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

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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 (*Chrysoschus sps.*) (Laberytie 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 (Faroqqui et al., 2020). *Calotropis* sps. contain toxic cardiac glycosides which are...
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 milkweed plant (Asclepias spp. 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 (Frick and Wink, 1995). Glazier, 1975) or into the extra dorsal space (Scudder et al., 1986). Lygaeid bugs sequester the cardenolides into the integument (Brower and 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 (Prick and Wink, 1995). Aphid neri (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 scraping the leaf region (Figure 1A 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 manufacturers’ protocol. The mitochondrial cytochrome oxidase subunit I gene (COI) was amplified using universal primer pair LCO1480 (5’GGTCAACAAATCATAAGATATTGG-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. toulgoetii; O. koslandana; O. ghatnatha; O. sederi; O. sasyamal) 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

![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.](image-url)
enoenzymes used. The dried leaves were powdered and Soxhlet extraction method was followed with 90% methanol as solvent. On the other hand, two evenly distributed leaves 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 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 and 1 M PTO, 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 Hyperion 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 *Hypantria 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.
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 Calotropis 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 2. Unrooted phylogenetic tree analysis of Olepa species obtained from NCBI database along with query sequence. Bootstrap values shown at branch point.](image)

![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.](image)
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 (C27H44O) (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-LCMS 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 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.
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 5. Strophanthidin, a cardenolide compound was observed in *Calotropis gigantea* leaf sample and *Olepa* ssp. 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* ssp. 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* ssp. 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.
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 equilibrium 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 spp. larva feeding gregariously on Calotropis leaves that are rich in toxic cardenolides specifically strophanthidin. Olepa spp. were able to feed, survive, oviposit and continue their population cycle on Calotropis leaves. The enzyme activity of GST was higher in Olepa spp. 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 6).

Olepa spp. 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 Gramma geneura and Eustigmene acrea sequester plant pyrrolizidine alkaloids and exploit them as drug store compounds for pheromone biosynthesis (Hartmann et al., 2005). In the present study, Olepa spp. 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 spp. fitness (Figure 3A and B). These suggest that the toxic strophanthidin can be sequestered or metabolised by Olepa spp. 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 LD10 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 spp. larvae revealed glutathione-strophanthidin conjugate presence in larval body tissue. The induced GST enzymes are primarily involved in catalyzing the conjugation between sulphydryl (–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 nucleophilic 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
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 ω-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 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 cholesten-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.

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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

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