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Potential of Azadirachtin and Neem *(Azadirachta indica)* Based Saponins as Biopesticides for *In vitro* Insect Pests Cellulase (Beta-1,4-Endoglucanase) Enzyme Inhibition and *In vivo* Repellency on *Tribolium castaneum*

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Authors' contributions

This work was designed and carried out in collaboration between both authors. Author AJS performed the experimental work, designed the protocol and wrote the first draft of the manuscript. Analyses of the study was done in collaboration. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The work was undertaken to identify the role of Neem derived compounds (saponins and azadirachtin) on the digestive cellulose hydrolyzing enzyme activity of red flour beetle (*T. castaneum)*, rice grasshopper (*Oxya chinensis*) and red pumpkin beetle (*Aulacophora foveicolis*).
Place and Duration of Study: The work was carried out at the Institute of Biochemistry and Biotechnology University of the Punjab Lahore Pakistan.
Methodology: Total cellular proteins were isolated from the insect's gut and salivary glands and were tested for cellulose hydrolyzing activity on substrate agar plates. Saponins and Azadirachtin were isolated from *Azadirachta indica* tissues and used for enzyme inhibition studies. Repellancy test was performed for *T. Castaneum*, using saponins and Azadirachtin. For computational studies sequence of endoglucanase gene was identified from *T. castaneum* genome and protein structure was deduced.

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Results: Saponins were able to inhibit beta-1,4-endoglucanase enzyme activity, present in all the three insect pests. A computational dissection of *T. castaneum* endoglucanase enzyme, deduced from *T. castaneum* genome, showed that there were five cys involved in the formation of disulphide bridges in the molecule. The disulfide bridges did not provide any protection to endoglucanase active site. Azadirachtin had no effect on cellulase activity of *Oxya chinensis* and *Aulacophora foveicolis*, while beta-1,4-endoglucanase activity of *T. castaneum* was inhibited. Repellency test for *T. castaneum* revealed that each group of compounds (Saponins and Azadirachtin) was able to repel the insect.
Conclusion: Neem derived compounds had a considerable inhibitory effect on the digestive cellulose hydrolyzing enzyme of *T. castaneum*.

Keywords: Tribolum castaneum; azadirachtin; saponins; cellulase inhibition; Oxya chinensis; Aulacophora foveicolis.

1. INTRODUCTION

Plants in nature produce a diverse variety of organic compounds, the majority of which does not participate directly in growth and development. Various insects depend on carbohydrates (starch and cellulose) during different developmental stages of their lives including *Tribolium castaneum, Aulacophora foveicollis* and *Oxya chinensis*.*T. castaneum* (Herbst) is one of the chief storage pests that rely on starch and is responsible for the severe loss of stored grains [1]. As the insects are totally dependent on starch hydrolyzing enzymes for their survival, these enzymes are good candidates for the inhibition by plant derived molecules. The plant derived substances are usually referred to as secondary metabolites [2]. They are thought to function as biochemical defenses or allelochemicals acting as a defense against the pathogens and insects [3]. The natural insecticides based on plant extracts are considered as an attractive alternatives to synthetic chemical insecticides for pest management [4]. A number of Neem plants derived compounds have been studied in this regard [5,6,7]. Earlier we have reported compounds isolated from the leaves of *Psidium guajava* (guava) showed complete inhibition of endo-1, 4-beta-D-glucanase enzyme activity and insect repellency for *Aulacophora foveicolis* [8]. *Azadirachta indica* also known as the Indian Neem plant has been found to be a promising source of natural pesticides. The seeds and leaves of neem have been traditionally used for centuries to control pests [9] and have shown marked insect control potential [10-13]. The most prominent insecticidal constituent of neem seed kernels is reported to be azadirachtin [1] and related structural compounds including saponins [14]. Azadirachtin is a complex tetran or tri-terpenoid limonoid (Fig. 1) from the neem seeds [15]. It is considered most important due to its various effects on insects [10,16]. Azadirachtin is known to have adverse effects on more than 200 insect species [17]. Neem seed extracts are considered to be very suitable insecticides for pest as they are non-toxic to warm-blooded organisms [18].

Azadirachtin from neem, acts as an antifeedant, an insect growth regulator, and sterilant against a broad spectrum of pest insects [20]. Bioassays were run for the larval growth inhibition and antifeedant activity on the variegated cutworm *(Peridroma saucia)* and milkweed bug *(Oncopeltus fasciatus).* There are reports on the inhibition of a number of enzymes including nervous and digestive system enzymes of the insects by neem derived compounds. Earlier, we reported that series of plants including *Melia azedarach, P. guavaja,* and *A. indicia* had a potential to inhibit insect cellulases [13]. The active components in the plant extract caused inhibition were identified to be flavonoids, which are good insect

repellant too [8,13,21]. The effects of two glycosylated flavonoids: cyanidin-3-glycosides and quercetin-3-rutinosid (isolated from mango leaves) on *A. foveicolis cellulase* causes inhibition [21]. It was concluded that, the natural glycosylated flavonoids stand good merit as potential insect cellulase inhibitors. Nathan et al. [22] studied the effects of neem limonoids azadirachtin, salannin, deacetylgedunin, gedunin, 17-hydroxyaz-adiradione and deaceytlnimbin on the enzymes present in the gut of the larvae of the rice leaf folder. The activities of acid phosphatases (ACP), alkaline phosphatases (ALP) and adenosine triphosphatases (ATPase) of rice leaf folder larvae gut were affected when they were fed on rice leaves treated with limonoids. Azadirachtin proved to be most effective in inhibiting all enzymes. Various compounds present in Neem include Azadirachtin and saponins which act as enzyme inhibitors (Figs. 1A and 1B). The compounds present in the neem plant also acts on nervous enzymes of insects. Subrahmanyam et al. (23) reported that the antifeedant azadirachtin is responsible for the reduction in food consumption in female *Locusta migratoria.* However, azadirachtin is involved in producing disturbances in various nervous and endocrine functions of the insect [24]. *Tribolium castaneum* (Insecta: Coleoptera: Tenebrionidaei) is an omnivorous beetle pest of stored agricultural products. It has been estimated that between one quarter and one third of the world's grain crop is lost each year during storage. Neem plant has a secondary inhibitory effects on live insects, their enzymes and for their antifeedant activities. *T. castaneum* is a powerful model organism for the study of generalized insect development, and an important pest. Genome of *T. castaneum* has been reported as a representative of insect pests, as it reflects gene contents and their structure-function relationship. The availability of sequenced *T. castaneum* genomes, has allowed the discovery and functional characterization of novel genes and proteins. *T. castaneum* (Herbst) genome was used to identify, clone, express, and characterize a novel endo--1, 4-glucanase we named TcEG1 *(T. castaneum* endoglucanase 1) [25]. Cellulase activity produced by the insect, play a major role in its stored grain destruction. Most natural pesticides such as Neem dried leaves or other Neem products are used as pesticides against *T. castaneum* on foods like grains, flour and rice. The Neem derived compounds has not been evaluated individually for their potential as bio pesticide, as a cellulose hydrolyzing enzyme inhibitor, though methods has been established for their separation and isolation. Here, we report evaluation of *in vitro* cellulase inhibitory potential of azadirachtin and saponins and repellency on insects including *T. castaneum, Oxya chinensis, and A. foveicolis.* Such evaluation will be useful in developing natural bio-pesticides on a commercial basis.

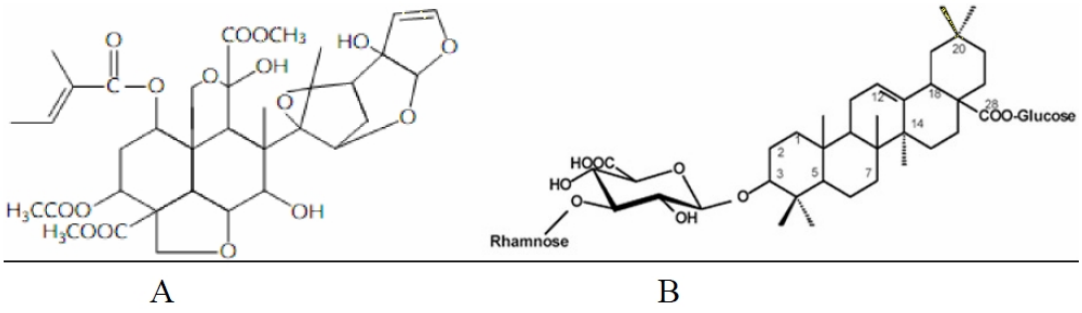

Fig. 1. Structure of azadirachtin (A) [2] and saponin (B) [19].

2. MATERIALS AND METHODS

The entire reagent used was of analytical grade, and all the experiments were repeated at least twice in triplicates.

2.1 Extraction of Azadirachtin

For the extraction of azadirachtin from neem seed oil, the same method was used as described by Dai [26,27]. 1000 g of neem kernel was purchased from a local shop in Anarkali Lahore, and oil was extracted. 100ml of oil was defeatted with 100 ml of diethyl ether. The lower layer was extracted with 200 ml of methanol and the filtrate was dried, in dark overnight. Next morning, yellow liquid (10 ml) was obtained which was dissolved in dichloromethane. The solution was tested for the presence of azadirachtin, using vanillin reagent [26]. Vanillin reagent was prepared as follows: 1 ml of 20 mg/ml solution of vanillin in methanol was mixed with 300 µl of concentrated sulfuric acid. A range of 10 to 100 µl of azadirachtin was used for estimation. The amount of azadirachtin was estimated by using a standard curve [27]. An absorption maxima for vanillin azadirachtin complex was determined on spectrophotometer to confirm the presence of azadirachtin [26,27]. The purity of the extracted compound was checked with the aid of a thin layer chromatography (TLC) using two dimensional systems.

Commercially prepared silica gel plates were used 2 cm above the bottom edge, two spots were applied (crude neem kernel extract and purified azadirachtin) to the plate. The TLC plate was dried and placed in the chamber containing chloroform-methanol-water (65:35:10) solvent system and was sprayed with 1% vanillin solution in ethanol. The plate was dried and sprayed with 20% sulfuric acid solution in methanol and placed at 110ºC till the appearance of spots. After performing single dimensional TLC, same plate was run in second dimension using different solvent system containing butanol-acetic acid-water (4:1:5). The plate was dried and sprayed with the same reagents used for single dimensional TLC.

2.2 Extraction of Saponins

Saponins were extracted from the neem powder [28]. For this purpose 100.0 g of neem powder was defatted in 200 ml of chloroform. The mixture was filtered and dried; precipitates were added to 200 ml methanol-water mixture (60:40). The mixture was extracted twice with 500 ml of butanol, using a separating funnel. Saponins were isolated by drying the butanol layer and the precipitates were dissolved in 50 ml of water. The upper layer of the mixture was separated and evaporated in a petri dish. Precipitates were again dissolved in distilled water. To check the purity of the extracted compound, thin layer chromatography was employed. Commercially prepared silica gel plates (Sigma) were also used, 20 µl samples were applied using a solvent system, chloroform methanol-water (65:35:10) solvent system. After completion, the plates were removed, dried and sprayed with 10% sulfuric acid in ethanol.

2.3 Inhibition Assays

A colony of *T. castaneum* was maintained in the laboratory in a plastic bucket in the dark and live insects were picked for experimental studies; while *O. chinensis* and *A. foveicolis* were collected from the fields of Punjab University New campus Lahore. Inhibition of

cellulase enzymes was monitored on carboxymethyl cellulose (CMC) agar plates, as described previously [13] to determine the inhibitory effect of neem derived compounds on the enzyme activity of the whole body of *T. castaneum* extract. Reaction mixtures were prepared by mixing 0.5 ml of buffer (pH 8.0), 0.5 ml of neem derived saponins (0.5 mg/ml) or azadirachtin, 0.5 ml of *T. castaneum* enzyme source and 1 ml of substrate (2% CMC). The flasks were incubated at 50°C overnight. After incubation, reducing sugars was estimated by the dinitrosalicylic acid (DNS) Miller et. al. [29] method and absorbance was read at 546 nm against the blanks.

2.4 Repellency Assays

Laboratory repellency test of *T. castaneum* for purified saponins and purified azadirachtin was carried out [30]. For this purpose, a filter paper disc of the same size was placed inside the petri dishes. A small filter paper disc of 2.5 cm diameter was soaked in 0.5 ml of saponins and azadirachtin extracts and was dried. The discs soaked with the inhibitors were placed in their respective petri dishes and 20 insects were introduced at zero time. Mobility of the insects was monitored after every 30 s for a time span of 7 min.

2.5 Computational Analysis

For computational analysis, sequence of *T. castaneum* TcEG1 (*T. castaneum* endoglucanase 1) was used as reported by Willis [25] using Visual Molecular Dynamics (VMD) and Protein Data Bank (Swiss PDB Viewer).

3. RESULTS AND DISCUSSION

Insects are the major cause of crop damage worldwide. For this purpose, the main enzyme system utilized by insects is cellulase. Many plants have been screened for the presence of repellants and antifeedants to form an effective bio-insecticide against insect pests of crops [30]. In this regard, neem is one of the most important and potent plants. Neem plant has a variety of compounds that act as insecticides, repellents, antiseptics etc. There are three main classes of bioactive compounds present in neem namely limonoids, saponins, and azadirachtin. Neem derived compounds are a source of inhibitors against cellulases present in various insects [13]. Azadirachtin was isolated from the neem kernel [26]. Saponins were purified from neem leaf powder [28].

Azadirachtin is a complex tetran or tri-terpenoid limonoid from the neem plant [5-7,15,26,27]. Azadirachtin was extracted from neem kernel oil. The purity of the compound was checked on thin layer chromatography, as described in materials and methods. Results are shown in Fig. 2A and 2B. For single-dimensional chromatography, results are shown in Fig. 2A, lane 1 and 2. The defatted sample from leaf extracted showed trailing band, while methanol extract of the defatted sample showed a single spot conforming the removal of most of the unwanted material. For the second dimension, solvent system B (butanol-acetic acid-water; 4:1:5) was employed, azadirachtin was confirmed by spraying the TLC plate with vanillin reagent in methanol. Different neem derived inhibitors, that is, crude neem Seed oil, crude neem powder extract, saponins and azadirachtin were incubated with the enzyme of *T. castaneum* and substrate at 50ºC overnight.

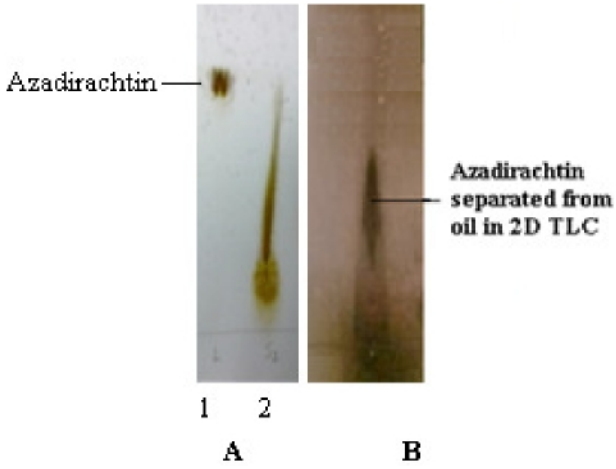

Fig. 2. Single dimension TLC (A), lane 1 shows purified azadirachtin and lane 2 shows a crude extract sample, second dimension TLC (B) showing position of azadirachtin

After the qualitative test, quantitative estimation of azadirachtin was carried out [27]. Azadirachtin complex showed absorption maxima at 577 nm in agreement with the results previously reported [27]. The extracted azadiractin was used for inhibition of digestive cellulose hydrolyzing enzyme activity and repellency test in vivo for *T. castaneum*. Results of enzyme inhibition studies are shown in Fig. 3a to3f; CMC agar plate assay showed that azadirachtin was able to inhibit *T. castaneum* cellulase while it had no effect on *A. foveicolis* and O. chinensis Fig. 3c to 3e.

Saponins and azadirachtin both were able to inhibit the enzyme about 80%. Earlier, we have reported that neem leaf extract has an ability to inhibit the cellulase activity [13]. Ximenesa [31] reported inhibition of cellulases by phenols. Confirmation of inhibition of endo-cellulases, Exo-cellulases, and - glucosidase by vanillin, syringaldehyde, trans-cinnamic acid and hydroxybenzoic acid was done. Both the enzymes were then subjected to inhibition by neem extracts. Neem extracts were reported to be effective in inhibiting insect enzymes [13].

Saponins are naturally occurring surface-active glycosides. Main production has been reported in plants but also in lower marine animals and some bacteria [32]. Saponins are named because of their soap-like properties. Saponins consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose [33]. The sugar moiety is glycosidically linked to a hydrophobic aglycone which may be triterpenoid or steroid in nature. Recently we reported that two glycosylated flavinoids from mango were able to inhibit cellulose hydrolyzing activity present in a beetle *A. foveicolis* [21]. The possible mechanism of inhibition can be related to the structure of saponins that have terpenoid along with an attached sugar moiety in the chair confirmation (Fig. 1). As the cellulose hydrolyzing enzymes are bound to the glucose moiety of cellulose molecules. The glycosidic compounds could mimic the structural identity and may inhibit the enzyme. It is thought that the carbohydrate molecule fit into the active/binding site of the enzyme and could lock its hydrophobic aglycone by forming hydrogen bonds with the relative amino acid residues. It was reported that, a sugar residue bound at the catalytic site of cellulase undergoes a distortion that renders it in a planar half chair conformation during catalysis of cellulose [34]. Wang [33] investigated the effects of steroidal saponins isolated from *Yucca schidigera* (Spanish Dagger, a flowering plant) extract, on three species of ruminal bacteria

and two species of fungi. Steroidal saponins inhibited all cellulolytic activities of both bacterial and fungal species. This showed that saponins may be useful in targeting cellulose-digesting ruminal microorganisms.

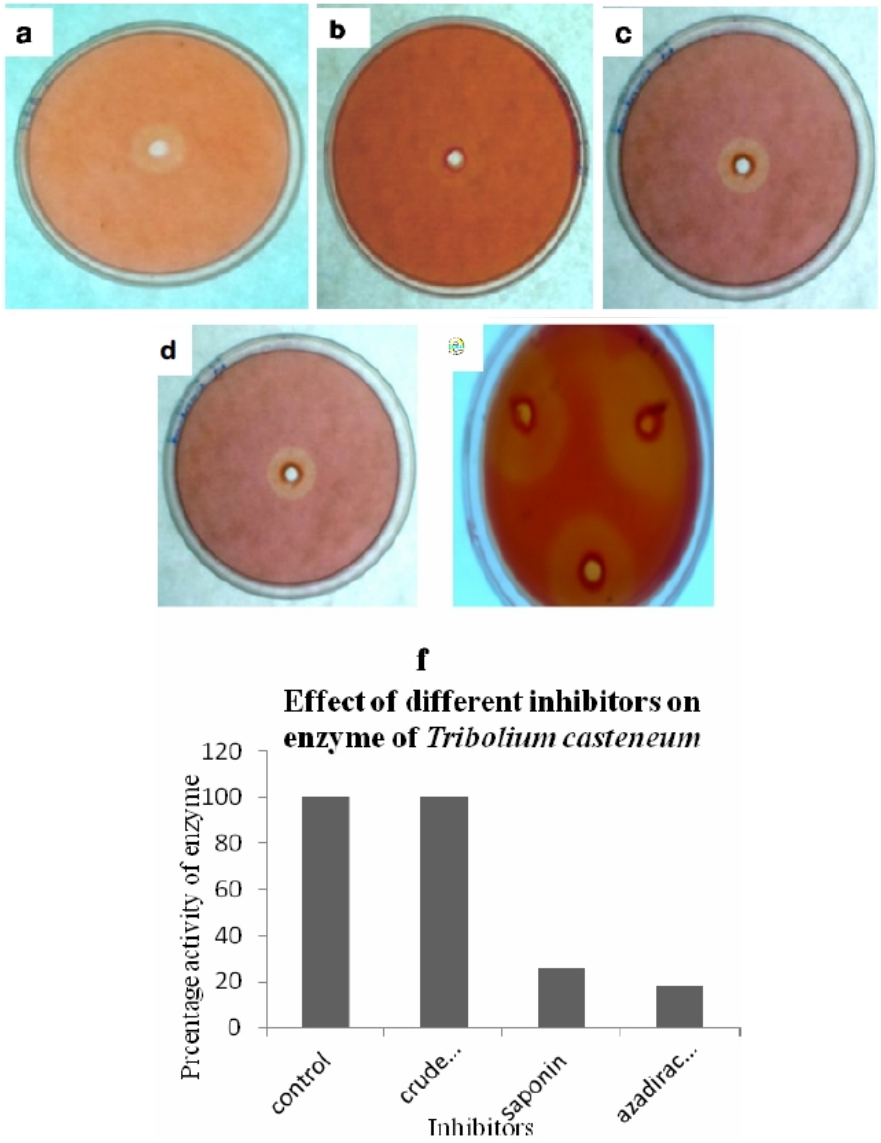

Fig. 3. The percentage inhibition of cellulose hydrolyzing enzyme of *T. castaneum, O. chinensis and A. foveicolis* by neem derived purified saponins and Azadirachtin: (a) *T. castaneum* cellulase without Azadirachtin, (b) cellulase with azadirachtin, inhibition is clearly visible, (c and d) *O. chinensis* cellulase with and without Azadirachtin, no inhibition was observed, (e) *A. foveicolis* with gut and salivary gland cellulase in the presence and absence of Azadirachtin, no inhibition was observed and (f) percentage inhibition of *T. castaneum* cellulase by crude leaf extract, saponin and azadirachtin

Earlier, it was confirmed that a steroid like compounds isolated from the Neem seeds acts a growth regulator and had an impact on the development of insect pest of stored products [1]. Azadirachtin was also able to inhibit the cellulase activity to a similar extent (Fig. 3) and proven to be a good inhibitor of enzyme activity. The structural geometry of azadirachtin was revealed by Nakanishi [35]. As far as the structure is concerned, there is no structural similarity between azadirachtin and substrate (cellulose) molecule, as it was found in case of saponins and glycosidic flavonoids (due to the attachment of carbohydrate moiety), as reported earlier for mango flavonoids [21]. For insect cellulases, it is considered that they contain a higher percentage of Cysteine (Cys) residues as compared to other and they could clip the active site of cellulase enzyme, as in case of *Apriona germari* [34] perhaps to protect active site. The effect of reducing agent beta-mercapto ethanol on enzyme activity could provide some clues in this regard. Earlier, we have reported that the reducing agent showed a positive effect by increasing the reaction rate, but at higher concentrations it reduced the enzyme activity. Possible explanation is the relaxation of the active site by reducing Cys disulfide bridges at lower concentrations; but at higher concentrations, reduction of disulfide bridges is involved in the stabilization of globular structure of the protein which had a detrimental effect on the enzyme activity [36]. *O. chinensis* and *A. foveicolis* cellulase were not affected by azadirachtin. Its explanation could be that most *O. chinensis* and *A. foveicolis* solely fed on cellulose, and binding of azadirachtin to the enzyme active site, in any form could be detrimental to the insect. As far as *T. castaneum* is concerned, the genome sequence of insect *T. castaneum*, has been revealed. It was decided to dissect the molecular structure of cellulose hydrolyzing enzyme protein present in the *T. castaneum*.

Molecular model of the molecule was drawn and it was shown that aspartic acid (Asp) 54, Asp 57 and glutamic acid (Glu) 412 are the active site residues in endoglucanase from Nasutitermes takasagoensis. Sequence homology showed that the same active site residues occupy the positions Asp 68, Asp 71, and Glu 425 in the endoglucanase from *T. castaneum*. Thus, this sequence alignment confirms that Asp68, Asp71, and Glu425 are the active site residues in the cellulase (Endo-β-1, 4-glucanase) present in the *T. castaneum*. It has several hydroxyl groups in its structure which can form hydrogen bonds with side total of 5 Cys residues at positions Cys 120, Cys 293, Cys 326, Cys 378, and Cys 385 in the molecule. It was revealed that Cys 120, 293, and Cys 326 were present in Alpha helix and thus, unable to form any kind of disulfide bond which restricts their role merely to the structural purposes. While two other amino acid residues at Cys 378 and Cys 385, were in a position to form a disulfide link, which may not shield the enzyme active site due to its lateral position (Fig. 4). Perhaps, there is no protection provided by disulfide bridges, as in case of *A. germari* [34] to the active site of cellulose enzyme present in the *T. castaneum*.

The insects were fed on food items such as flour, rice, dried fruit, nuts, and beans, not totally relying on cellulosic materials, as in case of many other insects. Thus, the inhibition of cellulase molecule may not completely deprive the insects from food, as it can also utilize starch and other carbohydrates. Considering the structure of azadirachtin molecule, it is clear that azadirachtin has a complex structure, and has a number of possibilities for interactions with hydrophobic amino acid residues present in the enzyme protein. The binding of the enzyme in its micro environment with azadirachtin could alter the enzyme activity. Gangadhara [37] reported the inhibition of rice bran lipase by azadirachtin Nathan [22] reported the effect of azadirachtin on the mid gut enzymatic profile of *Spodoptera litura* Fab. Similarly, the effect of neem limonoids on lactate dehydrogenase of an insect, rice leaf folder (*Cnaphalocrocis medinalis*) was also reported [38].

Azadirachtin is an antifeedant, and is involved in the inhibition of glutathione synthesis and inhibition of various neuroendocrine processes in the brain of locust (*Schistocerca gregaria*) [39]. Najat [40] evaluated the potential of insect growth regulator in the growth and development of Tribolium confusum azadirachtin, it was proven as insect growth regulators after testing its chitin inhibitor activity in adults of *T. confusum* (Coleoptera: Tenebrionidae). It was evaluated on fecundity, hatchability, and viability of eggs, longevity and morphometric of oocytes. Azadirachtin has been reported as an inhibitor for a number of enzymes. A glycosylated triterpenoid saponin from peas (*Pisum sativum*) was reported to be specific and a highly potent inhibitor of diguanylate cyclase [41] which is a key regulatory enzyme of cellulose synthesizing apparatus in the bacterium *Acetobacter xylinum*. The Neem plant contains compounds that are insect repellant (Fig. 5) possibly due to inhibition of nervous enzymes of insects and are anti eedant [39].

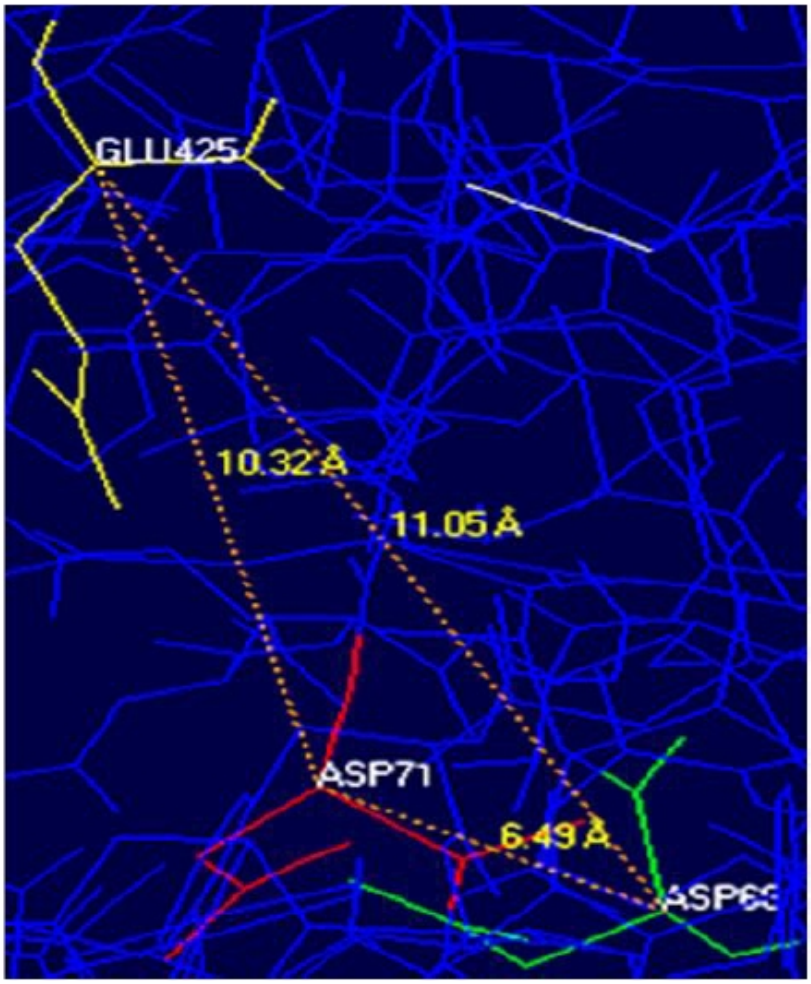

Fig. 4. Location of active site of endoglucanase of *T. castaneum* using computational tools. Active acid amino acid residues Asp 71 (red), Asp 68 (green), Glu 425 (yellow) are visible along with their distances. Cys residues at position 378 and 385 are visible in yellow

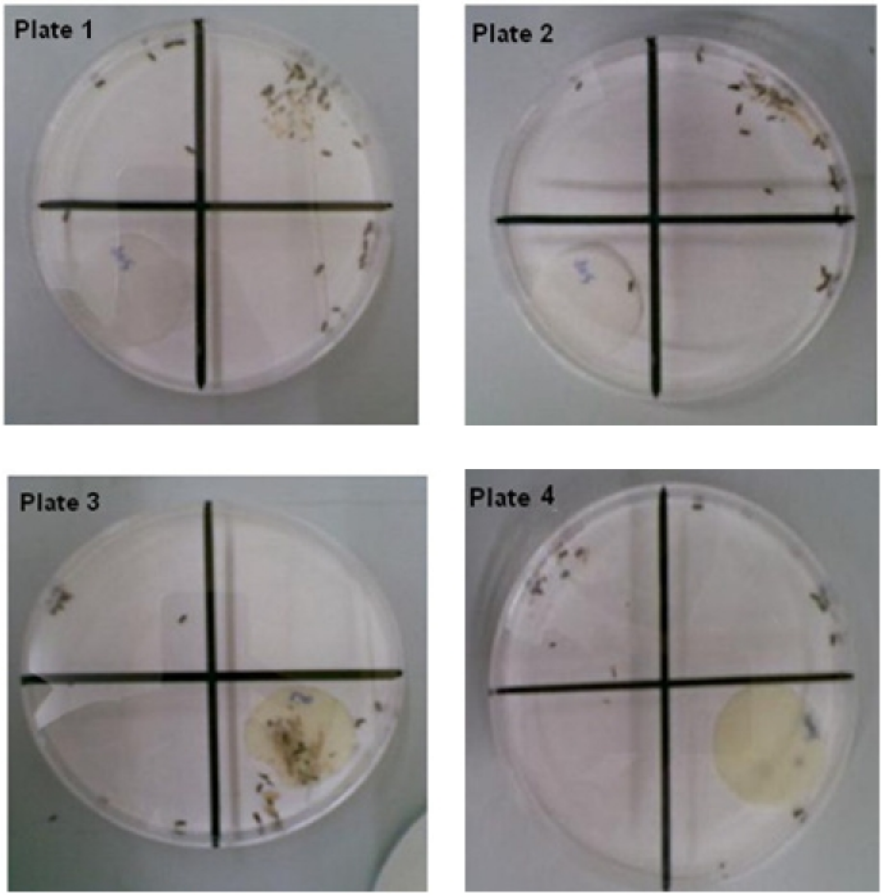

Fig. 5. Plates showing movement of live *T. castaneum* in response to different inhibitors. Plate 1: 2 insects in the zone containing saponins disc after 30 s. Plate 2: 1 insect in the zone containing saponins disc after 5 min. Plate 3: 9 insects in the zone containing azadirachtin disc after 30 s. Plate 4: 1 insect in the zone containing azadirachtin disc after 5 min

Repellency test was performed by introducing insects on a petri dish containing filter paper disk dipped in saponins and azadirachtin, the movement of insects was observed. Results are shown in Figs. 6 and 7, both the compounds were able to repel the insects within 5 min and proved to be a potent repellant.

The functions of saponins common in a large number of plants and plant products were investigated [42]. Saponins were found to be significantly functioning as antifeedants, and affect growth and reproduction in lower animals. Results showed that azadirachtin, saponins and neem extract were able to inhibit the enzyme activity as described in the results section (Fig. 3). In a study it was demonstrated that azadirachtin has a latent effect on *T. confusum* progenies as manifested by the reduced growth and development of immature and mature stages, and lesser production of adults [40].

The phytochemical test results of the neem plant indicated high scores for saponins, moderate scores for tannins; while alkaloids, terpenes, and flavonoids had low scores [14].

Many saponins are known to be antimicrobial, to inhibit mold, and to protect plants from insect attack. They may be considered as a part of the plant defense system [43]. It has been reported that saponins can be activated by the plant enzymes in response to tissue damage or pathogen attack [44].

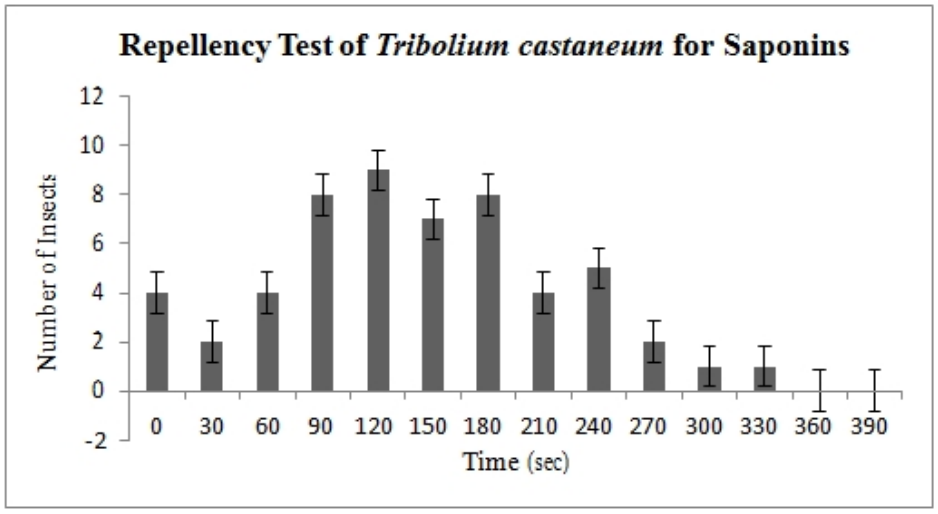

Fig. 6. Graph showing amount of insects present in the zone containing saponins on the petri dish. The graph shows a succesive decrease in the amount of insects around inhibitor with the passage of time

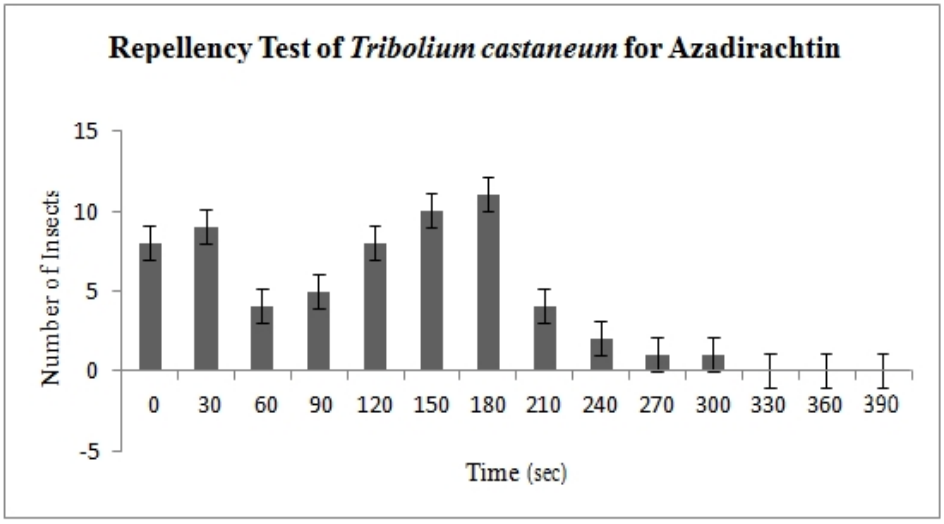

Fig. 7. Graph showing amount of insects present in the zone containing azadirachtin on the petri dish. The graph shows a succesive decrease in the amount of insects around inhibitor with the passage of time

4. CONCLUSION

It can be concluded that neem compounds are potent insect repellant, antifeedant, and enzyme inhibitor. It implies that the use of natural products like azadirachtin, as pesticide would help in controlling the storage pests in an environmental-friendly way. Azadirachtin was observed as a repellant of stored grain insect (*T. castaneum*); whereas, saponins were effective as inhibitor for both insects. Therefore, neem extract can be employed for the production of biopesticides which is effective against insects but have no detrimental effects on humans.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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