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RESEARCH ARTICLE

Potential induction of apoptosis and acetylcholinesterase inhibition in *Aedes* mosquitoes by *Streptomyces*-derived ethyl acetate extract

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ABSTRACT

The use of *Streptomyces* secondary metabolites for mosquito control has recently received positive attention. Accordingly, this study was performed to elucidate the cellular, genomic and biochemical responses of *Aedes* mosquitoes to *Streptomyces* sp. KSF103 ethyl acetate (EA) extract, a mixture previously characterized for its potential bioactivity. Through flow-cytometry based apoptosis assay, EA extract elicited apoptosis-mediated cell death in C6/36 cells of *Aedes albopictus*. Microarray analysis on *Ae. aegypti* larvae and adults revealed the potential involvement of tropomodulin and sestrin, which was validated by quantitative real-time RT-PCR, suggesting apoptosis induction in response to the EA extract. Functional analysis suggested that MAPK and Notch signaling pathways are linked to apoptosis. On the other hand, biochemical assays demonstrated acetylcholinesterase (AChE) inhibition in both larvae and adults, suggesting the toxicity disrupted their nervous system. In conclusion, this study has revealed the promising bioactivities of a *Streptomyces*-derived insecticide, providing insights into the mechanisms involved and emphasizing its potential significance in mosquito control.

Keywords: Bioinsecticide; mosquito control; actinobacteria; *Streptomyces*; natural product.

INTRODUCTION

An increasing corpus of published data demonstrates that *Streptomyces* secondary metabolites possess significant insecticidal activities against various insects including mosquitoes (Amelia-Yap *et al*., 2022). *Streptomyces* sp. KSF103, isolated from terrestrial soil of tropical rainforest, has demonstrated significant insecticidal effects against several medically important mosquito species. Notably, it exhibits larvicidal, adulticidal, and ovicidal activities without causing harm to non-target organisms (Amelia-Yap *et al*., 2023). Nevertheless, its underlying mode of action has not been identified. Therefore, identifying the underlying causes of cell death and potential genes associated with its insecticidal activities is critical. This knowledge will deepen our understanding of the precise mechanisms behind its action against mosquitoes. Armed with this understanding, we can strategically design more effective and environmentally friendly mosquito control strategies.

Utilizing a proteomic approach, Tan *et al*. (2023) demonstrated that the ethyl acetate (EA) extract of *Streptomyces* sp. KSF103 affects mosquitoes through a complex mechanism involving the upregulation of specific proteins associated with metabolism, energy production, and cellular organization. Molecular docking further suggests that fructose-bisphosphate aldolase (FBA) could serve as a promising target for innovating new insecticides. To comprehensively understand its mechanism of action, however, it is crucial to employ multiple investigative approaches. Thus, this study aimed to understand the cellular, genomic and biochemical responses of *Aedes* mosquitoes to this extract through flow cytometry, enzymatic and microarray analyses, in order to examine how the extract interacts with mosquito cells and influences their biological processes.

MATERIALS AND METHODS

Flow cytometry-based apoptosis assay

Apoptotic and necrotic cells were distinguished using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences). *Aedes albopictus* C6/36 cells were plated at a density of 4×10^6 cells per flask and maintained for 24 h prior to *Streptomyces* sp. KSF103 EA extract exposure for 48 h at CC_{25}, CC_{50}, and CC_{75} concentrations. Heat-treated cells produced from a heat treatment at 80°C for 1 min were used as positive control, while DMSO-treated cells served as a negative control. Cells were centrifuged at 300 rpm for 5 minutes and then resuspended in 1x Annexin-V binding solution. A 100 µL sample of the cell suspension was transferred into a FACS tube and stained with 5 µL of PI and 5 µL of Annexin-V-FITC for 15 minutes in the dark (CyAn ADP Analyzer, Beckman

Coulter, USA). Each experiment recorded 10,000 events per sample.

Biochemical assays

The inhibitory activities of the extract on acetylcholinesterase (AChE), non-specific esterase (EST), glutathione-S-transferase (GST) and mixed-function oxidase (MFO) of mosquitoes were assessed using biochemical assays with non-blood-fed female adults aged three to five days and fourth instar larvae. Prior to the biochemical assays, mosquito adults and larvae were treated with the EA extract using their respective LD_{50}/LC_{50} concentrations obtained from Amelia-Yap *et al*. (2023). LD_{50}/LC_{50} is the lowest concentration recorded to measure the acute toxicity of a material (Erhirhie *et al*., 2018), thus they were used in the subsequent experiments. Treatments using acetone and 1% DMSO were negative controls for the mosquito adults and larvae, respectively. Mosquito adults and larvae yielded from experimental and negative control groups with treatment durations of 0, 1 and 24 h were kept separately. To compare the variations in enzyme levels among individual *Ae. aegypti*, the *in vitro* activities of all enzymes were assessed according to Hemingway and Brogdon (1998) with minor modifications. The mosquitoes from two cohorts were individually tested for the activities of α-EST, β-EST, AChE, GST and MFO.

Twenty-four larvae and adult mosquitoes were each homogenized in 200 µL of ice-cold distilled water. A 25 µL portion of this homogenate was reserved for the AChE assay. The rest of the homogenate was centrifuged at 14,000 rpm for one minute at 4°C, and the resulting supernatant was utilized for the other enzyme assays. All experiments were carried out in triplicates using 96-well plates. The Infinite 200 Pro microplate reader (Tecan Trading AG, Mɲnnedorf, Switzerland) was used to measure the absorbances [optical density (OD) values].

The normalized data were analyzed using a two-way ANOVA in GraphPad Prism, with subsequent Bonferroni testing for significance ($P < 0.05$).

The details for each assay are provided below.

(i) Acetylcholinesterases (AChE) assay

A 25 µL sample of mosquito homogenate was combined with 145 µL of Triton phosphate buffer and 10 µL of 0.01 M dithiobis 2-nitrobenzoic acid solution. The reaction was started by adding 25 µL of 0.01 M acetylthiocholine iodide. One reaction was blocked by adding 0.05 µL of 0.1 M propoxur, while the other reaction continued without inhibition. After incubating for 1 hour at room temperature, the absorbance was measured at 405 nm (Leong *et al*., 2019). The AChE activity was determined based on the percentage of insensitivity to AChE activity following propoxur inhibition (Saelim *et al*., 2005).

(ii) Non-specific esterase assay

Non-specific esterases (EST) enzyme assays were conducted according to established protocols (Brogdon *et al*., 1988; Lee, 1990). In duplicates, 20 µl of mosquito homogenates were added to each well. Next, 200 µL of 30 mM α-naphthyl acetate was introduced to one set of samples in the 96-well plate, while another set received 200 µL of 30 mM β-naphthyl acetate. The plate was left at room temperature for 15 min before adding 50 µl of 3mM of fast-blue B salt. The following 15 min of incubation were terminated by adding 50 µl of 10% acetic acid. At 570 nm, the OD values were measured. Using standard absorbance curves for known concentrations of α-naphthol or β-naphthol, EST activity for each substrate was determined. The enzymatic activities were reported as nmol of α-naphthol or β-naphthol/min/mg protein.

(iii) Glutathione-S-transferases (GST) assay

First, 10 µL of mosquito homogenate supernatant was mixed with 200 µL of 63 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 10 mM reduced glutathione. The mixture was incubated for 20 minutes at room temperature, and OD values were measured at 340 nm. GST activity was calculated using Beer's Law (A = €cl) and expressed as CDNB/min/mg protein. The OD value was converted to ɔmol of CDNB conjugates using an extinction coefficient (€) of 4.39 mM^{-1}, with a path length of 0.6 cm (Leong *et al*., 2019).

(iv) Mixed function oxidases (MFO) assay

MFO enzyme assays were conducted following Brogdon *et al*. (1997). In duplicate wells, 2 µL of supernatant was combined with 80 µL of 0.625 M potassium phosphate buffer (pH 7.2), 200 µL of 2 mM TMBZ (dissolved in methanol), and 25 µL of 3% hydrogen peroxide (Leong *et al*., 2019). After 2 hours of incubation at room temperature, the OD was measured at 650 nm. MFO activity was calculated using a standard curve for cytochrome C and expressed as equivalent units of cytochrome P450/min/mg protein.

(v) Protein assay

All enzyme analyses were normalized for mosquito size variations using protein concentration as a correction factor. A BSA standard curve, created with a commercial protein assay kit (Bio-Rad, Hercules, CA, USA), was used to determine protein concentrations. For the assay, 10 µL of mosquito homogenate was mixed with 300 µL of Bio-Rad dye reagent and incubated for 5 minutes at room temperature. OD was then measured at 570 nm (Leong *et al*., 2019).

Microarray analysis

Total RNA was extracted from four groups: 15 EA extract-treated 4th instar larvae, 15 DMSO extract-treated 4th instar larvae, 15 EA extract-treated 3-day-old female mosquitoes, and 15 acetone-treated 3-day-old female mosquitoes, using the NucleoSpin RNA Mini Kit (Macherey-Nagel). RNA quantity and quality were assessed with a NanoDrop 1000 spectrophotometer and gel electrophoresis, selecting samples with a 230/260 to 260/280 ratio of 1.9-2.1. RNA integrity was further evaluated with the Agilent 2100 Bioanalyzer, with samples having RIN values above 5.8 used for microarray experiments, as optimal RIN for *Ae. aegypti* is around 6.5 (Fabrick & Hull, 2017).

The microarray experiment was outsourced to a biotechnology company. The custom high-density 60K array was created with 60-mer oligos targeting 28,165 gene transcripts from gene builds AaegL5_1, AaegL5_2, and AaegL5_3 (www.vectorbase.org); and GCF_002204515 (https://www.ncbi.nlm.nih.gov/) of *Ae. aegypti* LVP_AGWG. A set of 1319 random computer-generated probes were added as negative controls. Additional eArray-based quality controls were applied, leading to a microarray with a total of 62,976 features, printed in an 8 × 60K format. Two replicates per treatment were utilized.

The concentration of all RNA samples was normalized to 50 ng before the preparation of Cyanine- 3 (Cy3) labelled cRNA using the One-Color Low Input Quick Amp Labeling Kit (Agilent, Valencia, Ca) according to manufacturer's instructions. Cy3 labelled cRNA was then purified by RNeasy Mini Kit (Qiagen, Valencia, CA). Dye incorporation and cRNA yield were assessed using the NanoVue™ Plus Spectrophotometer (GE Healthcare, UK). Cy3-labeled cRNA (0.825 µg, ≥6 pmol Cy3/µg cRNA) was fragmented at 60°C for 30 minutes in a 25 µL reaction containing 25X Agilent fragmentation buffer and 10X Agilent blocking agent. Following fragmentation, 25 µL of the mixture was combined with 25 µL of 2× Hi-RPM Hybridisation Buffer and hybridized to the custom 8 x 60K array for 17 hours at 65°C in an Agilent hybridization oven. Post-hybridization, microarrays were washed

with GE Wash Buffer 1 (room temperature) and GE Wash Buffer 2 (37°C) for 1 minute each. Slides were scanned using the Agilent SureScan Microarray Scanner (G4900DA) at 532 nm with a 3 µm resolution and extended dynamic range (10–100%). Normalized intensities were extracted with Agilent Feature Extraction Software and analyzed using Agilent GeneSpring Software.

Microarray data were analyzed using Agilent GeneSpring Analysis Software version 14.9.1. The normalization method for the raw data employed percentile shift, where the location of all the spot intensities in an array was adjusted. Raw intensity data were then subtracted. The default in GeneSpring is to threshold the expression values to 1.

Fold change was calculated as a difference between pair(s) of samples/conditions. GeneSpring transforms linear intensities into log scale during experiment creation. The statistical test, moderated t-test, was used throughout the analysis in this project. The moderated t-test is a modification of the unpaired t-test, which was optimized to estimate the variance, vglobal and a degree of freedom, dglobal of the assumed normal distribution across genes.

Volcano plots were used to visually represent differential expression between two different conditions to provide a visual summary of p-values and fold-change values. Moderated t-test analysis was utilized to obtain genes whose fold change between treated and untreated mosquitoes was > 2.0 or < -2 (with a p-value cut-off of < 0.05).

Quantitative real-time RT-PCR (qRT-PCR)

Seventeen mosquito samples, including two biological replicates used in microarrays and fifteen from new batches, were used to validate, by real-time PCR, some of the over-expressed and repressed genes in the non-blood fed 3-day-old EA extract-treated female mosquitoes and EA extract-treated fourth instar mosquito larvae compared to their respective control groups. Total RNA from each sample was extracted as described earlier. Subsequently, cDNA for each sample was synthesized by reverse transcription using the qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, UK) with 0.4 µg of total RNA added as a template. PCR primers were designed using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the sequences were listed in Table 1. qRT-PCR reactions were performed using 10 µL of 2× qPCRBIO SyGREEN Mix (PCR Biosystems, London, UK), equal amounts of forward and reverse primers (10 µM) and 2 µL cDNA in a final volume of 20 µL. The RpS17 housekeeping gene (40S Ribosomal protein S14, AAEL005266) was used as an endogenous control (Dzaki *et al*., 2017). Each qRT-PCR run included measurements of RpS17 and the target gene in the samples. Standard amplification conditions were 2 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C. Dissociation curves were analyzed after each reaction to confirm the presence of a single peak for the desired amplicon, excluding contaminated DNA or primer dimers. Samples were analyzed in duplicate using the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems). Triplicate reactions were conducted on each sample, and the data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Functional characterization

Gene ontology (GO) analysis, a useful technique for functional gene annotation, provides support for the computational representation of biological systems with high-throughput genome or transcriptome data. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a set of databases designed to manage biological pathways, diseases, genomes, chemical compounds, and drugs. Functional characterization of DEGs detected from the moderated t-test, involving the upregulated and downregulated groups from both *Ae. aegypti* larvae and adults were included. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/), a bioinformatics-based web, was used for functional gene annotation in this experiment (Dennis *et al*., 2003). The DEGs were independently input into DAVID to obtain the GO and KEGG analyses, using P < 0.1 (the default setting) as the selection threshold.

RESULTS

Flow cytometric analysis on apoptosis

Flow cytometry analysis after Annexin V and PI labeling classified cells into four categories: Annexin V+/PI- (early apoptotic), Annexin V+/PI+ (late apoptotic), Annexin V-/PI- (live), and Annexin V-/PI+ (necrotic) (Pumiputavon *et al*., 2017). When apoptosis is measured over different concentrations of treatment, cells could be tracked from FITC Annexin V and PI negative (viable, or no measurable apoptosis) to FITC Annexin V positive and PI negative (early apoptosis, membrane integrity is present), and finally to FITC Annexin V and PI positive (end stage apoptosis and death). The movement of cells through these three stages suggests apoptosis. The DMSO-treated cells showed the percentage of the apoptotic cells as recorded at 81.75 ± 1.05 % after 48 h of incubation.

The EA extract showed varying percentages of intact and apoptotic cells on the treated cells, depending on the concentrations (Figure 1). The percentage of intact cells significantly decreased following treatment with the extract in a concentration-dependent manner. In C6/36 cells, the treatment with the lowest concentration (CC_{25}= 46.7 µg/mL) of the EA extract for 48 h yielded higher percentage of early apoptotic cells at 44.1 ± 1.56 %. Meanwhile, cells undergoing late apoptosis increased markedly as the concentration increased. Treatment with the sublethal concentration (CC50 = 152.2 µg/mL) led to a higher number of cells in the late apoptosis stage compared to early apoptosis, recorded at 51.5 ± 2.40% for late apoptosis and 39.8 ± 0.85 % for early apoptosis; whereas the treatment with the highest concentration (CC_{75}= 496.2 µg/mL) induced the highest percentage of late apoptosis at 92.5 ± 0.85 %. All concentrations showed significant differences compared to the negative control (P < 0.05). However, there was no notable change in the percentage of necrotic cells following treatment with the extract after 48 hours (Figure 2).

Biochemical assays

Changes in the activity of the detoxifying enzymes varied according to the exposure duration and the examined enzymes (Figure 3). After treatment with the EA extract at LC_{50} on the larvae, AChE activity showed a slight drop at 1 h compared to 0 h but rocketed at 24 h. Despite observing a fluctuating trend, 0 h (P < 0.0001), 1 h (P < 0.0001) and 24 h (P= 0.0105) showed a significant reduction of AChE activity in larvae compared to their respective control groups. In response to treatment with the EA extract at LD_{50} on the adult mosquitoes, the activity of AChE had significantly increased at 0 h (P = 0.0008) and 1 h (P < 0.0001) compared to the control groups. However, a fall of AChE activity was observed at an exposure time of 24 h, showing a significant reduction (P = 0.0414) relative to the control group.

Compared with their respective control groups, the activities of α-EST were significantly decreased at 0 h (P < 0.0001) and 1 h (P = 0.0078) with the EA extract at LC_{50} for the larvae. There was an initial increase in the overall activity of α-EST with time, followed by a decrease and a sharp increase. At 24 h, the inhibitory activity of the EA extract did not differ significantly relative to the control group. In response to the treatment with the EA extract at LD_{50} for adult mosquitoes, there were no significant differences recorded for the activity of α-EST between

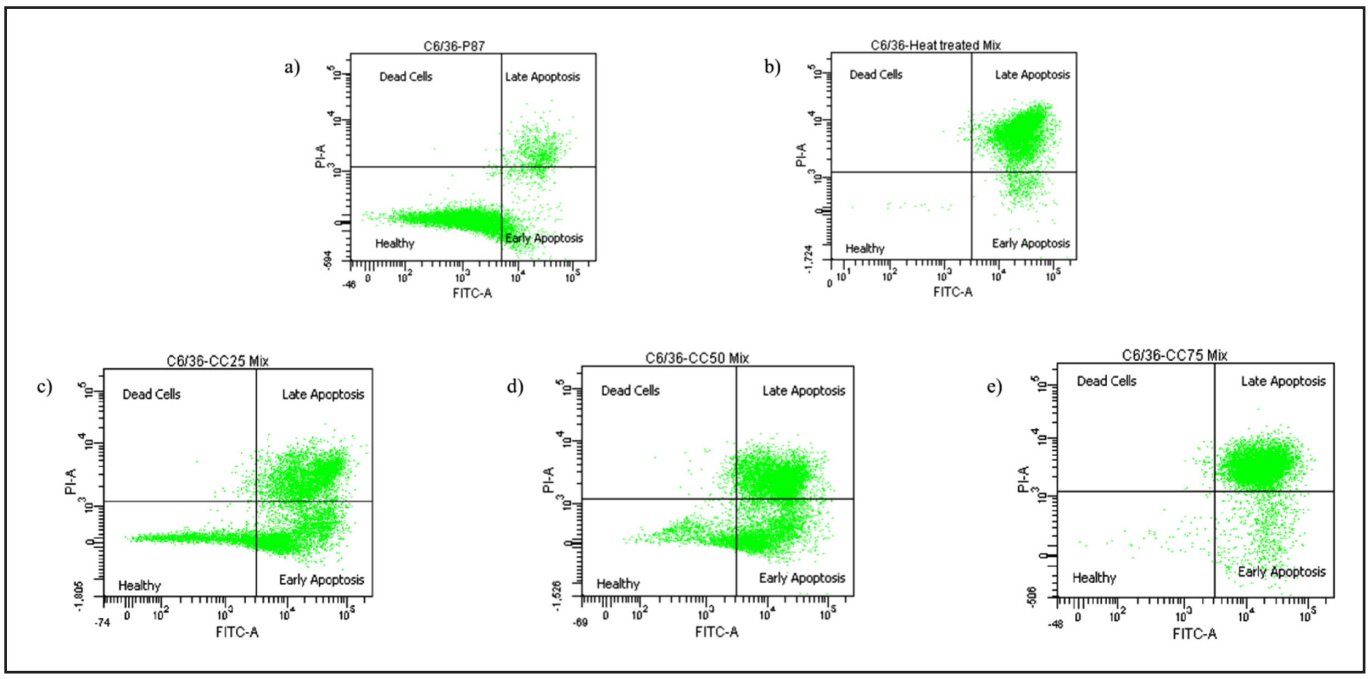

Figure 1. *Streptomyces* sp. KSF103 EA extract induced apoptosis in C6/36 cells. Apoptosis was evaluated using an annexin V-FITC apoptosis detection kit and flow cytometry. The X-and Y-axes represent fluorescence intensity (on log scale) of annexin V-FITC staining and PI, respectively. These plots can be divided into four regions corresponding to: 1) viable cells (AV/PI -/-; Q3); 2) early apoptotic cells (AV/PI +/-; Q1); 3) late apoptotic cells (AV/PI +/+; Q2); 4); and necrotic cells (AV/PI -/+; Q4). These figures represent the flow cytometric apoptosis analysis in C6/36 cells with different treatments: a) DMSO-treated cells; b) heat-treated cells; c) CC_{25}; d) CC_{50}; and e) CC_{75}.

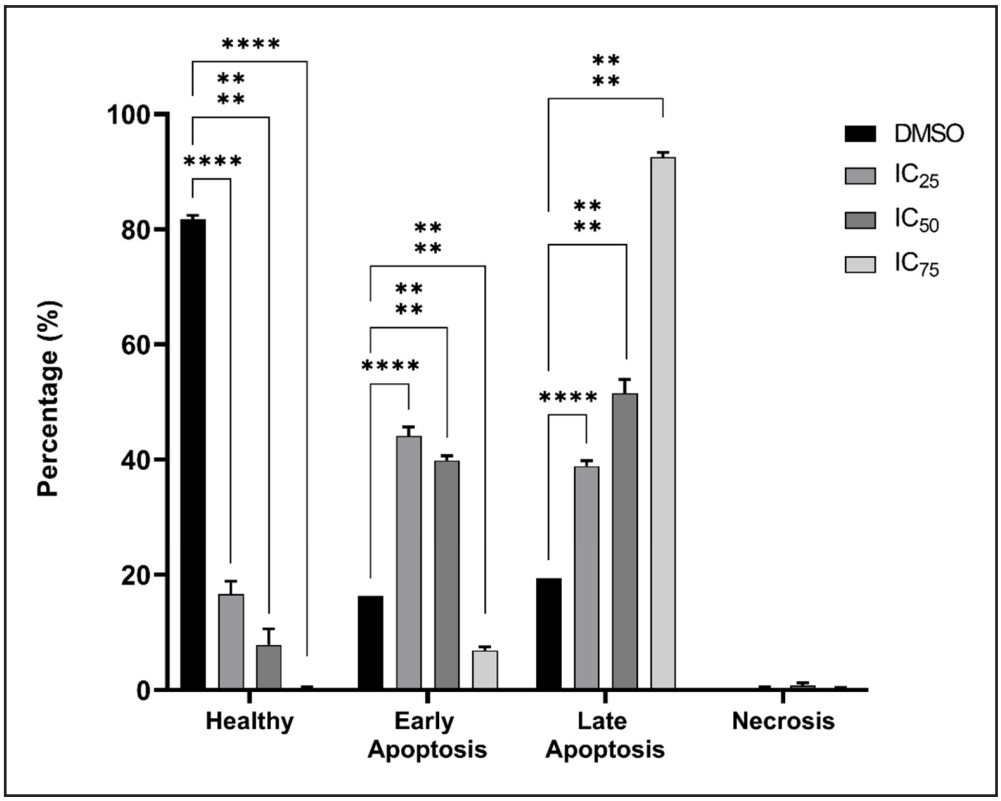

Figure 2. Bar graph of apoptosis, quantified by FACS, after Annexin V-FITC and PI labelling. The results were shown as mean ± SD of three independent experiments with ****P < 0.0001 indicated a significant difference compared to the DMSO-treated control analyzed by the t-test.

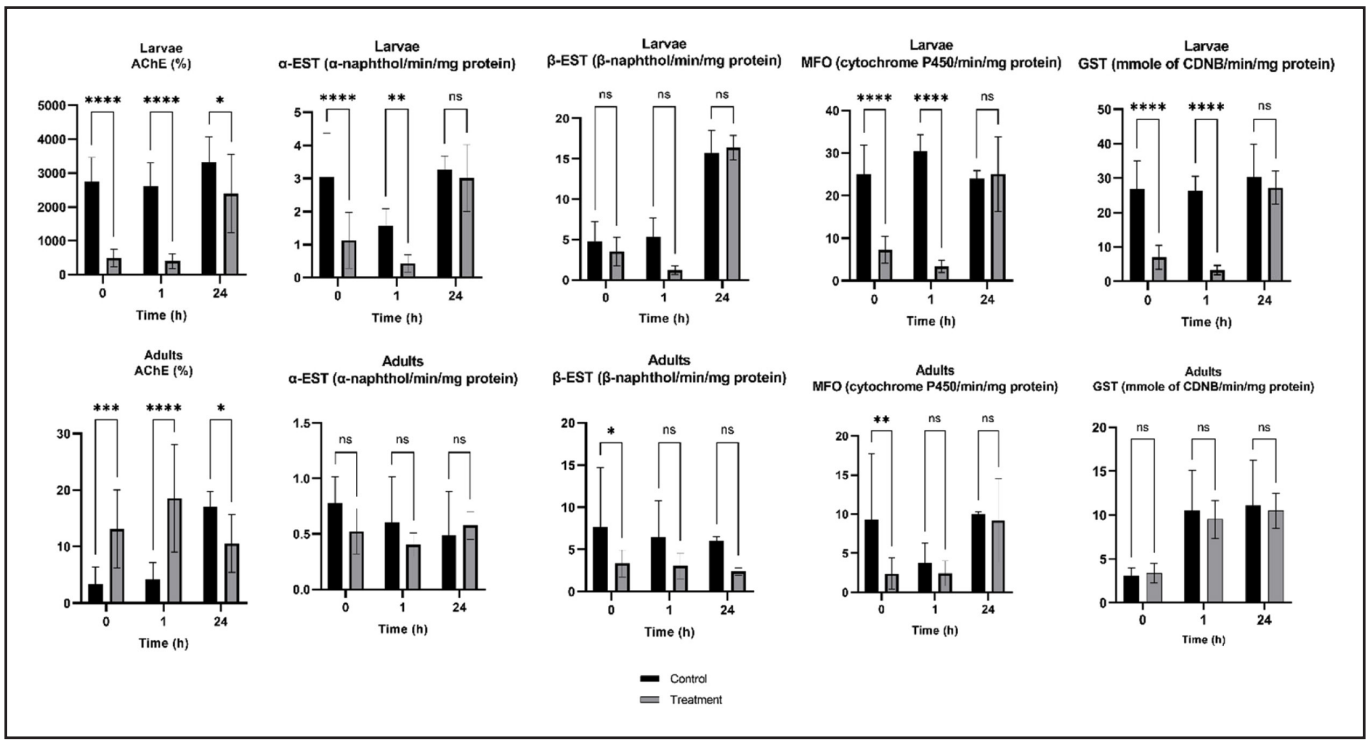

Figure 3. Biochemical effects of *Streptomyces* sp. KSF103 EA extract on *Ae. aegypti* larvae and adults. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, no significant difference, using Bonferroni's multiple comparisons test.

the treatment and control groups across all three timepoints. There were no significant differences on the β-EST activities between the treatment and the control groups at 0 h, 1 h and 24 h, after the treatment with the extract at LC_{50} on the mosquito larvae. The activity of β-EST was significantly decreased at 0 h (P = 0.0224) of the treatment with the EA extract at LD_{50} for the adult mosquitoes compared with the control group but showed no significant differences when exposure durations were increased to 1 h and 24 h.

In response to treatment with the EA extract at LC_{50} on the mosquito larvae, the MFO activity initially decreased at 1 h, and subsequently increased again with a prolonged exposure time. With an observable fluctuation trend of the MFO activity in the treatment groups, only 0 h (P < 0.0001) and 1 h (P < 0.0001) demonstrated significance reduction in comparison to the control groups. At 24 h, no significant difference was demonstrated in the treatment group compared to the control group. After treatment with the EA extract at LD_{50} on the adult mosquitoes, the activity of MFO was significantly reduced at 0 h (P = 0.0025) of exposure duration relative to the control group. At 1 h and 24 h of exposure duration, the inhibitory activity of the EA extract did not differ significantly in contrast to their respective control groups.

Based on the LC_{50} treatment on the larvae, there were significant differences at 0 h (P < 0.0001) and 1 h (P < 0.0001) in GST inhibition by the test extract, in comparison to their respective groups. However, the inhibitory activity of the EA extract did not differ significantly relative to its control group at 24 h. In response to the treatment with the EA extract at LD_{50} for adult mosquitoes, no significant differences were documented for GST activity between the treatment and control groups across all three timepoints.

Microarray analysis of differential expression

To identify significant differences in gene expression, fold change > 2 or < -2 and p-value < 0.05 obtained from moderated t-test were used as the threshold. Based on this parameter, 164 genes were differentially expressed in *Ae. aegypti* larvae. Among the differentially expressed genes (DEGs), 123 genes were upregulated, and 41 genes were downregulated in response to the EA extract. The number of upregulated genes was more than the number of downregulated genes. After performing moderated t-test analyses, 169 genes were found to be differentially expressed in adults *Ae. aegypti*. Among the differentially expressed genes, 106 genes were upregulated whereas 63 genes were downregulated in response to the EA extract. The number of upregulated genes was also more than the number of downregulated genes. The complete list of differentially expressed genes in *Ae. aegypti* were presented in Supplementary Table 1.

Table 1. Primers used for quantitative real-time reverse transcriptase polymerase chain reaction

Genes	Sequences	Product size (bp)
Tropomodulin	(Tmod_Aeg) Forward: 5'-CAATATCGTCATTCGCCCGC-3' Reverse: 5'-GCGTCAAACTAGCAGCATGAG-3'	143
Sestrin	(Ses_Aeg) Forward: 5'-CTGGCCCACTTCCATTCCTT-3' Reverse: 5'-GATTGCGGTGGGATGATTGC-3'	185
Multidrug resistance protein 4	(MRP4_Aeg) Forward: 5'-TACTTCCTTCTGGTGGTGCG-3' Reverse: 5'-TGTCGCGGTTCATGATGAGT-3'	114
Ribosomal s17	(Rps17) Forward: 5'-AAGAAGTGGCCATCATTCCA-3' Reverse: 5'-GGTCTCCGGGTCGACTTC-3'	200

Genes differing in expression were represented in volcano plots, which Figure 4 (a) represented larvae while Figure 4 (b) represented adults. The volcano plots showed the negative $\log_{10}$ of p-value versus $\log_2$ of fold change. The genes were colored depending on their passing the default cut-offs. Genes upregulated which pass both p-value and fold change cut-offs were depicted in red, and genes downregulated which pass both p-value and fold change cut-offs were shown in dark blue. Light blue represented genes downregulated which pass the p-value cut-off but fail to pass the fold change cut-off, while orange represented genes upregulated which pass the p-value cut-off but fail to pass the fold change cut-off. Grey represented genes in the array neither pass the p-value cut-off nor the fold change cut-off, which were not found to differ significantly between the mosquitoes treated with the EA extract and the control mosquitoes.

The data on the top 10 genes with the highest fold changes in *Ae. aegypti* larvae and adults compared with the controls were listed in Tables 2 and 3, respectively. Overlapping genes between *Ae. aegypti* larvae and adults were listed in Table 4.

Validation of microarray results by quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed to validate the differential expression of three candidate genes (Table 1), in which tropomodulin and sestrin were detected as significantly upregulated in the larvae and adults *Ae. aegypti* treated with EA extract compared to their respective untreated groups. These genes were selected based on previous associations with apoptosis, their expression levels in microarrays, or the overlapping genes discovered from the results of mosquito larvae and adults. MRP4, a multidrug resistance (MDR) protein which

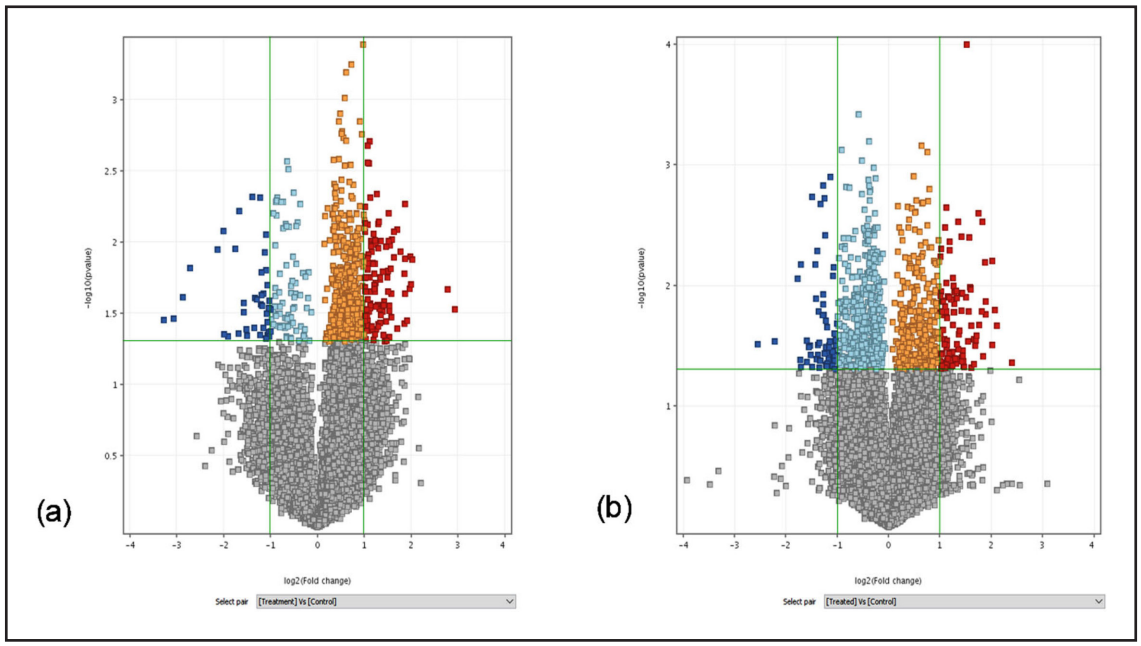

Figure 4. Volcano plots of probe sets differing between *Ae. aegypti* larvae (a) and adults (b) treated with *Streptomyces* sp. KSF103 EA extract and negative controls. The representations are as follows: x-axis, $\log_2$ of fold change; y-axis, $-\log_{10}$ of p-value. Thresholds are shown as green lines. For a gene to be differentially expressed, it must satisfy the criteria of p-value < 0.05 and fold change > 2 or < –2. For larvae, 154 expressed genes (DEGs) are detected, with 123 upregulated shown in red, and 41 downregulated genes were shown in dark blue on the plot. For adults, 169 differentially expressed genes (DEGs) are detected, with 106 upregulated shown in red, and 63 downregulated genes were shown in dark blue on the plot.

Table 2. Top 10 genes found to be significantly upregulated and downregulated in larvae *Ae. aegypti* compared with negative controls

Accessions	Gene Title	Fold Change Abs	Regulation	P-value
XM_021844388.1	sodium/hydrogen exchanger 9B2 (LOC5574378)	7.300	Down	0.024
XM_001654666.2	neural/ectodermal development factor IMP-L2 (LOC5573583)	6.858	Up	0.021
XM_001663184.2	arrestin homolog (LOC5577143)	4.064	Up	0.013
XM_001662479.2	multidrug resistance-associated protein 4 (LOC5576192)	4.038	Down	0.008
XM_021841155.1	transmembrane protein 184B (LOC5566232)	3.973	Up	0.020
XM_021857708.1	zinc finger C2HC domain-containing protein 1C (LOC5580206)	3.658	Up	0.013
XM_001654701.2	3-ketoacyl-CoA thiolase, mitochondrial (LOC5573712)	3.645	Up	0.023
XR_002500887.1	mitochondrial carrier protein Rim2 (LOC5575850)	3.406	Up	0.012
XM_021853964.1	lysine-specific demethylase 3B (LOC5568859)	3.361	Down	0.011
XM_021845000.1	sodium/potassium-transporting ATPase subunit beta-2 (LOC5572931)	3.242	Up	0.041

Table 3. Top 10 genes found to be significantly upregulated and downregulated in adults *Ae. aegypti* compared with negative controls

Accessions	Gene	Fold Change Abs	Regulation	P-Value
XM_001661346.2	HD domain-containing protein 2 (LOC5574363)	5.256	Up	0.044
XM_001654826.2	protein rolling stone (LOC5573844)	4.284	Up	0.022
XM_021847450.1	tropomodulin (LOC5568680)	4.191	Up	0.016
XM_021848757.1	ataxin-1 (LOC5580105)	4.062	Up	0.031
XM_001650495.2	leucine-rich repeat-containing protein 58 (LOC5566166)	4.052	Up	0.006
XM_001650735.2	sugar transporter SWEET1 (LOC5566373)	3.664	Up	0.006
XM_001662618.2	uncharacterized LOC5576475 (LOC5576475)	3.583	Up	0.039
XM_021852488.1	OTU domain-containing protein 7B (LOC5567349)	3.553	Up	0.003
XM_021845830.1	nicotinate phosphoribosyltransferase (LOC5565689)	3.503	Up	0.016
XM_021848506.1	ras-interacting protein RIP3 (LOC5569142)	3.420	Down	0.009

Table 4. Overlapping genes of *Ae. aegypti* larvae and adults treated with *Streptomyces* sp. KSF EA extract

Accessions	Gene	Fold Change Abs	Regulation	P-Value
XM_021847450.1	tropomodulin (LOC5568680)	4.191	Up	0.016
XM_021841318.1	sestrin homolog (LOC5574799)	2.358	Up	0.046

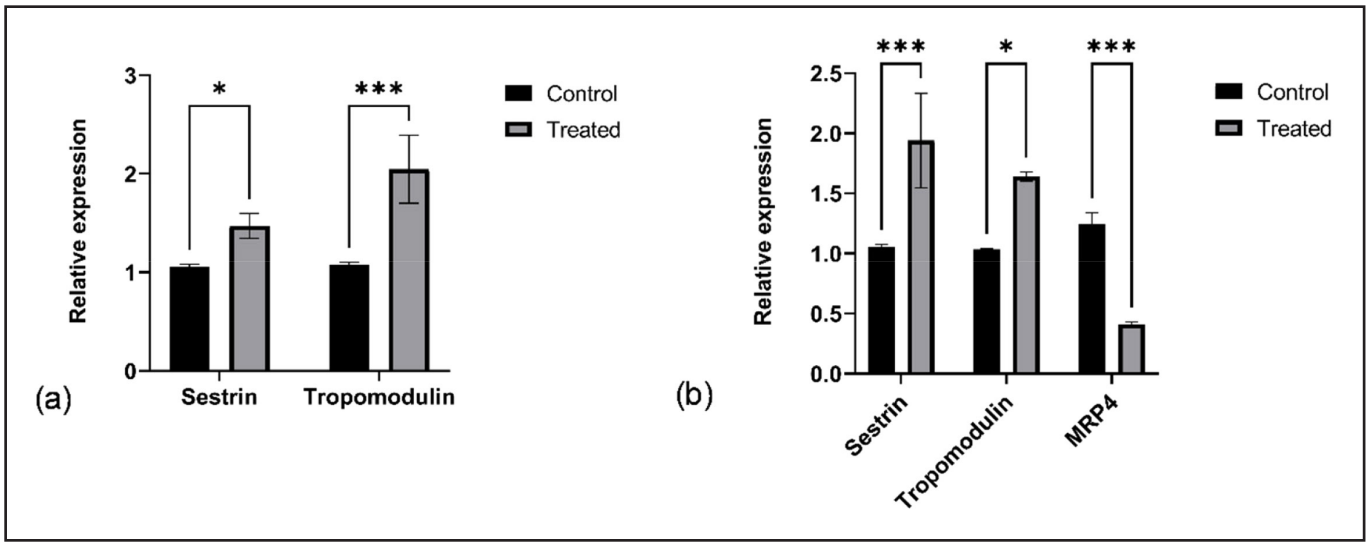

Figure 5. qRT-PCR analysis of DEGs in adults (a) and larvae (b). *P < 0.05, ***P < 0.001 indicated a significant difference compared to the control analysed by the t-test.

was downregulated, was also selected as a candidate gene for *Ae. aegypti* larvae. All candidate genes validated in treated *Ae. aegypti* had demonstrated significant differences relative to the control groups (P < 0.05). The estimates from qRT-PCR verified the same expression patterns of the tested genes, thus providing validation of the significance indicated by microarrays (Figure 5).

Functional analysis

The results from the GO term enrichment analysis (Table 5) showed differences in expression levels and GO classification of DEGs between larvae and adult *Ae. aegypti*. The genes were categorized into three GO categories, namely molecular function (MF), biological process (BP), and cellular component (CC). By analyzing GO enrichment of these DEGs of adults *Ae. aegypti* via DAVID, the DEGs in MF were principally enriched in ATP binding, ATP-dependent microtubule motor activity, and ATPase activity coupled to transmembrane movement of substances. In larvae *Ae. aegypti*, BP analysis uncovered that the DEGs are responsible for regulating DNA-templated transcription. The DEGs in CC were mainly enriched in mitochondrion. Among the MF terms, the DEGs were enriched in ATP binding and protein serine/threonine kinase activity.

The selected DEGs were functionally classified based on the KEGG pathway analysis via DAVID (Table 6). In adults *Ae. aegypti*, the KEGG enrichment results showed that the DEGs were enriched in citrate cycle (TCA cycle) and carbon metabolism. In larvae *Ae. aegypti*, the DEGs were enriched in five pathways, which mainly included MAPK signalling pathway – fly (Figure 6), biosynthesis of amino acids, notch signalling pathway (Figure 7), citrate cycle (TCA cycle) and Wnt signalling pathway (Figure 8). Due to the limitation of DAVID, MAPK signalling pathway of fly was used as a comparison in this contex.

Table 5. The GO annotation of DEGs of adults and larvae *Ae. aegypti* treated with *Streptomyces* sp. KSF103 EA extract

Experimental group	Category	Term	Count	P-value
Adults	GOTERM_MF_DIRECT	GO:0005524: ATP binding	14	0.007
	GOTERM_MF_DIRECT	GO:0003777: ATP-dependent microtubule motor activity	3	0.025
	GOTERM_MF_DIRECT	GO:0042626: ATPase activity, coupled to transmembrane movement of substances	3	0.082
Larvae	GOTERM_BP_DIRECT	GO:0006355: regulation of transcription, DNA-templated	4	0.092
	GOTERM_CC_DIRECT	GO:0005739: mitochondrion	4	0.053
	GOTERM_MF_DIRECT	GO:0005524: ATP binding	14	0.036
	GOTERM_MF_DIRECT	GO:0004674: protein serine/threonine kinase activity	3	0.05

Table 6. KEGG pathway analysis of DEGs associated with adults and larvae *Ae. aegypti* treated with *Streptomyces* sp. KSF103 EA extract

Experimental group	Category	Term	Count	P-value
Adults	KEGG_PATHWAY	Citrate cycle (TCA cycle)	3	0.047
	KEGG_PATHWAY	Carbon metabolism	4	0.096
Larvae	KEGG_PATHWAY	MAPK signaling pathway – fly	5	0.017
	KEGG_PATHWAY	Biosynthesis of amino acids	4	0.022
	KEGG_PATHWAY	Notch signaling pathway	3	0.032
	KEGG_PATHWAY	Citrate cycle (TCA cycle)	3	0.047
	KEGG_PATHWAY	Wnt signaling pathway	4	0.051

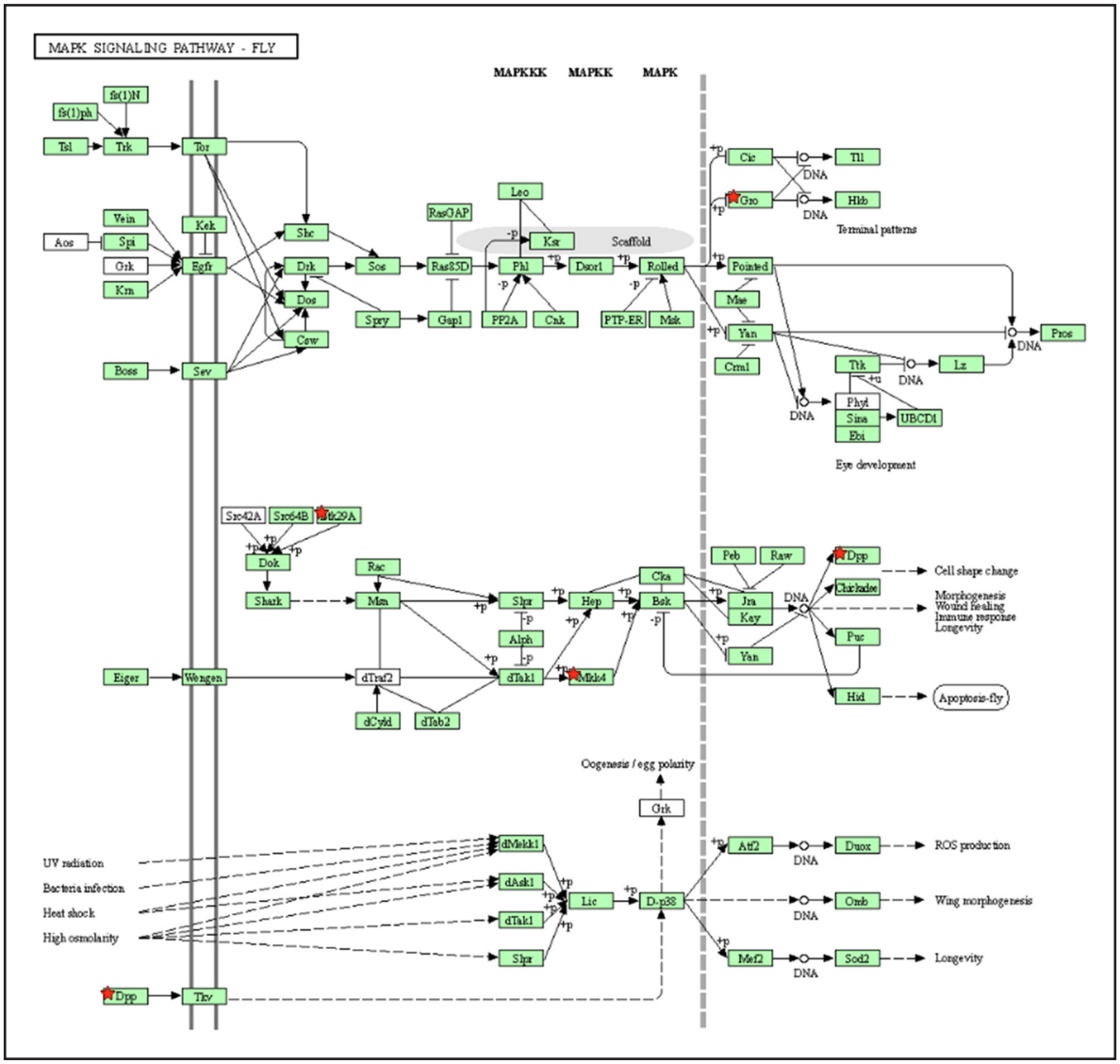

Figure 6. KEGG pathway for MAPK-signalling pathyway- fly. DEGs from the microarray results are starred in red.

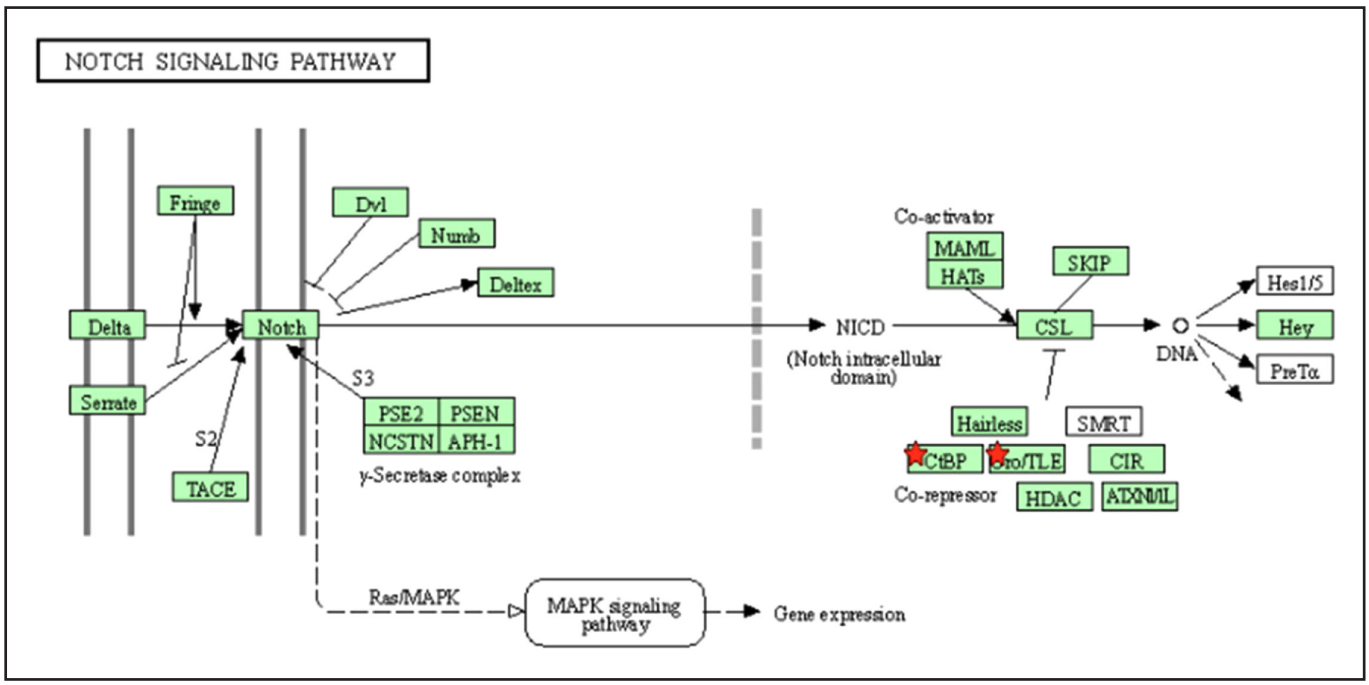

Figure 7. KEGG pathway for Notch signalling pathway. DEGs from the microarray results are starred in red.

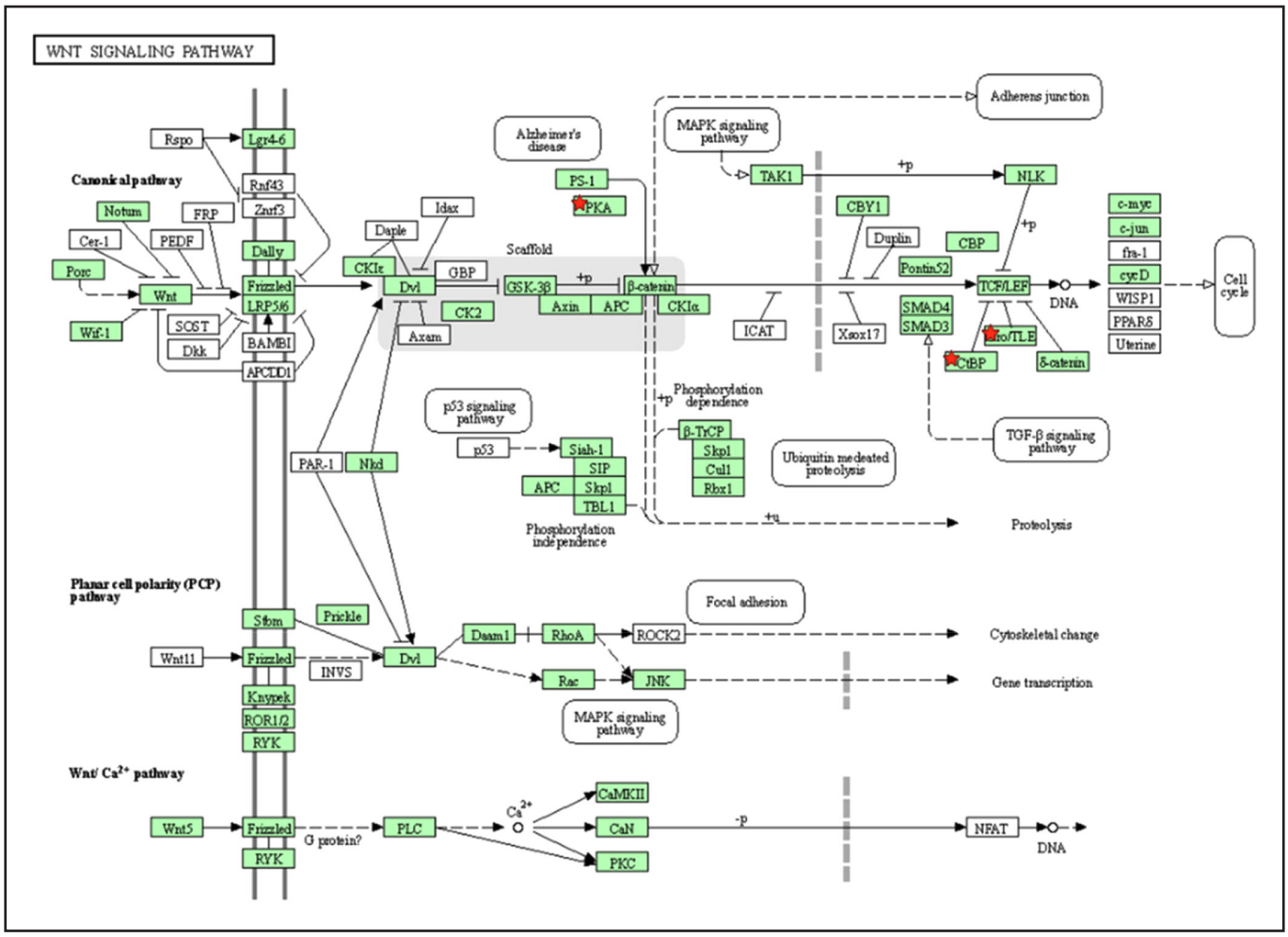

Figure 8. KEGG pathway for Wnt signalling pathway. DEGs from the microarray results are starred in red.

DISCUSSION

Flow cytometric analysis on apoptosis

Apoptosis confirmation was performed using the simultaneous staining of cells with FITC-Annexin-V/PI, which enabled bivariate analysis to distinguish between intact cells (FITC– PI–), early apoptotic (FITC+ PI–), late apoptotic (FITC+ PI+), and necrotic cells (FITC– PI+) (Abdolmohammadi *et al*., 2009; Juan *et al*., 2012). When the integrity of the cytoplasmic membrane is compromised, PI can enter the nucleus and stain the DNA (Hawley & Hawley, 2004). In a concentration-dependent manner, treatment with *Streptomyces* sp. KSF103 EA extract exhibited that it induced the permeabilisation of plasma membrane of C6/36 cells which permitted the binding of PI to double-stranded DNA by intercalating between base pairs. This indicated that the EA extract could elicit cell death via induction of apoptosis, which is a key regulator of physiological growth control and regulation of tissue homeostasis (Elmore, 2007).

Apoptosis is a programmed cell death process that occurs spontaneously in response to adverse stimuli; this process is crucial in getting rid of abnormal or unwanted cells and plays an essential role in the stability of the internal environment and the balance of development in a multicellular organism (Suganuma *et al*., 2011; Huang *et al*., 2013). Recently, the significance of apoptosis in insect growth and development has attracted attention to its application in pest management (Rhee *et al*., 2002; Parthasarathy & Palli, 2007). Insecticides may function in part by inducing apoptosis. As a protective strategy, apoptosis occurs, for instance, in the midguts, salivary glands, and ovaries of honeybee larvae following insecticide exposure (Gregorc & Ellis, 2011). Moreover, after exposure to sublethal doses of imidacloprid, apoptosis and autophagy were significantly increased in the brain of worker honeybees (*Apis mellifera*) (Wu *et al*., 2015). A number of botanical insecticides also revealed the same function to induce apoptosis in diverse insect species. An increase in the expression of programmed cell death protein 11, a protein involved in the process of apoptosis, has been linked to camptothecin-induced apoptosis in the midgut epithelial cells of *Spodoptera litura* (Gong *et al*., 2014). A famous botanical insecticide, azadirachtin, triggered apoptosis in the cell lines of insects and has been investigated in greater depth during the past few decades. In Sl-1 cell lines, azadirachtin induced the caspase-dependent mitochondrial pathway via activating the cleavage of caspase-3 (Huang *et al*., 2011). Azadirachtin also induced apoptosis in midgut epithelial cells of *S. litura* (Shu *et al*., 2015). In addition, the cry toxins from *Bacillus thuringiensis*, promoted mitochondrial permeabilisation and caused apoptosis in *Ae. aegypti* larvae (Lemeshko & Orduz, 2013).

The results confirmed that *Streptomyces* sp. KSF103 EA extract induced apoptosis in C6/36 cells. With a dearth of related research, it is unclear, nevertheless, whether apoptosis induction may be classified as a unique mechanism or as one of the many targets of synergistic processes.

Biochemical assays

An investigation of the preliminary mode of action and resistance mechanisms of insecticides from the bacteria origins can yield valuable insights for the development of streamlined mosquito control alternatives with novel target sites and lower dosages with higher toxicities, and also assessing the most suitable formulations and delivery mode to be used for future commercialisation and resistance management (Isman, 2006). The target sites and mechanisms behind the insecticidal activities of secondary metabolites, including several bacterial derivatives and plant-derived secondary metabolites (Isman, 2006), have received much attention. For example, avermectin and its analogues selectively prevent electrical impulse transmission in the membranes of pharyngeal muscles, motor nerves, female reproductive tracts, excretory/secretory (ES) pores of nematodes, and muscle and nerves of insects and crustaceans by amplifying glutamate effects on glutamate-gated chloride ion channels (Bloomquist, 2003; Subbanna *et al*., 2019). Spinosad demonstrates effects on target insects consistent with nicotinic acetylcholine receptor activation. It also affects GABA receptors (dos Santos Dias *et al*., 2017). Moxidectin works by opening the chloride channel in the plasma membrane of the neurological and neuromuscular systems. In mosquito larvae, prodigiosin isolated from *Serratia marcescens* displayed differences in the enzyme activities: elevated esterases, reduced AChE, reduced phosphatases, and reduced proteases (Suryawanshi *et al*., 2015).

Studies show that essential oils work by reversibly inhibiting acetylcholinesterase (AChE) enzymes isolated from houseflies (Jankowska *et al*., 2017; Chintalchere *et al*., 2020). Honokiol inhibited AChE moderately and significantly increased cyclic AMP levels, revealing that it may act on both AChE and octopaminergic receptors (Wang *et al*., 2019). Asaricin and isoasarone extracted Piperaceae plant significantly suppressed AChE in the Indian meal moth and American wheat weevil (Hematpoor *et al*., 2017). Aqueous extract of soapnut kernel with demonstrated mosquitocidal efficacy (Koodalingam *et al*., 2009) significantly decreased AChE and carboxylesterase levels in mosquito larvae (Koodalingam *et al*., 2011). Target site insensitivity, which diminishes the sensitivity of sodium channels in the nervous system to pyrethroids; or sensitivity of the key enzyme AChE to OP and carbamates, as well as enhanced metabolic detoxification in multiple groups of insecticides, are the major mechanisms of resistance to insecticides presently used in mosquito control (Hemingway *et al*., 2004). Several SMs are particularly efficient against insecticide-resistant mosquitos (Perumalsamy *et al*., 2010; Wang *et al*. 2012), suggesting that they could be useful in resistance management strategies. α-asarone, (–)-asarinin, and pellitorine, for instance, can control the field populations of *Cx. pipiens pallens* and *An. sinensis*, which have already developed high levels of resistance to AChE inhibitors chlorpyrifos, fenitrothion, and fenthion; axonic nerve poisons α-cypermethrin and deltamethrin; and mitochondrial uncoupler chlorfen (Perumalsamy *et al*., 2010).

The suboptimal effects of insecticides on different insect species involve insect behavioural changes, development, reproduction, and insecticide resistance. Insect response to insecticide exposure is a complicated detoxification mechanism that involves several enzymes. AChE biochemical testing demonstrated the inhibition of this enzyme in the presence of *Streptomyces* sp. KSF103 EA extract. AChE activities were significantly reduced in the treated mosquitos. This finding suggested that the EA extract directly affected the nervous systems of mosquito larvae and adults. Likewise, previous study has also demonstrated that *Streptomyces rimosus* affected *Cx. quinquefasciatus* through AChE inhibition (Ganesan *et al*., 2018).

No significant difference was shown in EST, MFO and GST. The role of enhanced EST activity was associated with the degree of malathion resistance (Hemingway & Georghiou, 1983). ESTs and GSTs are often overexpressed in pyrethroid-resistant *Ae. aegypti* (Amelia-Yap *et al*., 2018). MFO and, to a lesser extent, EST are the most prevalent enzymes responsible for detoxifying DDT and pyrethroids (Scott & Wen, 2001). The finding may possibly reveal that the active ingredients in the present study differs from that of pyrethroids, DDT, and organophosphates. In addition, the roles of esterases, oxidases, and GST in insect detoxification relied on the structure and concentration of the insecticides and probably the age of the insect under investigation (Thom *et al*., 2001). The pyrethroid-resistant Puerto Rican strain of *Ae. aegypti*

demonstrated that non-specific esterase did not correlate with a quantitative change in esterase activity (Hemingway & Georghiou, 1983).

It has been stated that natural products, which comprise a blend of compounds, are more complex than synthetic insecticides and, as a result, postpone the emergence of resistance (Vollinger, 1987). A previous study reported that the diamondback moth quickly develops resistance to all major classes of synthetic insecticides. On the contrary, after 42 generations of selection, acquiring resistance to neem was unsuccessful because of its complicated mode of action and constituents (Vollinger, 1987; Schmutterer, 1988). The interplay of behavioural and physiological effects of natural insecticides inhibits the emergence of resistance (Rice, 1993). Thus, insecticides with novel modes of action are constantly in demand to combat resistance issues. Previous studies stated that the presence of multiple resistance mechanisms (e.g., target-site insensitivity, reduced penetration, and insecticide detoxification) suggest that resistance to any class of insecticide may occur given sufficient time, constant selection pressure, and a sizable population for selection to take place (Sun, 1992; Rattan, 2010). However, novel crude compounds derived from nature with particular or several target sites have a lower incidence of developing resistance (Sun, 1992).

Microarray analysis

Differentially expressed genes (DEGs) of different functional classes were modulated by the EA extract in mosquitoes. Among the modulated genes, genes associated with apoptosis, namely tropomodulin (tmod) and sestrin, were upregulated in both life stages of mosquitoes.

The microarray analysis identified overlapping DEGs in both mosquito larvae and adults, with both sets of DEGs associated with apoptosis. Tmod is the only protein known to cap the pointed end of tropomyosin-coated actin filaments, which typically consist of a tropomyosin-binding helix region at the N-terminus, an actin-binding region, and LRR regions at the C-terminus (Rao *et al*., 2014). Tmod plays a vital role in regulating actin dynamics and cytoskeletal structure, further determining cell morphology, cytomechanics, contraction, and dendritic processes (Gray *et al*., 2018). Tmod has also been found to be crucial for apoptosis (Desouza *et al*., 2012). It has been discovered that Tmod expression restores thymosin β-10-mediated apoptosis in SKOV-3 ovarian cancer cells (Rho *et al*., 2004).

Aside from tmod, the EA extract triggered apoptosis through the expression of sestrin, representing another overlapping DEG discovered in mosquito larvae and adults. Sestrin expression is induced upon DNA damages such as UV-irradiation, and oxidative stress like hypoxia (Kim *et al*., 2013). A previous study has reported that sestrin led to apoptosis in cancer (Budanov *et al*., 2002). Moreover, quercetin, a plant flavonoid, was found to induce apoptosis via sestrin 2/AMPK/mTOR pathway in HT-29 colon cancer cells (Kim *et al*., 2013).

Azadirachtin is a tetranortriterpenoid limonoid derived from *A. indica* which could regulate growth and cocooning in *Bombyx mori* L. by triggering apoptosis in the prothoracic gland (Zhang *et al*., 2017). Furthermore, Shu *et al*. (2018) reported that azadirachtin inhibited the growth of *S. litura* larvae by causing the midgut cells to undergo apoptosis (including intestinal wall cracking, abnormal cell structure, and cell death). The analysis comparing untreated and Bin-treated *Cx. quinquefasciatus* larvae showed variations in the expression of transcripts related to mitochondria-mediated apoptosis (Tangsongcharoen *et al*., 2017). Another study comparing susceptible and Bin-resistant larvae found a remarkable differential expression of transcripts involved in apoptosis (Rezende *et al*., 2019). Similar to the findings from past studies of azadirachtin and Bin toxin produced by *Lysinibacillus sphaericus*, the current results demonstrated that the preliminary mode of action of EA extract could possibly involve apoptosis induction in *Ae. aegypti*.

Although arthropods lack the adaptive response of vertebrates, an unfavorable input stimulates a variety of effective cellular and humoral mechanisms. Various forms of cell death, including apoptosis, are crucial for arthropod immune defense, as apoptosis acts as a protective mechanism by eliminating damaged cells. Apoptosis can be driven by a variety of factors which include signaling via death receptors (DR); DNA damage by UV, γ-irradiation, or genotoxic drugs; the load on endoplasmic reticulum (ER); withdrawal of growth or trophic factors; oxidative stress; and a large number of other factors (Kook *et al*., 2014).

Several proteins are predicted to play roles in regulating apoptosis in *Ae. aegypti* are IAP1, Ark, Dronc, the IAP antagonists Mx and IMP, a number of effector caspases, and several other proteins more peripheral to the core apoptosis pathway (Bryant *et al*., 2008). A detailed study of the processes governing apoptosis in disease vectors like mosquitoes may help improve disease management strategies that target the apoptosis pathway.

One of the many mechanisms by which insects react to xenobiotic insult, such as insecticide exposure, is metabolic detoxification and subsequent excretion (Panini *et al*., 2016). Phase I and II reactions involve the metabolisation of destructive molecules by enzymes such as EST, GST, and MFO to reduce their toxicity or make them more easily transported, whereas phase III involves elimination and sequestration by transmembrane efflux transporters, such as multidrug resistance (MDR) proteins (Favell *et al*., 2020). The multi-transmembrane protein known as multidrug resistance protein 4 (MRP4), sometimes referred to as ATP-binding cassette subfamily C member 4 (ABCC4), is able to transport a wide range of compounds (both endogenous and xenobiotic) out of the cell and plays a role in determining drug sensitivity (Errasti-Murugarren and Pastor-Anglada 2010). This versatile transporter has been associated with extracellular signalling pathways, drug resistance in cancers and cellular protection (Hardy *et al*., 2019). One unexplored possible source of insecticide detoxifying activity in *Ae. aegypti* is the MDR genes. ABC transporters, including MDRs, have been identified as resistance factors in insecticide resistant strains of tobacco budworm, *Heliothis virescens* (Lanning *et al*., 1996), spider mite, *Tetranychus urticae* (Riga *et al*., 2014), small brown planthopper, *Laodelphax striatellus* (Sun *et al*., 2017), and the leaf beetle *Chrysomela tremula* (Pauchet *et al*., 2016), all of which are agricultural pests. In this study, a downregulation in MRP4 in *Streptomyces* sp. KSF103 EA extract-treated mosquitoes was found, suggesting that the extract can potentially prevent insecticide resistance. Strategies aimed at halting the emergence of insecticide resistance will benefit greatly from a deeper understanding of how phase III excretion mechanisms might be enhanced to aid in the metabolism of insecticides.

MAPKs have been wide studied in both vertebrates (Krens *et al*., 2006) and invertebrates, for examples, *Caenorhabditis elegans* (Sakaguchi *et al*., 2004), *Anopheles gambiae* (Horton *et al*., 2011) and *D. melanogaster* (Ragab *et al*., 2011), however with minimal effort done in *Ae. aegypti*. MAPKs are responsible for transmitting extracellular signals into cell nucleus, which leads to cell differentiation, proliferation, and apoptosis (Farkhondeh *et al*., 2020). Under stress, MAPK signaling pathways also govern apoptosis. Organophosphorus compounds (OPCs) may induce apoptosis by interfering with the regulation of the MAPK signaling pathway (Farkhondeh *et al*., 2020). An insect hormone-modulated MAPK signaling cascade contributes as a general switch to trans-regulate differential expression of diverse midgut genes in *P. xylostella* during insecticidal action of Cry1Ac toxin, a toxin synthesised by *Bt* (Guo *et al*., 2021). Moreover, it has been

demonstrated that phoxim triggered upregulations in MAPK and PI3K/Akt signaling pathways, leading to apoptotic death and preventing protein synthesis in silkworms (Tian *et al*., 2017). However, comprehensive studies on MAPK signaling are needed for functional characterisation.

Cellular identity, differentiation, proliferation, and apoptosis are regulated by the Notch signaling pathway (Artavanis-Tsakonas *et al*., 1999). A previous study revealed that Notch1 directly stimulates the expression of c-MYC and that inhibition of Notch1 using small molecule inhibitors of the γ-secretase complex resulted in cell cycle arrest and apoptosis and decreased c-MYC levels (Sharma *et al*., 2006). Dang (2012) demonstrated that connections with other signaling pathways allow the Notch pathway to regulate the apoptotic response in various developing species. The Notch signaling pathway positively regulates the expression of ATP-binding cassette transporter, which is essential for ATP-driven efflux systems that transport xenobiotics (Wu *et al*., 2019). At the transcriptomic level, the sea louse *Caligus rogercresseyi* selectively responds to the delousing drugs deltamethrin and azamethiphos by differentially activating mRNA of the Notch signaling pathway and of ABC genes (Boltaɒa *et al*., 2016). Notch signaling pathway was also correlated with the mortality of *Ae. aegypti* treated with engineered *Saccharomyces cerevisiae* (Mysore *et al*., 2021). The exposure of EA extract in *Ae. aegypti* in the present study may also promote interactions with Notch signaling pathway. However, in-depth experimental studies are needed to reveal the critical function of Notch signalling in *Ae. aegypti* following insecticide exposure.

Some annotated genes were also involved in Wnt signalling pathway in addition to the aforementioned signaling pathway. Wnt signaling pathway is a protein-based signal transduction pathway that is crucial for embryonic development. Recent genetic and molecular research in *D. melanogaster* shows that these signaling pathways are functionally conserved and take part in a variety of developmental events (Su *et al*., 1998; Wu & Nusse, 2002; Xu & Gridley, 2012).

This study identified a significant number of molecular players involved in mosquitoes treated with natural products. Despite the inconsistencies of the genes involved in apoptosis compared to previous research, it reflects the complexity of apoptosis in mosquitoes. The observed DEGs may provide the basis for developing novel insecticides as a screening biomarker of natural products intended to be used as an insecticide, to formulate the EA extract with ideal formulation, and to reduce the risk of insecticide resistance development.

CONCLUSIONS

This study shed light on the mechanism behind the use of *Streptomyces*-based insecticide for mosquito control, offering valuable insights into its cellular, genomic, and biochemical effects on *Aedes* mosquitoes. Through apoptosis induction via EA extract, involvement of key genes such as tropomodulin and sestrin was identified, with validation through real-time RT-PCR. Furthermore, this study highlighted the disruption of MAPK and Notch signaling pathways, alongside acetylcholinesterase inhibition, suggesting a multi-faceted approach to insecticidal action.

Declaration of competing interest

The authors declare no conflict of interest.

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