Expression profiles of glutathione S-transferases genes in semi-engorged *Haemaphysalis longicornis* (Acari: Ixodidae) exposed to *Cymbopogon citratus* essential oil

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Short report

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Abstract

Background: The tick *Haemaphysalis longicornis* is well known as vector of several zoonotic pathogens responsible for various clinical conditions, increasingly threatens the veterinary and public health. It is mainly distributed in East Asia, New Zealand, Australia, and several Pacific islands, and has been expanded rapidly in United States since its first founding on a nonimported domestic sheep in New Jersey. Glutathione S-transferases (GSTs) are phase II detoxification enzymes, which function via combining with pesticidal molecules and catalyzing the conjugation of molecules by thiol of glutathione, so as to protect tissues from oxidative stress damage. In the tick *H. longicornis*, glutathione S-transferases (*HlGST* and *HlGST2*) have been previously identified. However, the relationship between the expression of glutathione S-transferases and the essential oil treatment in ticks remains unexplored. Hence, in the present study, the expression profiles of *HlGST* and *HlGST2* mRNAs were evaluated in *H. longicornis* after exposure to *Cymbopogon citratus* essential oil.

Results: At 24 h post-exposure of *H. longicornis* to different sublethal concentrations of *C. citratus* essential oil, ANOVA results revealed significant difference ($F_{2,6} = 55.94$, $P = 0.0001$) in the expression of *HlGST*. Tukey's test showed that *HlGST* was significantly induced after treatment with 1% *C. citratus* essential oil ($P = 0.0002$); whereas no significant difference ($P = 0.3551$) was detected after treated by 2% *C. citratus* essential oil. No significant difference ($F_{2,6} = 0.8990$, $P = 0.4555$) in the expression of *HlGST2* between the treatment and the control group of 50% ethanol. Nevertheless, the under-expression of HlGST2 in the treatment groups versus the untreated control group was not significant ($F_{3,8} = 2.643$, $P = 0.1208$).

Conclusion: The results implied that GST mRNA is a potential molecular target for *C. citratus* essential oil in *H. longicornis.* Further understanding of the underlying mechanisms of the GST at the molecular level could contribute to develop effective control measures for ticks and tick-borne diseases.

Background

Ticks, a major public health and veterinary ectoparasites, require vast amount of blood for development and reproduction. During blood feeding, they can inflict great harm on humans and animals [1, 2] causing skin lesions, irritation, allergic reactions, and severe anaphylactic reactions [3]. Ticks can transmit pathogens such as bacteria, viruses, fungi, protozoans, helminthes, and protists which cause various clinical conditions such as theileriosis, babesiosis, dermatophilosis, anaplasmosis, ehrlichiosis, tularaemia, tick-borne encephalitis, West Nile fever, Crimean-Congo hemorrhagic fever, Severe Fever with Thrombocytopenia Syndrome (FLTS), among others [1, 2, 4]. Apart from mosquitoes, they are the topmost vectors of human diseases [1].

The tick Haemaphysalis longicornis (Acari: Ixodidae) is considered to be native to East and central Asia, where it thrives under temperate conditions and is of significant medical and veterinary importance in a number of countries [5, 6]. It is regarded as major invasive livestock pest in New Zealand, parts of Australia, and several Pacific islands – including Tonga, Fiji, Vanuatu, New Caledonia, and Western Samoa [7], and has been expanded rapidly in United States since its first founding on a nonimported domestic sheep in New Jersey [8]. H. longicornis is known as vectors of rickettsiae causing Q fever, viruses causing Russian spring-summer encephalitis, and protozoa causing theileriosis and babesiosis, respectively [9], and consequently, responsible for huge economic loss experienced by livestock producers [10]. Recently, H. longicornis has been implicated as the vector and reservoir of severe fever with thrombocytopenia syndrome (SFTS) virus which causes a new type of hemorrhagic fever in East Asia [11].

The use of chemical acaricides has been the conventional and most popular method of tick control to which can be applied directly to the host animals. However, due to environmental implications and the development of resistance by ticks, attention of researchers has shifted to the development of other eco-friendly alternatives such as plant derived formulations [12]. Essential oils of the genus Cymbopogon have demonstrated insecticidal/acaricidal properties as well as repellant activity against mosquitoes, flies and ticks [13]. Cymbopogon citratus (lemongrass) essential oil is obtained from an aromatic grass belonging to the botanical family of Poaceae (Gramineae) [14]. GC-MS analysis revealed that the overall biochemical components of C. citratus essential oil include geranial (citral-A), neral (citral-B), geraniol, myrcene, limonene, linalool, geranyle acetate, caryophyllene oxide, 2-undecanone, γ-cadinene, 6-methylhept-5-en-2-one, Camphene, Citronellal, n-decanal, (E)-caryophyllene, and 2- tridecanone [15]. Nevertheless, chemical studies of lemongrass in different habitats around the world identified citral as the major volatile constituent [16]. Though the toxicity and repellence of plant essential oils and their terpenoid constituents have long been recognized, the exact biochemical mechanism of action is yet to be fully understood, particularly in ticks [17].

Glutathione S-transferases (GSTs) are phase II detoxification enzymes that use the thiol of glutathione to catalyze the conjugation of molecules with an electrophilic center [18, 19]. GSTs are responsible for phase II xenobiotic detoxification via combining with pesticidal molecules through either chelation or conversion of the lipid metabolites resulting from the induction of the pesticidal materials so as to protect tissues from oxidative stress damage [18, 20]. Increased metabolic detoxification has been reported as one of the basic mechanisms underlie pesticide resistance [21], and GSTs play a major role in this regard. In the tick H. longicornis, two glutathione S-transferases (HlGST and HlGST2) have been previously characterized [22]. HlGST and HlGST2 was upregulated upon exposure to sublethal doses of flumethrin and cypermethrin, respectively [23]. However, the relationships between the expression of glutathione S-transferases and the essential oil treatment in ticks remains unexplored. Hence, in the present study, the expression profiles of HlGST and HlGST2 mRNAs were evaluated in H. longicornis after exposure to C. citratus essential oil, in the hope of further understanding the molecular mechanism underlying the impacts of essential oil on ticks.

Methods

Cymbopogon citratus (lemongrass) essential oil

The essential oil of *C. citratus*, used in this study was acquired from AYUS GmbH-Oshadhi Hautpflege (Bühl, Germany; www.oshadhi.com). The essential oil was diluted with 50% ethanol to sublethal concentrations of 1% and 2% for tick treatment.

Sample collection, tick rearing, treatment and dissection

The tick *H. longicornis* were collected from the vegetation using flag dragging method at Xiaowutai National Natural Reserve Area in China. The ticks were reared on domestic rabbits *Oryctolagus cuniculus*, as described by Liu et al. [24]. Rabbits were sustained at 20-25 ºC, 50% RH, and natural daylight cycles, with each used for a single infestation. The adult ticks were allowed to feed up to semi-engorged (with body-weight ranges of 0.11-0.14g) for 4 days with mating and were collected for subsequent essential oil treatment.

The essential oil was diluted in an aqueous solution containing 50% ethanol and was gently agitated. The semi-engorged ticks were randomly divided into 20 survived ticks per group of 3 replicates of which two experimental

groups were treated with 1% and 2% sublethal concentrations of *C. citratus* essential oil, respectively, and two control groups with 50% ethanol treatment and an untreated. The LC50 is 2.9% *C. citratus* essential oil (unpublished data) for adult *H. longicornis* and sublethal concentrations of 1% and 2% were chosen so as to have sufficient live ticks for tissue dissection and RNA extraction.

The treatment groups were immersed into 5 mL of the respective concentrations by placing them directly into Eppendorf tubes (EP) tubes. The essential oil and ethanol solutions were decanted after 5 min and the ticks were dried in tissue paper towel. Then, they were kept separately in petri dishes with moisturized cotton wools and placed inside an incubator for 24 h at an optimum temperature of 27±2 °C, relative humidity of 80±5% and 16 h light/8 h darkness photoperiod. Thereafter, each tick was dissected in 0.1 mol/L PBS at pH 7.2 and the midguts were harvested and remnants of blood were removed, frozen quickly with liquid nitrogen and stored at -80 ºC for further analysis.

RNA extraction and cDNA synthesis

Total RNA from the tick midguts was extracted using the TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China) following the manufacturer's protocol. The Nano Drop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the RNA concentration and purity, and agarose gel electrophoresis was used to evaluate the RNA integrity. The extracted RNA samples were stored at -80 ºC until use. The complementary DNA (cDNA) was synthesized by reverse transcription reaction from 4 µg of total RNA using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China), and this ensures the exclusion of DNA contamination. Thereafter, the synthesized cDNA samples were stored at -20 ºC until the subsequent protocol is ready.

Primer design and amplicon sequence analysis

The primers for the genes and actin gene (control) used in this study were designed and obtained from Hernandez *et al*. [23]. Polymerase chain reaction (PCR) was carried out according to the manufacturers directions. The PCR cycle program used is as follows: 95 ºC for 10 min, denaturation step of 40 cycles at 95 ºC for 15 s, and an annealing/extention step at 60 ºC for 20 s. The PCR products were separated on 1% agarose gels and stained with ethidium bromide. Purified products were sent to Invitrogen (Beijing, China) for sequencing. The obtained sequences were analyzed by a BLASTn search in GenBank and the use of Clustal W method in the MEGA-X software (Pennsylvania State University, Pennsylvania, USA).

Quantitative real-time PCR (qPCR)

Transcript analysis of *HlGST* and *HlGST2* genes was performed through real-time PCR in a Mx3005P qPCR system (Agilent Technologies, Santa Clara, USA) using TransStart® Top Green qPCR SuperMix (TransGen Biotech) according to the manufacturer's directions. The qPCR assays were carried out in 96-well polypropylene plates (Axygen, San Jose, USA) in a 20 µL reaction volume containing 1 µL of cDNA template, 10 µL of 2× TransStart® Top Green qPCR Supermix, 0.4 µL of each 10 µM primer, 0.4 µL of Passive Reference Dye (TransGen Biotech) and 7.8 µL of H_2O. The thermal cycling program used is as follows: 94 ºC for 30 s, then 40 cycles of 94 ºC for 5 s and 60 ºC for 30 s. Verification of primers with high amplification specificity were done by the observation of different peaks in corresponding melting curves. Triplicate reaction for each DNA sample were contained in each plate. After each assay, melting curves were traced to confirm that the fluorescence signal was

retrieved from specific PCR products and also ensure that there are no primer dimers. Ct represents the relative gene expression levels for each gene in each sample and it is transformed into relative values or it is relatively quantified (RQ) by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T = (C_{T,\,Target} - C_{T,\,Actin})_{\,sample} - (C_{T,\,Target} - C_{T,\,Actin})_{\,control}$ [25].

Statistical analysis

Differences in mRNA expression among samples were compared by ANOVA and Tukey *post hoc* test while the differences in mRNA expression between the two control groups used were compared using unpaired t-test. The differences were considered statistically significant when $P < 0.05$. All the data of all groups were analyzed using GraphPad Prism 8 software (GraphPad Software, Inc.).

Results

Primer verification and qPCR conditions

To confirm each primer pair, non-specific amplification was performed using the Tm of 59 ºC and 60 ºC with the cDNA template of target genes prepared before. Amplification products were analyzed by agarose gel electrophoresis where bright bands were observed (Fig. 1). To confirm the amplicon sequences for all genes examined in this study, PCR amplicons were sequenced.

HlGST and *HlGST2* expression in response to *C. citratus* essential oil

At 24 h post-exposure of *H. longicornis* to different sublethal concentrations of *C. citratus* essential oil, ANOVA results revealed statistically significant difference ($F_{2,6} = 55.94$, $P = 0.0001$) in the expression of *HlGST*. After treated by 1% *C. citratus* essential oil, *HlGST* was increased more than 6 times (6.47 ± 0.39) than in the control group of 50% ethanol. However, *HlGST* was decreased after 2% *C. citratus* essential oil treatment (1.84 ± 0.57). Tukey *post hoc* test revealed that expression of *HlGST* was significantly increased ($P = 0.0002$) after treated by 1% *C. citratus* essential oil, when compared with that in the control and in 2% *C. citratus* essential oil treatment ($P = 0.0004$); whereas there was no significant difference between the expression level in 2% *C. citratus* essential oil treatment and in the control ($P = 0.3551$) (Fig. 2A).

In the group that used untreated control, ANOVA result showed significant difference ($F_{3,8} = 54.18$, $P < 0.0001$) in the expression of *HlGST*. The fold difference of gene expression for *HlGST* was very high (720.8 ± 37.70 and 207.4 ± 64.07) after treatment with 1% and 2% *C. citratus* essential oil, respectively. Tukey's test showed greater significant variation in the expression level of *HlGST* at 1% *C. citratus* essential oil treatment more than in the control ($P < 0.0001$). There was also greater significant variation in the expression level of *HlGST* at 2% *C. citratus* essential oil treatment more than in the control group ($P = 0.0383$) but less than in 1% *C. citratus* essential oil treatment (Fig. 2B).

No significant difference ($F_{2,6} = 0.8990$, $P = 0.4555$) was observed in the expression of *HlGST2* between the treatment groups and the control group of 50% ethanol (Fig. 3A). However, *HlGST2* was under-expression in the treatment groups versus the untreated control group although it was not significant ($F_{3,8} = 2.643$, $P = 0.1208$) (Fig. 3B). Tukey *post hoc* test showed that *HlGST2* was under-expressed after treated by 1% *C. citratus* essential oil with a fold difference of 0.10 ± 0.02 less than in control ($P = 0.6688$). There was no significant difference ($P =$

0.9981) in the under-expression level of *HlGST2* at 1% *C. citratus* essential oil treatment group versus untreated control group (Fig. 3B).

To evaluate the effect of the use of ethanol as control on *H. longicornis*, the expression of *HlGST* and *HlGST2* in *H. longicornis* at 24 h post-exposure to 50% ethanol were relatively quantified *vis-à-vis* the untreated group. Tukey *post hoc* test showed no significant difference in the relative gene expression of both *HlGST* and *HlGST2* in 50% ethanol treated group (Fig. 2B and 3B). However, t-test result showed a significant difference in the expression of *HlGST* (t_4 = 2.82, P = 0.0477) to 50% ethanol treatment *vis-à-vis* the untreated group. *HlGST* exhibited 123.3 ± 43.34 folds difference of gene expression in the 50% ethanol treated group (Fig. 4A). Conversely, there was no significant difference in *HlGST2* expression between the 50% ethanol treatment group and the untreated control group (Fig. 4B).

Discussion

The expression of two GST genes (HlGST and HlGST2) were investigated in the midgut of H. longicornis after exposure to sublethal concentration of C. citratus essential oil. The glutathione S-transferases is one of the main enzymes in the midgut of arthropods that play major role in the detoxification process [26, 27]. GSTs come in a number of classes and they contribute to the detoxification of various endogenous and exogenous compounds. To understand the role of HlGST in enzymatic detoxification, we explored the expression profile of HlGST and HlGST2 between treated and untreated H. longicornis under different sublethal concentrations of C. citratus essential oil, and further evaluated the expression levels of these genes in response to ethanol which was used to dilute the esential oil and also used to treat one of the negative control groups. From the quantitative PCR (qPCR) results, HlGST mRNA was found to be induced more than normal after exposure to sublethal concentration of C. citratus essential oil. HlGST2 mRNA induction was lower compared to that of HlGST. To our knowledge, this is the first report on the expression profile of GSTs in H. longicornis in response to different treatments of C. citratus essential oil. The expression levels of HlGST and HlGST2 in H. longicornis were previously investigated using sublethal doses of chemical acaricides (flumethrin, chlorpyrifos, and Amitraz) which revealed over-expression of HlGST and HlGST2 to flumethrin and chlorpyrifos, respectively [23].

The metabolic processing of citral, the main constituent of C. citratus essential oil which is mainly made up of a combined constituent of the stereoisomers geranial and neral have not been previously examined in ticks especially H. longicornis. Generally, there has been a couple of studies on the molecular targets of bio-insecticides/acaricides in arthropods. Some of these molecular targets have been implicated in insects and ticks resistance to insecticides/acaricides [21]. An essential step towards the achievement of the purpose of developing an effective eco-friendly control measures for ticks lies in the understanding molecular mechanisms behind tick resistance to acaricides at the molecular levels. Various studies have reported different genes involved in drug resistance. However, much is still yet to be known about the overall pathway involved in drug resistance process. Three classifications of mechanisms behind pesticide resistance include target-site mutations (target site resistance), increased metabolic detoxification (metabolic resistance), and decreased penetration of acaricide (penetration resistance) through the outer protective layers of tick's body [21].

In this study we examined the underlying expression patterns of two genes that codes for one of the enzymes that confer metabolic resistance of acaricides in ticks. Metabolic resistance is believed to involve three methabolic pathways which are mediated by three enzyme families namely carboxylesterases, monooxygenases (cytochrome

P450s), glutathione S-transferases (GSTs) [23]. Glutathione S-transferases are multifunctional intracellular enzymes involved in the detoxification of endogenous and xenobiotic compounds [28, 29] via subjecting the electrophil to reductive or conjugative modification [18]. In the present study, there was six folds difference increase in the expression level of H. longicornis HlGST at 24 h post-exposure compared to the control group of 50% ethanol treatment, and a highy elevated fold difference expression level of above 700 vis-à-vis the untreated control group. The significant up-regulation of GST indicates that this gene might be involved in metabolic activation pathways and detoxification of essential oils [20, 30, 31]. Similarly, elevated levels of GST activity have been observed and associated with insecticidal resistance in different insects [32]. In the Musca domestica (housefly), over-expression of one or more GSTs were implicated in the resistance to organophosphates [33]. The pyrethroid resistant strains of Nilaparvata lugens (planthopper) was orchestrated by the elevated levels of GST expression [34]. Also, the metabolic resistance caused by GST over-expression has been implicated as the main mechanism responsible for dichlorodiphenyltrichloroethane (DDT) resistance in mosquitoes [35].

Our results revealed a reduction in the expression levels of HlGST and HlGST2 as the sublethal concentration was increased indicating a negative relationship between concentration and expression of GST. Aditionally, HlGST2 was more under-expressed vis-à-vis the untreated control group. Possible explanation could be an increase in oxidative stress in the ticks as the inhibition in the activity of GST increases with increased concentration of C. citratus essential oil as observed in previous studies [36]. One of the common explanations for the mechanism of toxic actions of pesticides is oxidative stress which is caused by the disruption in the equilibrium between antioxidant defenses in the body system and the quantity of free radicals such as reactive oxygen species (ROS). So oxidative stress is the resultant effect when the level of ROS increases far more than the antioxidant defense mechanisms [36, 37]. This metabolic phenomenon was observed when bio-insecticide was administered to experimental rats and fishes which resulted in the inhibition in GST activity and decrease in the activity of glutathione peroxidase [37, 38]. Similarly, increased abamectin concentratin in rats led to the reduction in the activity of glutathione (GSH) and GST with increase in oxidative stress markers [39, 40, 41].

To our knowledge, no studies have been reported on the impact of ethanol on GST expression level in arthropods. GST exhibited some level of sensitivity to ethanol in this study. We comparatively evaluated the expression levels of GST between the two control groups of 50% ethanol treated group and the untreated group. Significant expression levels of H. longicornis GST was observed in the 50% ethanol group which recorded a fold difference of over 100 to that of the untreated group unlike in H. longicornis GST2 whose expression was not significant. This corroborates a previous study which found an increase in GST activity after long-term ethanol treatment in the liver of the rat. Both the Alpha and Mu class GST activities were significantly stimulated by 36% and 44%, respectively [42]. The study of the expression levels of GSTs to ethanol treatment is vital because ethanol is frequently used in laboratory experiments and researches as solvent for water-soluble substances. Another previous study examined the effects of solvents and surfactant agents on the female and larvae of cattle tick Boophilus microplus; and reported that methanol and ethanol caused 45.3 and 14.2% of mortality, respectively on the ticks [43]. Thus, it is paramount not to use ethanol as solvent in experiments in which inducing effects of the solvent is required to be avoided.

Conclusion

The above results implied that GST is a potential molecular target for C. citratus essential oil in H. longicornis, which were induced more than normal after exposure to sublethal concentration of C. citratus essential oil.

Although not much work has been done on the effects of GSTs on bio-acaricides such as plant essential oils, an understanding of the underlying mechanisms of action of detoxification enzymes at the molecular level could play a major role in developing an effective and eco-friendly control measures for ticks and other vectors. More transciptomic research should be carried out to explore the molecular targets of different plant-derived products because this could be the key in discovering new effective and eco-friendly alternative tick control measures.

Abbreviations

GST: Glutathione S-transferase; *HlGST*: *Haemaphysalis longicornis* glutathione S-transferase; *HlGST2*: *Haemaphysalis longicornis* glutathione S-transferase 2; RH: relative humidity; qPCR: quantitative real-time PCR; RQ: relative quantity; Tm: melting temperature; DDT: dichlorodiphenyltrichloroethane.

Declarations

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Availability of data and materials

The data supporting the conclusions of this article are included in the article. Raw data used or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JL and DOA designed the study. DOA and TP performed the experiments. DOA and XW fed and dissected the ticks. DOA analyzed data and wrote the manuscript. ZY and JL edited and reviewed the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Hebei Normal University as complying with the Animal protection law of the People's Republic of China (Protocol Number: IACUC-157031).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

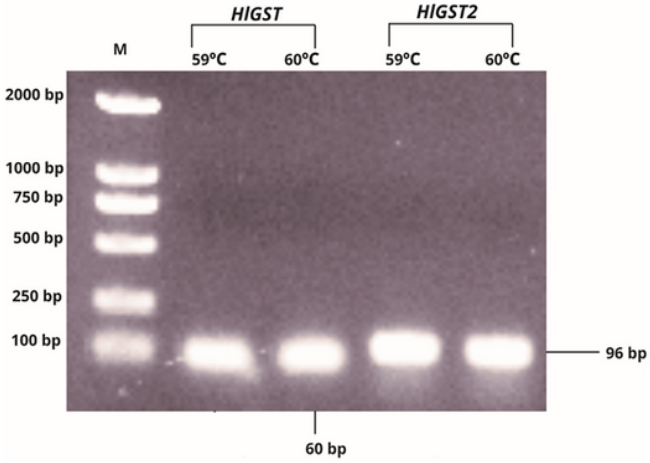

Figure 1

Primer verification by PCR amplification from HlGST and HlGST2. Two melting temperatures (Tm) of 59°C and 60 °C were used for each of the genes. HlGST: Haemaphysalis longicornis GST; HlGST2: Haemaphysalis longicornis GST2.

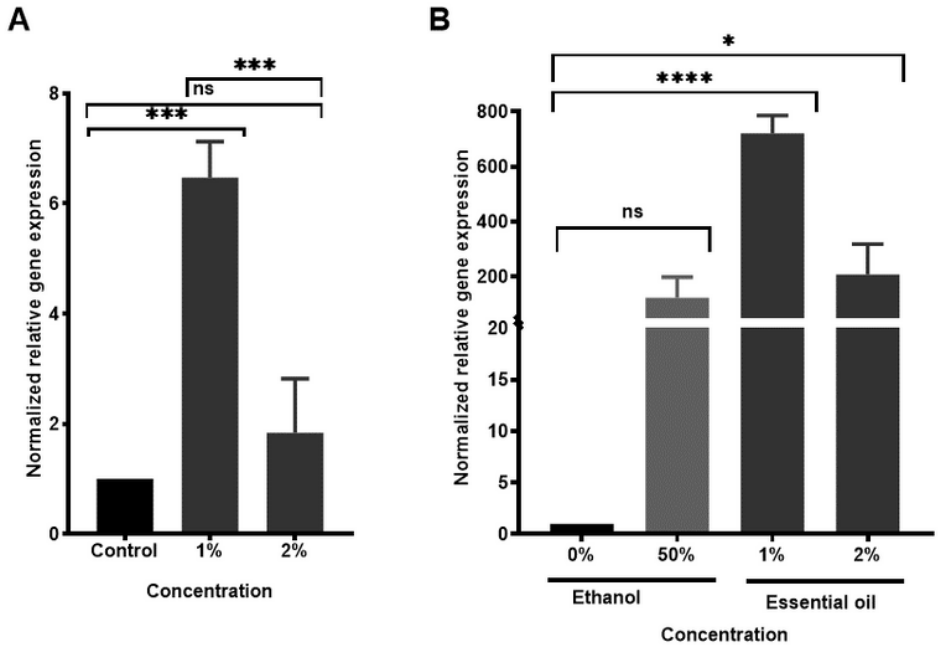

Figure 2

Relative expression of HlGST in H. longicornis at 24 h post-exposure to two different sub-lethal concentrations of C. citratus essential oil. (A) Exposure to C. citratus essential oil using 50% ethanol treatment group as control; (B) Exposure to C. citratus essential oil using untreated group as control. Data were presented as the means (n = 3) ± SE. Statistical significance was calculated at 5% probability using one-way ANOVA followed by Tukey's test. Asterisks above bars indicate significant difference between groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: not significant.

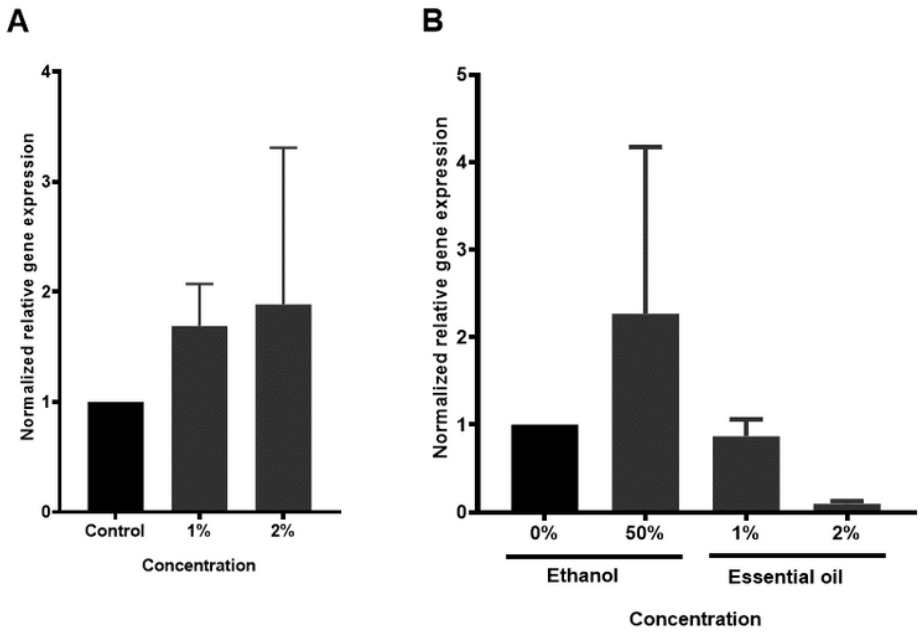

Figure 3

Relative expression of HlGST2 in H. longicornis at 24 h post-exposure to two different sub-lethal concentrations of C. citratus essential oil. (A) Exposure to C. citratus essential oil using 50% ethanol treatment group as control; (B) Exposure to C. citratus essential oil using untreated group as control. Data were presented as the means (n = 3) ± SE. Statistical significance was calculated at 5% probability using one-way ANOVA followed by Tukey's test.

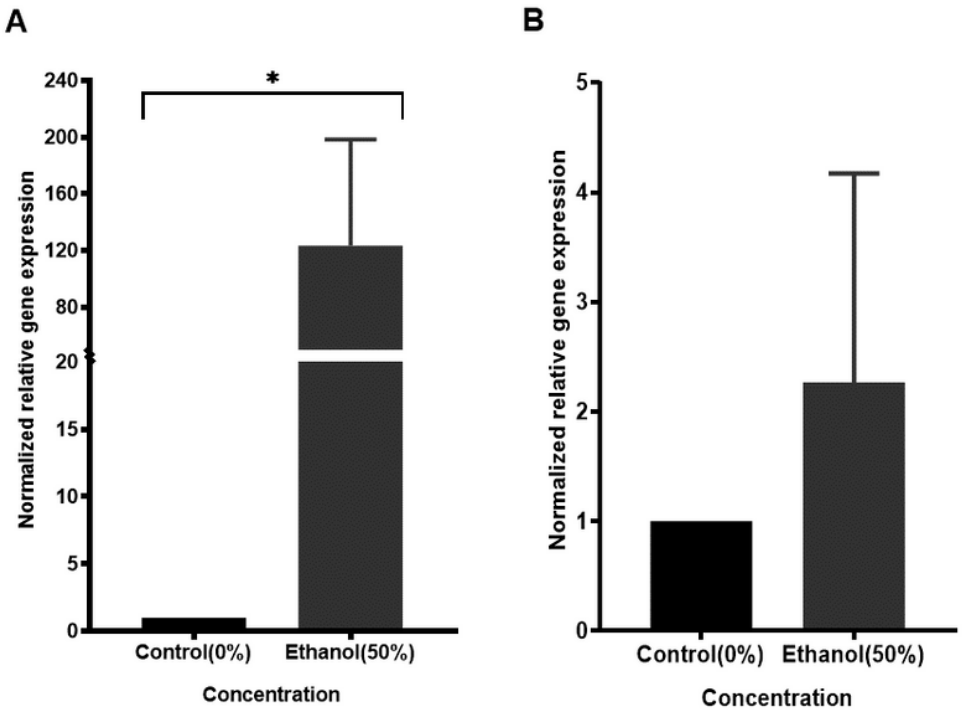

Figure 4

Effect of 50% ethanol on the expression levels of HlGST and HlGST2 in H. longicornis at 24 h post-exposure vis-à-vis the untreated group. (A) Expression levels of HlGST in 50% ethanol treated group compared to the untreated control group; (B) Expression levels of HlGST2 in 50% ethanol treated group compared to the untreated control group. T-test results compared the HlGST and HlGST2 expression levels between 50% ethanol treated group and untreated group. Data were presented as the means (n = 3) ± SE. Statistical significance was calculated at 5% probability using one-way ANOVA followed by Tukey's test. Asterisks above bars indicate significant difference between groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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