of the gene	Forward primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Product length	Tm (˚C)
Wh	CATCAACGGAGCGCTTTTC	GGATAGGTGATCGAGGTGAAC	210	59
CYP 450	ATGCCGAAGGATACCGCCAA	CGTGCGCAGATTGTTCACCA	198	59
EST	CGGGCCGGATTTCTTGGTTC	TTCGGGTCCCTCCAAAGGC	186	59.1
GST	CCAACGCCGACAACGAGAAG	CTTCTTGCACCGCTCCAACC	200	59
ACTIN	ATCGACAATGGGTCGGGCAT	GTCCTTCTGGCCCATTCCGA	126	59

Abbreviations: *Wh, white; CYP450, Cytochrome P450 monooxygenase; Est, Esterase; GST, Glutathione transferase.*

Rna Extraction And Cdna Synthesis

For gene expression analysis, 12 hrs treated third instar larvae were collected. RNA was extracted using the Nucleospin RNA Plus kit (TAKARA, Japan) in triplicates for each sample from treated ((4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone), negative control (water), and positive controls (Temephos and azadirachtin). The concentration RNA was evaluated via NanoDrop Spectrophotometer (ND-2000, Thermo Scientific, USA). cDNA was synthesized using 500 ng each total RNA sample using QuantiTec reverse transcription kit with genomic DNA wipeout option (Qiagen, USA).

Quantitative Reverse-transcriptase Pcr (Qrt-pcr)

The qRT-PCR was performed utilizing four different gene-specific primers and one reference gene *actin* for *An. stephensi* and *Cx. quinquefasciatus* mosquitoes. For quantification, 10 µL reactions were employed containing 5 µL of SYBR green Master mix (Bio-Rad Laboratories, USA), 1 µL of F & R primer mix (500 nM each primer), and 4 µL of cDNA (1:50 dilution). The qRT-PCR cycling conditions were activation at 95 °C for 30 sec, 35 cycles of denaturation at 95 °C for 5 sec, annealing and extension at 59 °C for 30 sec. Finally, melting curve analysis was included by increasing the temperature from 65 to 95 °C with 0.5 °C increments followed by signal capture. Gene expression profiles were evaluated using the Cycle threshold (Ct) values of the *actin* gene for all *An. stephensi* and *Cx. quinquefasciatus* mosquitoes using the formula $2^{-\Delta\Delta}$Ct. Three biological replicates and each consisting of three technical replicates were used for each sample.

Statistical analysis

The observed mortality data were corrected for each concentration of larvicidal using Abbott's formula (Sakuma 1998) to estimate lethal concentrations (LC_{50} and LC_{90}). Three replicates were done for each treatment. Data from three replicates in each treatment (n = 3×3 = 9) were expressed as mean ± standard deviation. For RNA isolation and cDNA synthesis, three biological replicates and each consisting of 3 technical replicates were used for each sample. One-way ANOVA was used to determine significant differences among mean values. The data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and the values were considered as significant at $P < 0.05$.

Results

Characterization of isolated compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone

Crystallization of the active fraction 4 from hexane gave the active principle (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone as colourless crystals.

Isolation Of Active Constituent (4r, 5s) 4-hydroxy-7-angeloyloxycarvatoneacetone

The hexane extract of the leaves of *B. axillaris* 40g was chromatograph over silica gel (acme's 100–200 mesh) packed in hexane. The column was eluted with hexane-ethylacetate and finally with ethyl acetate. Similar fractions were obtained, the fraction 3 eluted with

hexane: ethylacetate 3:1 was found to be an active. The active fractions was dried and washed with acetone-methanol mixture. The residue was subjected to preparative HPLC cosmosil, 5C18-MS- II 250×2.0mm, 50µm, JASCO Gulliver system, gradient elution with acetonitrile-water 40–60%, 15ml/min, 215mm). The compound obtained was dried when a colourless gummy residue was uptained. α-D -193^{o} (CHCl2) and It analysed for $C_{15}H_{22}O_4$ by HRESI-MS (m/z).

IR

IR: ν_{max}KBr cm^{-1} 3438 (hydroxyl), 2958, 2873, 1707, (α, β-unsat. ester carbonyl), 1676 (α, β –unsat. Keton carbonyl), 1650 (olefinic), 1458, 1417, 1384 (methyl bending), 1230 (ester C–O str.), 1147, 1083, 948, 928, 849, 761 (olefinic) cm^{-1} showed in supplementary Fig. S1. The active compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone) was obtained as colourless gummy residues.

HR-ESI-MS gave the molecular formula $C_{15}H_{22}O_4$ (Fig. S5). The IR spectrum showed hydroxyl (3438 cm^{-1}), α, β unsaturated ester carbonyl (1707, 1230 cm^{-1}), α, β unsaturated keton carbonyl 1676 cm^{-1}, and olefinic fiction (948, 928, 849, 761 cm^{-1}).

The NMR spectrum showed (Fig S2) the presence of 4 methyl groups, 2 secondary methyl groups H-9 and H-10 of the isopropyl group at C-3 appeared as 3 proton doublets (J = 6.6 Hz) at δ 1.04 and 0.96. The two trans methyl group of an angelate moiety H-4' and H-5' appeared at δ 4.55 (J = 6.1 and 3.3 Hz) while the adjacent proton H-3 appeared as multiplet δ 1.82. The methylene protons H-2 adjacent to the ketone appeared at δ 2.52 as double doublets (J = 16.5 and 12.1 Hz, H-2 β) and δ 2.55 as a double doublets (J = 16.5 and 4.8 Hz, H-2α). The methylene proton H-7, each appeared as doublets J = 14.9 Hz) due to germinal coupling at δ 4.83 and 4.87. H-3' appeared as dq at δ6.11, J = 5.3 and 1.7 Hz). The broad singlet's 2.23 are assigned to hydroxyl group; the C^{13} NMR values also confirmed structure of the compounds (Fig. S3 and S4).

The 2-D NMR spectra (COSY, HSQC, HMBC and ROESY) also confirmed the structure. Thus the compound was identified the (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone). The spectral data are comparable with Bhlman et al. (1985) who have isolated the compound from the aerial parts of *Blumea mollis* (B. *axillaris*) previously. The compound has also been reported from the essential oil of *Laggera alata* (D.Don) Sch.Bip. ex Oliv. by GC-MS analysis (Onyayade et al 1990).It can be noted that the corresponding tiglate has also been reported from *Blumea mollis* by Bohlman et al. 1979. We have reported from anti-plant fungal activity and mosquitocidal activity of both the tiglate and acetate and its acetate (Subramaniyan et al. 2021)

Larvicidal activities of the crude hexane extract of B. axillaris

Fraction-4 crude hexane extract of *B. mollis* was tested for larvicidal activity against mosquito larvae *An. stephensi* and *Cx. quinquefasciatus.* The hexane extract of *B. mollis* showed good larvicidal activity against *An. stephensi* and *Cx. quinquefasciatus.* The LC_{50} and LC_{90} values of the extract were recorded as 155.811 and 566.763 ppm respectively against *An. stephensi* and 77.215, and 99.653 ppm respectively against *Cx. quinquefasciatus* at 24 hours (Table 3) and azadirachtin used for comparison (Table 4). In negative control (water) no mortality were found and they exhibited active and normal movement.

Table 3
Larvicidal activity (in ppm) of hexane crude extract from *B. axillaris* against *An. stephensi* and *Culex quinquefasciatus*

Mosquito	Treatment	LC_{50} (ppm)	95% Confidence limit		LC_{90} (ppm)	95% Confidence limit		Intercept ± SE	Slope ± SE	X^2
			LL	UL		LL	UL			
An. stephensi	B. axillaris	155.811	126.404	189.720	566.763	414.869	938.129	0.01 ± 0.7	1.6*	155.811
Cx. quinquefasciatus	**hexane crude extract**	77.215	51.965	99.653	336.656	248.655	566.936	1.2 ± 0.7	2.2*	77.215

Table 4
Larvicidal activities (in ppm) of standard compound Azadirachtin against *An. stephensi* and *Culex quinquefasciatus*

Mosquito	Treatment	LC_{50} (ppm)	95% Confidence limit		LC_{90} (ppm)	95% Confidence limit		Intercept ± SE	Slope ± SE	X^2
			LL	UL		LL	UL			
An. stephensi	**Azadirachtin**	3.64	2.87	3.99	9.91	7.88	14.77	0.6 ± 0.5	1.0 ± 0.1	0.3*
Cx. quinquefasciatus		3.17	2.59	3.65	9.67	7.76	14.10	3.6 ± 0.2	3.0 ± 0.6	0.3*

Larvicidal Activities Of (4r, 5s) 4-hydroxy-7-angeloyloxycarvatoneacetone

The compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone was tested for larvicidal activity against *An. stephensi* and *Cx. quinquefasciatus* larvae. The compound showed a promising larvicidal activity against 3rd instar larvae of *An. stephensi* and *Cx. quinquefasciatus* at 2 ppm concentrations. The LC_{50} and LC_{90} values of the compound were recorded as 1.19 and 3.678 ppm, respectively against *An. stephensi*, and 0.85 and 3.59 ppm respectively, against *Cx. quinquefasciatus* after 24 hrs of exposure and azadirachtin and temephos were used as positive control for comparison (Tables 5, 6). In negative control (water) no mortality were found and they exhibited active and normal movement.

Table 5
Larvicidal activities (in ppm) of phytocompound against the 3rd instar *An. stephensi* larvae compared with azadirachtin and temephos.

Mosquito	Treatment	LC_{50} (ppm)	95% Confidence limit		LC_{90} (ppm)	95% Confidence limit		Intercept ± SE	Slope ± SE	X^2
			LL	UL		LL	UL			
An. stephensi	(4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone	1.19	1.03	1.76	3.678	2.715	6.452	4.6 ± 0.1	2.8 ± 0.4	0.9*
	Azadirachtin	0.34	0.22	0.43	1.04	0.90	1.27	2.6 ± 0.3	6.2 ± 0.1	3.7*
	Temephos	0.65	0.56	0.73	1.62	1.42	1.93	3.2 ± 0.3	5.5 ± 0.1	1.7*

LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, LL lower limit (95% confidence limit), and UL upper limit (95% confidence limit). $*P \leq 0.05$, level of significance of chi-square values.

Table 6
Larvicidal activity (in ppm) of phytocompound against the 3rd instar *Cx. quinquefasciatus* larvae compared with azadirachtin and temephos.

Mosquito	Treatment	LC_{50} (ppm)	95% Confidence limit		LC_{90} (ppm)	95% Confidence limit		Intercept ± SE	Slope ± SE	X^2
			LL	UL		LL	UL			
Cx. quinquefasciatus	(4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone	0.85	0.60	1.02	3.59	2.43	8.447	2.1 ± 0.3	5.1 ± 0.1	4.1*
	Azadirachtin	0.28	0.12	0.37	0.55	0.46	0.66	4.3 ± 1.1	7.4 ± 0.3	0.1*
	Temephos	0.92	0.11	1.66	1.82	1.17	646.1	4.3 ± 0.9	5.1 ± 0.1	2.4*

LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, LL lower limit (95% confidence limit), and UL upper limit (95% confidence limit). $*P \leq 0.05$, level of significance of chi-square values.

Ovicidal Activities Of (4r, 5s) 4-hydroxy-7-angeloyloxycarvatoneacetone

The isolated (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound was tested for ovicidal activity against eggs of *An. stephensi* and *Cx. quinquefasciatus*. The ovicidal activities of results are given in Tables 7 and 8. The results showed 84% and 80% of ovicidal activities at 2 ppm against *An. stephensi* and *Cx. quinquefasciatus*, respectively. The positive control azadirachtin showed 74% and 18% and temephos showed 54% and 10% of ovicidal activity at 2 ppm concentration against *An. stephensi* and *Cx. quinquefasciatus*, respectively. The compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone treated eggs of *An. stephensi* and *Cx. quinquefasciatus* were found to be shrunken after 120 hrs post-treatment, and many eggs did not hatch. In water control (negative), most of the eggs hatched.

Table 7
Percent ovicidal activity of (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound against the eggs of *An. stephensi* (Mean ± STD)

Mosquito	Treatment	Concentrations (ppm)			
		0.5	1	1.5	2
An. stephensi	(4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone	23 ± 2.82^{b}	41 ± 2.82^{b}	53.8 ± 3.34^{b}	84.2 ± 3.34^{b}
	Azadirachtin	32.19 ± 2.21^{b}	44.47 ± 1.95^{b}	66.46 ± 1.26^{b}	74.59 ± 2.38^{b}
	Temephos	4.7 ± 2.07^{d}	8 ± 3.18^{d}	13.79 ± 2.96^{d}	18.13 ± 2.15^{d}

Values are mean ± standard deviation (SD). Means are separated by Tukey's test of multiple comparisons, One-way Analysis of Variance (ANOVA). *$p \leq 0.01$, level of significance.

Table 8
Percent ovicidal activity of (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone **compound against the eggs of** Cx. quinquefasciatus **(Mean ± STD)**

Mosquito	Treatment	Concentrations (ppm)			
		0.5	1	1.5	2
Cx. quinquefasciatus	**(4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone**	21.8 ± 1.78^{b}	33.4 ± 2.19^{b}	45.6 ± 4.38^{b}	81.0 ± 2.82^{b}
	Azadirachtin	16.72 ± 1.12^{b}	31.56 ± 1.45^{b}	39.54 ± 1.12^{b}	54.85 ± 1.17^{b}
	Temephos	4.84 ± 2.64^{d}	5.04 ± 2.16^{d}	8.22 ± 2.02^{d}	10.44 ± 2.10^{d}

Values are mean ± standard deviation (SD). Means are separated by Tukey's test of multiple comparisons, One-way Analysis of Variance (ANOVA). *$p \leq 0.01$, level of significance.

Effect Of (4r, 5s) 4-hydroxy-7-angeloyloxycarvatoneacetone On Non-target Organisms

The effect of (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone was treated against *Poecilia reticulate* (guppy fish). The results showed lethal susceptibility at 2 ppm concentration with LC_{50} = 2.78 and LC_{90} = 4.81 of *P. reticulata.* The results were compared with the azadirachtin and temephos positive controls. The azadirachtin and temephos positive controls showed least susceptible of LC_{50} = 2.34 and LC_{90} = 3.56 and LC_{50} = 1.74 and LC_{90} = 2.36, respectively against *P. reticulata* non-target organism (Table 9).

Table 9

Effect of (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone **(in ppm) on non-target organism**

Non-target organism	Treatment	LC_{50}	LC_{90}
P. reticulata	(4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone	2.78	4.81
	Azadirachtin	2.34	3.56
	Temephos	1.74	2.35

LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larva

Histopathology

The *An. stephensi* and *Cx. quinquefasciatus* larvae exposed to (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone revealed severe damages in the midgut epithelial columnar cells (CC). The peritrophic membrane (pM) looks completely ruptured on compound treated *An. stephensi* and *Cx. quinquefasciatus* larvae while comparing with the control larvae. Further, midget content (MC) of both treated larvae was found to be oozed out compared to control larvae. But, adipose fabric (A.F) and circular muscles (CM) appeared normal (Figs. 1, 2).

Expression patterns of functional genes in An. stephensi **larvae treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone and controls**

In the gene expression analysis, the *An. stephensi* larvae was exposed with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound, negative control (water), and positive controls (temephos and azadirachtin) (Fig. 3). The *Wh* gene was found to be upregulated in the *An. stephensi* larvae treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound when compared to the positive control azadirachtin and temephos. The *Wh* gene of *An. stephensi* larvae exposed to positive controls (temephos and azadirachtin) showed similar downregulation. The *Wh* gene was found to be upregulated in the *An. stephensi* larvae treated with negative control (water) (Fig. 3). Interestingly, *GST* gene was found to be upregulated in the *An. stephensi* larvae treated with negative control water than the other *An. stephensi* larvae exposed with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound and positive controls (temephos and azadirachtin). The expression level of the *GST* gene in the *An. stephensi* larvae treated with temephos showed two-fold higher expression than that in *An. stephensi* larvae treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound (Fig. 3). The *GST* gene was found to be downregulated in the *An. stephensi* larvae treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone (Fig. 3). The *CYP50* gene was found to be downregulated in the *An. stephensi* larvae treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound while comparing with the *An. stephensi* larvae treated with positive control temephos. The *CYP50* gene was found to be downregulated in the *An. stephensi* larvae treated with positive control azadirachtin. The *CYP50* gene was found to be upregulated in the *An. stephensi* larvae treated with negative control water (Fig. 11). Interestingly, the *EST* gene was not expressed in the *An. stephensi* larvae treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound. The *EST* gene of *An. stephensi* larvae treated with positive control temephos and negative control water showed upregulation. The *EST* gene was found to be downregulated in the *An. stephensi* larvae treated with positive control azadirachtin (Fig. 3).

Expression patterns of functional genes in Cx. quinquefasciatus **larvae treated with compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone and controls**

In the gene expression analysis, *Cx. quinquefasciatus* larvae was exposed with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound, negative control (water), and positive controls (temephos and azadirachtin) (Fig. 4). The *Wh* gene was found to be two-fold upregulated in the *Cx. quinquefasciatus* larvae treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound than the positive control azadirachtin, temephos, and water. The *Wh* gene of *Cx. quinquefasciatus* larvae exposed to positive controls (temephos and azadirachtin) showed similar downregulation. The *Wh* gene was found to be upregulated in the *Cx. quinquefasciatus* larvae treated with negative control water than the *Wh* gene of *An. stephensi* larvae exposed to positive controls (temephos and azadirachtin) (Fig. 4). Interestingly, the *GST*, *CYP50*, and *EST* genes did not express in the *Cx. quinquefasciatus* larvae exposed to (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound. The *GST* gene in the *Cx. quinquefasciatus* larvae treated with positive control temephos and negative control water showed similar upregulation. The *GST* gene in the *Cx. quinquefasciatus* larvae treated with positive control azadirachtin was found to be downregulated (Fig. 4). The *CYP50* gene in the *Cx. quinquefasciatus* larvae treated with positive control temephos and negative control water showed similar upregulation. The *CYP50* gene in the *Cx. quinquefasciatus* larvae treated with positive control azadirachtin was found to be downregulated (Fig. 4). The *EST* gene in the *Cx. quinquefasciatus* larvae treated with

positive control temephos and negative control water showed similar upregulation. The *EST* gene in the *Cx. quinquefasciatus* larvae treated with positive control azadirachtin was found to be downregulated (Fig. 4).

Discussion

India has a great wealth of many naturally occurring plant bioactive compounds, which have great potential for insecticidal properties. Numerous plant compounds have been identified as natural insecticides against vector-borne mosquitoes. The plant extracts and plant phytocompounds are easy to use, free of risk, cost-effective products in controlling vector-borne mosquitoes and vector-borne diseases.

In the present study, the fraction 4, which was isolated from the hexane extract of *B. axillaris* was found to be the most effective treatment against *An. stephensi* and *Cx. quinquefasciatus*. The LC_{50} and LC_{90} values of the fraction-4 hexane extract were recorded as 155.811 and 566.763 ppm, respectively against 3rd instar larvae of *An. stephensi*, and 77.215 and 99.653 ppm, respectively against 3rd instar larvae of *Cx. quinquefasciatus.*

Our study revealed that the isolated compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone from *B. axillaris* exhibited the lowest LC_{50} value of 1.19 and LC_{90} value of 3.678 against the 3rd instar *An. stephensi* larvae. The isolated compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone exhibited the lowest LC_{50} value of 0.85 and LC_{90} value of 3.59, against the 3rd instar *Cx. quinquefasciatus* larvae. This result was compared with our previous study of isolation of monoterpein ester from *B. axillaris* identified as (4R, 5S)-4-hydroxy-7-tigloyloxy carvotanacetone and converted into acetyl derivative, that is (4R, 5S)-4-acetoxy-7-tigloyloxy carvotanacetone. These compound showed good larvicidal activity against *Cx. quinquefasciatus* larvae. The observed LC_{50} and LC_{90} values of compounds 1 and 2 on *Cx. quinquefasciatus* larvae were 1.73, 1.27 and 4.59, 3.33 ppm mortality in 24 h, respectively (Subramaniyan et al. 2021). This result was also compared with the previous report by Xiao et al. (2012). They reported that the lignan compound isolated from the *Phryma leptostachya* plant exhibited LC_{50} values of 1.21 and LC_{90} of 4.2 against the *Cx. pipiens* (Xiao et al., 2012).

Compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone from *B. axillaris* showed better ovicidal activity of 84%, 80% against an eggs of *An. stephensi* and *Cx. quinquefasciatus*, respectively than the positive control temephos and azadirachtin at 2 ppm concentration. Similarly, the compound (4R, 5S)-4-hydroxy-7-tigloyloxy carvotanaceto and synthesized compound (4R, 5S)-4-acetoxy-7-tigloyloxy carvotanacetone from our previous study exhibited 68% and 77% ovicidal activity against eggs of *Cx. quinquefasciatus* at 120 h post-treatment. This result was also compared with the previous result published by Suman et al., 2013. The isolated compound pyriproxyfen and diflubenzuron showed only 47.3% and 25.5% ovicidal activity against *Ae. aegypti* mosquitoes (Suman et al. 2013). Overall, the activity of (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone showed better activity at 2 ppm concentration against *An. stephensi* and *Cx. quinquefasciatus* when compared to temephos and azadirachtin (Tables 7 and 8). These overall studies,

Previous results have reported that the compounds derived from the plant source produce damages in the midgut cells of mosquito larvae. In the present study, we use the (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone treated *An. stephensi* and *Cx. quinquefasciatus* larvae for histopathology. The results revealed serious damages in the midgut cells of the treated larvae (Figs. 1 and 2). Previously, several isolated compounds from plants like nilocotin, triterpenoid, and limonoids revealed severe damages in the midgut cells of targeted vector-borne mosquitoes (Schmutterer and Ascher 1987; Reegan et al. 2014). Further, the current results showed (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone is safe for non-target organism *P. reticulata.* The compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone is non-toxic, up to the tested concentration of 2 ppm. The *P. reticulata* showed normal movement (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound, azadirachtin, and temephos. From the result, we suggest that the isolated compound can be used without harming *P. reticulata* predators in Integrated Mosquito Management (IMM).

In this study, we used *isolated* compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone, positive controls (temephos and azadirachtin), and negative control (water) against the 3rd instar larvae of *An. stephensi* and *Cx. quinquefasciatus* and analyzed the influence of novel compound on the expression of functional genes in both *An. stephensi* and *Cx. quinquefasciatus* 3rd instar larvae, which was not reported before. The *Wh* gene that we targeted plays a classical role in eye pigmentation (Anaka et al. 2008; Xiao and Robertson 2017). No early reports have been reported for this white gene. The *Wh* gene encodes the ATP-binding cassette (ABC) transporter. Our results revealed upregulation of the genes for both *An. stephensi* and *Cx. quinquefasciatus*, which would block ATP-binding transporters and make mosquitoes to lose its pigment cells in the eyes. *CYP50* gene is a most important gene that enhances resistance in mosquitoes to digest the pesticides. Xu et al. (2005) reported that the *CYP50* gene is an important permethrin resistance property in *An. gambiae* and *Cx. quinquefasciatus* mosquitoes (Xu et al. 2005). In the resistance-developed mosquitoes against permethrin, *CYP50* gene was found to be downregulation. Downregulation of *CYP450* genes linked to the homeostatic response and the upregulation of *CYP450* involved in the synthesis of protein for energy and oxygen (Xu et al. 2005). Our result showed downregulation in

the expression level of *CYP50* gene in *An. stephensi* mosquitoes when exposed to the novel compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone. Whereas, *CYP50* gene in *Cx. quinquefasciatus* did not show any expression. Therefore, we conclude that our isolated compound has the capability to make changes in homeostatic responses of the mosquitoes. Ransen et al. (2001) reported the expression of *GST* gene in *An. gambiae* mosquitoes exposed to insecticide resistance (Ranson et al., 2001). They reported that totally three *GST* genes (*aggst-8*, *aggst-9*, and *aggst-10*) were responsible for the insecticide resistance when mosquito exposed to the DDT insecticides (Ranson et al., 2001). In 2003, Ortelli et al. found that the *GST* gene is responsible for the resistance in malarial vector when *An. stephensi* exposed to the 1,1,1-tricholoro-2, and 2-bis-(p-chlorophenyl) ethane (Ortelli et al. 2003). Therefore, our expression analysis of *GST* gene in the *An. stephensi* and *Cx. quinquefasciatus* larvae treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound clearly showed that it would create a heritable change in the *An. stephensi* and *Cx. quinquefasciatus*. Paton et al. (2000) found that the expression of the *EST* gene present in the mosquitoes develop their own resistance when mosquitoes exposed to the organophosphorus-mediated insecticide (Paton et al., 2000). However, there is no expression pattern found when *An. stephensi* and *Cx. quinquefasciatus* larvae exposed to the (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound.

Overall, these studies, proved for the first time that the 4-hydroxycarvotanacetone (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone isolated from *B. axillaris* plant having good mosquitocidal properties.

Conclusion

Over the past decades, insecticides have been extensively utilized against vector-borne mosquitoes. In this chapter, the compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone derived from *B. axillaris* showed good larvicidal, ovicidal activities against *An. stephensi* and *Cx. quinquefasciatus.* The compound (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone did not affect the non-target organisms. Overall, our results clearly revealed that (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone from *B. mollis* plant have the most effective treatment against the *An. stephensi* and *Cx. quinquefasciatus*.

Declarations

Author contribution

S.A.C., S.S., V.E.H., K.B., planned and designed the article; V.E.H., and S.S., wrote the article; S.A.C., S.S., and K.B. edited and revised the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

The authors are thankful to Entomology Research Institute, Loyola College, Chennai, Tamil Nadu, India for financial assistance and for providing laboratory facilities. The authors are partially supported for the instrument facility; Laboratory of Pharmacognosy and Natural Products Chemistry, Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Japan and Department of Chemistry, Bioscience Research Foundation, Kandamangalam, Kanchipuram District, Tamil Nadu, India

Data accessibility

This article did not use any field or experimental data that can be archived.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Figures

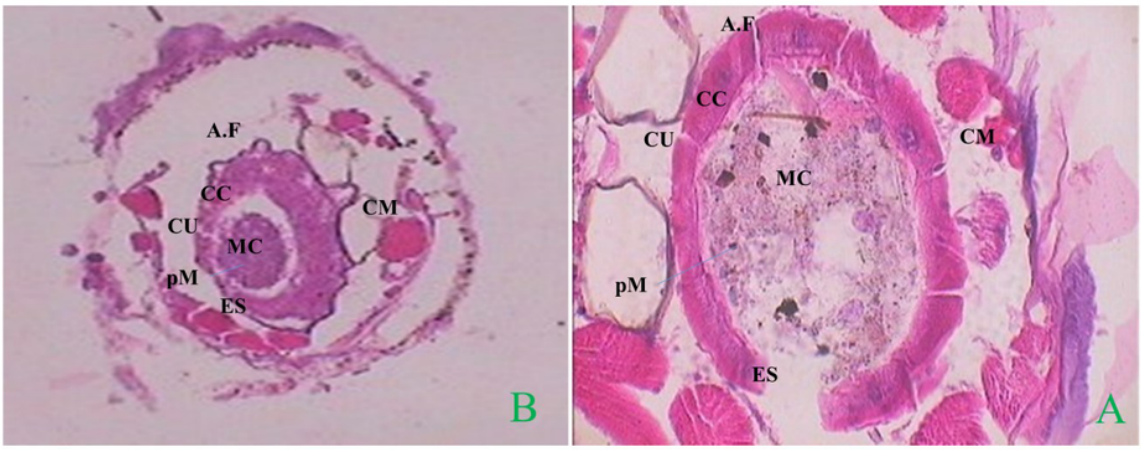

Figure 1

Cross section of the midgut 3rd instar larvae of *An. stephensi* treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone **(B) compared with control (7A)**

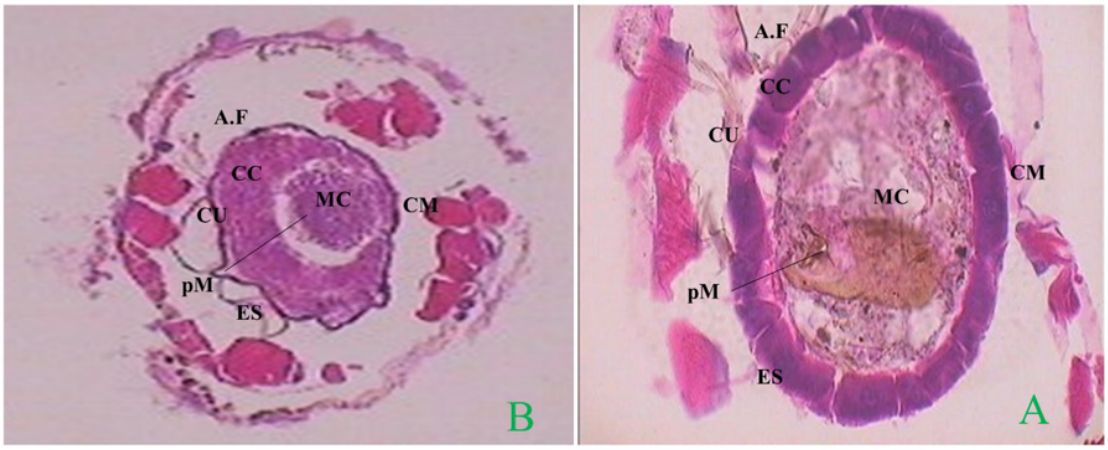

Figure 2

Cross section part of the midgut 3rd instar larvae of *Cx. quinquefasciatus* treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone (B) compared with control (A)

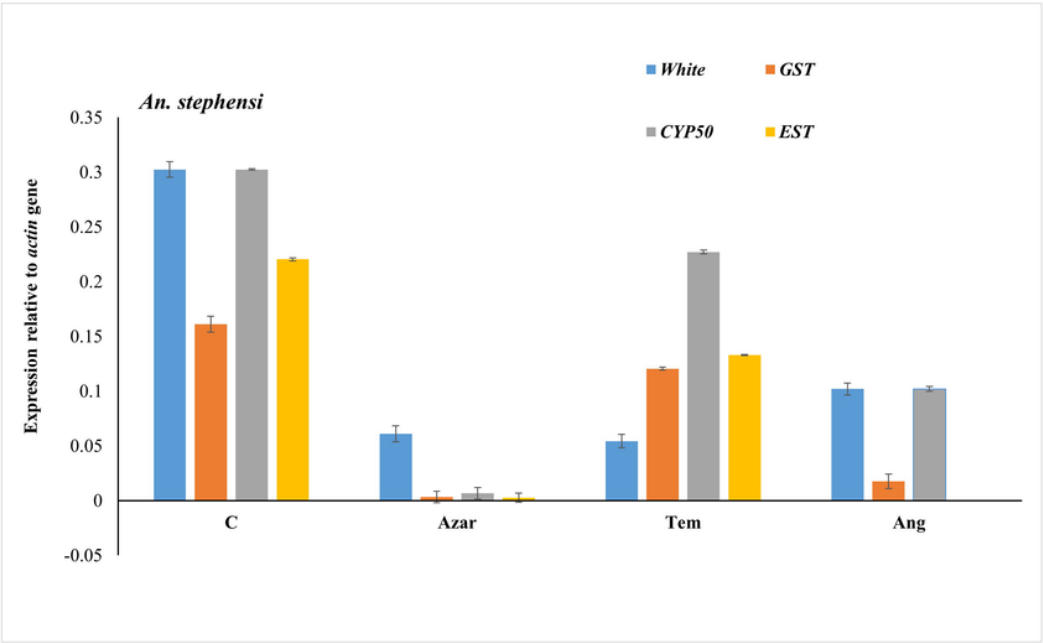

Figure 3

Quantitative real time RT-PCR (qRT-PCR) analysis of eight functional genes of *An. stephensi* larvae mosquitoes treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone **compound, positive controls (temephos and azadiractine), and negative control (water**). The actin gene was chosen as a reference gene for the normalization of expression levels of different functional genes (*Wh*, *GST*, *CYP450*, *and EST*) in 3rd instar *An. stephensi* larvae mosquitoes treated under with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound, positive controls (temephos and azadirachtin), and negative control (water). The Cycle threshold (Ct) values of the treated larvae of mosquitoes were obtained by qRT-PCR and analysed using using the formula $2^{-\Delta\Delta}$Ct. Three biological replicates and each having three technical replicates of (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound infected larvae were used for qRT-PCR analysis.

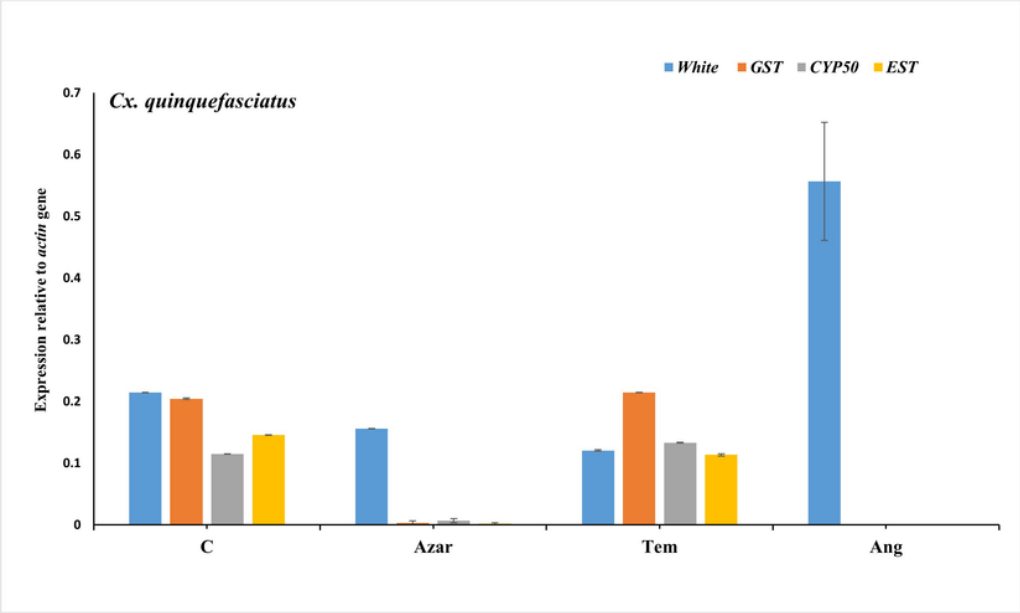

Figure 4

Quantitative real time RT-PCR (qRT-PCR) analysis of eight functional genes of *Cx. quinquefasciatus* larvae mosquitoes treated with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone **compound, positive controls (temephos and azadiractine), and negative control (water**). The actin gene was chosen as a reference gene for the normalization of expression levels of different functional genes (*Wh*, *GST*, *CYP450*, *and EST*) in 3rd instar *Cx. quinquefasciatus* larvae mosquitoes treated under with (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound, positive controls (temephos and azadirachtin), and negative control (water). The Cycle threshold (Ct) values of the treated larvae of mosquitoes were obtained by qRT-PCR and analysed using using the formula $2^{-\Delta\Delta}$Ct. Three biological replicates and each having three technical replicates of (4R, 5S) 4-hydroxy-7-angeloyloxycarvatoneacetone compound infected larvae were used for qRT-PCR analysis.

Supplementary Files

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- GraphicalabstractJPEG600DPI.jpg
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