Heliyon 8 (2022) e10596

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Polyphagous insect *Olepa* sps. feeding on cardenolide rich *Calotropis gigantea* (L.) leaves and detoxification mechanism involving GST

Krishnamanikumar Premachandran, Thanga Suja Srinivasan *

Centre for Climate Change Studies, International Research Centre, Sathyabama Institute of Science and Technology, Chennai 600119, Tamil Nadu, India

ARTICLE INFO

Keywords:
Olepa
Calotropis gigantea
Sequester
GST
Glutathione-strophanthidin conjugate
Strophanthidin
Detoxification

ABSTRACT

Cardenolides, a group of cardiac glycosides are potent inhibitors of Na^+/K^+ ATPase pump in mammals, animals including insects. Some insects can circumvent the toxicity of cardenolides by mechanisms like target site resistance and metabolic resistance resulting in enhanced tolerance or adaptation. In this paper, we report an intriguing observation of a polyphagous feeder feeding gregariously on the leaves of *Calotropis gigantea* (L.) without any apparent adverse effect. No choice feeding assay showed higher larval biomass and reduced number of days to develop on *C. gigantea* leaves compared to *Ricinus* and banana. We found the activity of GST higher in *C. gigantea* fed larva and HR LC-MS analysis of *Olepa* sps. revealed the presence of glutathione-strophanthidin conjugate in larval body tissue. *In silico* molecular simulation results confirmed strong interaction between delta variant GST and glutathione-strophanthidin complex. The sequestration site and cost benefit of glutathione-strophanthidin sequestration in body tissues of *Olepa* sps. needs further investigation.

1. Introduction

Plants and insects have co-existed and shaped each other in evolution for more than 350 million years. The evolutionary arms race between plants and insects has resulted in the development of diverse plant defensive traits and counter-adaptive features in insects to recognize and activate an efficient immune response. Herbivores insects have evolved to overcome plant defence traits through behavioural like contact avoidance (*Helicoverpa zea*) (Musser et al., 2002) and molecular processes (Birnbaum et al., 2017) like target site resistance (*Chrysochus* sps.) (Labeyrie and Dobler, 2004) and metabolic resistance including rapid excretion (*Bombyx mori*) (Luque et al., 2002), sequestration (monarch butterflies) (Després et al., 2007), and enzymatic detoxification (*Myzus persicae*) (Francis et al., 2005).

Of all the defence strategies, the metabolic resistance including detoxification mechanism (biochemical conversion or metabolism) is most extensively studied (Després et al., 2007; Wouters et al., 2016). The mechanism of detoxification primarily varies among insect species depending on their host plant nutrition and insect physiology.

The most important detoxification enzymes of insects include cytochrome P450s monooxygenases (CYP 450s), glutathione S-transferases (GSTs) and esterase's (ESTs) which are mainly involved in the catalyses of various plant allelochemicals and harmful xenobiotics (Després et al., 2007). These enzymes are induced after ingestion of toxic compounds relating to resistance or tolerance: CYP450 resistance in *Culex quinquefasciatus* against insecticides (Liu et al., 2011); GST resistance in *Myzus persicae*, against isothiocyanates from Brassicaceous plants (Francis et al., 2005). These detoxification enzymes also interact and mediate the transport of toxic compounds across the insect midgut and store them into less toxic forms or excrete (Ali and Agrawal, 2012; War et al., 2018). In *Manduca sexta*, a cytochrome P450 gene (*CYP6B46*) product interacts with plant-derived nicotine converting them into an intermediate-transport form to cross the midgut and is then converted back to nicotine and excreted (Morris, 1983; Kumar et al., 2014). GSTs catalyses the conjugation of xenobiotics with hydrophilic groups such as glutathione, converting them to less reactive and more soluble form facilitating excretion or sequestration by specific carriers (Enayati et al., 2005; Aidlin Harari et al., 2020).

Olepa sps., a moth (Erebidae family) first described by Watson in 1980 (Kalawate et al., 2020). It is a polyphagous insect with a wider host range (banana, *Ricinus*, sunflower, cotton, castor, gingelly, maize, ivy gourd, brinjal etc.) and possesses the ability to deal with diverse phytochemical defence systems (Heckel, 2018). *Calotropis procera* was reported as a new host plant for the polyphagous lepidopteran pest, *Olepa ricini* (Fabricius) from the Indian region with 9 ± 0.5 days adult longevity (Farooqui et al., 2020). *Calotropis* sps. contain toxic cardiac glycosides which are

* Corresponding author.
E-mail addresses: thangasuja.cccs@sathyabama.ac.in, sujasree07@gmail.com (T.S. Srinivasan).

https://doi.org/10.1016/j.heliyon.2022.e10596
Received 6 April 2022; Received in revised form 14 August 2022; Accepted 6 September 2022

produced by plants for protection against pests. These toxic steroidal phytochemicals are distributed in more than 30 genera and 9 families of angiosperms including the milk weed plant (*Asclepias* sps. Apocynaceae family) (Morsy, 2017). The primary pharmacological effect of cardiac glycosides is to inhibit the Na^+/K^+ ATPase exchanger in cardiac muscles which is required for the active transport of Na^+ out and K^+ into the cell (Pirahanchi and Aeddula, 2019). Insects employ different molecular mechanisms for adaptation against toxic cardenolides like target site insensitivity, and metabolic resistance like detoxification, rapid excretion, efflux carriers and sequestration of cardenolides (Petschenka et al., 2013).

Monarch butterfly (*Danaus plexippus*), queen butterfly (*Danaus gilippus*), and plain tiger butterfly (*Danaus chrysippus*) ingest the cardenolides contained within the milkweed plant (*Asclepias*) and sequester them for defence against their predators (Nishida, 2002). *Danaus plexippus* and lygaeid bugs sequester the cardenolides into the integument (Brower and Glazier, 1975) or into the extra dorsal space (Scudder et al., 1986) facilitated by carrier-mediated process (Frick and Wink, 1995). *Aphid nerii* (milkweed aphid) on feeding milkweed plant differentially express several canonical insect detoxification genes, including genes encoding cytochrome P450s (CYP450s), glucuronosyltransferases (UGTs), uridine diphosphate (UDP), ATP-binding cassette transporters (ABC transporters), and glutathione S-transferases (GSTs) (Birnbaum et al., 2017).

The present study reports, *Olepa* sps. ability to detoxify toxic cardenolide compound strophanthidin present in *Calotropis gigantea* (L.) leaves. *Olepa* sps. larva was exposed to different host plant leaves and their adult development and longevity was assessed by no choice feeding assay. The detoxification enzyme activity of *Olepa* sps. associated with different host plant leaves including *C. gigantea* (L.) was studied. HR-LCMS analysis was performed to evaluate the fate of ingested cardenolide compound in *Olepa* sps. Finally, we discuss the results in the light of possible mechanism of GST detoxification enzyme interaction with cardenolide and sequestration in larval body based on HR-LC MS results and molecular dynamic simulation studies.

2. Materials and methods

2.1. Insect rearing

Olepa sps. first to second instar stage larvae approximately 20 nos. were collected from ivy gourd plants (*Coccinia grandis* (L.)) planted at the vegetable garden, Sathyabama Institute of Science and Technology (SIST) (12.8725° N, 80.2184° E), Tamil Nadu, India in August 2019. The larvae were immediately transferred to the agriculture lab of SIST in Petri plates (90 × 20 mm) with the natal host plant. The polyphagous larvae moved freely from the partially open Petri plates and started feeding on *C. gigantea* (L.) leaves which were kept for feeding *Danaus chrysippus* (plain tiger moth). Compared to the natal host plant, *Olepa* larva preferred to feed on *C. gigantea* (hereafter will be mentioned as *Calotropis* throughout the text). The larvae were further grown on *Calotropis* leaves for two generations before the experiments were carried out. The first and second instar larva avoided the vein region rich in glycosides and fed on the leaves by scrapping the leaf region (Figure 1 A and B). On reaching third instar, the larvae started feeding the entire leaf region including veins and the fully matured pre-pupal larva were gregarious feeder and left only the midrib region of leaves. *Calotropis* leaves were provided in excess and replaced daily. The fully developed larvae (pre-pupal stage) reached the top of the cages and pupated in the rearing cages. Newly emerged adults were transferred into new cages and provided with sucrose solution 0.1% as feed and were permitted to mate freely. They were provided with a fresh twig of *Calotropis* as mating and oviposition site. Newly laid eggs on *Calotropis* leaves were collected daily and transferred into new rearing cages under the same conditions as above.

2.2. Cytochrome c oxidase (COI) gene sequencing and evolutionary studies

Total genomic DNA was extracted from the head and thorax region of the larval sample excluding abdomen region mainly to avoid gut microbial DNA contamination using HighPrep Insect DNA kit from MagBio genomics by following the manufactures' protocol. The mitochondrial cytochrome oxidase subunit I gene (COI) was amplified using universal primer pair LCO1480 (5′GGTCAACAAATCATAAAGATATTGG-3′) and HCO1298 (5′TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). The amplified products were visualized using 1 per cent agarose gel and the high-intensity bands were eluted. The eluted PCR products were sequenced directly in both directions using ABI Prism 3730 Genetic Analyzer based on Big Dye Terminator Chemistry at Eurofins genomics, Bangalore. The raw sequences were aligned using BioEdit software version 7.2.5. The obtained sequences were deposited in NCBI and sequence similarity search was performed using BLAST algorithm NCBI for species identification. Sequences of related eight *Olepa* sps. (*O. schleini; O. ricini; O. toulgoeti; O. koslandana; O. ghatmatha; O. zedesi; O. suryamal*) were downloaded from the NCBI database. A phylogenetic tree was constructed using the maximum likelihood method on MEGA software version X (Kumar et al., 2018). Bootstrap value kept as 1000.

2.3. No choice feeding assay

Olepa sps. larvae used in the experiments were obtained from the second-generation colonies grown on *Calotropis* leaves.

To assess adult development and longevity on different host plants, a no-choice bioassay was conducted using neonate larvae. *Olepa* sps. is a secondary pest of banana, *Ricinus* and few other crop plants and accordingly *Calotropis*, *Ricinus* and banana leaves were chosen for the bioassay. The natal hostplant ivy gourd was not included as enough leaf samples for conducting the experiment was not available during the

A) B)

Figure 1. Stereo microscopic images of different stage instars of *Olepa* sps. larva. Image A and B were represented at 200 μm scalebar A) First instar larva on *Calotropis gigantea* (L.) leaf immediately after hatching. B) Feeding pattern of first instar larva leaving behind leaf veins.

experimental period. Immediately after hatching, seven neonates were placed on leaves kept inside a 500 mL wide-mouth beaker wrapped with perforated transparent plastic cover for air circulation. Five grams of fresh leaves were placed inside each beaker on daily basis. Faecal waste and plant debris were removed frequently to avoid contamination. The larvae were monitored carefully for survival and number of days taken to reach fourth instar stage. Once the larva attained fourth instar stage they were carefully removed and weighed for their biomass build-up. They were further allowed to develop as adults and the number of days for development was observed. The experiment was randomly replicated three times.

2.4. Enzyme preparation and estimation

The experiment was replicated three times with live insects. *Olepa* sps. reared on *Calotropis*, *Ricinus* and banana host plant leaves were used in the experiment. Fourth-instar larva from each host plant leaf was taken for the preparation of whole larval homogenate using homogenization buffer (100 mM phosphate buffer, pH 7.0 containing 1 mM EDTA, 1 mM PTU, 1 mM PMSF and 20% glycerol). The larvae were homogenized thoroughly and centrifuged at 10,000 rpm for 20 min at 4 °C, and the supernatant was used as an enzyme source for carboxylesterase and glutathione S-transferase assay. For cytochrome P450, the extract was prepared from larval mid-guts. The larvae were dissected under a stereo microscope and the midgut region was collected. The collected extract was subjected to further centrifugation and served as the enzyme source. The three biological replicates of *Olepa* sps. fed with host plant leaves were pooled together to form bulk sample for further analysis. Protein concentration was determined using the Bradford estimation method and bovine serum albumin (BSA) was used as the standard. The protein content was measured at 595 nm absorbance in EnSpire™ Multimode Plate Reader by PerkinElmer, Inc.

2.5. Activity of detoxification enzymes

Carboxylesterase, glutathione S-transferase and cytochrome P450 enzymes are major detoxification enzymes present in the insect body. Estimation of carboxylesterase enzyme activity was done by the protocol adopted from (Hosokawa and Satoh, 2001). The activity of glutathione S-transferase (GST) was measured by following Kranti (2005) protocol against substrate 1-chloro-2, 4-dinitrobenzene (CDNB) (Srivastava and Karuppaiah, 2017). Estimation of cytochrome P450 enzyme activity was carried out by fluorescence assay (Cao and Liu, 2018). The enzyme estimation was technically replicated thrice.

2.6. HR-LCMS analysis

Olepa sps. larva fed with *Calotropis* leaves and their excreted faecal waste and fresh *Calotropis* leaves were subjected to HR-LCMS analysis to confirm the cardenolide presence. *Calotropis* leaves were freshly collected and cleaned with distilled water and kept in a hot air oven at 50 °C for 24 h. The dried leaves were powdered and Soxhlet extraction method was followed with 90% methanol as solvent. On the other hand, two evenly weighed larvae were taken and the midgut portion was removed from the larva particularly to avoid leaf extracts. The remaining portion of the larvae and 100mg of faecal pellets were separately homogenized with methanol and chloroform and kept for 16 h incubation at room temperature. Further, it was centrifuged and supernatant was discarded. Both chloroform and methanol pellets were dissolved with fresh methanol and pooled together and was taken for HR-LCMS analysis.

Q-Exactive Plus Biopharma (Thermo Scientific) instrument was used for analysis and Hypersil Gold 3 micron 100 × 2.1 MM C18 column was used. 0.1% formic acid in milli q water served as solvent A and methanol served as Solvent B. Runtime was set to 30 min and the flow rate was 3 μL/min. The acquisition method was set to be minimum range 80 (M/Z) and maximum 1200 (M/Z) with scanning rate for each spectrum per second. Thermo Scientific Xcalibur, Version 4.2.28.14 software used for data acquisition and Compound Discoverer 2.1 SP1 software used for data processing.

2.7. Molecular docking

Protein-ligand docking was done to predict the interaction between GST sigma and delta variant with GSH-strophanthidin complex. GST sigma and delta variants of insects are mainly involved in insect detoxification mechanism (Enayati et al., 2005; Hassan et al., 2019). Sigma variant GST enzyme of *Hyphantria cunea* and delta variant GST enzyme of *Bombyx mori* sequence was obtained from UniProt Database (https://www.uniprot.org/) and 3D structure was predicted with SWISS-MODEL homology modelling web server (https://swissmodel.expasy.org/) (Waterhouse et al., 2018). Strophanthidin structure was taken from the PubChem database. Auto Dock Vina software (Version 1.1.2) (Trott and Olson, 2009) used to dock the sigma variant GST enzyme along with GSH-strophanthidin complex (Model 1) and delta variant GST enzyme along with GSH-strophanthidin complex (Model 2).

2.8. Molecular dynamic (MD) simulation

Molecular dynamic (MD) simulation was performed to know the physical motions of atoms in protein-ligand interaction at the real atmosphere level. GST sigma - GSH-strophanthidin complex (model 1) and GST delta – GSH-strophanthidin complex (model 2) was further taken for MD simulation studies. The MD simulation for the complexes were carried out for 50 nanoseconds (ns) at GROMACS Version 5.1.2 (Abraham et al., 2015) using the OPLS-AA/L force field. The simulation started with solvating the GST sigma – GSH-strophanthidin complex (model 1) and GST delta – GSH-strophanthidin complex (model 2) in the triclinic box using TIP3P water model. The counter ions were added to neutralize the system. The system was energy minimized using the steepest descent algorithm with a maximum step size of 0.01nm and tolerance of 1000 kJ/mol/nm. System was equilibrated by using NVT and NPT ensemble for 100 picoseconds (ps). Finally, 50 ns production MD was performed for the system. Root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), hydrogen bond and minimum distance results were observed using GROMACS inbuilt tools (Abraham et al., 2015).

2.9. Statistical analysis

All the data were stated as mean ± S.E value. One-way analysis of variance (ANOVA) was performed to compare the data between different feeds and detoxification enzyme activity using SPSS 26.0.

3. Results

3.1. Cytochrome c oxidase (COI) mitochondrial gene sequencing

Morphological observations confirmed the genus as *Olepa* and a voucher specimen submitted at the insect collection box, agriculture lab, Sathyabama Institute of Science and Technology. The larval samples were subjected to COI mitochondrial gene sequencing for species identification. Sequence similarity search of the query sequence using BLAST analysis showed 70% similarity with *Olepa schleini* collected from Israel confirming the genus as *Olepa*. However, species-level information was not obtained due to sequence quality and query coverage. Phylogenetic analysis was done to identify the evolutionary relationship with other *Olepa* sps. Two different clades were observed: *O. schleini, O. ricini and O. toulgoeti* fall under clade I and *O. koshlandana, O. ghatmatha, O. zedesi and O. suryamal* fall under clade II (Figure 2). The query sequence falls outside the clades and considered as a different *Olepa* sps. Further in-depth sequence information is required for obtaining species-level information.

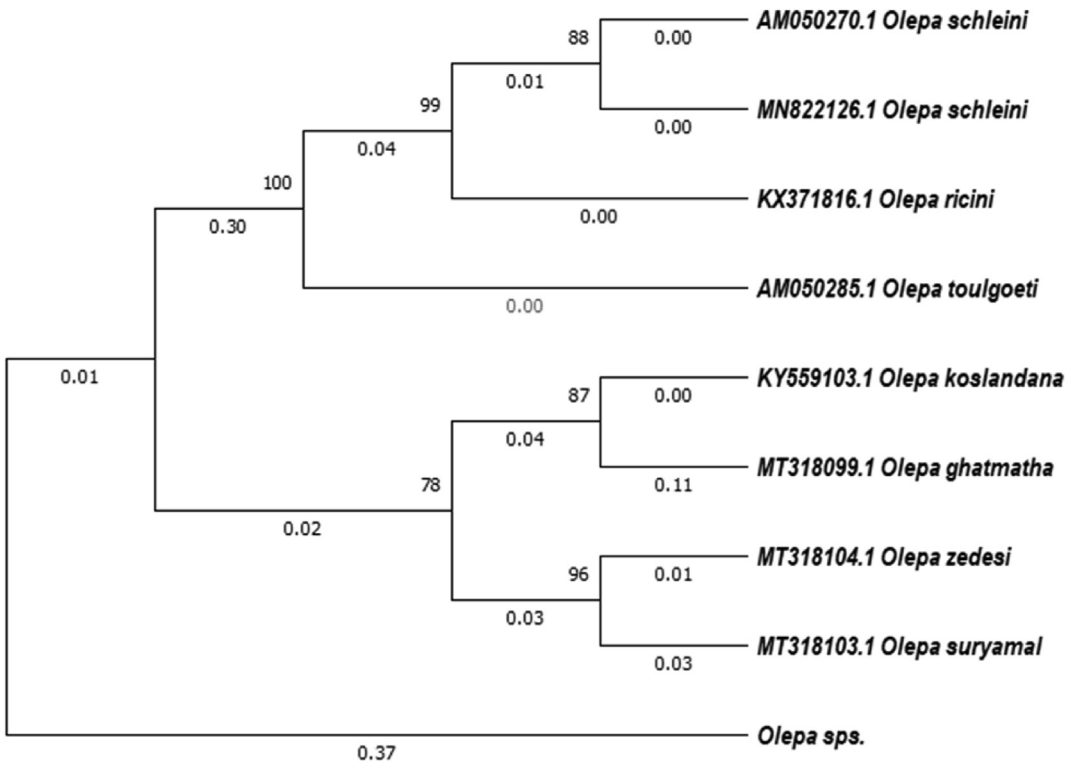

Figure 2. Unrooted phylogenetic tree analysis of *Olepa* species obtained from NCBI database along with query sequence. Bootstrap values shown at branch point.

3.2. No choice feeding assay

Olepa sps. showed more preference towards *Calotropis* than *Ricinus* and banana leaves. Average larval biomass build-up and days to develop was significantly higher in *Olepa* sps. fed with *Calotropis* leaves compared to *Ricinus* and banana leaves (Figures 3A and 3B). The larval biomass build-up of *Olepa* sps. fed with *Calotro*pis was rapid and took a week time (6.8 ± 0.2) to develop into an adult moth (Figures 3A and 3B). Degrees of freedom (*df*) value for the one-way ANOVA was 8 and P value of larval biomass (0.00) and average days to develop (0.00) are lower than the P calculated value (0.05%) which shows that the results are statistically significant.

3.3. Enzyme assays

The activity of CYP450, GST, and carboxylesterases was estimated from larval samples of *Olepa* sps. fed with different host plant leaves (Figure 4A, B, C). The activity of GST increased significantly in *Olepa* sps. larva fed with *Calotropis* compared to other host plant leaves (Figure 4B). Degrees of freedom (*df*) value for the one-way ANOVA was 8. P value of

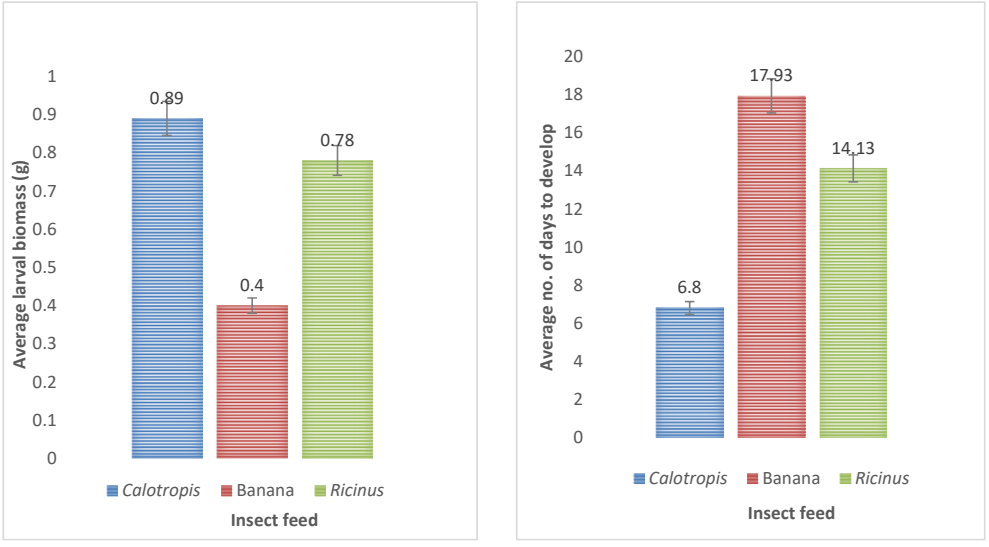

Figure 3. A) Average larval biomass build-up of *Olepa* sps. on different host leaves. B) Average number of days to develop as adults on different host leaves. Blue, red and green color denotes the *calotropis,* banana and *ricinus* respectively.

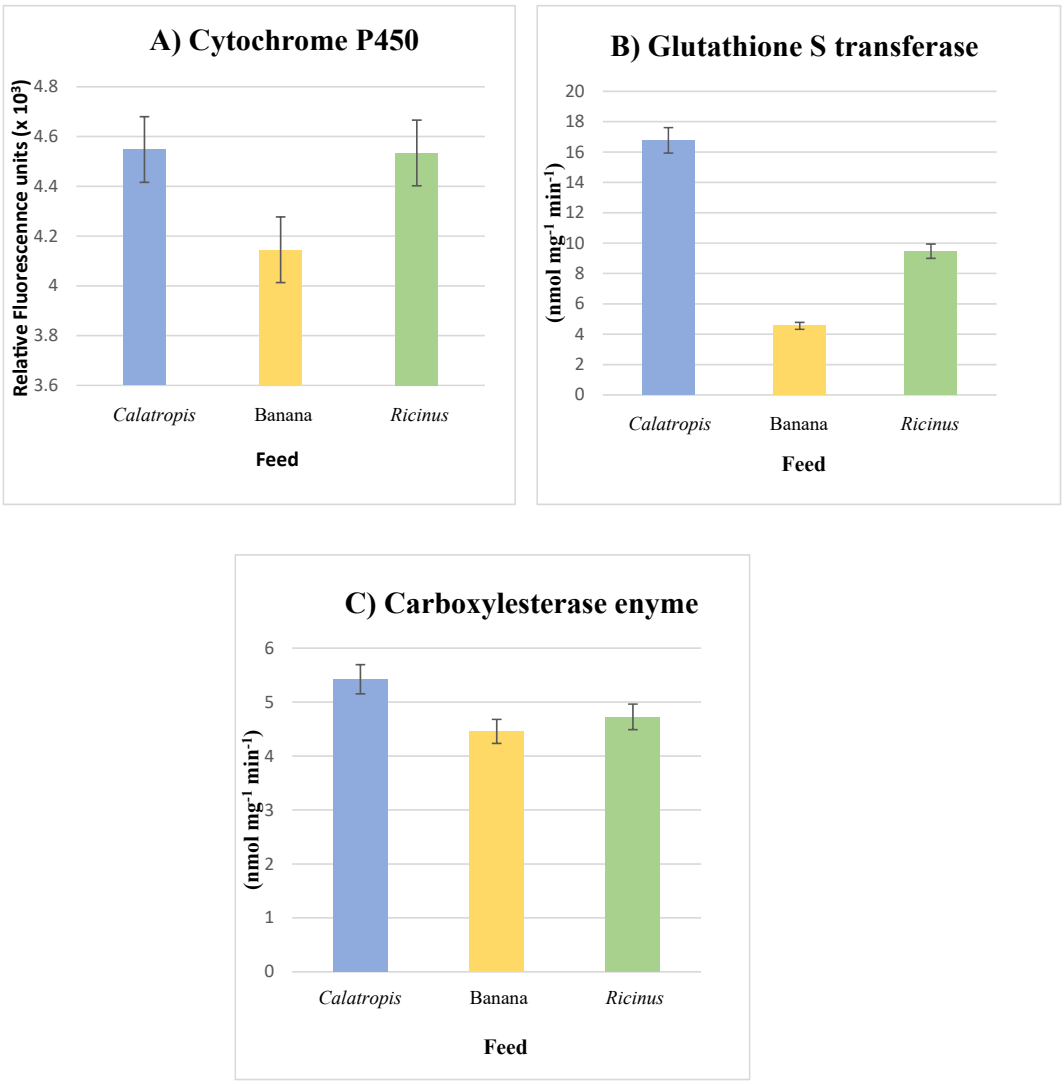

Figure 4. Estimation of detoxifying enzymes activity of *Olepa* sps. feeding on different host plant leaves. A) Cytochrome P450 enzyme B) Glutathione S transferase enzyme C) Carboxylesterase enzyme. Error bars represent the standard deviation of means (3 replicates). Blue, orange and green color denotes the enzyme activity of insects fed *calotropis,* banana and *ricinus* as food source.

GST (0.00) was lower than the calculated P (0.05%) value and was statistically significant. P value of cytochrome P450 (0.46) and carboxylesterase (0.38) was higher than the calculated P value (0.05%) and statistically non-significant. It is interesting to note that the activity of carboxylesterase was higher in larva fed with *Calotropis* compared to *Ricinus* and banana but was not statistically significant (Figure 4C).

3.4. Cardenolide compounds identified from HR-LCMS

HR-LCMS analysis of 90% methanolic extract of *Calotropis* leaves, *Olepa* sps. larvae fed with *Calotropis* leaves and faecal extracts had different components like metabolites, amino acids, small molecules, and polypeptides. A total of 111 compounds were identified in *Calotropis* leaves which includes eight steroidal compounds: meprednisone, desoxycortone, 17α-hydroxyprogesterone, strophanthidin, testosterone acetate, deoxycorticosterone 21-glucoside, 5α-Dihydrotestosterone and deoxycorticosterone acetate (Data not shown). Of the eight steroids, strophanthidin is a cardenolide with a five-membered furanone ring. On the other hand, larval samples of *Olepa* sps. had a total of 96 compounds including strophanthidin and cholest-4-en-3-one ($C_{27}H_{44}O$) (Figure S1). Cholest-4-en-3-one is an oxidised metabolite of cholesterol which was not observed in *Calotropis* leaf sample but present in the larval sample of *Olepa* sps. at a retention time of 25.75 min (Figure S1). Apart from that, many fatty acid related compounds were identified in the chromatogram of *Calotropis* sps. of which few fatty acid compounds were observed in common (Data not shown). In the faecal waste of *Olepa* sps. 41 different compounds were identified and no cardenolide related toxic compounds or metabolized steroidal compounds were detected in HR- LCMS (Figure S1).

Strophanthidin cardenolide was observed at 12.46 and 12.36 RT min in *Calotropis* leaf sample and *Olepa* sps. fed with *Calotropis* leaves respectively. The peak at 12.46 and 12.36 RT min represents MH+ of strophanthidin with 405.22 *m/z* ratio (Figure 5, Figure S1). In addition to strophanthidin, HR- LC MS results of *Olepa* sps. fed with *Calotropis* leaf samples had glutathione and glutathione-strophanthidin conjugate at 14.28, and 26.72 RT min with 308.22 and 694.49 *m/z* respectively (Figure 6). The thiol group (SH) of reduced glutathione 307.33 *m/z* (MH+ 308.22 *m/z*) interacts with electrophilic hydroxy group (–OH) of strophanthidin 404.49 *m/z* (MH+ 405.22 *m/z*) by enzymatic reaction of glutathione S-transferase and forms glutathione-strophanthidin conjugate (711.82 *m/z*). Due to the loss of water molecule (Δ18 Da) during conjugation of glutathione with strophanthidin, glutathione-strophanthidin conjugate was obtained at the peak of MH+ 694.49 *m/z* at 26.72 RT min (Figure 6).

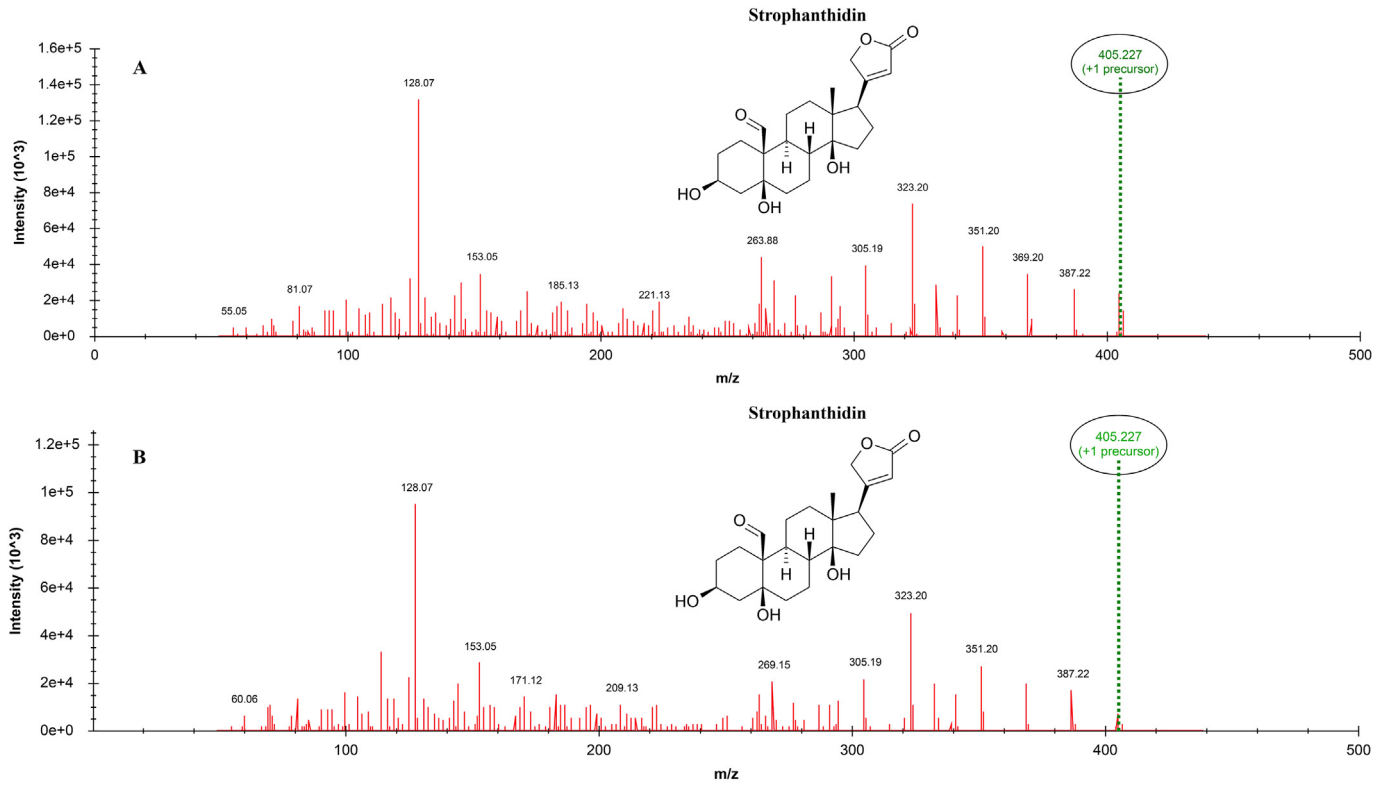

Figure 5. Strophanthidin, a cardenolide compound was observed in *Calotropis gigantea* leaf sample and *Olepa* sps. fed with *Calotropis* leaves. A) Strophanthidin (MH+ 405.22 *m/z*) from *Calotropis gigantea* leaf sample obtained at 12.46 RT min. B) Strophanthidin (MH+ 405.22 *m/z*) from *Olepa* sps. fed with *Calotropis* leaves obtained at 12.36 RT min.

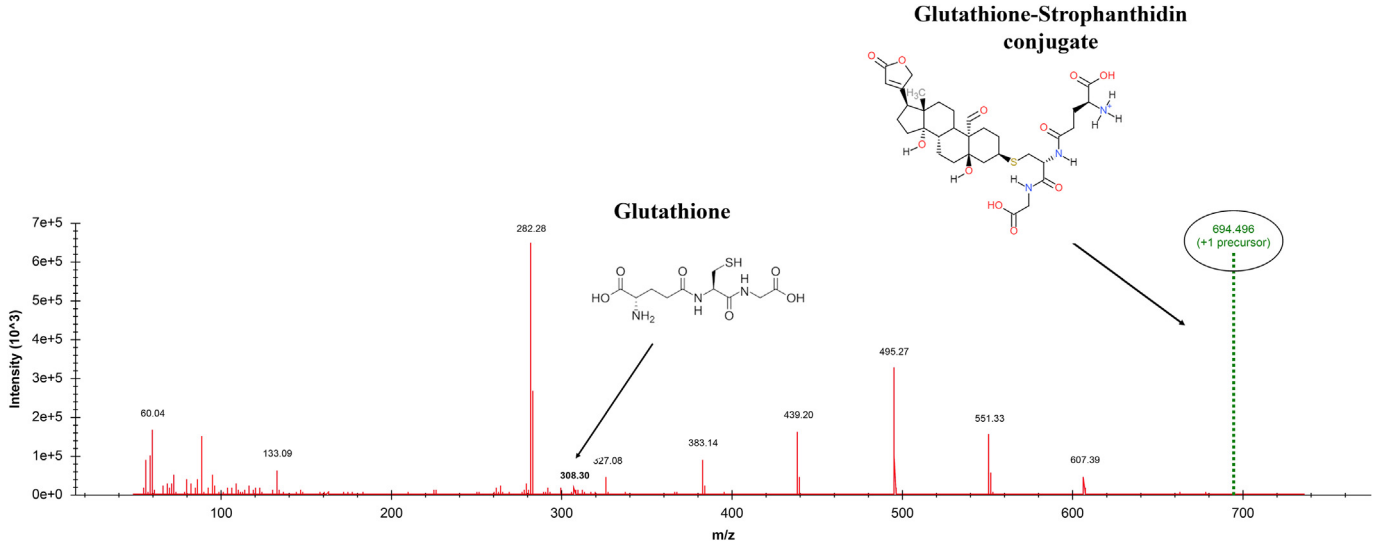

Figure 6. The chromatogram represents the presence of glutathione-strophanthidin conjugate (MH+ 694.49 *m/z*) at 26.72 RT min in *Olepa* sps. larval body fed with *Calotropis* leaves. The thiol group (SH) of reduced glutathione 307.33 *m/z* (MH+ 308.22 *m/z*) interacts with electrophilic hydroxy group (–OH) of strophanthidin 404.49 *m/z* (MH+ 405.22 *m/z*) and forms glutathione-strophanthidin conjugate (711.82 *m/z*). Due to the loss of water molecule (Δ18 Da) during conjugation of glutathione with strophanthidin, glutathione-strophanthidin conjugate was obtained at the peak of MH+ 694.49 *m/z* at 26.72 RT min.

3.5. Molecular docking and simulation

GST enzyme (sigma & delta) docked with GSH-strophanthidin complex individually. GST delta - GSH-strophanthidin complex (Model 2) shows higher number of hydrogen bonds (10) than GST sigma - GSH-strophanthidin complex (Model 1). The energy score of both docked models were −8.1 (Data not shown). Interaction of GSH-strophanthidin complex with GST delta enzyme (model 2) was higher than GST sigma enzyme (model 1). In model 1, strophanthidin interacted with tyrosine and phenylalanine amino acid at 8 and 9th position of GST whereas GSH interacted with methionine, serine, cysteine, glutamic acid, threonine, proline, asparagine and arginine at 158, 63, 67, 17, 64, 14, 95 and 98th position respectively of GST (Figure 7A). In model 2 strophanthidin interacted with valine and histidine at 8 and 36th position of GST

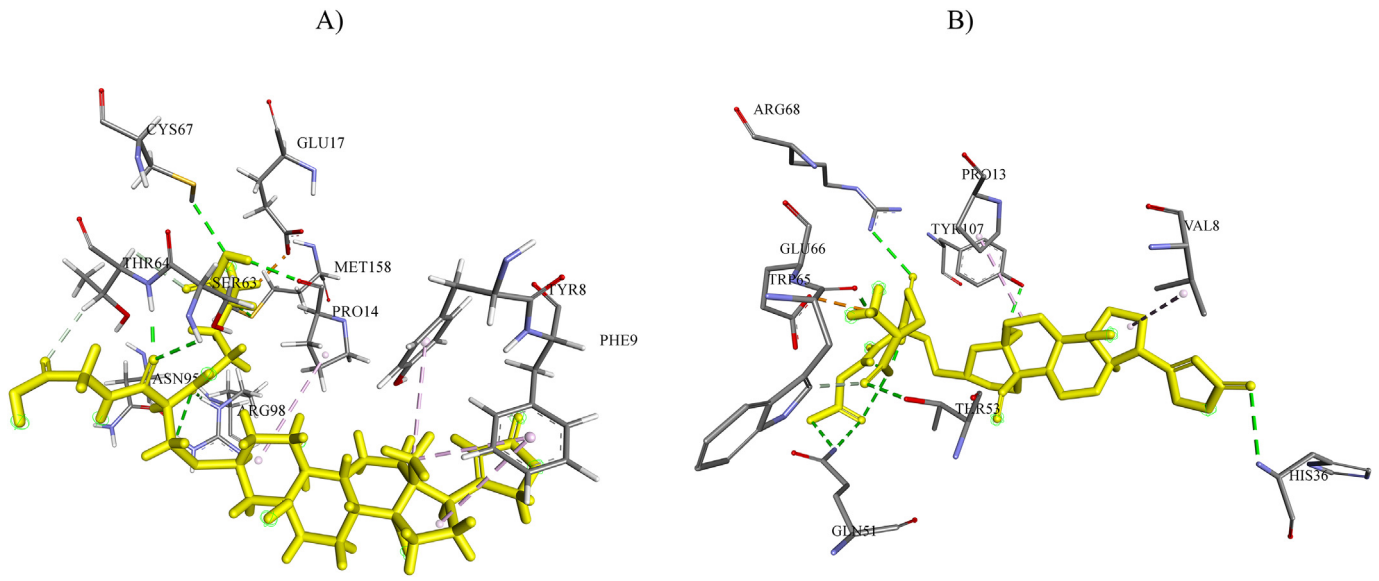

Figure 7. Molecular docking interaction of GST sigma and GST delta enzyme with GSH-strophanthidin complex. A) GST sigma with GSH-strophanthidin complex. B) GST delta with GSH-strophanthidin complex. Yellow color structure represents the GSH-stropahnthidin complex.

whereas GSH interacted with proline, tyrosine, arginine, glutamic acid, tryptophan, threonine and glutamine amino acid at 13, 107, 68, 66, 65, 53 and 51st position respectively of GST (Figure 7B). Further confirmation of the interaction was carried out by MD simulation.

MD simulation for model 1 and model 2 was carried out in the solvated states for 50 ns. The RMSD values of model 1 and model 2 were calculated with respect to the initial structure as a frame reference (0–50 ns). The RMSD values were steadily increased from 0 to 5 ns for both models and reached equilibration with few oscillations. Model 2 was steady throughout the simulation period whereas model 1 had more deviation (Figure 8). The amino acids dynamic movement change in protein complexes were analysed using RMSF. The overall fluctuation of model 1 was way higher than the model 2. Interactive sites of the model 2 was less fluctuated throughout the simulation period (Figure 8). The average Rg value of model 1 was 1.82 ± 0.01 and model 2 was 1.69 ± 0.01 nm. Model 2 was more compact than model 1 and average hydrogen bond maintained as 6 for model 2 and 2 for model 1. Minimum distance of model 1 (0.22 ± 0.09 nm) and model 2 (0.19 ± 0.09 nm) replicated the compactness of the docked models throughout the molecular interaction (Figure 8).

4. Discussions

The present study reports *Olepa* sps. larva feeding gregariously on *Calotropis* leaves that are rich in toxic cardenolides specifically strophanthidin. *Olepa* sps. were able to feed, survive, oviposit and continue their population cycle on *Calotropis* leaves. The enzyme activity of GST was higher in *Olepa* sps. larva fed with *Calotropis* and glutathione-strophanthidin conjugate was identified in the larval body tissue (Figure 6). *In silico* molecular dynamic simulation studies further suggested the interaction between GST and glutathione-strophanthidin complex (Figure 8).

Olepa sps. is a polyphagous feeder and polyphagous insects normally tolerate vast plant defence traits and possess mechanisms to manipulate host plant defence pathways (War et al., 2018). In addition, some polyphagous can consume remarkable quantities of toxic compounds which is either metabolized or sequestered into its non-toxic form (War et al., 2018). The polyphagous arctiids *Grammia geneura* and *Estigmene acrea* sequester plant pyrrolizidine alkaloids and exploit them as drug store compounds for pheromone biosynthesis (Hartmann et al., 2005). In the present study, *Olepa* sps. larva fed with *Calotropis* exhibited higher larval biomass (0.89 ± 0.3) (Figure 3A) and reduced number of days to develop into adult (6.8 ± 0.2) (Figure 3B) compared to *Ricinus* and banana. Despite the fact, *Olepa* larva has no prior physiological adaptation on *Calotropis* host plant, the larva exhibited higher survival, biomass build-up and fecundity on *Calotropis* in all generations of rearing (Data not shown) indicating no effect of ingested strophanthidin on insect fitness. Further, strophanthidin, which is an analogue of ouabain has a high affinity for Na^+/K^+ ATPase enzyme inhibiting their activity (Tobin and Abramson, 1975), however, the presence of strophanthidin in *Calotropis* leaves had no effect on *Olepa* sps. fitness (Figure 3A and B). These suggest that the toxic strophanthidin can be sequestered or metabolised by *Olepa* sps. larva without affecting insect fitness. This is contrary to the common observation of polyphagous insects exhibiting lower biomass build-up and increased development time on more toxic plants (Gols et al., 2008).

In the study, GST enzyme activity was significantly higher in larva fed with *Calotropis* compared to *Ricinus* and banana leaves. GST enzyme activity in the generalist insect *Myzus persicae* (green peach aphid), increased rapidly upon ingestion of toxic isothiocyanate rich *Brassica* leaves (Francis et al., 2005). GST enzymes of delta and epsilon subclasses are involved in the metabolism of λ-cyhalothrin insecticide in codling moth (*Cydia pomonella*) larvae (Hu et al., 2019; Wang et al., 2019). The expression levels of GST genes were upregulated on exposure to LD_{10} dose of *lambda*-cyhalothrin in codling moth and the levels were considerably higher in *lambda*-cyhalothrin-resistant codling moth populations (Hu et al., 2022). HR-LC MS analysis of *Olepa* sps. larvae revealed glutathione-strophanthidin conjugate presence in larval body tissue. The induced GST enzymes are primarily involved in catalyzing the conjugation between sulfhydryl (–SH) group of glutathione and primary hydroxyl group (–OH) of strophanthidin forming glutathione-strophanthidin conjugate (694.49 m/z ratio) (Figure 6). On the other hand, –OH group of cardenolide steroidal skeleton forms hydrogen bonds with specific amino acid residues of Na^+/K^+ ATPase enzymes inhibiting Na^+/K^+ channels in cardiac muscles (Ogawa et al., 2009). However, the conjugation reaction between electrophilic –OH group of strophanthidin and nucleophilic –SH group of glutathione neutralizes the primary electrophilic site and protects from further attack of strophanthidin on neutrophilic sites of enzymes and DNA. Further, glutathione conjugated with xenobiotics is more water soluble and is safely sequestered or excreted from insect body tissues (War et al., 2018).

Interestingly, ingested strophanthidin was present in the body tissue of the insect and was not detected even in traces from the faecal waste of

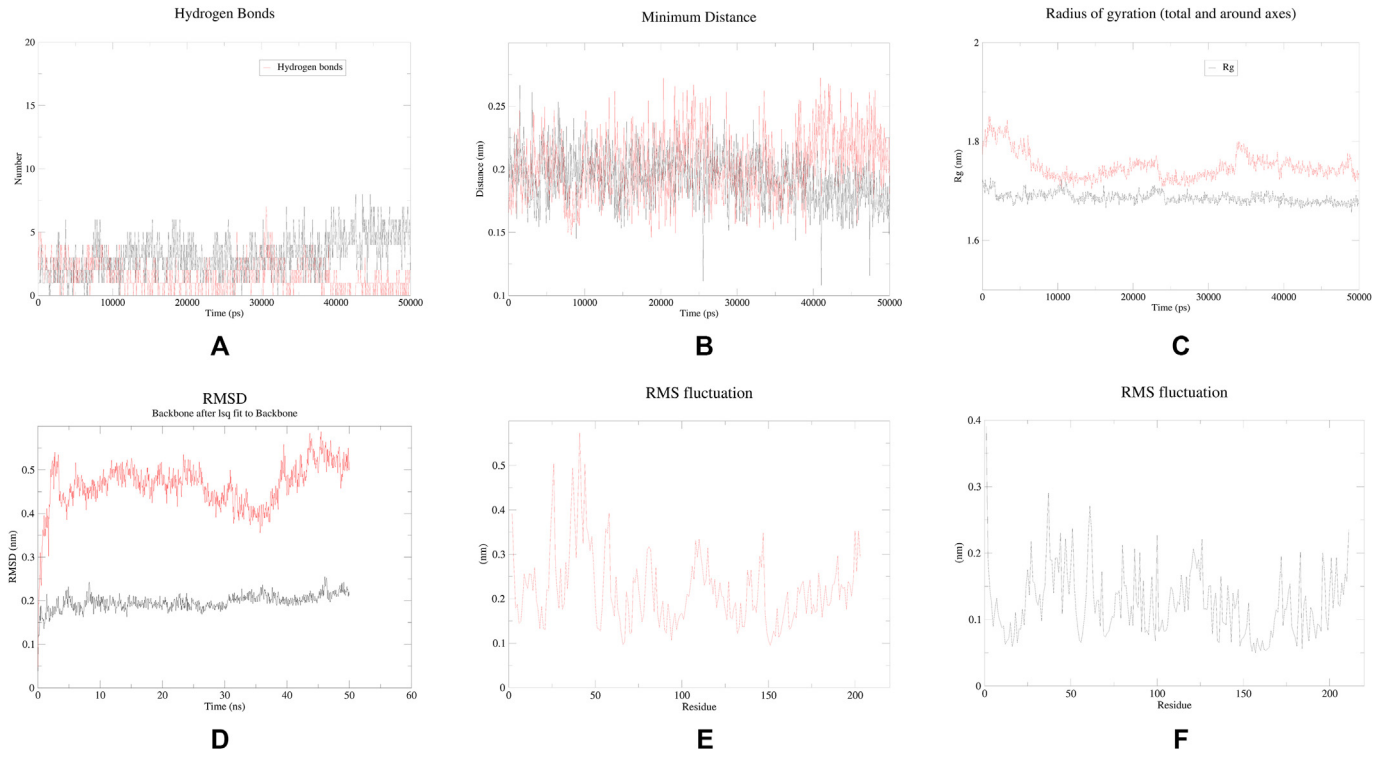

Figure 8. Molecular dynamic simulation results of GST sigma and GST delta enzyme with GSH-strophanthidin complex. A) Hydrogen bonds, B) Minimum distance, C) Radius of gyration (Rg), D) Root-mean-square deviation (RMSD), E) Root-mean-square fluctuation (RMSF) of GST sigma with GSH-strophanthidin complex and F) Root-mean-square fluctuation (RMSF) of GST delta with GSH-strophanthidin complex. Red color and black color denotes the GST sigma and GST delta enzyme respectively.

Olepa sps. (Figure S1). Thus, the cardenolide ingested by the larval stages are retained in the insect adulthood and can contribute for insect fitness and chemical defense against predatory insects and mammals. These results indicate *Olepa* sps. ability to ingest and tolerate toxic cardenolide strophanthidin by specialized biochemical mechanism involving GST in strophanthidin sequestration as glutathione-strophanthidin conjugate facilitating insect fitness. Recombinant soluble GST proteins and *in silico* simulation studies confirmed the interaction and conjugation reaction of GST proteins with λ-cyhalothrin insecticide as a means of tolerance mechanism in codling moth (Hu et al., 2022). Molecular docking and dynamic results predict a stronger interaction between delta variant of GST and glutathione-strophanthidin conjugate (Figures 7 and 8). However, glutathione-strophanthidin conjugate sequestration in various body tissues and costs and benefits of sequestration needs further investigation.

Additionally, cholest-4-en-3-one, oxidised metabolite of cholesterol is observed as a major peak in the chromatogram of *Olepa* sps. but not in *Calotropis* leaves (Figure S1). Apart from cardenolide strophanthidin, numerous fatty acid derivatives were observed in the chromatogram of *Calotropis* leaves (Data not shown). Generally, insects cannot synthesize sterols and are dependent on phytosterols for dietary supplementation of nutrients for various physiologically active processes like ecdysteroids. The observed cholest-4-en-3-one may be a metabolized product of the phytosterol fatty acids found in leaf tissues and can serve as precursor for the synthesis of insect pheromones and metabolites. In the study, *Olepa* sps. fed with *Calotropis* leaves had a higher larval biomass and reduced developmental time compared to other host plant leaves. Cholest-4-en-3-one accumulation in *Olepa* sps. had no effect on adult survival and fecundity. In contrast, ketosteroids like cholestan-3-one and cholest-4-en-3-one supplemented in the diet are shown to exhibit negative effects on *M. persicae* survival and fecundity (Bouvaine et al., 2014). The discrepancies in chloest-4-en-3-one accumulation on *Olepa* sps. fitness needs further investigation. Our results are the first to report strophanthidin sequestration by *Olepa* sps. Nevertheless, the functional and ecological significance of the sequestered cardenolides has not been analysed in the study. We are currently involved in understanding glutathione-strophanthidin sequestration and the relative quantification of strophanthidin from different body parts of *Olepa* sps.

Declarations

Author contribution statement

Krishnamanikumar Premachandran: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Thanga Suja Srinivasan: Conceived and designed the experiments; Wrote the paper.

Funding statement

This work was supported by the Science and Engineering Research Board (SERB), DST, (Grant No. ECR/2017/001081).

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e10596.

Acknowledgements

The corresponding author is acknowledging Sathyabama Institute of Science and Technology for support and author Premachandran K acknowledging Mr. Carlton Ranjith W.A for the constant support.

References

Abraham, M., Hess, B., van der Spoel, D., Lindahl, E., 2015. Gromacs 5.0.7. Www.Gromacs.Org.

Aidlin Harari, O., Santos-Garcia, D., Musseri, M., Moshitzky, P., Patel, M., Visendi, P., Seal, S., Sertchook, R., Malka, O., Morin, S., 2020. Molecular evolution of the glutathione S-transferase family in the Bemisia tabaci species complex. Genome Biol. Evol. 12, 3857–3872.

Ali, J.G., Agrawal, A.A., 2012. Specialist versus generalist insect herbivores and plant defense. Trends Plant Sci. 17, 293–302.

Birnbaum, S.S.L., Rinker, D.C., Gerardo, N.M., Abbot, P., 2017. Transcriptional profile and differential fitness in a specialist milkweed insect across host plants varying in toxicity. Mol. Ecol. 26 (23), 6742–6761.

Bouvaine, S., Faure, M.L., Grebenok, R.J., Behmer, S.T., Douglas, A.E., 2014. A dietary test of putative deleterious sterols for the aphid Myzus persicae. PLoS One 9 (1).

Brower, L.P., Glazier, S.C., 1975. Localization of heart poisons in the monarch butterfly. Science 188 (4183), 19–25.

Cao, H.H., Liu, T.X., 2018. Evaluation and Optimization of the Protocols for Measuring Cytochrome P450 Activity in Aphids bioRxiv.

Després, L., David, J.P., Gallet, C., 2007. The evolutionary ecology of insect resistance to plant chemicals. Trends Ecol. Evol. 22 (6), 298–307.

Enayati, A.A., Ranson, H., Hemingway, J., 2005. Insect glutathione transferases and insecticide resistance. Insect Mol. Biol. 14, 3–8.

Farooqui, S.A., Siddiqua, K.S., Parwez, H., Joshi, R., 2020. First report of Olepa ricini Fabricius, 1775 (Erebidae: Arctiinae: Arctiini) feeding on weed plant calotropis procera (Aiton) W.T. Aiton (Apocynaceae) from India. Trans. Am. Entomol. Soc. 146 (3), 459–461.

Francis, F., Vanhaelen, N., Haubruge, E., 2005. Glutathione S-transferases in the adaptation to plant secondary metabolites in the Myzus persicae aphid. Arch. Insect Biochem. Physiol. 58 (3), 166–174.

Frick, C., Wink, M., 1995. Uptake and sequestration of ouabain and other cardiac glycosides in Danaus plexippus (Lepidoptera: Danaidae): evidence for a carrier-mediated process. J. Chem. Ecol. 21, 557–575.

Gols, R., Bukovinszky, T., Van Dam, N.M., Dicke, M., Bullock, J.M., Harvey, J.A., 2008. Performance of generalist and specialist herbivores and their endoparasitoids differs on cultivated and wild Brassica populations. J. Chem. Ecol. 34, 132–143.

Hartmann, T., Theuring, C., Beuerle, T., Bernays, E.A., Singer, M.S., 2005. Acquisition, transformation and maintenance of plant pyrrolizidine alkaloids by the polyphagous arctiid Grammia geneura. Insect Biochem. Mol. Biol. 35, 1083–1099.

Hassan, F., Singh, K.P., Ali, V., Behera, S., Shivam, P., Das, P., Dinesh, D.S., 2019. Detection and functional characterization of sigma class GST in Phlebotomus argentipes and its role in stress tolerance and DDT resistance. Sci. Rep. 9, 1–15.

Heckel, D.G., 2018. Insect detoxification and sequestration strategies. In: Claudia, Voelckel, Georg, Jander (Eds.), 47. Annu. Rev. Plant. Biol., pp. 77–114.

Hosokawa, M., Satoh, T., 2001. Measurement of carboxylesterase (CES) activities. Curr. Protoc. Toxicol. 10, 1–14.

Hu, C., Liu, J.-Y., Wang, W., Mota-Sanchez, D., He, S., Shi, Y., Yang, X.-Q., 2022. Glutathione S-transferase genes are involved in lambda-cyhalothrin resistance in Cydia pomonella via sequestration. J. Agric. Food Chem. 70, 2265–2279.

Hu, C., Wang, W., Ju, D., Chen, G., Tan, X., Mota-sanchezhi, D., Yang, X., 2019. Functional characterization of a novel λ-cyhalothrin metabolizing glutathione S-transferase, CpGSTe3, from the codling moth Cydia pomonella, 76. Pest Manag. Sci., pp. 1039–1047.

Kalawate, A.S., Pawara, S., Shabnam, A., Dinesh, K.P., 2020. DNA barcode reveals the occurrence of Palearctic Olepa schleini Witt et al., 2005 (Lepidoptera: Erebidae: Arctiinae) from Peninsular India with morphological variations and a new subspecies. J. Threat. Taxa 12 (9).

Kumar, P., Pandit, S.S., Steppuhn, A., Baldwin, I.T., 2014. Natural history-driven, plant-mediated RNAi-based study reveals CYP6B46's role in a nicotine-mediated antipredator herbivore defense. Proc. Natl. Acad. Sci. U. S. A 111, 1245–1252.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. Mega X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35 (6), 1547–1549.

Labeyrie, E., Dobler, S., 2004. Molecular adaptation of Chrysochus leaf beetles to toxic compounds in their food plants. Mol. Biol. Evol. 21 (2), 218–221.

Liu, N., Li, T., Reid, W.R., Yang, T., Zhang, L., 2011. Multiple cytochrome P450 genes: their constitutive overexpression and permethrin induction in insecticide resistant mosquitoes, Culex quinquefasciatus. PLoS One 6, 6–13.

Luque, T., Okano, K., O'Reilly, D.R., 2002. Characterization of a novel silkworm (Bombyx mori) phenol UDP-glucosyltransferase. Eur. J. Biochem. 269 (3), 819–825.

Morris, C.E., 1983. Uptake and metabolism of nicotine by the CNS of a nicotine-resistant insect, the tobacco hornworm (Manduca sexta). J. Insect Physiol. 29, 807–817.

Morsy, N., 2017. Cardiac glycosides in Medicinal plants. Aromat. Med. Plants - Back to Nat.

Musser, R.O., Hum-Musser, S.M., Eichenseer, H., Peiffer, M., Ervin, G., Murphy, J.B., Felton, G.W., 2002. Herbivory: caterpillar saliva beats plant defences - a new weapon emerges in the evolutionary arms race between plants and herbivores. Nature 416 (6881), 599–600.

Nishida, R., 2002. Sequestration of Defensive Substances, pp. 57–92.

Ogawa, H., Shinoda, T., Cornelius, F., Toyoshima, C., 2009. Crystal Structure of the Sodium-Potassium Pump (Na ·/.. , K ·/.. -ATPase) with Bound Potassium and Ouabain.

Petschenka, G., Pick, C., Wagschal, V., Dobler, S., 2013. Functional evidence for physiological mechanisms to circumvent neurotoxicity of cardenolides in an adapted and a non-adapted hawk-moth species. Proc. R. Soc. B Biol. Sci. 280.

Pirahanchi, Y., Aeddula, N.R., 2019. Physiology, Sodium Potassium Pump (Na+ K+ Pump). StatPearls.

Scudder, G.G.E., Moore, L.V., Isman, M.B., 1986. Sequestration of cardenolides in Oncopeltus fasciatus: morphological and physiological adaptations. J. Chem. Ecol. 12 (5), 1171–1187.

Srivastava, C., Karuppaiah, C.V., 2017. Variation in insecticide detoxification enzymes activity in Spodoptera litura (Fabricius) of different geographic origin. J. Entomol. Zool. Stud. 5 (3), 770–773.

Tobin, T., Abramson, H., 1975. Irreversible inhibition of 3H-ouabain binding to Na++K+-ATPase by digoxigenin-3,12-dibromoacetate, an alkylating derivative of digoxigenin. Eur. J. Pharmacol. 32, 243–250.

Trott, O., Olson, A.J., 2009. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem. 31 (2), 455–461.

Wang, W., Hu, C., Li, X., Wang, X., Yang, X., 2019. Biotechnology and Biological Transformations CpGSTd3 Is a Lambda-Cyhalothrin Metabolizing Glutathione S-Transferase from Cydia Pomonella (L.).

War, A.R., Taggar, G.K., Hussain, B., Taggar, M.S., Nair, R.M., Sharma, H.C., 2018. Special Issue: using non-model systems to explore plant-pollinator and plant-herbivore interactions: plant defence against herbivory and insect adaptations. AoB Plants 10, 1–19.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., De Beer, T.A.P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T., 2018. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 46 (W1), W296–W303.

Wouters, F.C., Blanchette, B., Gershenzon, J., Vassão, D.G., 2016. Plant defense and herbivore counter-defense: benzoxazinoids and insect herbivores. Phytochemistry Rev. 15 (6), 1127–1151.