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Docking-Based Virtual Screening Ascertaining β-Sitosterol-Induced Alterations in the *Helicoverpa armigera* Hübner Gut Enzymes

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ABSTRACT

Present investigation attempts to study binding of β-sitosterol with *Helicoverpa armigera* midgut enzymes; alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP); through docking-based virtual screening. Extraction of the protein sequence of the enzymes revealed a respective linear chain of 535, 522 and 430 amino acids for ALP, ALT and AST. The binding energy for ALT-ligand complex was lowest as compared to the AST and ALP-docked complexes. The ALT-docked complex had ligand efficiency of (-) 0.32 with an inhibition constant of 104.01, more hydrogen bonds and hydrophobic interactions leading to a more stable complex. However, unfavored bumps in AST and ALP complexes may have led to comparatively unstable complexes. The dietary β-sitosterol exhibits differential binding with midgut enzymes of *H. armigera* larvae. The strong binding of β-sitosterol with ALT indicates the highest inhibition of ALT activity due to the activity-stability trade-off. The enzymes, AST and ALP exhibited relatively higher activity as a resultant of lesser stabilization of the β-sitosterol-enzyme complex. *In silico* studies have indicated that β-sitosterol can be used an effective control agent against *H. armigera.*

Keywords: Helicoverpa armigera, β-sitosterol, alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase (ALP), docking-based virtual screening.

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INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a serious threat to the global agricultural economy causing extensive annual crop damage worldwide (Craig & Jorge, 2013). Application of chemical-based interventions has led to the everlasting occurrence of insecticide residues in the foodstuff and drinking water, development of pest resistance against multiple pesticides and severe damage to the cultivation and beneficial organisms (Gill & Garg, 2014). All these issues have shifted the focus towards the development of phytochemical-based green chemistries and identification of the biochemical targets implicated in pest control and resistance management (Mouden, Klinkhamer, & Choi, 2017).

Bio-pesticides are considered safer and effective options owing to their non-detrimental nature, pest-specificity and biodegradability. Therefore, isolation, identification and characterization of bioactive phytocomponents from diverse flora inducing toxic, repellency/attraction and growth regulatory effects in insects is necessitated (Hikal Baeshen & Said-Al Ahl, 2017). *Thevetia neriifolia*, the yellow oleander plant, is an evergreen tropical shrub/small tree with antifungal, antibacterial properties and growth inhibitory potential against lepidopteran pest (Singh, Agarwal, Mishra, Mubbin, & Shukla, 2012; Mishra et al, 2015a, b; 2018; 2019). Our investigations on the effects of β-sitosterol on the physiological fitness and midgut enzymes of *H. armigera* revealed developmental abnormalities in *H. armigera* induced by the dietary β-sitosterol, an active phytocomponent identified from *T. neriifolia* stem extract (0.1-10 ppm) (Mishra et al, 2020). The larval and pupal weight gain reduced, the adult emergence dropped to 82.05% to 57.89% in comparison to 95.02% emergence in controls and the midgut biochemistry also altered significantly diminishing activity of three midgut enzymes; alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP) (Mishra et al, 2020).

The GC-MS (Gas Chromatography-Mass Spectrometry) analysis of the extract discovered a number of phytocomponents with β-sitosterol as one of the major bioactive constituents. Though, β-sitosterol is used by the phytophagous insects for their growth and reproduction, it has also been reported as a feeding attractant (Hamamura, Hayashiya, & Naito, 1961).

Thus, the present investigation studied the β-sitosterol binding activity with the target midgut enzymes; ALT, AST and ALP through Docking-based virtual screening. The ligand-docking method saves us from technical complications, associated long time and high cost of massive experimental testing of millions of compounds (Koh, 2003; Tuccinardi, 2009). It plays a significant role in designing new drugs effective against pests during which a chemical library of existing ligands is docked into the target binding site and scored with the aim to generate a sub-library rich in potential binders (Cavasotto & Orry, 2007; Loza-Mejía, Salazar, & Sánchez-Tejeda, 2018).

We propose that the current study would help us to understand the affinity of β-sitosterol with target proteins alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP) of *H. armigera via*

construction of a ligand library and, identify potential structural templates for efficient use in *H. armigera* management.

MATERIALS AND METHODS

Selection of enzymes

The earlier work has shown the inhibitory effect of β-sitosterol on three midgut enzymes - alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP) of *H. armigera* in the order of ALT > AST > ALP with percent inhibition of 59.17, 42.51 and 29.31, respectively (Mishra et al, 2020). Thus, these enzymes were selected for docking-based studies to ascertain the effects of β-sitosterol in *H. armigera*.

Ligand preparation

The 3D SDF structure of the ligand, β-sitosterol was retrieved from PubChem database and later imported in discovery studio visualizer as a pdb file (https://pubchem.ncbi.nlm.nih.gov).

Target preparation

The peptide sequence of all the three midgut enzymes of *H. armigera*, ALT, AST and ALP was used to investigate the structure and catalytic sites. The sequence was extracted from National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/protein). The FASTA protein sequence database was subsequently submitted for sequence alignment and homology modelling.

Sequence alignment and modelling

The comparative modelling involves construction of a protein model based on the alignment of a submitted sequence to the target sequence. The modelling of the 3D structure of the enzymes was performed by homology modelling program. The N-terminal sequence was submitted in SWISS-MODEL to build up the structure of enzymes (https://swissmodel.expasy.org/interactive). It helped in predicting the tertiary structure of protein based on the primary structure submitted (https://swissmodel.expasy.org). The best 3D protein structure was obtained according to the best suitable template structures using BLAST database for similarity (Altschu, 1997; Bienert et al, 2017). The 3D protein model was validated through Ramachandran plot (Ramachandran, Ramakrishnan, & Sasisekharan,1963) using PROCHECK Validation Software (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK) (Laskowski, MacArthur, Moss, & Thornton, 1993). The residues information retrieved ascertained the stereochemistry of protein and provided the information on torsion angles (*psi* [Ø] and *phi* [Ö]). The final protein structure obtained was stored in discovery studio visualizer as a pdb file.

Molecular docking studies

The ligand and the target structures obtained above were exported to Autodock 4.2; assignments of charges and ionization were based on the standard templates as

a part of the Autodock software. The docking studies were carried out based on the protocol described by Rizvi, Shakil, & Haneef (2013). Standard software procedure was used. Briefly, Ligand PDBQT file was prepared by setting number of active torsions and aromaticity criterion as 5 and 7.5, respectively. Similarly, target PDBQT files were constructed by adding hydrogen atoms and Kollman charges. The parameter file was prepared by using the X, Y, Z dimension as 60 x 60 x 60 grid while the genetic algorithms and docking parameters were set on the standard templates as a part of Autodock 4.2 software for docking parameters. LamarkianGA(4.2) was selected as the output.

After docking, the dock score was calculated as binding energy of ligand with the target to assess the affinity of the ligand for the three targets tested.

RESULTS

The protein sequence of the three midgut enzymes in *H. armigera* extracted from NCBI revealed a linear chain of 522 amino acids (accession number XP_021189399) for ALT enzyme (Protein 1) whereas, the respective sequence for AST (Protein 2) and ALP (Protein 3) consisted of 430 amino acids (accession number XP_021185960.1) and 535 amino acids (accession number XP_021194992.1).

The best suited 3D structures of the proteins generated using SWISS-MODEL software based on the query sequence submitted are presented in Fig. 1. Protein 1 was made of 42% α-helix, 12% β-strand and 44% coil (Fig. 1a). In comparison, Protein 2 comprised 41% α-helix, 13% β-strand and 44% coil whereas, Protein 3 contained 30% α-helix, 11% β-strand and 58% coil (Figs. 1b, c). Ramachandran plot for ALT showed the presence of 90.1% residues in the favoured region while 8.7% additional residues were allowed in the additional region (Fig. 2a, Table 1). On the other hand, Ramachandran plots for AST and ALP revealed respective 93.8% and 89.1% residues in the favoured region whereas, additional allowed regions were 5.6% for AST and 9.5% for ALP (Figs. 2b, c; Table 1). The binding pockets for the enzymes were visualized using Castp 3.0 online server and active amino acids were obtained.

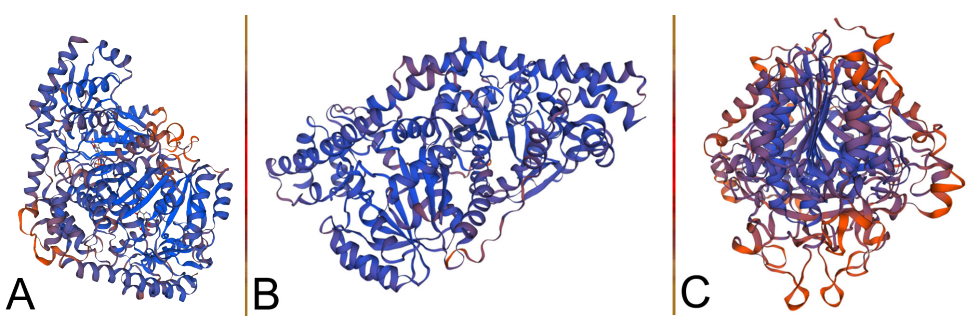

Fig 1. Best suited 3D modeled structure of (a) Alanine Aminotransaminase (ALT), (b) Aspartate Aminotransaminase (AST) and (c) Alkaline Phosphatase (ALP) enzymes using SWISS-MODEL Software.

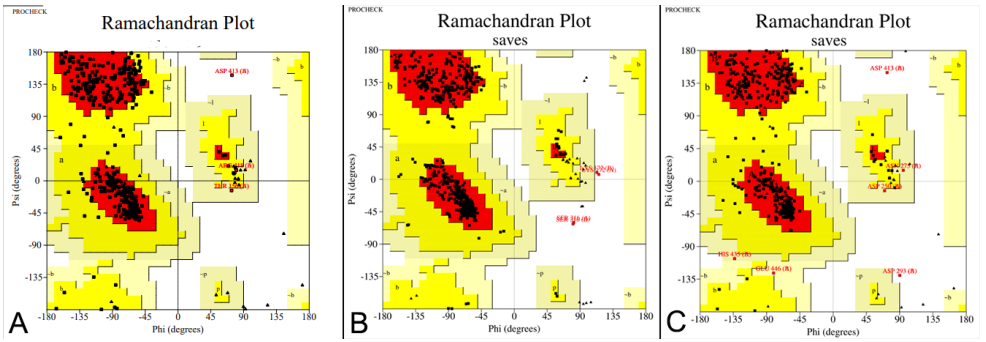

Fig 2. Ramachandran plots of (a) Alanine Aminotransaminase (ALT), (b) Aspartate Aminotransaminase (AST) and (c) Alkaline Phosphatase (ALP) enzymes obtained through Procheck validation server.

Table 1. Plot statistics based on Ramachandran Plot obtained using PROCHECK-Validation Server.

Residues	ALT	AST	ALP
Residues in most favoured regions [A,B,L]	736	653	754
Residues in additional allowed regions [a,b,l,p]	71	39	80
Residues in generously allowed regions [~a,~b,~l,~p]	7	0	8
Residues in disallowed regions	4	4	4
Number of non-glycine and non-proline residues	818	696	846
Number of end-residues (excluding Gly and Pro)	6	4	10
Number of glycine residues	76	60	78
Number of proline residues	50	40	36
Total number of residues	950	800	970

ALT = Alanine Aminotransaminase; AST = Aspartate Aminotransaminase; ALP Alkaline Phosphatase

The graphical analysis of the docked complexes for three tested proteins to view ligand-protein interactions using Autodock 4.2 software showed the best binding of the tested ligand in the binding pocket of ALT. The most important residues identified at the active binding pocket of ALT were Asp 134, Val 135, Gln 137, Arg 138, Asp 307 and Lys 311. The Asp 134, Val 135, ASP 307 was actively involved in formation of hydrogen bond with the ligand (Table 2, Fig. 3a). The binding energy obtained for ALT-ligand complex was (-) 9.53 kcal/mole whereas, the ligand efficiency was found to be (-) 0.32 with an inhibition constant of 104.01. On the other hand, the binding energies for AST (160.24 kcal/mole) and ALP (285.55 kcal/mole) complexes were quite higher. The docked complex for AST showed presence of 4 unfavored bumps (LEU 131, GLY 135, MET 314, TYR 315) whereas, ALP produced 7 unfavored bumps (SER 74, PRO 76, HIS 393, THR 396, ASP 470, ASP 471, GLY 468) produced due to amino acid group interactions (Figs. 3b, c). On the other side, presence of such bumps was not visualized in ALT complex.

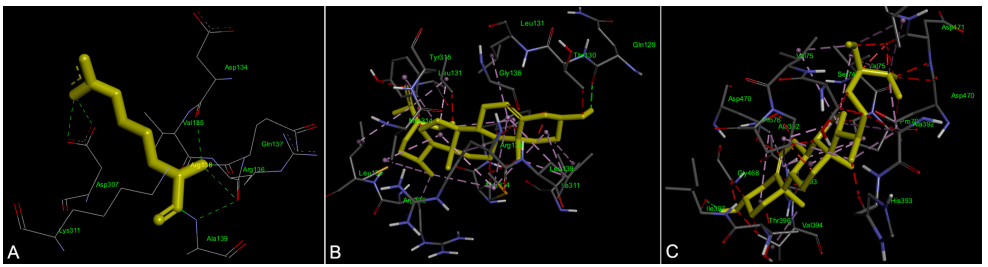

Fig 3. Ligand-protein complex of (a) Alanine Aminotransaminase (ALT), (b) Aspartate Aminotransaminase (AST) and (c) Alkaline Phosphatase (ALP) enzymes showing the actively participating amino acids binding with β-sitosterol.

Table 2. Interaction table of docking of Alanine Aminotransaminase (ALT), Aspartate Aminotransaminase (AST) and Alkaline Phosphatase (ALP) enzymes with β-sitosterol

Enzyme	H bond	Hydrophobic interactions	Hydrophilic interactions	Covalent interactions	Alkyl bond
ALT	ASP 134, VAL 135, ASP 307	ILE 58, LEU 61, VAL 135, ALA 139, ALA 310 ALA 369, MET 373,	Lys 130	ALA 139, GLN 137	LYS 311
AST	GLN 129	VAL 128, ALA 136, LEU 290	ARG 138, THR 130	NIL	ALA 136, ARG 138, LEU 290, VAL 128
ALP	ILE 397	ILE 118. MET 395, VAL 394	ASN 398, HIS 467	NIL	VAL 75, ALA 392, VAL 472

H-bonds = hydrogen bonds; ALA = alanine; ASP= aspartic acid; Val=Valine; ILE = isoleucine; LEU = leucine; ALA= alanine; MET = methionine; LYS=lysine; GLN= glutamine; ARG = arginine; THR = threonine

DISCUSSION

Plant-based insecticides offer a great means for the management of insect pests in a natural way. The pool of secondary metabolites in plants has been found to have a defensive role attributing to the diverse activities, such as antifeedant, insecticidal, growth regulatory etc. (Adeyemi, 2010). The multifarious bioactive molecules present in the plants are being explored continuously by the researchers owing to their non-detrimental effects on non-target hosts and nature (Adeyemi, 2010). The notable adverse effects of the dietary stem extract of *T. neriifolia* on the growth parameters, development and midgut enzymes of *H. armigera* larvae has already been demonstrated by us (Mishra et al, 2015a, b; 2018; 2019). The GCMS analysis of *T. neriifolia* stem extract revealed the presence of a number of components with β-sitosterol as one of the important bioactive constituents. As per our knowledge and reports available, the efficacy and probable use of β-sitosterol has not been investigated against *H. armigera,* though reported against a few other insects. Díaz et al, (2014) isolated β-sitosterol as the most active fraction from *Allophylus edulis* inducing growth regulatory effects on *Myzus persicae* and *Epilachna paenulata*. The antifeedant study against four lepidopteran pests viz., *H. armigera* (Hub.), *Spodoptera litura* (Fab.) (Fab.) and *Leucinodes orbonalis* revealed presence of steroids in the most effective fraction of the leaf extract of *Melochia corchorifolia* (Pavunraj, Baskar, & Ignacimuthu, 2012).

The bio-efficacy of sitosterol isolated from *Anemone pavonine* has been shown against *Pheidole pallidula* ants by Varitimidis, Petrakis, Vagias, & Roussis (2006).

Our earlier investigations showed the potential of β-sitosterol in disrupting physiological process and adequate growth and development which was proposed to be due to the accumulated effects of β-sitosterol and diminished midgut enzymatic activities (Mishra et al, 2020). As an extension of these studies, present investigations attempted to discover the exact binding sites of the target midgut enzymes with β-sitosterol through docking based virtual screening. We propose that the affinities and efficacy of the β-sitosterol against target enzymes can help in designing efficacious compound against *H. armigera*. Thus, β-sitosterol was used for construction of a ligand library aiming to identify potential structural templates.

The docked complex in our study showed more energetically favoured interaction of ALT with the ligand (β-sitosterol) as compared to AST and ALP protein. The presence of three hydrogen bonds and seven hydrophobic contacts in ALT-docked complex contributed to the stabilization with much lower binding free energy. The hydrogen bonds helped in tight gripping of the protein and ligand complex (Panigrahi, 2008; Rocher & Marchand-Geneste, 2008) while the hydrophobic and ionic interactions stabilized the docked complexes (Patil et al, 2010). Though AST-ligand docked complex also showed presence of Hydrogen bonds along with hydrophobic interactions, the unfavourable bumps caused due to different amino acids interaction may have attributed to a higher binding energy leading to an unstable complex. Similarly, ALP-docked complex displayed highest unfavored interactions that may have resulted in a very high binding energy of the complex as compared to other docked complexes. It has been reported that enzymes lose activity due to activity-stability trade-offs as they achieve enhanced stability by losing conformational dynamics (Siddiqui, 2017). On the other hand, enzymes gain additional dynamics and activity at the cost of stabilizing interactions. Present results are supported by our previous results that dietary β-sitosterol caused highest suppression of ALT enzyme activity (> 50%) in *H. armigera* larvae in comparison to the AST and ALP (Mishra et al, 2020). This proposes that decreased activity of ALT was accompanied by a concomitant increased stability caused by interactions and *vice-versa* in remaining two enzymes.

The expression of targeted genes has been studied earlier by applying *in silico* approaches by many researchers against various insects (Singhal, Kumar, & Virdi, 2014; Wickramasinghe et al, 2017). Another class of sitosterol, γ-sitosterol isolated from the methanolic extracts of *Hyptis suaveolens* possessed high binding affinity against odorant binding protein (3N7H) of *Anopheles gambiae* and was found to be an important mosquito repellent compound (Gaddaguti et al, 2012). Using AutoDock vina, Ahmad & Alkali (2018) tested nine phytochemical ligands for binding affinity with peroxisome proliferator-activated receptor gamma (PPAR-γ) involved in Diabetes-2 and obtained the best binding of β-sitosterol. On the other hand, Ma et al, (2015) reported slightly higher binding energy of three ligands; cholesterol, β-sitosterol and palmitic acid to sterol carrier Protein-2 which is a non-specific lipid transfer protein involved in the absorption and transportation of steroid or lipids in *H. armigera*. Other than insects,

the effect of binding of β-sitosterol to different receptors in other organisms has also been studied. Indriyanti, Garmana, & Setiawan (2018) conducted *in silico* assays with bryophyllin A, β-sitosterol, and kaempferol-3-glucoside isolated from *Kalanchoe pinnata* and tested their binding affinity with glucocorticoid receptor in mice. They revealed β-sitosterol binded to the receptor at Arg 611 and Gln 570 with hydrogen bonds and with hydrophobic binds at Gln 738, Try 735, Phe 737, Leu 741, Thr 739.

Present study provides sufficient information to identify the differential binding of midgut enzyme proteins in *H. armigera* larvae with dietary β-sitosterol. The study concludes the efficiency of β-sitosterol as an effective control agent against *H. armigera* suppressing the activity of midgut enzymes with highest inhibition of ALT activity due to the activity-stability trade-off. Other two enzymes, AST and ALP exhibited relatively higher activity as a resultant of lesser stabilization of the β-sitosterol-enzyme complex. Further binding affinity simulation studies may provide more evidence regarding control efficacy of β-sitosterol against *H. armigera.*

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CONFLICT OF INTEREST DISCLOSURE

There is no conflict of interest.

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