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GENE EXPRESSION STUDY OF COMPOUND (DEHB) TREATED LARVAE ISOLATED FROM THE *Streptomyces rimosus* AGAINST THE FUNCTIONAL GENES OF *Culex quinquefasciatus* Say (Diptera:Culicidae)

PATHALAM GANESAN[1#], DANIEL MAGESH[1#], SEKAR ANAND[1#], HOST ANTONY DAVID[1], MICHEAL GABRIEL PAULRAJ[1] AND SAVARIMUTHU IGNACIMUTHU[1,2*]

[1]Entomology Research Institute, Loyola College, Chennai, Tamil Nadu, 600034, India.
[2]Xavier Research Foundation, St. Xavier's College, Palayamkottai 627002, India.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Authors PG, DM and SA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors HAD and MGP managed the analyses of the study. Author SI managed and guided the research and writing work. All authors read and approved the final manuscript

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ABSTRACT

The present study is aimed at checking the effect of compound (di-2-(ethyl hexyl) phthlate) isolated from *Streptomyces rimosus* on the expression of the genes from mosquitoes, namely, Cytochrome P450 (CYP450), glutathione S-transferase (GST), Esterase (EST) and Beta-actin (ACTIN) using RT-PCR and qTPCR. Nucleotide sequences of GST, EST, CYP and actin genes from *Cx. quinquefasciatus* were extracted from NCBI database. Primers were designed in the conserved regions of mosquito nucleotide sequence using Primer3 Input web tool. Mosquitoes were treated with the compound isolated from *Streptomyces rimosus*. Twenty early third instar larvae of *Cx. quinquefasciatus* were introduced into the containers. Concentrations of 2.0 ppm of the compound were prepared using dimethyl sulfoxide (DMSO) (249 ml water and 1 ml DMSO); five replicates were maintained for treated concentration. Total RNA was extracted using RNeasy kit, according to the manufacturer's instructions. Total RNA was converted into cDNA by using QuantiTect Reverse Transcription kit. The RT- PCR reaction was on the final volume of 20 µl. The reactions were performed under standardized qRT-PCR conditions by using gene specific primers. The results indicated that EST gene showed downregulation when compared to the standard controls. In addition GST gene showed differential expression levels and CYP 450 showed no variation in expression when compared to the controls. As per previous studies,

**Corresponding author: Email: imuthus@hotmail.com;*
[#]Contributed equally

EST and GST genes are responsible for insecticide resistance, but in our study the compound-treated larvae showed some up- and downregulation of genes in EST and GST. The present study clearly states that the isolated compound DEHP from *S. rimosus* may be used to disrupt the resistance development of *Cx. quinquefasciatus* against insecticides.

Keywords: *Cx. quinquefasciatus*; gene expression; cytochrome P450 (CYP450); glutathione S-transferase (GST); esterase (EST).

1. INTRODUCTION

Insect-borne diseases from mosquitoes cause death in most of the tropical areas which leads to the abundant influence on employment and other human activities. Controlling these mosquitoes and diseases itself is difficult for the government organization [1,2]. *Culex quinquefasciatus* is the main vector for causing filarial disease to humans and other mammals. Tropical areas are more susceptible to transmittable diseases and thus the risk of contracting insect-borne diseases has increased due to ecological impacts due to increasing global development [3,4]. Recently mosquito control program is suffering due to the development of resistance in mosquito immune system. There are many insecticides developed to control mosquitoes; however, they have developed resistance. Many synthetic insecticides that were developed to effectively control mosquitoes also have adverse effects on mammals and environment [5,6,7,8,9]. Resistance against insecticides is expected to be a pre-adaptive occurrence of resistance genes, which may be due to regular exposure to insecticides, and are passed through generations. Expression of these resistance genes allow mosquito to adapt with a resistance mechanism to the insecticide to which it was exposed. Several reports have indicated that mosquitoes have evolved with multiple resistance mechanisms for different insecticides [10-27].

As vectors, mosquitoes mainly transmit diseases to humans and other animals, when they imbibe the blood from the host. To identify the host specifically, mosquitoes have olfactory organs mainly for host seeking. Most of the vectors like mosquitoes, utilize carbon dioxide (CO_2) and 1-octen-3-ol as olfactory signals in host-seeking activities mainly present in the vectorial capability. Still, we could not identify the molecular and cellular basis of the olfactory responses [28]. They have three types of olfactory appendages, like antenna, proboscis, and maxillary palp. The antenna has the largest quantity and variety of olfactory sensilla; maxillary palp is less complex, protecting a single morphological type of chemosensory organ from cuticle [29,30,31]. Mostly insect's olfactory proteins are involved in the odorants reception, and they are odorant-degrading enzymes (ODEs), chemosensory proteins (CSPs), odorant receptors (ORs), ionotropic receptors (IRs), odorant-binding proteins (OBPs), and sensory neuron membrane proteins (SNMPs). They are responsible for the reception of host-seeking for blood feed [32].

Glutathione transferases (GSTs) are soluble dimeric proteins. Soluble dimeric proteins like GSTs are universal in nature. The main function of GSTs is involved in the metabolism, detoxification and excretion of a large amount of exogenous and endogenous compounds from the cell. Above 40 GST genes have been identified in the genomes of eukaryotes which is currently available in the sequence database. They have been classified into 13 different classes based on their amino acid sequences, immunological properties and substrate specificities. Certain classes of GST found across multiple eukaryotic phyla, like Zeta and Omega classes, others GST restricted to distribution in insect-specific, Delta and Epsilon classes [33,34,35,36,37]. Most of the research reports related to insect GSTs have focused on insecticide resistance, and recent reports mainly on protection against cellular damage by oxidative stress [38,39,40,41,42].

Enzymes like cytochrome P450 monooxygenases (P450), GSTs and carboxy/cholinesterases (CCE) from three families are mostly concerned in insecticide metabolism. These three enzymes are mostly involved in a wide range of reactions in detoxification. They play an essential role in enzymatic defense against xenobiotics, removal of many by-products of metabolism, multiple biosynthetic pathways and intricate in chemical communication. These gene families are very quickly developing in each insect species and have a unique supplement of detoxification genes, with very few insect species across orthologs [43,44,45,46]. Cytochrome P450s (monooxygenases) establish a universal and complex superfamily of hydrophobic, heme-containing enzymes. The main function of P450s is biosynthesis of numerous vital endogenous compounds and detoxification of many xenobiotics [47]. Acetylcholinesterase (AChE) is a key enzyme, which is mainly involved in the hydrolysis of neurotransmitter acetylcholine (ACh) in cholinergic synapses of neurons. AChE plays a vital role in every function of living organism including insects.

Nowadays main target of the insecticides is AChE [48,49,50,51].

Insect's juvenile hormones (JH) are critically involved in the functions like development, reproduction, caste determination, and behavior. Certain JH have developed chemical analogs and most of them have insecticidal activity against different species of insects. They have effects on insects which are similar to the exogenously applied JH are agonists, and useful in physiological studies. Insecticides of JHA have low toxicity against vertebrate animals [52,53,54,55,56,57]. There are many research studies on insecticides that inhibit the mechanism of different essential enzymes present in the insects and other animals.

The present study aims to study the effect of isolated compound (DEHP) on the expression of the genes from mosquitoes, namely, Cytochrome P450 (CYP450), glutathione S-transferase (GST), Esterase (EST) and Beta-actin (ACTIN) using RT-PCR and qTPCR.

2. MATERIALS AND METHODS

2.1 Isolation of Compounds

The isolation and characterization of compound (DEHB) from *Streptomyces rimosus* was previously published following the method of Ganesan et al. [58].

2.2 Gene Identification and Primer Design

Nucleotide sequences of GST, EST, CYP and actin genes from *C. quinquefasciatus* were extracted from NCBI database. These sequences were used as query sequence to analyze using BLASTn database through NCBI BLAST WEB service. The information regarding each sequence was analyzed using open reading frame (ORF) finder to identify the coding information of each sequence. Primers were designed in the conserved regions of mosquito nucleotide sequence using Primer3 Input web tool.

2.3 Mosquito Treatment with Isolated Compound

Mosquitoes (F1) were treated with the compound isolated from *S. rimosus*. Twenty early third instar larvae of *Cx. quinquefasciatus* were introduced into the containers. Concentrations of 2.0 ppm of the compound were prepared using dimethyl sulfoxide (DMSO) (249 ml water and 1 ml DMSO); five replicates were maintained for treated concentration. The same concentrations were used for the temephos (85%) and azadirachtin (76%). Water control was also maintained. The larvae were taken alive in the period of twelve and twenty-four hour intervals.

2.4 Extraction of the RNA from the Treated Larvae

Total RNA was extracted using RNeasy kit, according to the manufacturer's instructions. Briefly, 100 mg of the tissue sample was taken. The tissue was weighed and transferred into liquid nitrogen, and ground thoroughly with a mortar and pestle or homogenizer. The ground sample was transferred into 2 ml micro-centrifuge tube, 450 µl Buffer RLT or Buffer RLC (maximum of 100 mg tissue powder) were added and vortexed vigorously. The lysate was transferred to a QIA shredder spin column (lilac) placed in a 2 ml collection tube, and centrifuged for 2 min at full speed. The supernatant was transferred to a new micro-centrifuge tube without disturbing the cell-debris pellet in the collection tube. 0.5 volume of ethanol (96–100%) was added to the cleared lysate and mixed immediately by pipetting. The sample (usually 650 µl), including any precipitate that may have formed was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 s at 10,000 rpm. 700 µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at 10,000 rpm to wash the spin column membrane. The flow-through was discarded and 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 15 s at 10,000 rpm and the flow-through was discarded. 500 µl Buffer was added RPE to the RNeasy spin column and centrifuged for 2 min at 10,000 rpm. The RNeasy spin column was placed in a new 2 ml collection tube, and the old collection tube with the flow-through was discarded and centrifuged at full speed for 1 min. The RNeasy spin column was placed in a new 1.5 ml collection tube and 30–50 µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at 10,000 rpm to elute the RNA. Eluted RNA was stored in -80°C for further use.

2.5 cDNA Synthesis

Total RNA was converted into cDNA by using QuantiTect Reverse Transcription kit. The RT-PCR reaction was on the final volume of 20 µl. Genomic DNA elimination reaction containing gDNA wipeout buffer, template RNA and RNase-free water for 14 µl was incubated at 42°C for 2 min followed by addition of 6 µl reverse-transcription reaction master mix and further incubated at 42°C for 30 min with final heating for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase (Qiagen).

2.6 Quantitative RT-PCR

The reactions were performed under standardized qRT-PCR conditions by using gene specific primers as listed in Table 1 and Fig. 1. For quantitative real-time PCR (qRT-PCR) expression levels of four candidate genes of third-instar larvae of *Cx. quinquefasciatus* with two time intervals were analyzed. qRT-PCR was performed using Bio-Rad CFX96Real Time PCR system (CFX96 Optics Module). For quantification, 20 µl reactions were employed containing 10 µl of SYBR premix Ex Taq II (TaKaRa, Japan), 0.8 µl primers (400 nM each primer) and 5 ml diluted cDNA (1:50). Standard curves were constructed from an appropriate range of dilutions of cDNA. The cycling conditions of qPCR were: enzyme activation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 34 s, followed by the melting curve analysis with the temperature range of 50–90°C. The cycle threshold (Ct) values and starting quantity were calculated using Bio-Rad CFX Manager.

Table 1. Primers used for gene expression studies

S. no	Gene name	Prime sequence (5'-3')	Size	Tm ^{o}C
1	Cytochrome P450 (CYP450)	ATGCCGAAGGATACCGCCAA CGTGCGCAGATTGTTCACCA	198	58.99
2	Glutathione Stransferase (GST)	CCAACGCCGACAACGAGAAG CTTCTTGCACCGCTCCAACC	200	58.99
3	Esterase (EST)	CGGGCCGGATTTCTTGGTTC TTCGGGTCTCCTCCAAAGGC	186	58.99
4	Beta- Actin (ACTIN)	ATCGACAATGGGTCGGGCAT GTCCTTCTGGCCCATTCCGA	126	58.99

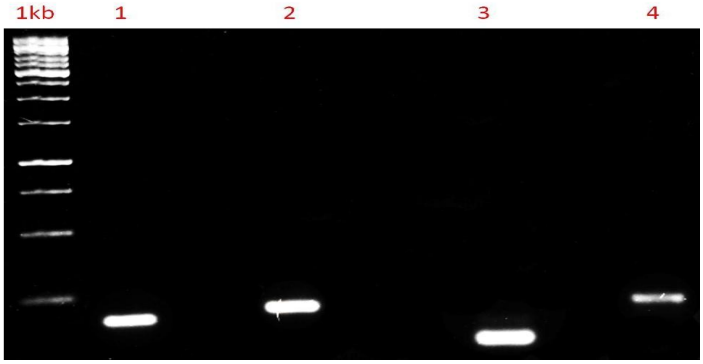

Fig. 1. Confirmation of amplification of primers using Genomic DNA isolated from *Cx. quinquefasciatus*. (Lane-1-EST, Lane-2- CYP, Lane-3-Actin, Lane-4-GST)

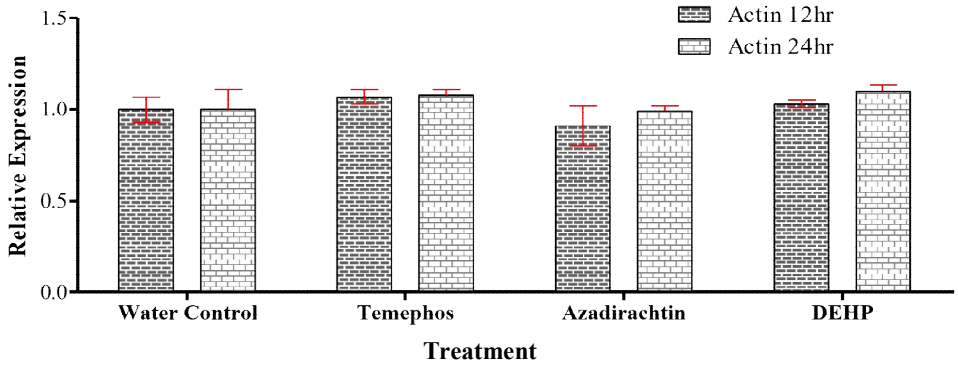

Fig. 2. Quantitative expression of Actin gene in 12 and 24 hrs treatments. Accumulation of genes transcript was determined using 1mg of total RNA. Actin gene was used as a normalizer

3. RESULTS

3.1 Isolation and Conversion of cDNA

The total RNA of the various compounds-treated larvae were extracted with RNA isolation kit. RNA purity was checked using Nanodrop (ND-2000Medex SKU: TSC-ND-2000). The expected quantity of the RNA was successfully converted into cDNA that was used for further analysis.

3.2 Quantitative RT-PCR

Expression pattern of these four gene were analyzed by using Quantitative RT-PCR analysis for 12 hrs and 24 hrs treatments showed differential expression patterns. The concluded results were compared with standard control. Expression of *CYP* gene did not show variation in regulation compared to water control in both 12 hrs and 24 hrs. Samples treated with Temephos showed upregulated expression in 24 hrs than 12 hrs and also compared with other samples. Azadirachtin showed downregulation in both 12 hrs and 24 hrs and compared with other samples (Fig. 3). EST gene showed downregulation in 24 hrs than 12 hrs and also compared with water and Temephos samples. Azadirachtin samples showed upregulation in both 12 hrs and 24 hrs (Fig. 4). GST gene expression showed different expression levels; water samples showed no variation in expression levels in 12 hrs and 24 hrs. Temephos samples showed downregulation in 24 hrs than 12 hrs. Azadirachtin showed upregulation when compared with other samples like water, Temephos and DEHP. Interval of 24 hrs showed upregulation compared to 12 hrs (Fig. 5 and Fig. 6).

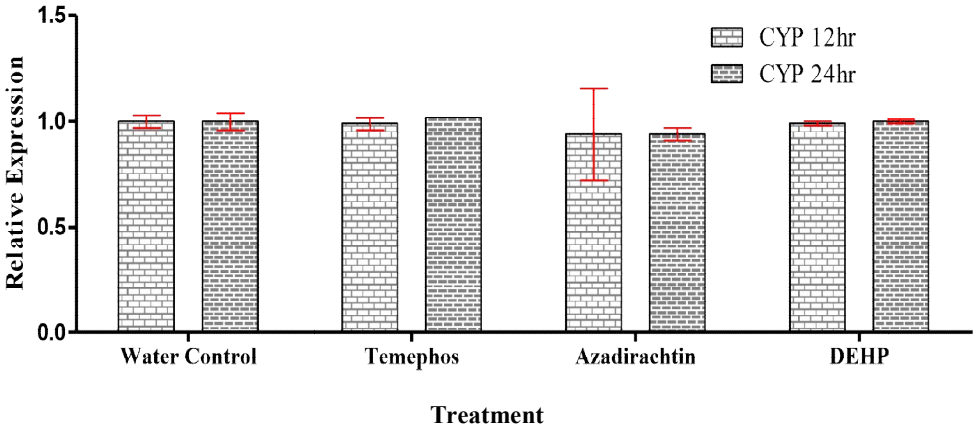

Fig. 3. Quantitative expression of CYP gene in 12 and 24 hrs treatments. Accumulation of genes transcript was determined using 1mg of total RNA. Actin gene was used as a normalizer

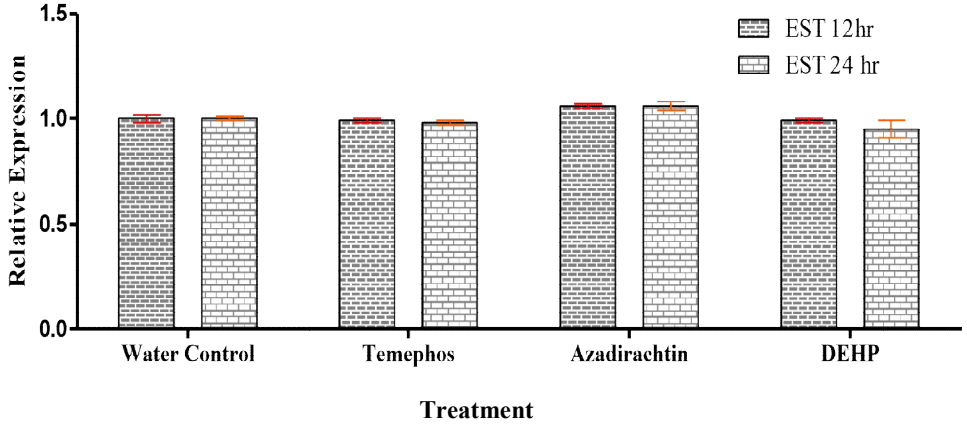

Fig. 4. Quantitative expression of ESTgene in 12 and 24 hrs treatments. Accumulation of genes transcript was determined using 1mg of total RNA. Actin gene was used as a normalizer

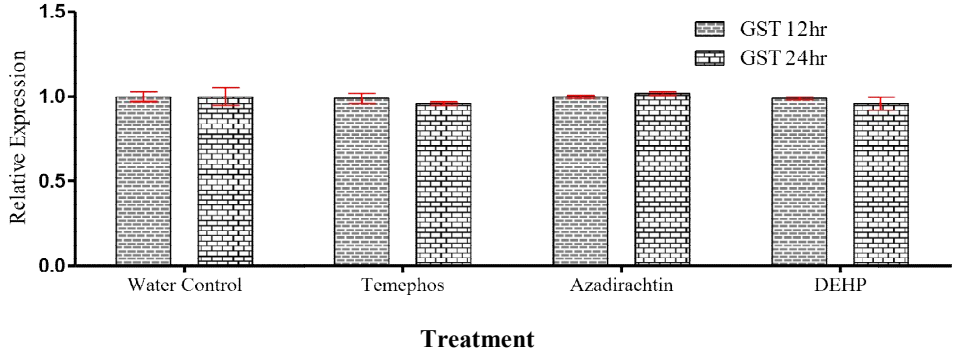

Fig. 5. Quantitative expression of GST gene in 12 and 24 hrs treatments. Accumulation of genes transcript was determined using 1mg of total RNA. Actin gene was used as a normalizer

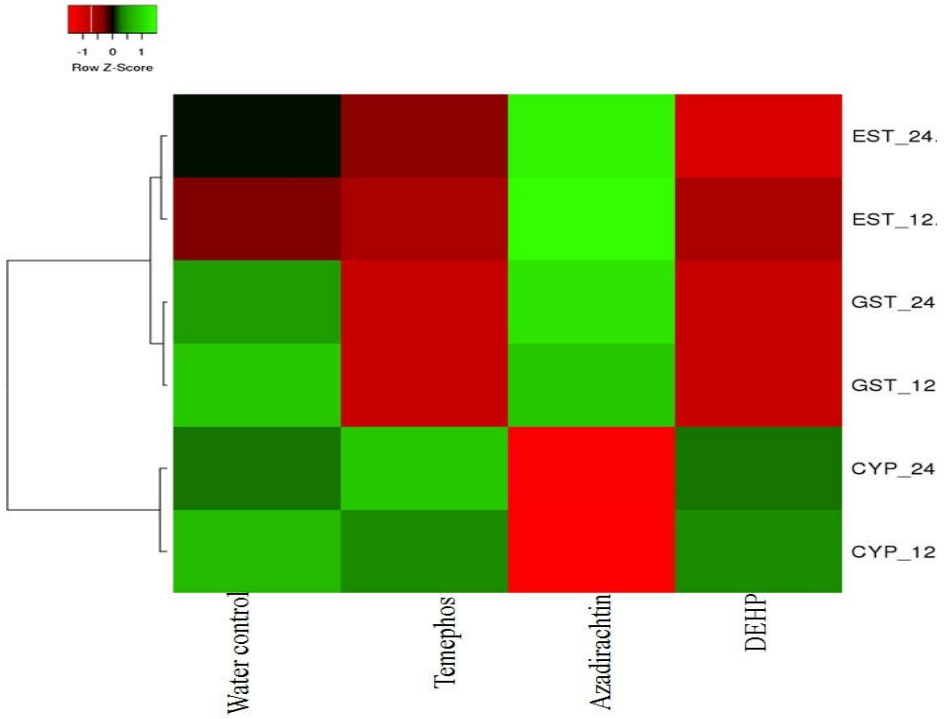

Fig. 6. Heat maps representing the genes using Heat mapper

4. DISCUSSION

Expression of gene in insects is a common determining event in the development of insect's ontogeny. Transcriptional factors are known to control and form the linkages and transcriptional factors during overexpression of genes in insect development. Resistance development against insecticides and pesticide is a common expression of genes in mosquitoes and other insects. Novel tactics are instantly needed to avoid or interrupt the resistance development in insects. Control of resistant development in mosquitoes will need to build the essential understanding of the genes and regulatory networks of resistance development [59]. In the present study four functional genes were checked against the DEHP, azadirachtin, temephos and water control. The four genes exhibited differential expression against DEHP and standard controls. The results were compared with the controls. EST gene showed downregulation in 24 hrs than 12 hrs when compared to the standard controls. GST gene showed differential expression level, Temephos and Azadirachtin expressed up- and downregulation.

CYP450 showed no variation in expression when compared to the controls in 12 hrs than 24 hrs. In previous studies Nikou, et al. [60] identified that African malaria vector, *Anopheles gambiae* developed resistance to Pyrethroid insecticides. The mosquitoes were exposed to pyrethroid which increased the expression of cytochrome P450 monooxygenase 6 (CYP6) P450 gene in both male and female mosquitoes. The expression (CYP6Z1) was higher in males than females. Ranson, et al. [61] described the expression of GST genes from *Anopheles gambiae*. Three GST genes such as aggst18, aggst1-9 and aggst1-10 were responsible for the GST-based resistance when mosquitoes were exposed to DDT-based insecticides against malarial vector *Anopheles gambiae*. Ortelli, et al. [62] presented four GST genes in the African malaria vector mosquito *Anopheles gambiae*. These four genes are responsible for the development of resistance in the malaria vectors which are exposed to insecticides with 1,1,1-trichloro-2,2-bis-(pchlorophenyl) ethane. Paton, et al. [63] noted that the over expression of the carboxyl esterase gene present in the *Culex* mosquito developed their own resistance when exposed to the oraganophosphorous insecticide.

Plants and animals having a number of genes that are working as development- and functional-oriented genes. Metabolic function of the insects was maintained by the genes which play a crucial role in the detoxification of both endogenous and exogenous toxic compounds and insecticides. Cytochrome P450s were increasingly expressed in *Culex quinquefasciatus* mosquitoes for the resistance against insecticides. When the mosquitoes are exposed to insecticides containing, the expression of the P450 genes is increased and the resistance against those insecticides is developed. In this case piperonyl butoxide was working as an inhibitor of cytochrome P450s in the filarial vector mosquito *Culex quinquefasciatus* [64]. Different genes like GSTs and P450s that were related with xenobiotic initiation and pesticide resistance were sex-dependently expressed [65]. Cytochrome P450s gene expression was both up- and down-regulated when the *Culex quinquefasciatus* mosquitoes were exposed to permethrin. Several genes of *Culex quinquefasciatus* were identified in up- and down-regulation in larvae and adult of mosquitoes [66]. In the resistance-developed mosquitoes against permethrin insecticides, multiple P450 genes were up- and down-regulated. Downregulation of P450 linked to the homeostatic response since insects want to defend the cell from the lethal effects of extra P450 derived oxidizing species and molecules from the up-regulated P450s and thus stable the usage of energy, O2, and other constituents required for the synthesis of proteins. They play a main role in insecticide resistance [67]. When exposed, P450s such as CYP9J24, CYP9J26 and CYP9J28 induced the resistance in *Ae. aegypti* and *Anopheles gambiae* against permethrin- and deltamethrin insecticides [68]. The previous studies clearly showed that resistance development of insects were linked to these functional genes. These functional genes are important for the metabolism of endogenous and exogenous compounds, contributing for the metabolization of toxics such as insecticides.

5. CONCLUSION

The present study showed that when *Cx. quinquefasciatus* was exposed to the compound DEHP expression of four functional genes (were noticed) present in the *Cx. quinquefasciatus*. EST gene showed downregulation when compared to the standard controls. GST gene showed differential expression levels and CYP 450 showed no variation in expression when compared to the controls. As per previous studies EST and GST genes are responsible for insecticide resistance, but in this present study the compound-treated larvae showed some up- and down-regulation of genes in EST and GST. Isolated compound DEHP from *S. rimosus* may be used to inhibit or disrupt the resistance development of mosquitoes against insecticides.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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