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A Comparative Study of Inhibitory Properties of Saponins (derived from *Azadirachta indica*) for Acetylcholinesterase of *Tribolium castaneum* and *Apis mellifera*

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ABSTRACT

The study describes the effect of saponins, isolated from a medicinal plant *Azadirachta indica* on the CNS enzymes of a stored grain pest, *Tribolium castaneum* and a socioeconomic insect, *Apis mellifera*. A comparative study was designed to identify the role of saponins on insect acetylcholinesterase (AChEs). The enzyme activities were tested for the effect of saponins. The AChE activity of *T. castaneum* was inhibited by the saponins and follows competitive inhibition kinetics. In case of *A. mellifera* enzyme activity was not inhibited. *In vitro* and *in vivo* inhibition was observed for *T. castaneum* at larval stages, in dose dependent and time dependent manner. LC50 was determined to be 0.7ppm. To investigate the different effects of saponins on *A. mellifera* and *T. castaneum* AChE, computational approach was employed. For this purpose a dissection of 3-D model of *A. mellifera* and *T. castaneum* AChE enzyme was studied which showed that change in amino acid sequence of primary structure of enzyme exists at the saponin binding site, resulting in weak interaction for *A. mellifera* as compared to *T. castaneum* enzyme protein. Computational studies inidicate that *A. mellifera* enzyme had a little binding affinity for saponin as compared to *T. castaneum* AChE. The amino acid residues of *T. castaneum* AChE identified at positions 259(SER), 176(SER), 173(GLY), and 502 (HIS) are involved in binding with saponin molecule to form four hydrogen bonds. Whereas in *A. mellifera* hydrogen bonds are formed at two positions by SER 171 and TYR104 with the saponin molecule indicating weak interaction as compared to *T. castaneum* Saponins derived from *A. indica* work as biosafe pesticides as it has no considerable effect on CNS enzymes of *A. mellifera* (a major plant pollinator and friendly insect) as compared to *T. castaneum*.

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Authors' Contribution
AJS designed the experimental work and analyzed the results. SB and MK helped in experimentation. TN performed the computational analysis. ARS helped in the preparation of manuscript.

Key words
***T. castaneum*, *Apis mellifera*, Acetylcholinesterase, Saponins, Pesticides, *Azadirachta indica*.**

INTRODUCTION

The widespread use of organophosphorous (OP) compounds and carbamates as pesticides generates adverse effects on non-target organisms like honeybees. The chemicals interfere with the nerve signaling and functions of the non-targeted friendly insect. Biopesticides have been applied on various plants for insect control due to their inhibitory effects on different digestive and central nervous system enzymes including cellulases and amylases (Zhu and Zhang, 2005; Sami and Shakoori, 2007; Sami, 2014; Sami *et al*., 2016; Gupta, 2006; Colovic *et al*., 2013; Dulin *et al*., 2012). The toxicity of these pesticides to insects is examined by their ability to inhibit acetylcholinesterase (AChE). Neem plant has long been studied for its medicinal, insecticidal, antibacterial properties and agricultural importance (Ashfaq *et al*., 2016; Benelli *et al*., 2017).

The most popular plant based biopesticides are extracted from neem that is applied on both food and cash crops. More than 50 neem based commercial biopesticides which are emulsified concentrate are sold in market. Neem is one of the 29 plants species which are used against diseases of medicinal plants and pest (Glare *et al*., 2012; Benelli *et al*., 2017; Guo *et al*., 2008). Azadirachtin, a compound isolated from Neem had shown inhibitory effects on AChE enzyme activity (Sami *et al*., 2016). The inhibitor may bind to the enzyme AChE reversibly, irreversibly or pseudo-irreversibly. To date, two different Ace genes (ace1 and ace2) that encode AChE have been found in various insects. It was reported that the expression of ace1 gene is much greater in insects as compared to ace2 and point mutations in ace1 cause insecticide resistance and reduced sensitivity, than ace2 (Lee *et al*., 2007). Different molecular forms of Ace were identified among insects. The deduced structure of AChE of *Tribolium*

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castaneum (Herbst) (Coleoptera: Tenebrionidae), suggests that it contains all conserved sequence motifs including a choline binding site, an acyl pocket and the catalytic triad (Kim *et al*., 2012; Lu *et al*., 2012). Overall structure of the protein is ellipsoidal. There are 14 α helices surrounding 12 mixed β chains. The central position is occupied by an active site gorge ~ 20 Å deep (Manavalan *et al*., 1985). *T. castaneum* is a pest that damages major stored grains, causing economic losses worldwide. AChE enzyme of *T. castaneum* can be targeted for pesticide development and effectively controlling crop damage by *T. castaneum* (Sallam, 2008). Most of the pesticides currently in use not only destroy pests but also affect friendly insects. Due to the socio economic value of *A. mellifera*, studies on the toxic effect of different pesticides on the honey bee is a matter of great importance, a pivotal pollinator in natural and commercial agriculture. Honeybees contact toxicity associated with AChE inhibitors are also studied (Dulin *et al*., 2012). Kinetic properties and molecular studies are reported on two AChEs from the honey bee, *Apis mellifera* (Kim *et al*., 2102). Reports on structural localization of AChE activity in the compound eye of the honey bee are also presented by Kral and Schneider (1981).

AChE enzyme is of extreme pharmacological and economic importance as it occurs as a chief CNS enzyme in insects and pests (Colovic *et al*., 2013; Kono and Tomita 2006). Organophosphates (OPs) and carbamates are two major classes of inhibitors used widely for inhibiting AChE. The potential of these compounds as inhibitors of AChE is both therapeutically as well as economically important. This mechanism of AChE inhibition is used both in the field of medicine for disease targeting (Alzheimers, Parkinson's disease, Glaucoma) and in the field of agriculture for pesticides development (Kuhr and Dorough, 1976; Gupta, 2006). In the process of inhibition of AChE by various classes of compounds different mechanisms are followed. OPs have the capability of phosphorylating Serine residues on the AchE in a non-reversible way thus rendering the enzyme inactive (Darvesh *et al*., 2008). Carbamates another important AChE inhibiting class are derived from carbamic acid. The structure of carbamates contain carbamate moiety along with oxygen or sulphur. Carbamtes cause carbamylation of important serine residues on the AChE molecule but in a reversible manner (Colovic *et al*., 2013; Gupta, 2006). Thus carbamates act as reversible inhibitors of AChE. Inhibition of AChE account for anti-feedant behaviors, repellancy, larval mortality and other nervous system defects in insects and pests. Various classes of compounds have been investigated as potent inhibitors of AChE and are currently in use as therapeutic agents as well as biopesticides. *A. indica* derived compound classified as saponins, is a heterogenous mixture of molecules varying both in their aglycone and sugar moieties. The main aglycone (sapogenin) moiety is quillaic acid, a triterpene of predominantly 30 carbon atoms (hydrophobic). The aglycone is bound to various sugars including glucose, glucuronic acid, galactose, xylose, apiose, rhamnose *etc.* (Guo *et al*., 2008).

The current research report is aimed at evaluating the potential of Neem based saponin as green pesticide, to control stored grain pests. Biochemical properties of the AChE and docking studies of saponins allowed a clear understanding of several amino acids in the overall inhibition/toxicity of the compounds on *T. castaneum* and *A. mellifera*. The inhibition and antifeedant properties of AChE in response to saponins are studied through bioassays and docking experiments. The results of this study validate the importance to consider the potential of neem derived saponins in the development of natural insecticides. This is the first study on neem based saponins and can be a stepping stone to generate potential natural biopesticides.

MATERIALS AND METHODS

Pest selection and rearing

Tribolium castaneum commonly known as Red flour beetle was reared in the laboratory. The cultures of *T. castaneum* were incubated with whole wheat flour as substrate. The cultures were maintained at 37 ± 1°C with suitable humidity level to facilitate *T. castaneum* growth for several months to get a healthy culture. *A. mellifera* insects were purchased from a local farm.

Crude extract preparation

The crude extract of *T. castaneum* (larvae and insects) and *A. mellifera* (insect stage) were prepared by homogenizing 0.15g insects in 0.1% $NaHCO_3$ solution (10mg/ml). The homogenate was centrifuged at 3000 rpm for 5 min and the supernatant was stored at -20°C till further use.

Preparation of inhibitor

For inhibition studies, Neem derived saponins were purchased from local market in Pakistan (United chemicals, Anarkali, Lahore), which were further purified by extraction with butanol. Saponins were estimated using a standard method. Digoxin (Sigma-Aldrich) was used as a standard.

Acetylcholinesterase (AChE) activity assay

The AChE activity was measured according to the method previously described by Ellman *et al*. (1961). The reaction mixture was prepared in test tubes containing 100 µl of homogenate and 2.6 ml of 0.1M phosphate buffer (pH

8). To this reaction mixture 0.01 M of the 5: 5-dithiobis-2-nitrobenzoic acid (DTNB) and 0.075 M Acetylthiocholine (ATC) was added. The reaction mixture was incubated at 30°C for 20 min and the absorbance was read at 412 nm.

Partial purification of AChE enzyme

The Protein preparations from *T. castaneum* (10 mg) was applied to gel filtration column (10×1.5 cm) using Sephadex- G- 100, pre-equilibrated with 20mM phosphate buffer at pH 8, and eluted by using the same buffer at a flow rate of 1 mL/min.

Void volume was calculated to be 10 ml and excluded from the protein fractions. One ml fractions were collected and were subject to Bradford assay and enzyme activity assay.

SDS-PAGE

The fractions from gel filtration indicating enzyme activity were pooled together. The pooled fractions were subjected to SDS-PAGE gel (12%) at 60 V for 5 h. The gel and running buffers contained 0.5% Triton X-100 (v/v). Afterwards the gel was subjected to coomasie staining for molecular weight determination.

NATIVE-PAGE

The pooled fractions were concentrated after precipitating with chilled acetone at -20ºC. The protein precipitates were dissolved in buffer pH 8.5 (0.05 M Tris -HCl) and were subjected to preparative PAGE without SDS, in a cold chamber with a continuous Tris-glycine buffer system.

Zymogram

Zymography is an electrophoretic technique which is performed for the detection of enzyme activity. The enzyme extract was subjected to electrophoresis on Native-PAGE. After PAGE the gel was sliced horizontally into 20, 1.0 mm slices. The gel was then transferred, on the 2% substrate-agar (ATC) plates in phosphate buffer pH 8.0. The gel was overlaid and was covered with cling film wrap to prevent evaporation and was incubated at 37°C for 10 min. After 10 mins, the color reagent DTNB was poured on to the plates and the AChE activity was observed.

In vitro *inhibition of AChE*

To determine the inhibitory effects of saponins, the enzyme was incubated with neem derived saponins of concentrations ranging from 0.05-1ppm and 2.6ml 0.1 M phosphate buffer pH 8, for 50 min at 30°C in a set of test tubes. 100 µl of 5-dithiobis-2-nitrobenzoic acid (DTNB) and Acetylthiocholine (ATC) substrate with concentrations (ranging 700 µM) was added in already incubated test tubes. The mixtures were incubated at 30°C for 20 min. The AChE activity was measured at 412 nm using a UV-visible spectrophotometer. Similar procedure was performed without inhibitor and enzyme kinetics was determined. All experiments were performed in triplicates. One unit of AChE activity is expressed as millimoles of AChE hydrolyzed per milligram of protein per minute.

In vivo *inhibition of AChE*

A mortality bioassay was set up to determine the concentration of saponins for insects and larval stage of *Tribolium castaneum*. The insects were exposed to different concentrations of saponins (0.05, 0.1, 0.5, 1 ppm) and mortality was observed for 48 h. The results were used for probit analysis using SPSS 16.0 software (Abbott, 1925). The mortalities were corrected using formula (Finney, 1947) and results were expressed as means with standard deviations of three replicates.

Bioinformatics studies

The structure for AChE of *T. castaneum* (accession no. EZ99262.1) and *A. mellifera* (accession no. BAE06051.1) were generated based on homology modelling. The sequences were submitted for modelling to Swiss Model at ExPASy (Expert Protein Analysis System) bioinformatics resource portal of the Swiss Institute of Bioinformatics (SIB), an automated homology modelling serve, where BLAST was used to search the ExPDB (Expert Protein Data Bank) database for templates. AChE from *Drosophila melanogaster* was used as a template, as it shares high homology with AChE of these three insect species. After completing sequence alignment and its manual refinement, the catalytic site of AChE along with active site residues were analysed by the inhibitor (α-D-glucopyranosyl-(1,3)-α-D-glucuronopyranosyl-(1,3))α-3-hydroxyolean-12-ene-28-oate, a Saponin molecule using Docking Server (Bikadi and Hazai, 2009; Morris *et al.*, 1998).

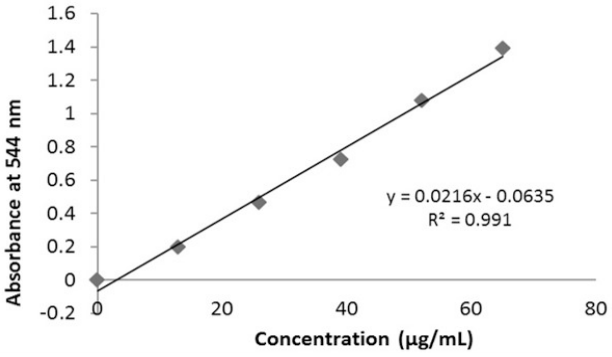

Fig. 1. Standard curve for the estimation of saponins, quantified using vanillin-sulfuric acid assay.

RESULTS AND DISCUSSION

Saponin Content

Saponins were quantified using vanillin-sulfuric acid assay (Hiai *et al*., 1976). To perform the test, 0.25mL of saponin solution was mixed with 0.25mL of 10% (w/v) vanillin, dissolved in methanol, on ice-bath. 2.5 mL sulphuric acid (72% w/v) was added and mixed followed by heating at 60°C for 10 min, cooled and taken absorbance at 544 nm. Standard graph of digitalis (digoxin) was built to use as reference for estimation of saponin content.

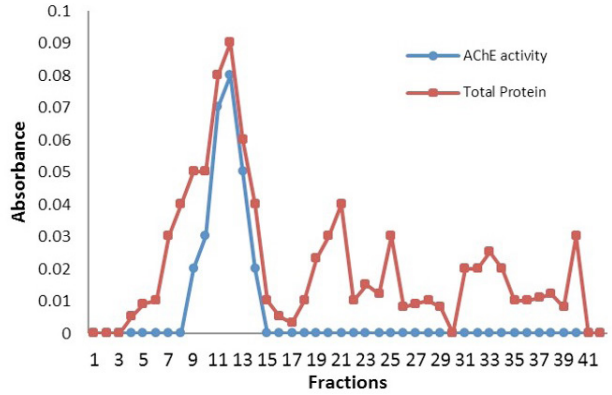

Fig. 2. Purification of *Tribolium castaneum* AChE by Gel filtration chromatography (Sephadex G100), protein estimation by Bradford and colorimetric enzyme activity assay. Fractions were collected after eluting a void volume.

Characteristics of AChE

The crude extract of *T. castaneum* (insect and larvae) was used for the total protein estimation and was determined to be 0.49 mg/ml. The rate of enzyme activity was 5.7 µmol/min/mg of protein. Gel filtration chromatography was performed for the partial purification of enzyme (AChE) (Fig. 2). All the fractions were tested for protein concentration and enzyme activity. Fractions showing enzymatic activity were collected and pooled together were subjected to preparative native-PAGE analysis. Following native-PAGE the gel was sliced horizontally into 1.0 mm slices, as described previously (Sami and Akhter, 1993). Each slice was tested for acetylcholinestrase activity by zymogram technique also. Results showed that Fraction No. 11, 12 and 13 had the enzyme activity as it appeared yellow on the Zymogram (Fig. 3), diffused activity of enzyme is visible in yellow due to higher rate of reaction. Protein was extracted from the native-PAGE and SDS-PAGE was performed to determine the molecular weight and test the immunogenic activity. The gel showed a band of 35 kDa, and immunogenic activity in an immunoblotting assay (Fig. 4).

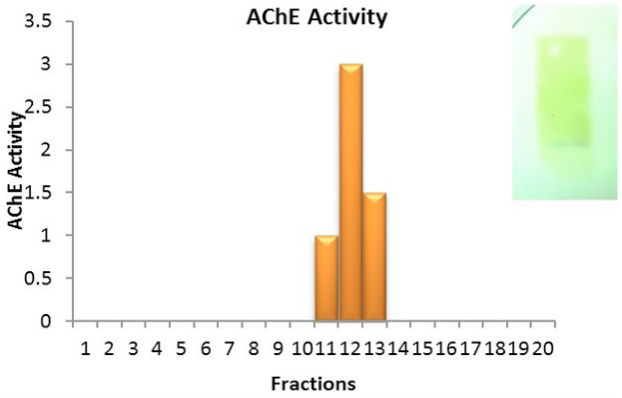

Fig. 3. AChE activity in preparative native-PAGE fractions. The fractions 11, 12 and 13 showing diffused enzyme activity on zymogram are inseat.

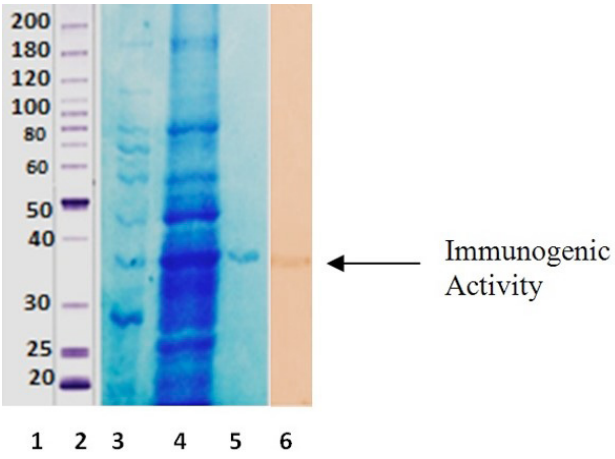

Fig. 4. Purified AChE of *Tribolium castaneum* on 12% SDS-PAGE and western blot. A band of 35kDa is visible. Lane 1, marker; Lane 2, marker in 1% SDS-PAGE; Lane 3, crude extract of insect; Lane 4, purified fraction from gel filtration and preparative PAGE; Lane 5, immuno-blot of purified protein.

Inhibition kinetics

AChE isolated from *T. castaneum* and *A. mellifera* were studied for the inhibition by Saponins. It was observed that AChE from *T. castaneum* was almost completely inhibited while there was little or no effect on *Apis mellifera* enzyme (Fig. 5).

Saponins were able to inhibit the AChE in *T. castaneum* in a linear fashion with the increase in the concentration of enzyme activity. The AChE is the target site of many organophosphate and carbamate insecticides in central nervous system of various insects and vertebrates. The main effects of neem and saponins in our experiments were the inhibition of AChE in higher doses of neem based

saponins treatment in *T. castaneum*. Further the results were compared with Honey bee acetylcholinesterase and it was observed that saponins do not inhibit AChE in *Apis mellifera* (Fig. 5). The results demonstrate that saponins can be very useful botanical insecticides which exert toxic affects to target species but do not affect non-target species of insects.

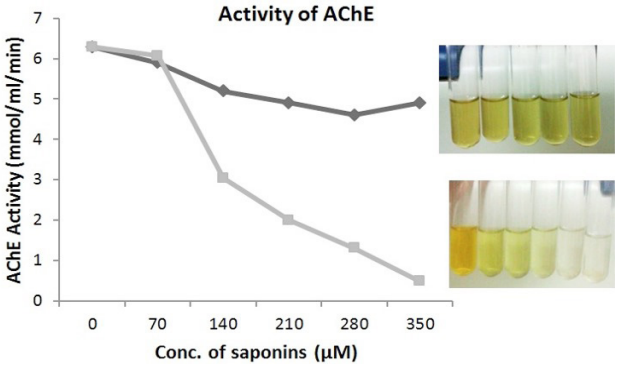

Fig. 5. Effect of different concentrations of saponin on the AChE activity of *T. castaneum* and *A. mellifera*. Black line shows *T. castaneum* and grey line for *A. mellifera*. Inhibitory effect of saponin on *T. castaneum* is clearly shown.

Inhibition kinetics was studied using Line-weaver Burk Plot. The Km was calculated to be 0.0463 M and 0.093 M with and without inhibitor, and the Vmax was 0.839µM/min. The Vmax with and without inhibitor remain unchanged indicating that the inhibitor binds at the catalytic site of enzyme. The Ki was calculated to be 2.5 M (Fig. 6).

The Km of enzyme changed when subjected to inhibitor and Vmax was same indicating that saponins can inhibit AChE competitively and has high affinity for enzyme's active site (D'Incao *et al*., 2012; Meena *et al*., 2011).

Effect of saponins on larvae

The LC_{25}, LC_{50} and LC_{90} were calculated by probit analysis as 0.03 ppm and 0.7 ppm and 2.0 ppm respectively, $P < 0.002$. The concentration of saponin (isolated from Neem) equal to LC_{50} also inhibited AChE of *T. castaneum*. The inhibition of AChE at various concentrations of saponins can be seen in Figure 7. The activity of AChE was inhibited considerably. The sprays made by saponin based formulations can be very useful to eradicate major pests of stored food grains such as rice, pulses and wheat products. These pests are widely distributed all around the world and cause extensive damage to stored food each year (Isman and Grieneisen 2014).

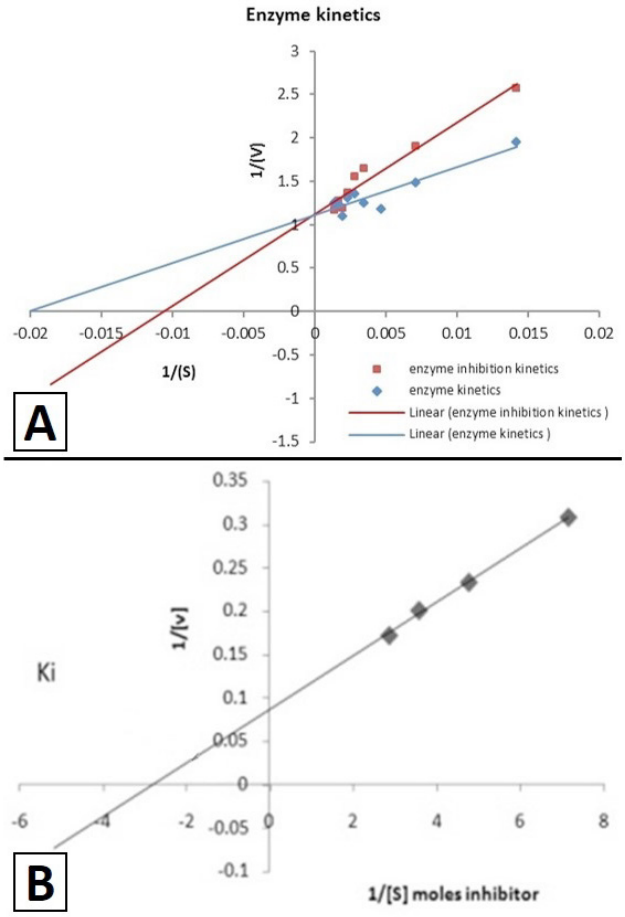

Fig. 6. A, Lineweaver-Burk plot for the AChE for *T. castaneum*; B, graph shows the inhibition constant for saponin.

The effect on AChE enzyme activity was dependent on inhibitor's concentration. Our studies revealed that pure forms of saponins are more effective against *T. castaneum,* as these insects are very resilient and survive even harsh conditions. More than 70% insects died above 0.7 ppm and 1 ppm was most effective over 24 h period (Fig. 7).

Repellency behaviour of insects to saponins

The repellency behaviour of live insects was recorded by using a filter paper disc as described by Sami *et al.* (2016).

For the determination of antifeedant behavior of insects in response to saponins live *T. castaneum* larvae were used. Commercially available neem seed extracts have diverse pest control properties, affecting insect growth, fertility, and metamorphosis in addition to direct toxicity and antifeedent and oviposition-deterrent effects. Recent studies indicate there are now over 500 species of

insects and mites resistant to biopesticides. There are over 1,000 insect/insecticide resistance combinations, and at least 17 species of insects that are resistant to all major classes of bioinsecticides (Corbet, 2006).

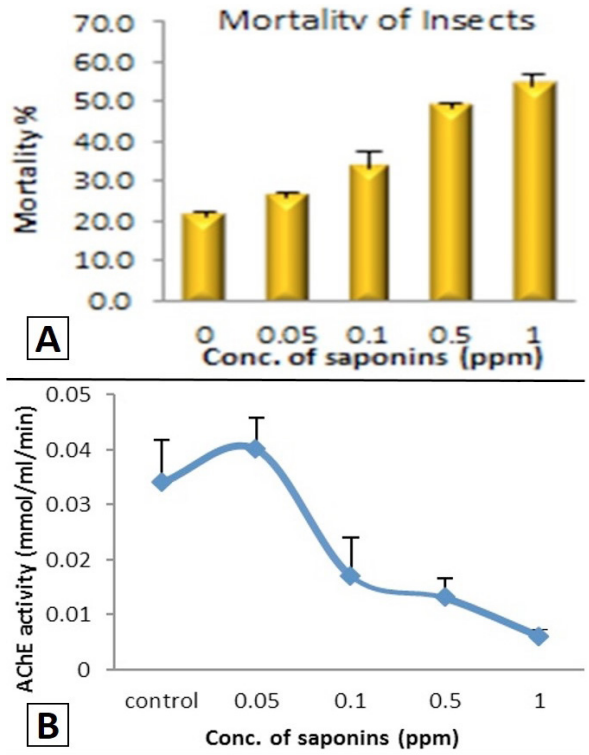

Fig. 7. A, LC_{50} determination using different concentrations of saponins; B, activity of Acetylcholinesterase at different saponin concentrations. The error bars indicate SDs with significant value ($P < 0.05$).

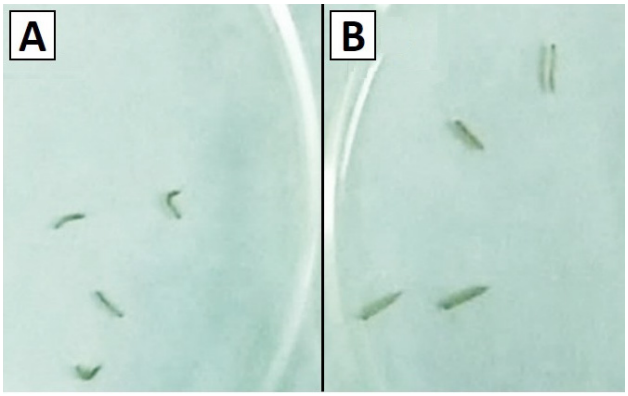

Fig. 8. Comparison of size of larvae after 24 h. Test larvae (A) treated with diet containing inhibitor, indicate diminution in size indicating negative effect of neem derived Saponins on *T. castaneum* larvae. Control larvae (B) appear normal in size with no saponins in the diet.

The results showed that there is an impact of neem derived compounds on the repellency of insects (Figs. 9, 10). Furthermore, the role of Neem derived compounds on the nervous system enzyme AChE was investigated.

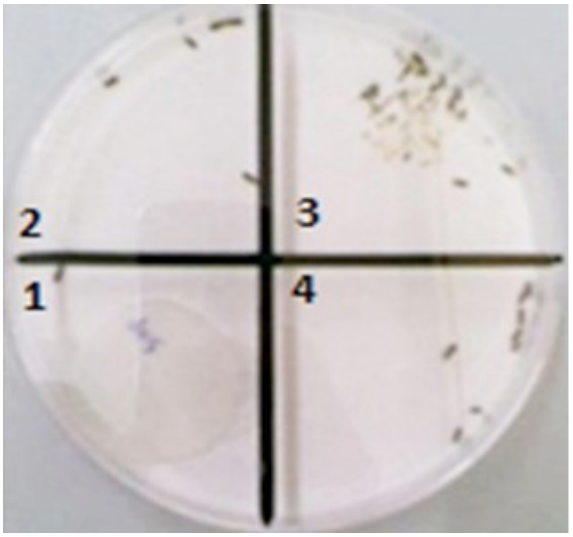

Fig. 9. Filter paper disc containing saponin (5 ppm) present in quadrant 1, acts as a repellent for *T. castaneum*, within 5 min of exposure, insects move towards the quadrant farthest from the inhibitor containing disc.

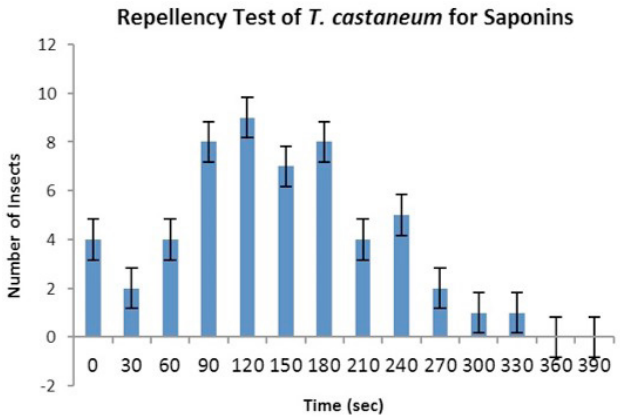

Fig. 10. Repellancy test of adult *T. castaneum* in response to Saponins. The graph indicates the number of insects present in the quadrant containing the inhibitor. The number of insects decreased with time.

Computational analysis

AChE model is built at SwissModel server using homology modeling, *Drosophila melanogaster* AChE was used as a template for modeling showing 62.02% homology. Further, Docking calculations performed using Docking Server.

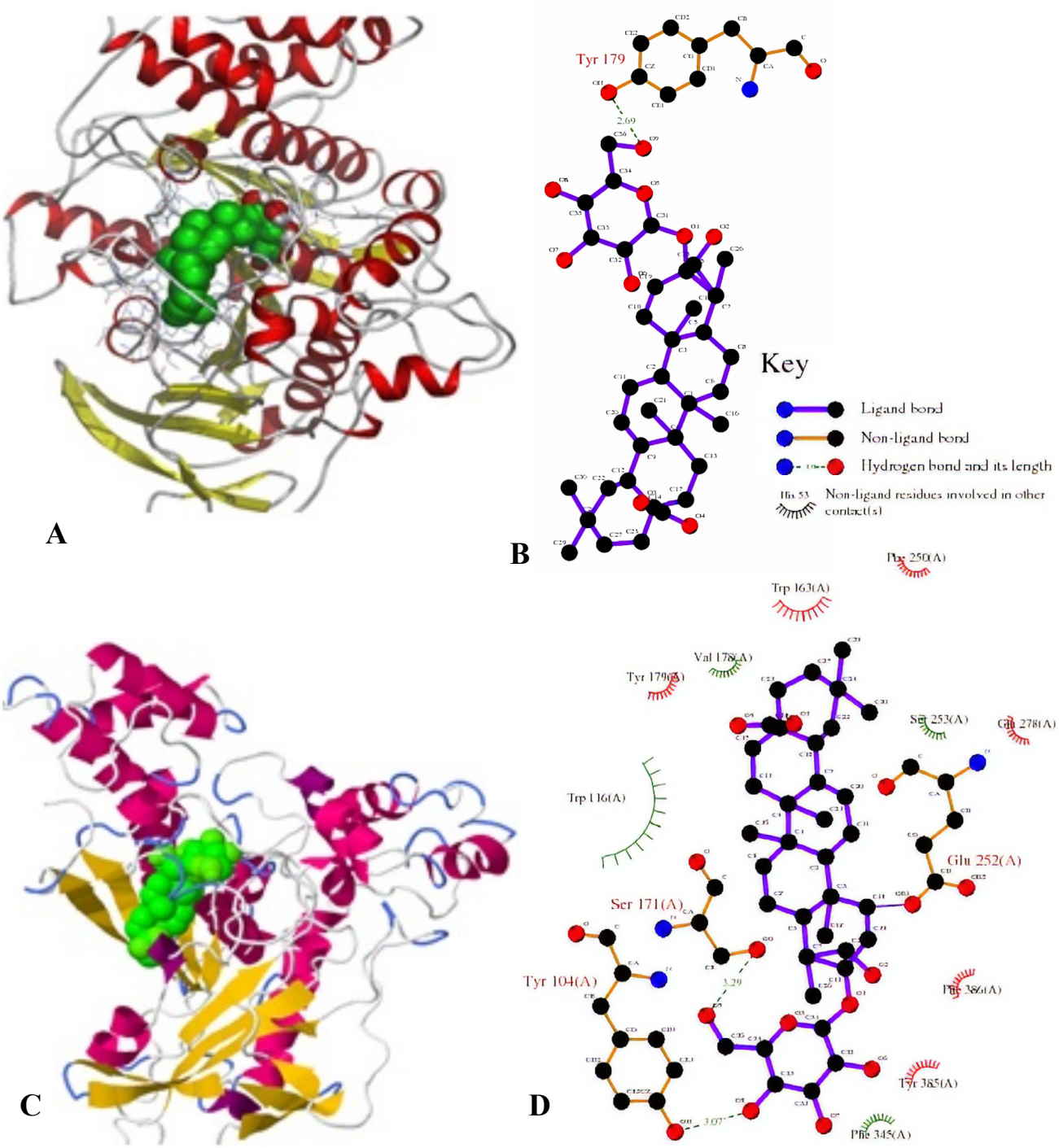

Fig. 11. Docking of saponin molecule with AChE of *T. castaneum* and *A. mellifera*. Dotted lines indicate the bonding of saponin with amino acid residues of the enzyme AChE present in both species (Saponin molecule highlighted in red dotted line).

Docking showed that saponins bind in the catalytic triad of 20 Aº deep active site gorge making the enzyme unable to bind the original substrate. However, the AChE are enzymes with high substrate specificities and the potential substrates are able to fit the catalytic centre, although AChE exhibits reduced sensitivity against insecticides. A dissection of binding/interaction sites of the enzymes with the target molecule (Saponin) showed

that the *A. mellifera* enzyme had a little binding affinity for saponin as compared to *T. castaneum* AChE. In *T. castaneum* AChE the amino acid residues identified at positions SER259 (-54.129 Kcal/mol), SER176 (-1.2678 Kcal/mol), GLY173 (1.0287 Kcal/mol), and HIS502 (10.6928 Kcal/mol) shows hydrogen bond with Saponin molecule. GLN111 (0.0921 Kcal/mol), ASN127 (0.1137 Kcal/mol) and TRP292 (3.1102 Kcal/mol) shows polar bond, hydrophobic interactions shows with amino acids LEU181 (-0.9041 Kcal/mol), TYR114 (-0.5178 Kcal/mol), LEU349 (-0.256 Kcal/mol) and PRO128 (0.6939 Kcal/mol), however surface binding studies shown that molecule is completely fit inside the AChE enzyme (Fig. 11A, B).

The sequence of *Apis mellifera* AChE was docked with saponin molecule. The estimated free energy of binding appear to be +221.7 kcal/mol. The frequency of binding was 40%. 2D plot for the docking shows following type of interactions: SER171 and TYR 104 formed hydrogen bond with binding energies (-3.458 and 10.041), respectively while TRP116, PHE 345, PHE 386, TYR385, were involved in cation-pi interaction. Hydrophobic interaction was formed by PHR350, TYR179, TRP163 (Fig. 11 C, D). It is revealed from the *in silico* study that this complex between the enzyme and inhibitor is less stable due to high energy of the required hydrogen bonds as compared to *Tribolium* AChE-I saponin complex (Kim *et al*., 2012). Summarily neem derived saponins could be used as economical and sustainable green biopesticides.

CONCLUSION

Neem derived saponins have higher affinity for insect pests by inhibiting the nervous system enzyme, Acetylcholinesterase. Saponins could be used as bio-safe green pesticides. Non interaction of *A. mellifera* nervous system enzyme is due to lack of binding affinity for saponins. Saponin based biopesticide treatments on plants is an economical technique that can be used to protect crops from damage and to increase biomass production.

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Statement of conflict of interest

The authors confirm that this article content has no conflict of interest.

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