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RESEARCH ARTICLE

Bio-efficacy of insecticidal molecule emodin against dengue, filariasis, and malaria vectors

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Abstract

Emodin, a compound isolated from *Aspergillus terreus*, was studied using chromatographic and spectroscopic methods and compound purity (96%) was assessed by TLC. Furthermore, high larvicidal activity against *Aedes aegypti*-AeA (LC_{50} 6.156 and LC_{90} 12.450 mg/L), *Culex quinquefasciatus*-CuQ (8.216 and 14.816 mg/L), and *Anopheles stephensi*-AnS larvae (6.895 and 15.24 mg/L) was recorded. The first isolated fraction (emodin) showed higher pupicidal activity against AeA (15.449 and 20.752 mg/L). Most emodin-treated larvae (ETL) showed variations in acetylcholine esterase, α and β-carboxylesterases, and phosphatase activities in the 4th instar, indicating the intrinsic differences in their biochemical changes. ETL had numerous altered tissues, including muscle, gastric caeca, hindgut, midgut, nerve ganglia, and midgut epithelium. Acute toxicity of emodin on brine shrimp *Artemia* nauplii (54.0 and 84.5 mg/L) and the zebrafish *Danio rerio* (less toxicity observed) was recorded. In docking studies, Emodin interacted well with odorant-binding-proteins of AeA, AnS, and CuQ with docking scores of − 8.89, − 6.53, and − 8.09 kcal mol^{-1}, respectively. Therefore, *A. terreus* is likely to be effective against mosquito larvicides.

Keywords *Aspergillus terreus* · Emodin · Mosquitocidal · Odorant binding proteins · *Artemia* nauplii · *Danio rerio*

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Introduction

Malaria, dengue, chikungunya, and Zika are the most common diseases spread by mosquitoes, which kill millions of people worldwide (Ward and Benelli 2017). They are the most frequent mosquito-borne illnesses in India and including encephalitis and filariasis, (Bhatia et al. 2014). Microbiological agents (with effective insecticidal activity) play a significant role in controlling disease spread by eliminating the devastating vectors while being environmentally friendly and specialized for target species (Tabanca et al. 2013). Many fungi produced substances, such as bioactive metabolites, industrial enzymes, and pigments have multiple biopotentials with low toxic and biodegradable properties (Kulkarni and Gupta 2013).

Environmental toxicological assays (using brine shrimp) are useful for assessing pesticides and toxicity of other chemicals before they employed in large-scale studies (Minguez et al. 2016. Long-term usage of synthetic chemicals has the potential to affect non-target organisms and the environment (Songa and Okonkwo 2016). It is very difficult to find novel and more selective compounds to combat the toxicity of target species. There have been plenty of studies dealt with bio-metabolite toxicity in the past against various model organisms such as *Oncorhynchus mykiss, Brachydanio rerio, Dicentrarchus labrax, Gambusia holbrooki* (Georgalas et al. 2007), and some invertebrates (*Artemia salina, Daphnia magna, Balanus amphitrite, Hippolyte inermis*) (Venkateswara Rao et al. 2007). Zebrafish can be used to test veterinary drugs, biocides, insecticides, feed additives, and any other new material for animal toxicity (*Danio rerio*) (Scholz et al. 2014). Acute fish embryo toxicity tests (EFT) are commonly performed, according to the OECD testing guideline (TG) 203 (OECD 1992). Because zebrafish embryos are transparent, scientists may study their development from the single-cell stage to the larval stage using stereomicroscopes (Braunbeck et al. 2015).

Insects communicate with their environment using biological signals such as pheromones, plant volatiles, and animal odors (Benton et al. 2009; Benton et al. 2009). Among insect species, odor is utilized to detect food (Foster and Hancock 1994), find a host (Takken 1991), complete the mating process (Cabrera and Jaffe 2007), the oviposition process (Bentley and Day 1989), and recognize predators (De Bruyne and Baker 2008). The odorant-binding proteins (OBPs) have a crucial role in pest and insect management, as well as olfactory signal transmission. They are released by accessory cells surrounding olfactory neurons and are found mostly in the sensillar lymph (Tegoni et al. 2004). Furthermore, the maxillary palp and proboscis are better adapted for sensing taste, carbon dioxide, octanol, and crucial chemical markers that distinguish the human host (Lu et al. 2007). Arthropod OBPs are water-soluble proteins with molecular weights ranging from 10 to 30 kDa that are distinguished by a highly conserved 6 α-helical domain that is specific to this protein family (Calvo et al. 2006). Previously, OBPs have been discovered in *An. gambiae* (Vieira and Rozas 2011).

Molecular docking is a powerful approach for determining how a ligand interacts with a protein with a known 3D structure. Understanding structural type communication and calculating inhibitor efficacy requires knowledge of binding modalities (Vijayakumari et al. 2016). In order to develop unique and strong mosquito repellents derived from fungal-based bioactive compounds, logistics predictions and hypotheses are generated using computer simulation methodologies. Emodin, also known as 1, 3, 8-trihydroxy-6-methyl anthraquinone, which possesses anti-cancer, anti-inflammatory, anti-oxidant, anti-ulcer, anti-fungal, anti-viral, and anti-parasitic agent found in a variety of fungi, plants, and lichens (Lin et al. 2009). The aims of this study were to isolate the bioactive component emodin and investigate how it kills larvae, pupae, and adults of *Ae. aegypti*, *An. stephensii*, and *Cx. quinquefasciatus*. After that, in silico docking studies were conducted. The present study is aimed to isolate and evaluate the insecticidal molecule from *Aspergillus terreus*. The more potent fraction was evaluated against target insect pest as well as non-targeted species. The insecticidal molecule Emodin were identified through UV–Vis spectroscopy, FTIR, HPLC, nuclear magnetic resonance (NMR) of protons (^{1}H NMR), carbon nuclear magnetic resonance (^{13}C NMR), and liquid chromatography-electrospray ionization mass spectrometer (LC–ESI–MS) analyses.

Materials and methods

The isolation, identification, and mosquitocidal capabilities of *Aspergillus terreus* mycelial ethyl acetate crude extract (ATMEAE) have been described (Ragavendran and Natarajan 2015; Ragavendran et al. 2018). The chemicals involved in enzyme assays, different analysis, and solvents were of analytical grade (Merck, Germany). The dechlorinated water was used throughout the bioassay. To clean glasswares and Petri plates, diluted nitric acid (HNO_3) was rinsed with distilled water and dried in a hot air oven.

Thin-layer chromatography

ATMEAE was separated from the bioactive compounds using thin-layer chromatography (TLC). Several solvents (methanol-hexane, ethyl acetate-acetone, methanol-ethyl acetate, and chloroform-acetone) were utilized in the separation of ME using a TLC plate. ATMEAE solutions were dissolved in ethyl

acetate and coated onto Silica Gel60 F254 TLC plates (Merck) with a size of 20 × 20 cm and a layer thickness of 0.20 mm and eluted with more volume of methanol and chloroform (95:5, 96:4, 97:3, 98:2, 99:1, and 100%), by drying each elution naturally. The front portion of the plate was marked immediately after it was removed from the TLC chamber. The plate was then allowed to dry naturally. UV light was used to spot the various fractions of ME on the TLC plate (254 nm). Rf was calculated as follows:

$$R_f = \textit{Distance moved by the solute} / \textit{Distance movedby the solvent}$$

R_f value was calculated for each fraction. Fractions with the same R_f values were mixed. Totally 3 fractions (F1, F2, and F3) were obtained as final from ATMEAE. In addition to its yield (milligrams), further each fraction was tested for initial screening of their larvicidal potential (Pandey et al. 2011).

Preparative TLC

The fraction with the highest larvicidal activity was considered further and its contents were separated on a 20-cm^2 silica gel plate using a chloroform–methanol mobile phase (97:3%). The zone eluted from the silica plate was scraped and collected separately. Each test tube received 100 mL of ethyl acetate. After dissolving the compounds, they were filtered twice using Whatman filter paper No. 1 to eliminate any suspended silica powder. Following evaporation of the extract, the purified samples were concentrated and kept at 4 °C for subsequent analysis. The pure component was obtained (84 mg) using an ethyl acetate.

Structural elucidation of the compound

UV and FTIR characterization of F1 fraction

In the UV–visible spectrophotometer (Shimadzu UV1800), the UV spectrum of the compound was measured at the λ between 300 and 700 nm using DMSO as a blank. An FTIR spectrometer (FT-IR; Brucker 4100) was used to determine the absorption spectrum of active fractions. The F1 fraction (1: 3 drop) was applied to potassium bromide (KBr) pellets that were dried at 50 °C. The FT-IR system was calibrated for background signal scanning with pure KBr (Deepika et al. 2012). Intensity versus wave number was used to create a spectrum and the compound analysis was conducted at St. Joseph College in Trichy, Tamil Nadu, India.

HPLC analysis

A modified earlier method (Anjum et al. 2012) was followed to evaluate *A. terreus* MEAE fraction-1 through a modified HPLC method. The sample was eluted with methanol (HPLC grade, Sigma Aldrich, USA) and prefiltered using a 0.22-µm membrane filter after injection (20 µL). A UV detector was attached to an instrument equipped with a Shimadzu LC solution No. 20 AD, Japan, to measure peak purity. For isocratic resolution, an LCGC C18 column with methanol: water (50:50) mobile phase at 1.0 mL/min and a head pressure of 25 kgf/cm^2 was used. Room temperature (30 °C) was maintained throughout the setup. Each HPLC analysis took 45 min to complete. A plate drier was used fter developing a plate to dry it completely, and analyzed with a UV detector (254 nm wavelength) (Zhang et al. 2013).

Nuclear magnetic resonance of proton (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR)

For the sample solution, the dried chemical was dissolved in deuterated DMSO. The solution was injected at a depth of 4.5–5 cm into the nuclear magnetic resonance (NMR) tubes. The spectrum was taken using the scale, and tetramethylsilane (TMS) was used as the internal standard on a 500-MHz Bruker Advance instrument (Khan et al. 2018). The number of carbon atoms in the sample was determined using ^{13}C NMR. Spectrum was produced using a sample prepared in DMSO at 500 MHz on scale (Khan et al. 2018). The samples were analyzed using an NMR spectrometer set at 100.52 MHz for ^{13}C and 400 MHz for ^{1}H, with DMSO as a solvent. This analysis was carried out at the Gandhigram Rural Institute (Deemed University) in Dindigul, Tamil Nadu, India, in the Department of Chemistry.

Liquid chromatography-electrospray ionization mass spectrometer analysis

In this experiment, an ESI source and an ion trap mass analyzer were used on a Bruker Dionex Ultimate (Thermo 3000) mass spectrometer. In brief, 20 µL of fraction-1 was introduced into the ESI. As shown, the gradient programme was used, and the solvent was eluted at a rate of 1 ml/min while the mass spectra were scanned between 10 and 40 m/z. Fraction-1 was subjected to the following conditions: capillary temperature of 300 °C, source voltage of 5.0 kV, source current of 100 mA, and capillary voltage of 22 V. All analyses were conducted in positive mode for 20 min with MS scans. The HPLC system with auto-sampler (HD Brucker) was used in conjunction with the mass spectrometer. A Zorbax Eclipse reversed-phase analytical column (LC18, particle size 5.0 µm, 150 mm × 4.6 mm) was used (da Silva et al. 2016). The analysis was performed at Indian Institute of Science (IISc) Division of Biological Sciences, Bangalore, India.

Larvicidal bioassay

All fractions (F1, F2, and F3) were dissolved in 10% dimethyl sulfoxide (1 mL DMSO) at prepared concentrations of 1 mg/mL to achieve the required doses (test stock solution 5 mL). The standard WHO procedure (1996) was used to assess the mosquito larvicidal efficacy of fractions, with minor modifications (Seetharaman et al. 2017). Twenty larvae of target mosquitoes were placed in a 150-mL glass beaker containing 100 mL of dechlorinated water and one ml of the respective sample concentrations (control, 50, 100, 150, and 200 mg/L). After 12 h of treatment, the proportion of larvae that died was calculated in triplicate for each concentration examined. A 10% DMSO solution added to the distilled water was employed as a negative control. Using Abbott's protocol, the mortality rate was calculated (Abbott 1925).

Pupal toxicity test

A. terreus isolated fractions were tested for pupicidal activity against target mosquitoes. The experiments were conducted in 150-mL beakers containing 99 mL of dechlorinated water and to each beaker, 20 freshly emerged pupae were kept with 1 mL of of the desired concentrations (control, 50, 100, 150, and 200 mg/L) respectively in all the fractions. To set up the control, 1 mL of 10% DMSO was added to 99 mL of dechlorinated water. To calculate the pupae mortality after 24 h, the Abbott formula was used (Abbott 1925).

Ovicidal activity

The ovicidal bioassay of the sample materials was carried out using a modified technique (Su and Mulla 1998). Samples of various concentrations were produced from the stock solution (50, 100, 150, and 200 mg/L). Before the treatment, each egg of the target insect was checked under a microscope. A total of 75 freshly developed mosquitoes eggs were treated with different concentration of fraction, respectively until they hatch out. Negative control was DMSO, while positive control was a commercial pesticide (Azadirachtin). The eggs were treated and counted under a microscope before being placed in distilled water for hatchability testing. Each test was carried out three times. Using the following calculation, the hatch rate after 48 h was calculated (Chenniappan and Kadarkarai 2008).

$$\text{Ovicidal activity} = \frac{\text{Number of unhatched eggs}}{\text{Total number of eggs introduced}} \times 100$$

Whole larval body homogenate preparation

The 4th instar larvae of treated and control groups were washed with sterile double H_2O and adhered water was removed from their surfaces using tissue paper. Individual larvae were homogenized, using a homogenizer, in Eppendorf tubes with ice-cold sodium phosphate buffer (pH 7.0, 20 mM) for determining enzyme activity. Upon centrifugation (8000 rpm at 4 °C) for 15 min, the homogenates were used to analyze enzymes in the subsequent steps.

Acetylcholinesterase assay

Three mosquito species (*An. stephensi, Cx. quinquefasciatus,* and *Ae. aegypti*) were tested to determine whether fraction-1 inhibited the enzyme acetylcholinesterase. According to Ellman et al. (1961), a modified acetylcholinesterase (AChE) assay was performed. The treated 4th instar larvae were used to examine how well the compounds blocked acetylcholinesterase.

Carboxylesterase assay

The activity of α- and β-carboxylesterases was evaluated on the three different mosquito larvae pre-treated with the fraction (Van Asperen 1962). A volume of 100 µL of undiluted and diluted (1:3) homogenates was incubated for 30 min at 30 °C in 1 mL sodium phosphate buffer (pH 7.0) containing 250 µM of α- and β-naphthyl acetate, respectively. The color was formed in an aliquot reaction with 400 µL of freshly prepared 0.3% Fast Blue B in 3.3% SDS for 20 min at 28 °C. Optical density was determined at 430 nm (α-carboxylesterase) and 588 nm (β-carboxylesterase) using the blank solution.

Acid and alkaline phosphatase assays

Acid and alkaline phosphatases were measured in the larvae of tested mosquitoes using the modified procedure (Asakura 1978). To evaluate acid phosphatase activity, 50 µL of larval homogenate was added to 450 µL of 50 mM sodium acetate buffer at 4.6 pH. To test alkaline phosphatase activity, 20 µL of larval homogenate was mixed evenly with 50 mM Tris–HCl buffer (pH 8.0) containing 12.5 mM p-nitrophenyl phosphate. The enzymatic reaction was arrested by adding 100 µL of 0.5N NaOH solution in an incubation vessel for 15 min at 37 °C in a hot water bath, followed by centrifugation (5000 rpm for 6 min). Using a Shimadzu UV-160A spectrophotometer, optical density (OD) was measured at 440 nm.

Histopathological study

The response of emodin was observed histopathologically by studying mosquito samples to observe the changes in morphological features. In addition, control larvae were initially fixed with 10% formalin and the 4th instar larvae

were treated with the pure compound. After dehydration in ethyl alcohol, the tissues were cleared in xylene, fixed in para-plast and sectioned (5 μm). By using the standard staining procedure, the sections were stained with hematoxylin and eosin (HE staining) (Kaewnang-O et al. 2011). In the end, midgut area of the control and treated larvae were viewed under a light microscope (at 40X magnification) and photographs were taken (Seetharaman et al. 2017).

Bio-toxicity assay of fraction 1 against *Artemia* nauplii

The brine shrimp biotoxicity test of emodin was performed by following OECD guidelines 236 (Busquet et al. 2014). A beaker containing 32 g of sea salt per liter (ppt) was used to hatch *Artemia* nauplii cysts. The beaker was covered with black polythene and placed under constant oxygen and light for 48 h. For this experiment, well-developed *Artemia* nauplii were collected and transferred into a glass container following an appropriate incubation period. A number of doses of emodin (dissolved in 10% DMSO) were tested (2, 4, 6, 8, and 10 mg/L). As a negative control, the equivalent volume of DMSO was added to the respective glass container. The LC_{50} value and percentage (%) of dead larvae were calculated with SPSS 20.0 software after 24 h of treatment. The formulae below mentioned was used to calculate the mortality (%) (Meyer et al. 1982).

$$\% \text{ death} = [(\text{test} - \text{control})/100)] \times 100$$

Danio *rerio* embryo test

According to the OECD (2013) guidelines, the embryonic acute toxicity test was conducted with some minor modifications based on the fish embryo toxicity test (FET). Various concentrations of emodin (0, 1.95, 15.6, and 62.0 mg/L) were prepared using *D. rerio* water. For exposure of zebrafish embryos, 2.0 ml of test solutions and two fertilized eggs were transferred to individual well of a 24-well microtiter plate. As a control, each plate contained four wells filled with 10% DMSO and the remaining wells with 3 concentrations were equally distributed. For each replication, ten embryos per concentration were used. Every 24 h, the emodin concentration and water quality were restored to maintain an appropriate level. At a temperature of 20 ± 1.0 °C and a 14:10 light/dark photoperiod, the embryos were monitored at intervals of 0, 24, 48, 72, 96, and 120 h. The body length, hatching rate, and mortality rate were studied using an inverted microscope (Nikon TF2000-U) (Li et al. 2016).

Homology modelling

The mosquito protein (odorant-binding protein) homology model from *An. stephensi* was developed using Modeler 9.20 (Eswar et al. 2006). The FASTA sequence for *An. stephensi* was retrieved from UniProtKB (Accession: B5A5T7). The corresponding PDB IDs, as determined by the BLAST search engine, must also be 2ERB, 3KIE, 3OGN, and 5DIC. Based on the DOPE score, the ideal model protein was selected. Additionally, the AMBERTOOLS 14 package was employed to reduce energy consumption (Case et al. 2014). To determine the best way to make the final structure as energy-efficient as possible, we used a Ramachandran plot and the Structural Analysis and Verification System (SAVES) (Ramachandran et al. 1963; Ramachandran and Sasisekharan 1968).

Molecular docking study

Preparation of odorant-binding proteins

The crystal structures of *Ae. aegypti* (PDB ID: 3K1E) and *Cx. quinquefasciatus* were obtained from the Protein Data Bank (PDB) (PDB ID: 3OGN). A mosquito Odorant Binding Protein structure was taken from the database in order to construct unique species proteins. Using Autodock 4.2, hydrogen atoms and kollman charges were added to the 3D structures, which were then saved as pdbqt files (Morris et al. 2009). The ligands have been given rotating bonds, torsional degrees of freedom, atomic partial charges, and non-polar hydrogen atoms. Docking simulations with various grid sizes including all residues relevant in compound identification were performed based on previous findings. For each docking simulation, a hybrid Lamarckian Genetic Algorithm (LGA) with a grid spacing of 0.375 Å and 30 docking runs was used.

Ligand preparation and molecular docking of the target protein

From PubChem (http://pubchem.ncbi.nlm.nih.gov/), we retrieved the ligand structure of emodin ($C_{15}H_{10}O_5$). In the next step, the geometry was optimized as B3LYP/6-311G**, using the Gaussian 03 package (Frisch et al. 2004). The docking analysis was performed using the prepared molecule. A molecular docking analysis has been conducted to understand how emodin binds to target proteins. *Ae. aegypti* (PDB ID: 3K1E), as well as *Cx. quinquefasciatus* (PDB ID: 3OGN) and a homology-modeled protein (*An. stephensi*), were docked with the emodin ligand. By binding the ligand to the protein, the the

altered protein structure will be confirmed, which eventually alters the function of the protein. The docked complex was viewed with PyMol (DeLano 2002) and the Discovery Studio visualizer (Dassault Systems 2016) to find out the key hydrogen and hydrophobic interactions between the related emodin-odorant binding protein complexes.

Statistical analysis

Probit analysis was used to calculate the median lethal concentrations (LC_{50} and LC_{90}) of the sample and chi-square χ^2 values (Finney 1971). The significance level for the (ANOVA) analysis was determined using the Tukey test at a $p < 0.05$. The data were analyzed using IBM SPSS 20.0 software (IBM, Armonk, NY, USA) as the mean ± standard deviation.

Results

Thin-layer chromatography of sample

Using different solvent combinations, TLC plate separation of bioactive molecules from ATMEAE (hexane:ethyl acetate, hexane:methanol, chloroform:acetone, ethyl acetate:acetone and chloroform:methanol). The chloroform:methanol solvent mixture was found to be significant in extracting the most active 3 fractions based on the unique resolution of the active components in the extracts with Rf values of 0.94, 0.81, 0.38, 0.62, 0.43, 0.28, and 0.26 cm (Fig. 1). Table S1 presents the Rf values of each fraction of *A. terreus*. In any of the tried concentrations (1 to 5%) of hexane in methanol solvent, no spots were visible. There was no separation seen in the mycelia extract during its movement through the mobile phase. The TLC purity of compound was determined by the Rf value of 0.38 cm (chloroform: methanol, 97:3%); the UV at 254 nm were used to spot the single band eluted; then the compound was weighed (84 mg).

Larvicidal activity of separated fractions

The results of the 4th instar larvae of target mosquitoes treated with different concentrations (50 100, 150, and 200 mg/L) of purified fractions (F1, F2, and F3) of *A. terreus* were presented in Table 1. Overall, the Fraction 1 treated *Ae. aegypti* (6.15 and 12.45 mg/L) showed mortality at the lowest LC_{50} and LC_{90} values, followed by *Cx. quinquefasciatus* (8.216 and 14.81 mg/L) and *An. stephensi* (6.95 and 15.24 mg/L). The isolated pure compound proved 100% effectiveness against the 4th instar larvae of *Ae. aegypti* (100%), *Cx. quinquefasciatus* (98%), and *An. stephensi* at a concentration of 200 mg/L (99%). With higher concentrations of the compound (200 mg/L), the mortality occured within 4 h after exposure. Approximately 50% of mortality occurred within 6 h of exposure. It was found that selected mosquitoes failed to develop as normal adults and died as a

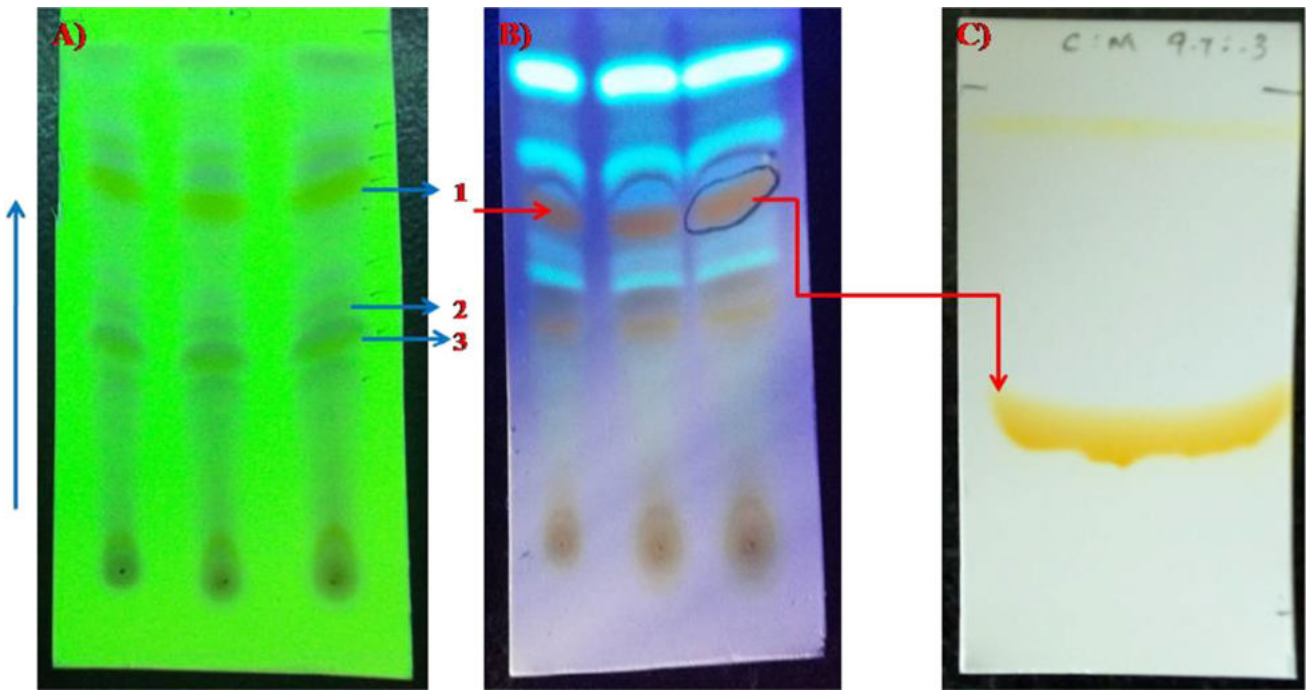


Fig. 1 (**a**) Thin-layer chromatography of *A. terreus* MEAE. Mobile phase (chloroform:methanol, 97:3%). (**b**) The obtained band was visualized by short and long UV 254 nm. (**c**) UV–visible spectrum of single band (emodin) and its Rf value is 0.38 cm

Table 1 Larvicidal efficacy of bioactive fractions from *A. terreus* against *Ae. aegypti, Cx. quinquefasciatus*, and *An. stephensi,* IVth instars larvae (after 12 h of exposure)

Mosquito species	Name of the fractions	Concentration of fractions (mg/L)	Percent mortality ± SD	LC_{50} (LCL-UCL)	LC_{90}(LCL-UCL)	χ^2 (*df* = 3)
Ae. aegypti	F1	50	54.0 ± 1.0	6.156 (5.418–15.378)	12.450 (10.312–29.914)	3.316
		100	66.0 ± 1.0			
		150	88.0 ± 1.1			
		200	100 ± 1.3			
		control	0.00 ± 0.00			
Cx. quinquefasciatus		50	30.0 ± 1.0	8.216 (5.494–21.367)	14.816 (13.915–30.449)	3.093
		100	46.1 ± 1.0			
		150	62.1 ± 0.5			
		200	98.1 ± 1.3			
		control	0.00 ± 0.00			
An. stephensi		50	22.1 ± 0.5	6.895 (4.421–27.994)	15.240 (8.345–43.997)	2.219
		100	38.0 ± 0.5			
		150	86.00 ± 1.0			
		200	99.1 ± 2.3			
		control	0.00 ± 0.00			
Ae. aegypti	F2	50	28.0 ± 0.5	7.917 (6.119–23.114)	29.217 (18.564–40.444)	3.094
		100	30.1 ± 1.0			
		150	38.0 ± 0.5			
		200	42.0 ± 1.0			
		control	0.00 ± 0.00			
Cx. quinquefasciatus		50	22.0 ± 0.5	10.211 (9.415–32.884)	29.414 (21.414–47.517)	2.324
		100	26.4 ± 0.5			
		150	34.0 ± 1.5			
		200	44.0 ± 0.5			
		control	0.00 ± 0.00			
An. stephensi		50	20.1 ± 0.5	12.855 (10.514–25.715)	23.565 (15.916–40.515)	2.456
		100	28.1 ± 0.5			
		150	30.0 ± 1.0			
		200	40.0 ± 0.5			
		control	0.00 ± 0.00			
Ae. aegypti	F3	50	22.0 ± 0.5	10.458 (9.242–28.747)	29.281 (20.531–42.325)	2.671
		100	26.1 ± 0.5			
		150	28.0 ± 0.5			
		200	30.0 ± 1.7			
		control	0.00 ± 0.00			
Cx. quinquefasciatus		50	16.0 ± 0.5	15.219 (10.311–29.573)	25.917 (20.621–45.271)	2.721
		100	20.0 ± 0.5			
		150	26.0 ± 0.5			
		200	30.0 ± 1.0			
		control	0.00 ± 0.00			
An. stephensi		50	20.0 ± 1.0	18.451 (14.560–27.871)	26.437 (18.374–52.575)	3.817
		100	24.1 ± 1.0			
		150	28.1 ± 1.1			
		200	32.0 ± 0.5			
		control	0.00 ± 0.00			
		Azadirachtin	100 ± 0.00	6.642 (3.916–19.708)	15.284 (14.313–33.649)	4.254

Control (deionized water with DMSO)—nil mortality; Reference, Azadirachtin (200 ppm)

LC_{50}, lethal concentration that kills 50% of the exposed larvae; LC_{90}, lethal concentration that kills 90% of the exposed larvae; *LCL*, lower confidence limit; *UCL*, upper confidence limit; *df,* degree of freedom, χ^2, chi-square values are significant at $p < 0.05$ levels. [a]Mean value of triplicates

result of morphological changes (Fig. S1a). The mosquitoes exposed to fraction 1 (F1) (200 mg/L at maximum concentration) demonstrated aggressive changes such as behavioral alterations (Fig. S1b), interference with coordination, up and down writhing activity, and forceful self-biting. Fraction 2 (F2) was tested against three mosquito larvae species: *Ae. aegypti* (LC_{50} = 7.91 and LC_{90} = 29.217 mg/L), *Cx. quinquefasciatus* (LC_{50} = 10.211 and LC_{90} = 29.41 mg/L), and *An. stephensi* (LC_{50} = 12.85 and LC_{90} = 23.56 mg/L). The fraction 3 (F3) performed the best against mosquitoes, notably *Ae. aegypti* (10.45 and 29.28 mg/L), *Cx. quinquefasciatus* (15.219 and 25.91 mg/L), and *An. stephensi* (18.45 and 26.43 mg/L). A negative control showed a 100% larval survival rate. Azadirachtin (200 mg/L) was the least toxic commercial insecticide (LC_{50} = 6.64 and LC_{90} = 15.28 mg/L). Significant chi-square values were found at the $p \leq 0.05$ level. At the low concentration of samples, F1 showed the highest larvicidal activity among the three fractions, hence it was selected for further spectral analysis.

Pupicidal toxicity test

The three isolated fractions tested for the pupicidal toxicity on target mosquitoes resulted in increased mortality rates at different levels of concentration (50, 100, 150, and 200 mg/L). The maximum concentration of fraction1 (F1) (200 mg/L) found to be effective (80%) against *Ae. aegypti* and *An. stephensi*. Based on the LC_{50} and LC_{90} values of F1, better results were found for *Ae. aegypti* (LC_{50} = 15.44 and LC_{90} = 20.75 mg/L), *An. stephensi* (LC_{50} = 16.83 and LC_{90} = 20.26 mg/L), as well as *Cx. quinquefasciatus* (LC_{50} = 19.52 and LC_{90} = 24.19 mg/L) (Table 2). Azadirachtin's LC_{50} and LC_{90} values against pupae were 14.31 and 26.05 mg/L, respectively. During the experiment (doses of F1), the pupal bodies moved restlessly, violently, and irregularly, and dead pupae were found in the bottom of the beakers (Figs. S2a and S2b). All the mosquitoes treated with F2 and F3 resulted significant mortality.

Ovicidal bioassay

The results of ovicidal potentials of *A. terreus* bioactive fractions (1, 2, and 3) are presented in Table 3. Presently, the bioactive fractions caused embryonic death and prevented eggs from hatching. The eggs hatchability rate is largely determined by the relationship between the doses of bioactive fractions and the egg size. Fraction 1 (F1) exhibited higher ovicidal effect than the other 2 fractions. At 150 mg/L concentration, *Ae. aegypti* shown low hatch rate (6.9%), followed by *Cx. quinquefasciatus* (13.4%), and *An. stephensi* (14%). The maximum concentration (200 mg/L) of F1 attained 0% hatchability of eggs and *Ae. aegypti* had a hatchability of 39%, *Cx. quinquefasciatus* had a hatchability of 36% and *An. stephensi* had a hatchability of 32% in the same concentration of fractions 2 and 3, respectively. Control eggs were reported to hatch at 97% with 10% DMSO. The concentrations at 200 mg/L of all the fractions were very harmful to eggs as compared to other concentrations. Also, the positive control, azadirachtin proved to be extremely harmful to mosquito eggs.

Biochemical assays

An interesting finding from the tested larvae is the changes in the activity of normal components either by increasing or decreasing compared to the control. Our study involved biochemical enzymatic assays of mosquito larvae, where, acetylcholinesterase, α- and β-carboxylesterase, and acid and alkaline phosphatases were measured.

Ae. aegypti (F_4 = 1434.070; $p < 0.01$) significantly suppressed the acetylcholinesterase (AChE) activity, which was measured using the control value of 2.61 mg protein/mL of the larval homogenate of *An. stephensi* (F_4 = 901.954; $p < 0.01$) and *Cx. quinquefasciatus* (F_4 = 1266.187; $p < 0.01$) (Fig. 2 a). AChE was inhibited by F1 in dose-dependent manner. A significant decrease in α-carboxylesterase activity was observed from F1 treatedlarvae of *An. stephensi* (2.61 to 0.61), *Ae. aegypti* (2.06 to 0.39) and *Cx. quinquefasciatus* (3.21 to 0.50 mg protein/mL homogenate), (Fig. 2 b). A similar type of activity was observed for the α-carboxylesterase (0.561 to 0.051, 0.521 to 0.151, and 0.621 to 0.097 µM β-naphthol released/mg/min), respectively (Fig. 2 c).

As a result of exposure to F1, the activity of acid and alkaline phosphatases was reduced slightly in larvae of *An. stephensi*, *Cx. quinquefasciatus*, and *Ae. aegypti*, from 0.448 to 0.230, 0.421 to 0.213, and 0.484 to 0.211 µM p-nitrophenol released/min/mg protein, respectively (Fig. 2 d). Furthermore, alkaline phosphatase significantly reduced the levels of targeted mosquitoes' larvae (0.361 to 0.142, 0.301 to 0.101, and 0.324 to 0.161 mg protein/mL of homogenate) (Fig. 2 e).

Histopathology profile of 4th instar larvae

The midgut epithelial columnar cells (EC) of 4th instar *An. stephensi* larvae were severely damaged after being exposed to F1 of ATMEAE. The lumen was encompassed by thin peritrophic membranes containing food particles in control larvae, whereas in treated larvae, the midgut contents, epithelial cells, and peritrophic membranes (PM) were ruptured (Fig. 3 a–c). Similarly, *A. terreus* compound (F1)-treated larvae had broken mid-gut epithelium and vacuolated cells (Fig. 3 d–f), whereas control larvae had a normal appearance in the mid-gut, hind-gut, muscles, brush border, and epithelial cells. The muscles appear slightly damaged and the brush border is disorganized. In larvae treated with the F1, the mid-gut was the most

Table 2 Pupicidal efficacy of *A. terreus* fractions (F1, F2, and F3) against *Ae. aegypti, An. stepheni*, and *Cx. quinquefasciatus* (after 24 h of exposure)

Mosquito species	Name of the fractions	Concentrations (mg/L)	Percent[a] mortality ± SD	LC_{50} (LCL-UCL) ppm	LC_{90} (LCL-UCL) ppm	χ^2 ($df = 3$)
Ae. aegypti	F1	50	60.0 ± 1.0	15.449 (10.243–25.992)	20.752 (19.560–39.414)	2.171
		100	72.0 ± 1.0			
		150	76.0 ± 0.5			
		200	82.1 ± 0.5			
		control	0.0 ± 0.0			
An. stephensi		50	56.0 ± 0.5	16.834 (10.812–21.915)	20.269 (18.642–32.416)	1.917
		100	64.0 ± 0.5			
		150	72.0 ± 1.0			
		200	82.2 ± 1.1			
		control	0.0 ± 0.0			
Cx. quinquefasciatus		50	46.0 ± 1.0	19.526 (13.316–23.519)	24.194 (20.912–37.580)	1.553
		100	54.0 ± 1.0			
		150	66.0 ± 1.0			
		200	72.0 ± 1.0			
		control	0.0 ± 0.0			
Ae. aegypti	F2	50	34.0 ± 0.5	21.388 (19.450–30.719)	43.518 (34.341–58.181)	1.593
		100	42.0 ± 1.0			
		150	46.0 ± 0.5			
		200	52.0 ± 0.5			
		control	0.0 ± 0.0			
An. stephensi		50	34.0 ± 0.5	25.418 (20.445–38.561)	51.591 (30.425–60.141)	1.385
		100	42.0 ± 1.0			
		150	46.1 ± 0.5			
		200	52.0 ± 0.5			
		control	0.0 ± 0.0			
Cx. quinquefasciatus		50	26.0 ± 1.1	31.672 (21.130–42.515)	55.450 (63.210–61.521)	2.436
		100	30.0 ± 1.0			
		150	38.0 ± 0.5			
		200	42.0 ± 1.0			
		control	0.0 ± 0.0			
Ae. aegypti	F3	50	26.0 ± 1.1	34.580 (27.150–42.436)	58.920 (50.230–65.587)	2.456
		100	30.0 ± 1.1			
		150	34.0 ± 0.5			
		200	42.0 ± 1.0			
		control	0.0 ± 0.0			
An. stephensi		50	22.0 ± 0.5	42.255 (31.720–48.514)	58.368 (46.512–69.151)	1.163
		100	26.0 ± 0.5			
		150	30.0 ± 1.0			
		200	34.0 ± 1.5			
		control	0.0 ± 0.0			
Cx. quinquefasciatus		50	26.0 ± 0.5	43.548 (32.250–49.469)	68.823 (50.450–73.107)	2.242
		100	38.0 ± 0.5			
		150	40.0 ± 1.1			
		200	41.0 ± 0.5			
		Control	0.0 ± 0.0			
		Azadirachtin	100 ± 0.00	14.316 (8.462–21.495)	26.052 (20.511–34.531)	3.809

Control (deionized water with DMSO)—nil mortality; Reference, Azadirachtin (200 ppm)

LC_{50}, lethal concentration that kills 50% of the exposed pupae; LC_{90}, lethal concentration that kills 90% of the exposed larvae; *LCL*, lower confidence limit; *UCL*, upper confidence limit; *df*, degree of freedom; χ^2, chi-square values are significant at $p < 0.05$ levels. [a]Mean value of triplicates

Table 3 Ovicidal activity of bioactive fractions (F1-F3) from *A. terreus* against *An. stephensi, Cx. quinquefasciatus*, and *Ae. aegypti.* Concentrations (mg/L)

Mosquito species		Egg hatchability (%)					
	Name of the fraction	Control	50	100	150		200
An. stephensi	F1	100.0 ± 0.0	56.0 ± 1.5	40.0 ± 1.5		14.0 ± 0.2	NH
Cx. quinquefasciatus		97.5 ± 0.0	61.0 ± 0.7	42.7 ± 0.7		13.4 ± 0.7	NH
Ae. aegypti		98.3 ± 0.0	51.3 ± 1.1	32.2 ± 0.5		6.9 ± 0.0	NH
An. stephensi	F2	99.0 ± 1.2	68.6 ± 0.5	55.2 ± 1.3		44.7 ± 0.0	32.8 ± 1.2
Cx. quinquefasciatus		100.0 ± 0.2	74.2 ± 1.1	63.2 ± 1.3		43.5 ± 1.2	36.6 ± 0.4
Ae. aegypti		98.0 ± 0.3	71.2 ± 1.5	60.0 ± 1.5		47.8 ± 0.5	39.0 ± 0.7
An. stephensi	F3	99.8 ± 0.0	86.1 ± 0.4	74.5 ± 1.2		68.9 ± 0.6	56.1 ± 1.2
Cx. quinquefasciatus		96.2 ± 1.2	88.0 ± 0.9	77.2 ± 0.0		55.0 ± 0.9	40.0 ± 1.6
Ae. aegypti		99.5 ± 0.4	76.3 ± 0.0	64.0 ± 0.0		57.1 ± 0.0	43.2 ± 0.9
	Azadirachtin	100.0 ± 0.0	89.2 ± 0.5	97.01 ± 0.2		NH	NH

Each value (X ± SD) represents mean of triplicate values

NH, no hatchability (100% mortality); Azadirachtin, positive control

affected tissue. After treatment with the F1, the histopathological alterations were observed in the 4th instar larvae of *Ae. aegypti*. Specifically, muscles, gastric caeca, hindgut, mid-gut, nerve ganglia, and mid-gut epithelium were damaged and collapsed. There were spoiled epithelial cells that contained the nuclei of the F1-treated larvae in vacuolation.

In a lethality study using brine shrimp, F1 was found to be moderately toxic to *Artemia* nauplii, with an LC_{50} of 54.0 mg/L and an LC_{90} of 84.51 mg/L ($\chi^2 = 5.321$, $p < 0.05$). Using F1 doses, the survival of *Artemia* nauplii was significantly decreased. In Fig. 4 a, the maximum mortality rate (64%) was reported at 10 mg/L, while the controls do not exhibit mortality. On the other hand, *Artemia* nauplii inside gut showed clusters of F1 after 24 h (Fig. 4 b–d) (Table 4).

A developmental study was conducted between 0 and 126 hpf. Based on the results, the treated and untreated embryos showed significantly different hatching rates at 96 hpf. As a control (96 h), 99.5% of the eggs hatched, and at lower concentrations (1.95 and 15.6 mg/L) of F1, more than 50% of the eggs were hatched. F1-treated embryos showed a dose-dependent reduction in body length. Compared to the control group, the maximum exposure (62.5 mg/L of F1) significantly reduced the length of embryos. Following 96 h of treatment with 62.5 mg/L of F1, embryos showed underdeveloped head regions and closed tails, with no heartbeat remaining in 120-hpf embryos (unhealthy tail). The concentrations of F1 had a significant effect on the mortality rate. The total body length of the embryos, mortality, as well as hatchability of F1-treated embryos (Fig. 5 and Fig. S3) were also significantly affected at the concentrations of 15.6 mg/L or higher of F1 (Fig. 5).

The UV spectra of isolated F1 were measured between 200 and 700 nm using DMSO as a blank. The (F1) had wide band with maxima at 443 nm and 291 nm (Fig. S4). The greatest UV absorption spectrum wavelength for F1 (with 0.856 OD value) is 443 nm, indicating the presence of both aromatic and methyl groups. TLC revealed that the pure compound was yellowish-orange in color. A single fraction was obtained and tested for purity by HPLC. There is a single prominent peak (Fig. S5, Table S2) indicating maximum purity and as a result, the compound has been separated at a retention time of 6.288 min and with a peak area of 95.90%.

The FTIR spectrum of fraction (F1) shows numerous peaks, including one at 3389.68 cm^{-1} that indicates stretching vibrations of OH alcohol (or) phenol groups. In the fractionated sample, a noticeable band forms at 3079.30 cm^{-1}, suggesting that the OH bond stretching frequency is most likely occurring in the protein or carbohydrates. The C-H alkanes are responsible for the peak of 2924.04 cm^{-1}. Anhydrides groups are detected in the symmetrical stretching peaks at 1815.89 cm^{-1}. At 2854.14 cm^{-1}, a peak was seen caused by methyl or methoxyl stretching vibrations. For the absorption peak at 1665. 98 cm^{-1}, bending vibrations of NH primary amines may be responsible for the medium-intense band. It is possible that aromatics -C–C stretching caused the medium peak at 1416.97 cm^{-1}. The strong band at 1272.84 cm^{-1} corresponds to C-O stretching and 1216.96 cm^{-1} is for C-N stretching, represents alcohols, carbohydrates or esters, and aliphatic amines. A prominent peak associated with O–H bending vibrations of carboxylic acids may be seen at 907.00 cm^{-1}, whereas NH primary amines can be found at 874.80 cm^{-1} (Fig. S6, Table S3).

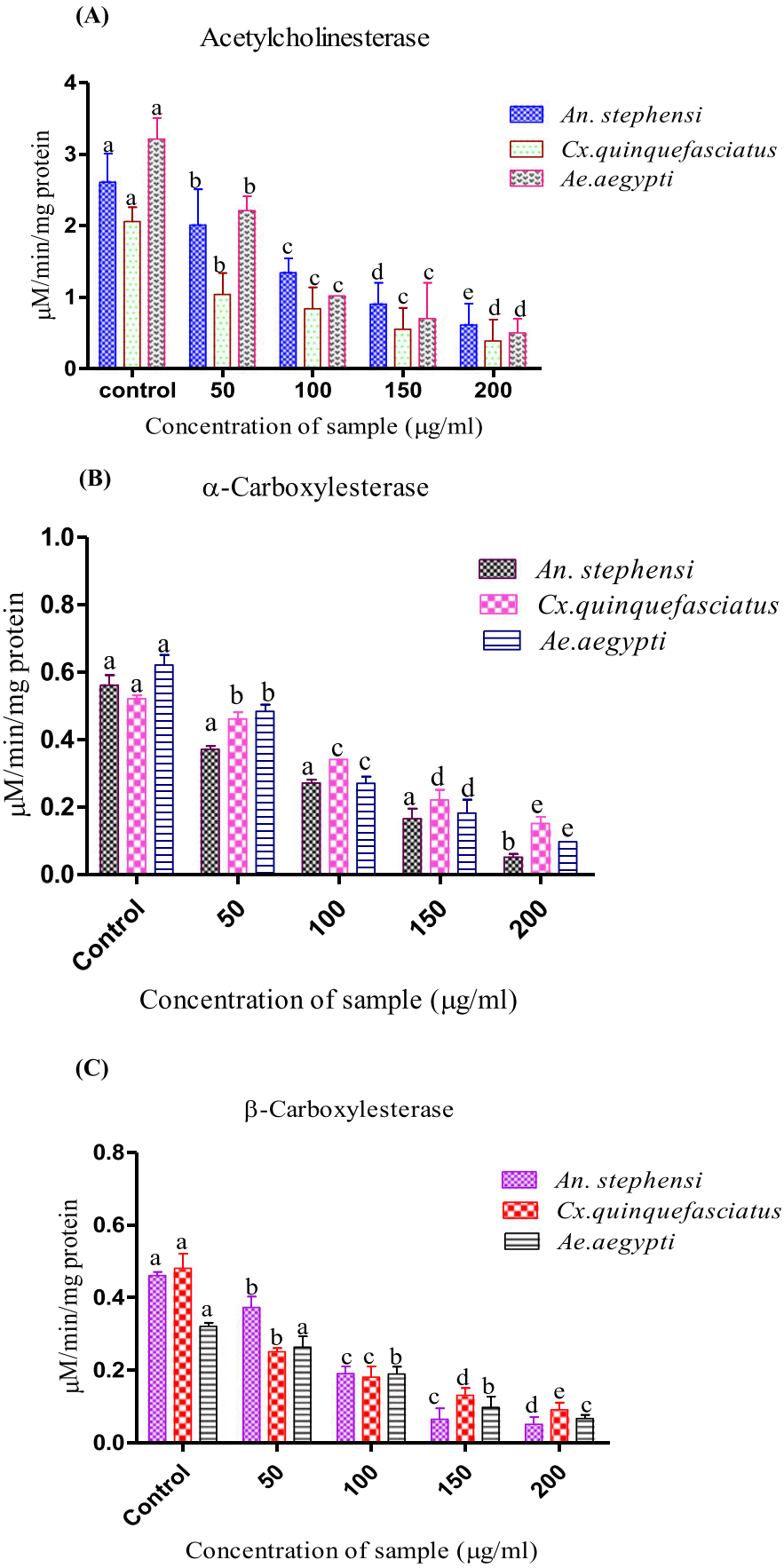
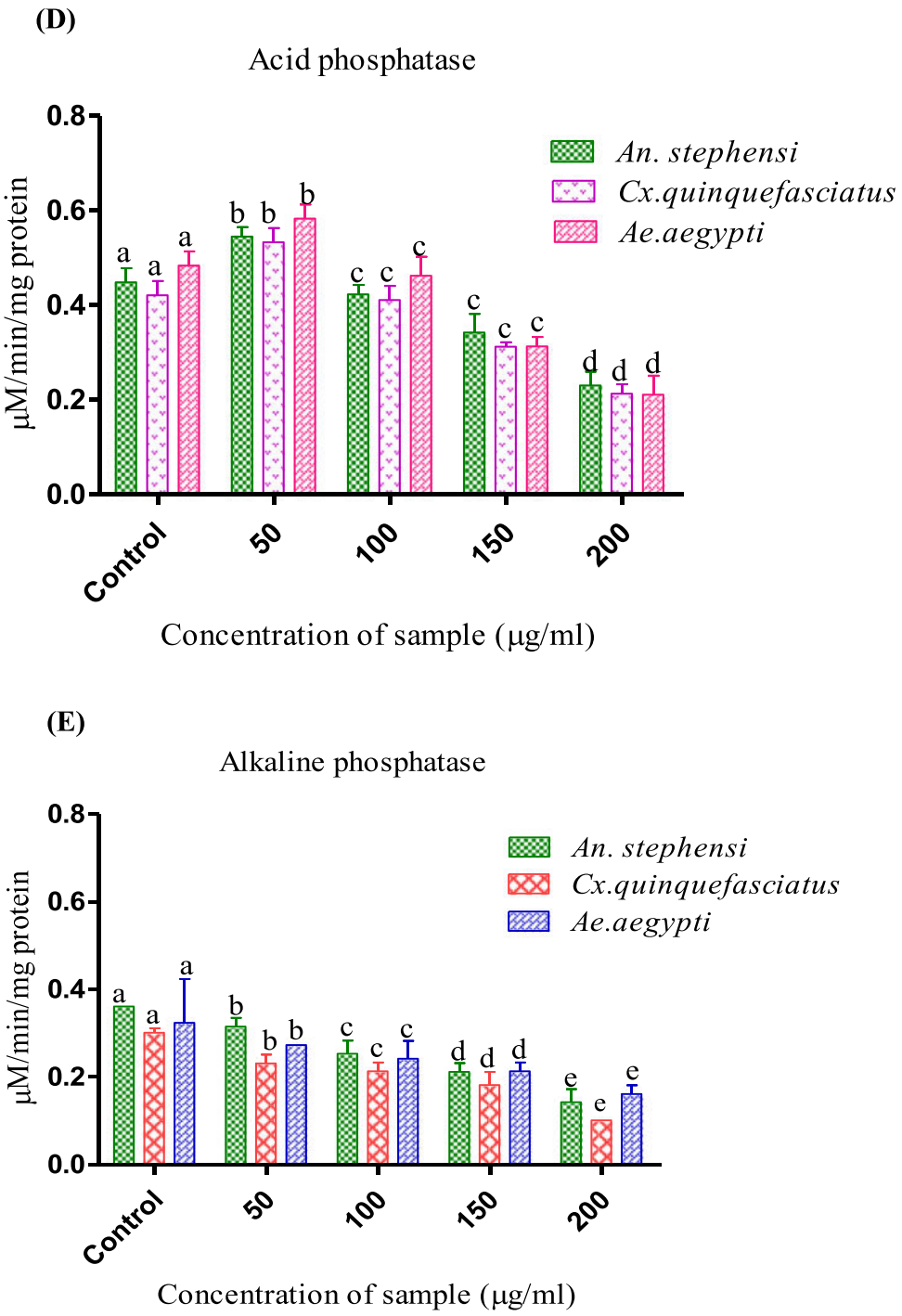


Fig. 2 Biochemical profile of emodin treatment on the larval enzyme activities. (**A**) Acetylcholinesterase, (**B**) α-carboxylesterase, (**C**) β-carboxylesterase, (**D**) acid phosphatise, and (E) alkaline phosphatise. Each bar represents mean ± SE of three replicates

Fig. 2 (continued)



To confirm, the compound's ^{1}H NMR spectrum in DMSO solution was obtained regarding Tetramethylsilane (TMS) as an internal standard. ^{1}H-NMR data (400 MHz, D6-DMSO): δ 12.11 (s, Ar(O–H), ^{1}H); 12.04 (s, Ar(O–H), ^{1}H); 11.41 (br, s, Ar(O–H), ^{1}H); 7.52 (s, ArH, ^{1}H); 7.19 (s, ArH, ^{1}H); 7.14 (s, ArH, ^{1}H); 6.61 (s, ArH, ^{1}H); 2.42 (s, Ar(CH3), ^{3}H) ppm. Also, the structure of the molecule was validated by ^{13}C NMR analysis, which was convincingly proven. The ^{13}C NMR spectrum indicates the characteristics of carbon position in the predicted area. ^{13}C-NMR data (100 MHz, D6-DMSO): δ 189.91 (C-9), 181.41 (C-10), 165.96 (C-8), 164.84 (C-1), 161.79 (C-6), 148.53 (C-3), 135.26 (C-12), 132.97 (C-14), 124.40 (C-4), 120.17 (C-2), 113.53 (C-13), 109.16 (C-11), 108.24 (C-5), 106.74 (C-7), 21.93 (C-15) ppm. The ^{1}H and ^{13}C NMR spectra of the molecule showed a noticeable resonance shift in comparison to the analogous protons and carbons in the unbound compound. Figure S7a shows the findings of measuring

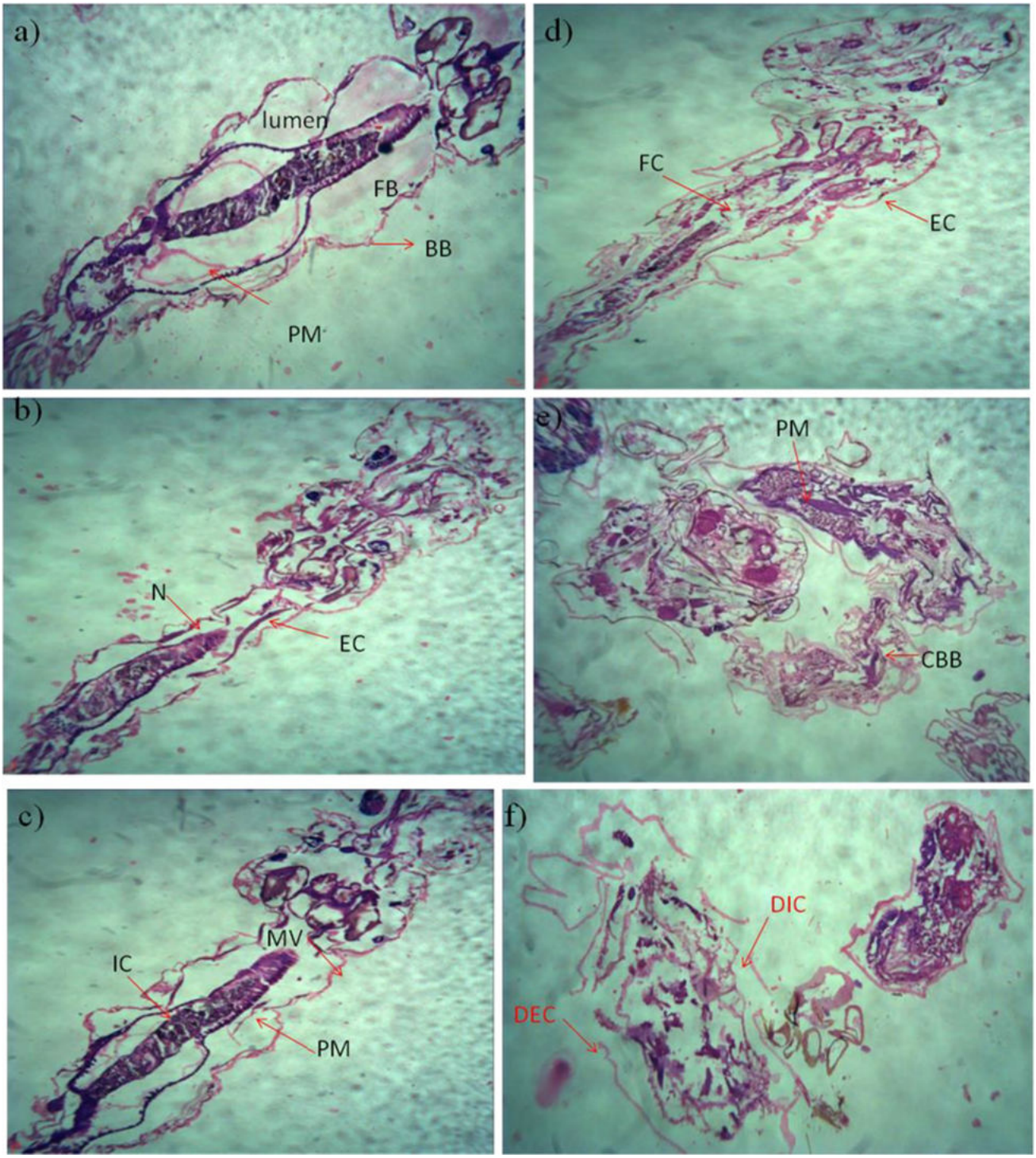


Fig. 3 Histological sections of 4th instar larvae of *An. stephensi*, *Cx. quinquefasciatus*, and *Ae. aegypti* after exposure to emodin compound: (**a**, **b**, **c**) Untreated 4th instar larvae; (**d**) Midgut of *An. stephensi* 4th instar larvae; (**e**) Midgut of *Cx. quinquefasciatus* 4th instar larvae; (**f**) midgut of *Ae. aegypti* 4th instar treated larvae, after 12 h of exposure to pure compound. (EP epithelial cells, MV microvilli, PM peritropic membrane, FC food column, CBB collapsed brush border, DEC degenerated epithelial cells, IC intestinal cells, DIC degenerated epithelial cells, N nucleus)

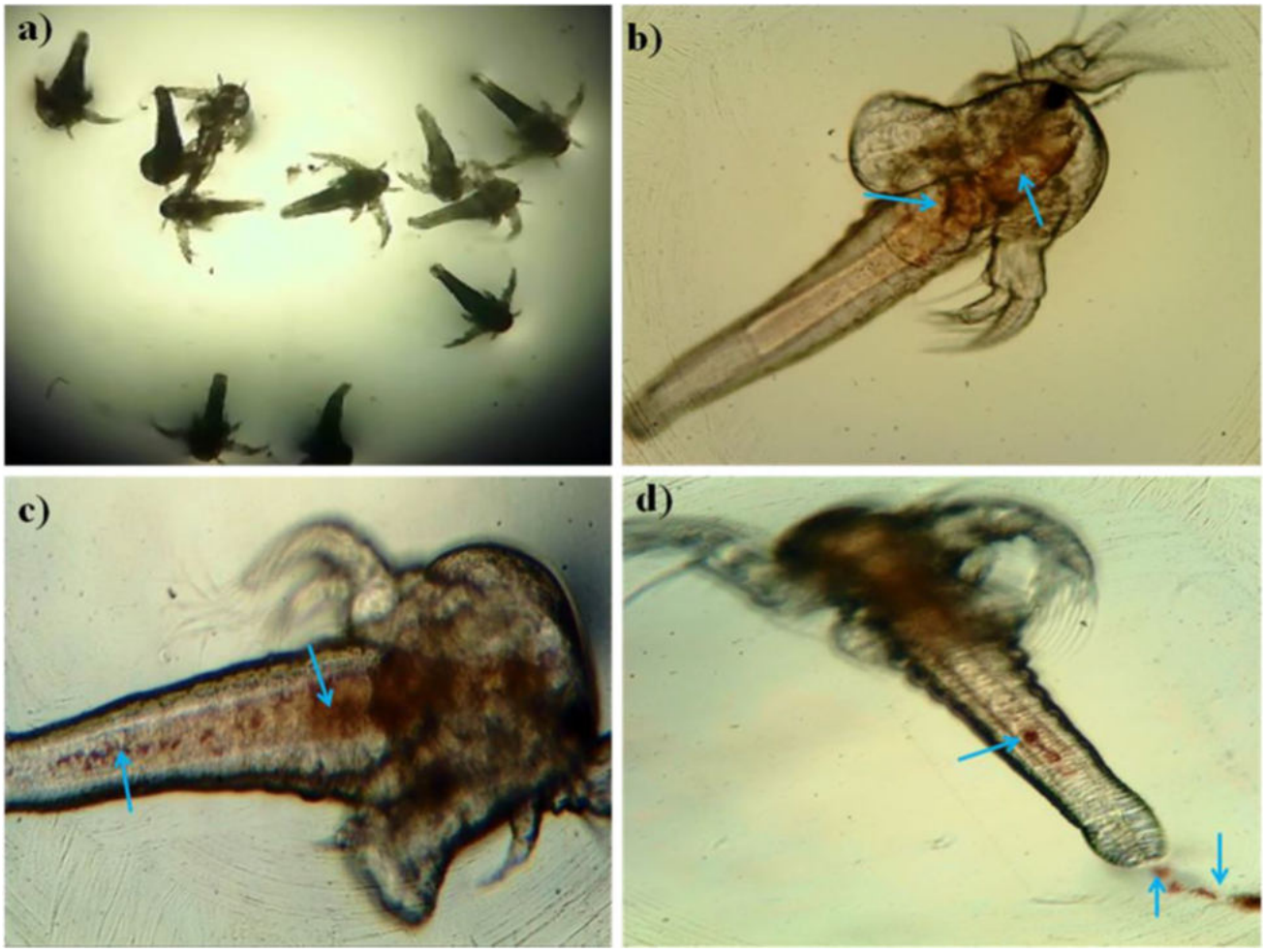


Fig. 4 Toxicity assessment of emodin on *A.* nauplii. (**a**) Control *A.* nauplii; (**b**) Treated with emodin. Blue arrow indicates the cluster of emodin compound

Table 4 Bio-toxicity assay of brine shrimp lethality using emodin

Concentrations (mg/L)	Percentage of mortality	24 h		
		LC_{50} (LCL-UCL) (mg/L)	LC_{90} (LCL-UCL) (mg/L)	$\chi^2(df=11)$
2.0	8.6 ± 0.5	54.0 (19.5–41.0)	84.51 (74.4 -96.3)	5.321
4.0	24.6 ± 2.5			
6.0	42.0 ± 1.0			
8.0	54.0 ± 1.0			
10	64.0 ± 2.0			
Control	0.0 ± 0.0			

emodin using ^{1}H NMR in D6-DMSO solvent. The phenolic proton has a wide singlet signal at 11.41 ppm (O–H). The phenolic protons caused peaks in the NMR spectrum of emodin at 12.11 and 12.04 ppm (O–H). Aromatic protons in four-meta locations were discovered at 7.52, 7.19, 7.14, and 6.61 ppm. Only one methyl (Ar-CH_3) functional group (at a quantity of 2.42 ppm) was expressed, according to database of emodin (Fig. S7b). Ketocarbons (C = O) had downfield shifts of 189.91 (C-9) and 181.41 (C-10) ppm, whereas phenolic carbons (Ar–C-(O–H)) had downfield shifts of 165.96 (C-8) and 164.84 (C-10) ppm, respectively (C-1). A forward shift of 21.93 ppm was observed for the methyl carbon (Ar (CH_3)) (Fig. S8). All chemical structures and closely similar compounds were compared to existing spectral data (Guo et al. 2011). A molecular ion peak at m/z 269.044 was discovered in emodin by LC–ESI–MS analysis (Fig. S9). Based on spectrum data, the molecule was identified as trihydroxy-6-methyl anthraquinone (emodin), with the molecular formula $C_{15}H_{10}O_5$. The pure compound has a boiling temperature of 263–267 °C and a melting point of 529.0 K.

Docking studies helped to find the different ways, that how ligands and receptors, enzymes, and other binding

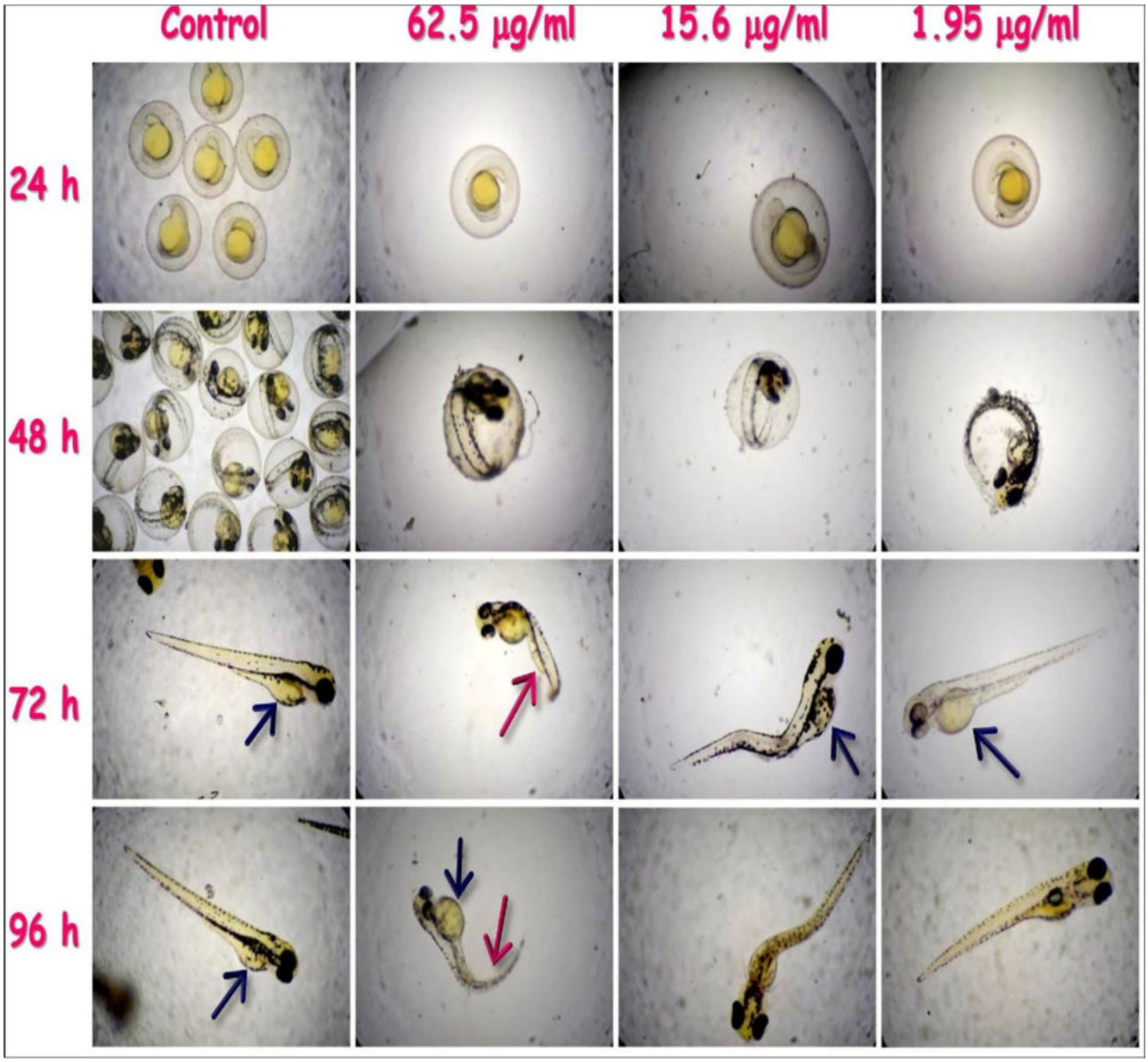


Fig. 5 Bio-toxicity of Zebrafish 96hpf embryos induced by emodin isolated from *A. terreus* at various concentrations (control, 1.95, 15.6, and 62.5 mg.L-1). Note: Pink arrow indicates malformation and blue arrow indicates yolk cysts

sites can bind to one other. The binding energy of the ligand inside the target protein was calculated using Autodock 4.2. The findings of the Ramachandran plot for *An. stephensi*, *Ae. aegypti* (3K1E), and *Cx. quinquefasciatus* (3OGN) model structures revealed that the most preferred residues are 93.6, 89.6, and 91.7%, respectively (Fig. S10). Table S4 shows the values of the dope score of *An. stephensi* Odorant binding protein (OBP). The emodin molecule interacts accurately with OBPs on the same active site, according to molecular docking research. *Ae. aegypti*-emodin (− 8.89 kcal/mol) complex forms hydrogen and hydrophobic interactions with active site residues (Table S5), which is higher than the other two complexes (− 6.53 and − 8.09 kcal/mol). PyMOL was used to analyze intermolecular interactions, and the results are shown in Fig. S11. A summary of binding energy and related parameters can be found in Table S6**.** At the best in Fig.S12, the binding energy value of the emodin *An. stephensi* complex was determined to be − 6.53 kcal/mol. Four hydrogen bonds are formed between emodin and the amino acid residues, Leu143 and His141. In the complex of emodin and *Cx. quinquefasciatus*, the binding energy is − 8.09 kcal/mol. With the amino acid residues, His111, Ala88, Met84 and Phe123, emodin forms four hydrogen-bonding

interactions. In Fig. 6 a–c shows the interaction between proteins and ligands obtained from PyMol software.

Discussion

Bioactive compounds are being isolated from plants and microbes which leads to the discovery of many essential natural compounds (McRae et al. 2007). The importance of understanding the effects of fungal compounds on the diffusion of host cuticles and larvicidal toxins have greatly increased in recent years (Demain and Fang 2000). *A. terreus* mycelial ethyl acetate extract (ATMEAE) and its fractions were tested against the 4th instar larvae of *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. stephensi*. *A. terreus* fractions with larvicidal activity against target vectors were screened. LC_{50} values of 6.15 to 15.24 mg/L were observed for fraction1 (F1) against all tested mosquitoes. In addition to the malformed pupae and deformed larvae, the different species of treated mosquito larvae displayed restless and irregular movements. Ragavendran et al. (2018) also reported similar behavioral observations. Sharma et al. (2015) examined the effects of *Achyranthes asperaon* extracts against *Ae. aegypti* larvae, which caused behavioral changes and excitation of the biting anal gills. During larval death, the respiratory muscles become paralyzed and causing the larvae to be unable to breathe properly, and they eventually die. Insect skin and cuticle pores allowed emodin to enter the larval body, where the compound interferes with molting, malformation, and other metabolic processes. The compound might collapsed the respiratory system, digestive system, and nervous system in mosquito larvae/pupa(Lee et al. 2017). Various researchers identified malformed larvae in mosquitos treated with triterpenoids, limonoids, niloticin, and isonimocinolide (Sengottayan 2013) (Reegan et al. 2016). Pradeep et al. (2015) isolated and evaluated the larvicidal property of 2, 3, 4, 5-tetrahydroisoquinolimidine-4-ol) from *Fusarium moniliforme* on third and fourth instar larvae of *Ae. aegypti* (LC_{50} = 237.0 and 276.4) and *An. stephensi* (LC_{50} = 335.6 and 258.1 mg/L). When tested against *Cx. quinquefasciatus* larvae in the fourth instar, catechin compounds yielded LC_{50} values of 3.76 and LC_{90} values of 9.79 mg/L (Elumalai et al. 2016). The *Streptomyces* sp. metabolite (5-(2, 4-dimethylbenzyl) pyrrolidin-2-one) was reported to be 100% effective against *An. stephensi* and *Cx. tritaeniorhynchus* (Saurav et al. 2013). Deepika et al. (2012) isolated (2S, 5R, 6R)-2-hydroxy 3, 5, 6-trimethyloctan-4-one from *Streptomyces* sp., which exhibited larvicidal activity against *An. subpictus* and *Cx. quinquefasciatus* at low doses. Using higher LC_{50} and LC_{90} values (110 and 200 mg//L). Murugesan et al. (2009) reported that *Trichophyton mentagrophytes* extracellular metabolites had proposed larvicidal potentials against *Ae. aegypti* larvae in the third instar.

The study showed that even when F1 is administered at low doses, it had remarkable pupicidal activity (with high mortality rates) against the tested mosquitoes. Furthermore, with an evidence of present study, Gandhi et al. (2016) discovered a good pupicidal agent, alizarin against pupae of *An. stephensii* and *Cx. quinquefasciatus* (LC_{50} and LC_{90} values of 1.97, 4.79, 2.05, and 5.50 mg/L). According to Geetha et al. (2010), *Bacillus subtilis* produces cyclic lipopeptides which show superior pupicidal properties against *An. stephensi*. According to the present study, emodin isolated from *A. terreus* had (0%) hatchability (higher concentration 200 mg/L) against selected mosquitoes. The emodin showed quite significant ovicidal potential similar to diflubenzuron

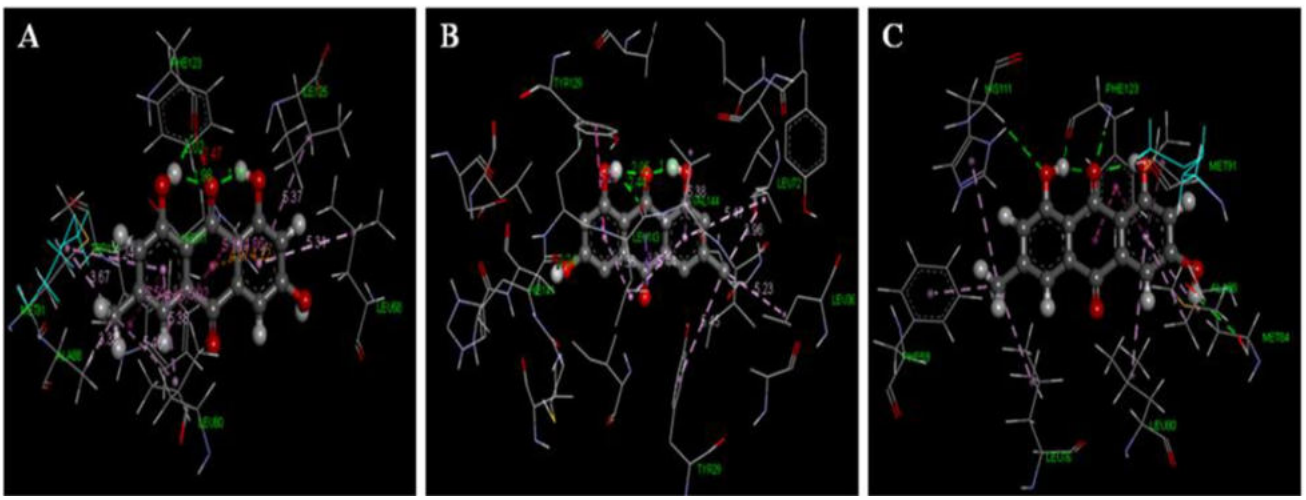


Fig. 6 In silico molecular docking analysis of emodin against three mosquito vectors. (**A**) *Ae. aegypti.* (**B**) *An. stephensi*. (**C**) *Cx. quinquefasciatus* odorant binding proteins (OBPs)

and penfluron, which are reported against four species of mosquitoes (Prakash 1993). Adversely, Karthik et al. (2011) reported that the metabolites of *Streptomyces* sp. had effect on the hatchability (0%) of eggs against *Cx.triaeniorhynchus* and *Cx.gelidus* when used at 1000 mg/L concentrations. Researchers, Su and Mulla (1998) reported that azadirachtin had zero ovicidal activity in the eggs of *Culex* species when administered at 10 mg/L.

In mosquitoes, acetylcholinesterase (AChE) plays a major role in resistance mechanisms against chemical insecticides (Solairaj and Rameshthangam 2017). AChE activity was significantly inhibited in three different mosquito larvae exposed to emodin in the current study. It is well-noted that emodin is toxicant to the larvae, as the AChE activity was drastically inhibited in the nerve junction, which eventually catalyzes the hydrolysis of acetylcholine. Koodalingam et al. (2011) showed a significant decrease in AChE levels in *Ae. aegypti* larvae that were treated with *Sapindus emarginatus* soap nut extract. In this line, Gade et al. (2017) confirmed the reduced AChE enzyme activity in *Aedes* and *Culex* larvae AChE enzyme level thrrough by stigmasterol and hexacosanol compounds. As evidenced by the earlier researchers, the emodin's possible mechanisms is because of interloping with the octopaminergic system and by inhibiting AChE activity in *Aedes* species. Thus, eventually resulted in an increased cAMP levels and leads to mortality (Perumalsamy et al. 2015; Selvaraj et al. 2021; Yang et al. 2003). Also for the *Culex* species, there might be an inhibition of NADH-ubiquinone oxidoreductase (Mitochodrial complex I) by emodin through catalyzing the transfer of 2 e^- from NADH to ubiquinone. Thus, resulted in the termination of ATP production and eventually larvae become weak and die (Liang et al. 2015). In case of *Anopheles* species, emodin may inhibiting directly the mechanisms on epidermal cells through hindering the production of enzymes responsible for the tanning or cuticular oxidation process (Kamaraj et al. 2011; Vinayagam et al. 2008).

Esterase enzymes are involved in dissolving carboxyl ester and phosphodiester bonds to develop resistance to insecticides. Studies with a variety of mosquito vectors focused the detoxification activities of α- and β-carboxylesterase as biomarkers (Agra-Neto et al. 2014); (Selin-Rani et al. 2016). During the development of the larvae, the levels of detoxifying enzymes (α and β-carboxylesterase activity) decreased significantly due to the emodin and the reduction. Similarly, *Serratia marcescens* (prodigiosin) metabolites inhibited their enzymatic activity and acetylcholinesterase activity in *Ae. aegypti* and *An. stephensi* larvae (Suryawanshi et al. 2015). In this line, Edwin et al. (2016) examined the inhibitory effects of andrographolide from *Andrographis paniculata* on the larvae of *Ae. aegypti,* particularly of carboxylate esterase activity. Notably, acid and alkaline phosphatases play a crucial role in metabolism and signaling processes, and their expression decreases during the developmental stages of insect larvae (Nathan et al. 2007). Compared to control, larvae exposed to emodin showed a decrease in acid and alkaline phosphatase activity. Fungal mediated salicylic acid-derived nanoparticles inhibited acid and alkaline phosphatase activity in larvae of *Ae. aegypti* (Ga'al et al. 2018). Likewise, the emodin-derived nanoparticles also will emerge with superior enzyme inhibitions.

The molting of midgut cells has been disrupted by bioactive components generated from natural resources (Kihampa et al. 2009; da Silva et al. 2013). The cuticle layer, fat body, brush boundary, and nuclei in the midgut and hindgut regions of treated 4th instar larvae were damaged, according to the histological profile of larvae treated with compound/fraction. In larvae treated with *Bacillus licheniformis* exopolysaccharides, Abinaya et al. (2018) evidenced the histological damage in the midgut, muscles, and abdominal regions shrinking. Similarly, Seetharaman et al. (2017) found that *Culex* sp. mosquito larvae showed damage in their microvilli, midgut lumen, peritrophic membranes and epithelial cells after exposure to limonoid compound from *Penicillium oxalicum*. As reported by Déciga-Campos et al. (2007), the toxicity of compound/fractions on brine shrimp was effective even at concentration (500 mg/L), whereas the present LC_{50} value for emodin was 54.0 mg/L, showing very low toxicity. Furthermore, in contrast to the present work, Lee et al. (2002) found that phrymarolin and ursolic compounds exhibited greater toxicity on brine shrimp larvae, and their LD_{50} values were 0.0013 and 27.0 mg/L, respectively.

In genetics, cell biology, and embryology, zebrafish (*D. rerio*) embryos are ideal models (Bakkiyanathan et al. 2012). Several investigations on embryotoxic, teratogenic substances, and also on prospective dietary ingredients have been conducted. The embryogenesis of zebrafish is comparable to that of higher vertebrates, such as humans (Busquet et al. 2008). At a concentration of 15.6 mg/L at 96 hpf, *A. terreus* derived emodin considerably impacted on zebrafish embryo body length, hatching rate, and tail deformity when compared to the control. Furthermore, Fan et al. (2015) reported that secondary metabolites from the marine-derived fungus *Penicillium expansum* Y32 who observed low heartbeat rate in zebrafish depending on the doses applied and the treatment period. Abutaha et al. (2015) studied the endophytic fraction of *Cochliobolus spicifer* for larvicidal effects and toxicity to zebrafish embryos, wherethe fungal fraction did not cause any symptoms of toxicity.

For hypothesizing and assembling logistic predictions, in silico computational simulation methods was employed (Gaddaguti et al. 2012; Koech and Mwangi 2013), to identify the possible binding residues responsible for biological functions. In this study, potential fungal emodin was examined for their repellent activity against an odorant-binding protein (OBP) of tested mosquitoes. Carvacrol, camphor ocimene,

α- and β-pinene, citronellal, geraniol, and linalool were shown to have a higher binding potential for the OBPs associated with mosquitoes' repellent effect (Müller et al. 2009). When compared to two other complexes, emodin—*Ae. aegypti* combination has high binding energy (−8.89 kcal/mol) and followed by *An. stephensi* (−6.53 kcal/mol, with hydrogen bonds formed by Leu143 and His141 amino acid residues) and *Cx. quinquefasciatus* (−8.09 kcal/mol, with hydrogen bonds formed by His111, Ala88, Met84, and Phe123 amino acid residues). Gaddaguti et al. (2016) reported that several chemicals from *Ocimum* sp., such as licopersin, γ-sitosterol, and benzene, 1, 2-dimethoxy-4-(2-propenyl)-, exhibited strong binding with high affinity to OBP of (3Q8I), with a G-score of −7.14 and TH57 amino acid residues. Similarly, OBP of (3N7H) revealed G-score of −4.54, ASN56, and CYS53 *An. gambiae* amino acid residues. According to Gopal and Kannabiran (2013), the camphor of *Nilaparvatha lugens* has a binding energy of −136 kcal/mol with OBP1 protein. The oleic acid showed the least binding energies with 1OOF, 2ERB, 3R1O, and OBP1. Carvacrol showed the least binding energies with 1QWV and 1TUJ proteins with −117.45 kcal/mol and −21.78 kcal/mol, respectively. *Calotropis gigantea* components (β-amyrin) were studied by Dhivya (2014), who recorded high glide scores (−6.73A°), made ^{1}H-bond with the target OBP, and comprised HIS111 amino acid residues. Di (2- ethylhexyl) phthalate (−8.66 A°) and α-amyrin (−5.7A°) are two components that show substantial binding characteristics with mosquito OBP of *Cx. quinquefasciatus*. Hydrophobic activity and hydrogen bonding were also seen in the isolated molecule when it was tested against comparable amino acids. High negative binding energy values showed the strongest binding affinity between the ligand and the target proteins, according to the current study. Also, a study is warranted to know more about the molecular processes driving natural mosquito repellents' interactions with OBP.

Conclusion

The research finding clearly shows that emodin (fraction-1), a bioactive compound (from *A. terreus*), was effectively extracted and identified using different spectral methods. Interestingly, the isolated fraction-1 (emodin) showed strong larvicidal activity against *Ae. aegypti* when compared to other mosquitoes (LC_{50} = 6.15 and LC_{90} = 12.45 mg/L). Hyper-excitation, severe paralysis, and aggressive self-biting movement were seen in emodin-treated larvae with anal gills that form a circle or ring structure. Fraction 1 (emodin) had the lowest LC_{50} and LC_{90} values (LC_{50} = 15.44 and LC_{90} = 20.75 mg/L) and had a pupicidal efficiency (80%) against *Ae. aegypti*. *A. terreus* derived emodin inhibited the enzyme activities such as acetylcholineesterase, and carboxyl esterases, and phosphatases in the treated larvae. Histological changes in emodin-treated 4th instar larvae of examined mosquitoes resulted in mild injured muscles, gastric caeca cell rupture, disordered brush border, and discharges of cytoplasmic debris in the gastric caeca lumen. At higher doses (200 mg/L), the emodin showed no egg hatchability from the target mosquitoes. Reasonable sensitive *Artemia* nauplii was found with mortality during the emodin biotoxicity experiment on non-targeted organisms. The bioactive compound (emodin) demonstrated a high binding contact with the odorant-binding proteins in all examined mosquitoes, according to the computational study. The bioassay of emodin treated with *A. nauplii* revealed that the toxicity is mostly determined by compound dosages. The survival and hatching rates of zebrafish embryos treated with emodin were found to be significantly higher. The obtained results will give better avenues for selecting the most relevant compounds for the design and development of efficient, safe, and environmentally friendly mosquito repellents soon than the current harmful synthetic repellents.

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Author contribution The general design and planning of the experiment, larval data processing, and result interpretation were all provided by CR, GB, MS, PW, VP, SSN, PK, and DN. The molecular docking investigation and interpretation were carried out by MS and CR. The brine shrimp test toxicity experiments were carried out by CR. The embryo toxicity test was carried out by VM. The manuscript was co-written by CR, GB, PW, VP, SSN, PK, and DN. The final document has been thoroughly examined and authorized for publication by all of the writers and special contribution by DA, PK, and DN.

Data availability During the present research entities, the datasets gathered and generated from the analysis after extraction/separation/isolation of compound emodin and evaluated biological results are available from the corresponding author on reasonable request.

Declarations

The present research entities does not contain any studies with human participants or animals performed by any of the authors.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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