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STUDY AND POSSIBLE INHIBITION MECHANISM OF NEEM DERIVED SAPONINS ON HONEYBEE BETA 1, 4 ENDOGLUCANASE (CELLULASE)

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ABSTRACT

Plants are known to produce certain insecticidal compounds as their secondary metabolites that act as self-defense system of plant. *Azadirachta indica* (Neem) is known to have insecticidal, fungicidal and nemicidal properties. Neem based compounds can be used as potential inhibitors of pest cellulases. Cellulases are the enzymes that hydrolyze the β-linkage in cellulose and generate carbohydrate residues. The inhibitory effect of Neem derived Saponins were estimated on Cellulase activity of salivary glands and gut of Honeybee and it was also confirmed by computational analysis. Saponins were found to inhibit the Cellulase, as Cellulase are not chief digestive enzyme of Honeybee so bio pesticide for Cellulase inhibition can be designed for pests and Neem derived compounds can also be used to control Honeybee mites, bacterial and fungal infections.

Keywords: *Azadirachta indica*, Neem, insecticidal, fungicidal, nemicidal, cellulases

INTRODUCTION

Azadirachta indica (Neem), a miraculous fast growing tree that holds many promising properties that have been benefiting mankind since ages. A number of secondary metabolites are believed to function as biochemical defenses or allelochemicals are produced by plants [1]. Neem plant is being

a source of great studies of more than 135 isolated compounds chemistry and diversity. These compounds are being divided into two major categories. Isoprenoids like diterpenoids and triterpenoids [2]. Like most of nor terpenoids Limonoids are exceedingly bitter. So they have attained tremendous attention due to their fungicidal, insecticidal and nemicidal properties [3]. These are multi-actions of these compounds against insects that are toxicity, antifeedant activity, antimitotic effects, growth regulation, sterilization, fecundity suppression, repellency of oviposition, includes destructive effects on damage of cuticle of larvae preventing them from moulting and endocrine system [4]. Azadirachtin and Saponins are reported to be potent insecticidal constituents of neem seed kernels [5]. Neem seed extracts are non-toxic to warm-blooded organisms so they are suitable insecticidal agents [6]. Many saponins are being studied to have antimicrobial activity that inhibits moulds and protecting plant from attack of the insects. They can be taken as defense system of the plant. Saponins have been reported to be effective against tissue damage and attack of the pathogens [7]. Azadirachtin and saponins are Neem based compounds which act as enzyme inhibitors. *Tribolium castaneum* (Insecta: Coleoptera: Tenebrionidaei) is an omnivorous beetle pest of stored agricultural products and is reported to be effected by Neem compounds. Neem plant has secondary inhibitory effects on live insects, their enzymes and for their antifeedant activities [8].

Cellulose, lichenin, cereal beta D glucans and hemicellulose metabolizing organism like bacteria, fungi and protozaons produce complex of enzyme known as Cellulases that catalyze cellulolysis by specifically hydrolyzing the 1,4 β D glycosidic linkages. Termites are also reported to produce cellulase enzyme [9]. Originally cellulolytic activity in the insects was attributed to the symbiotic gut bacteria. However first cellulase gene of the insect origin was discovered and cloned from the *Reticulitermes speratus* (Kolbe) (Isoptera: Rhinotermitidae) in late eighties. Currently the cellulolytic enzymes have been detected or identified in insects belonging to ten different orders including Blattodea, Coleoptera, Diptera, Hymenoptera, Isoptera, Lepidoptera, Orthoptera, Phasmatodea, Plecoptera and Trichoptera. Through the DNA and protein sequence homology 115 GH families (GHF) have been identified. Wood-eating insects such as termites or

cockroaches presumably require cellulases to digest their cellulose-rich foods [10]. Although, the presence of cellulase in hymenoptera is surprising.

Honey bee is the member of class Insecta. They are under the Apis genus having sub-family Apinae. 20,000 species of honey bees are discovered out of which 44 sub-species are recognized as honey bees. They are involved in production and storage of liquefied sugar commonly called honey [11]. Honeybee is considered to be the most important pollinator due to its broad diet, efficiently using scouting to locate and utilize patches of resources and longer foraging range. So the negative effect of crop fruit set due to isolation from natural lands may be neutralized by presence of honeybees in agriculture landscapes [12]. bees pollinate one or more cultivars of >66% of the world's 1,500 crop species [13] and are directly or indirectly essential for an estimated 15–30% of food production [14]. Currently, farmers that manage pollination on farms or in glasshouses rely on <11 of the 20,000–30,000 bee species worldwide. Per year rate estimated in United States alone is 5-14 billion dollars for the crop pollination by Honeybee [15,16]. Due to diseases, loss of subsidies, and insecticide poisoning [16]; along with increasing demand has declined beekeeping (≈50% since 1950) [17]. The honey bees population is declining due to various bee-associated infections. The causes may be pathogens (mites, viruses and bacteria) and parasites. Nevertheless Honeybee is important pollinator as many crops depend on it for pollination. Moreover it produces wonderful syrup known commonly as Honey that has many beneficial properties. The study was conducted to pinpoint the activity of cellulases in honeybee and effects of neem derived saponins and Azadirachtin on enzyme activity. In addition a neem based bio pesticide can be designed to control the threats that honeybee face as reported by [18].

MATERIALS AND METHODS

Insect

The honeybees (*Apis dorsata*) were collected from the local vegetation of Karkan Wahga during the month of May in the morning in a sterilized container. The Honeybees were stored at -20°C for further use.

Cruder extract of insect

The exoskeleton was removed from the honeybees as much as possible. Head and body was separated and weighed. The head was used as source of salivary gland enzymes and body was used as a source of

gut enzymes. The head and body was crushed using a clean mortar and pestle in 1% saline solution with the final concentration of 20mg/ml. Homogenized mixture was centrifuged at 3000 rpm for 10 minutes. Supernatant was stored at -20°C.

Screening for cellulase activity

Cellulase activity was found by 3.5 dinitosalicylic acid (DNS) assay [19]. 500µl crude extract of body region, 500µl 1% Carboxymethylcellulose and 500µl of buffer (pH 5), were used to prepare the reaction mixtures. Reaction mixtures were incubated at 50°C for 10 minutes, 3 ml DNS reagent was added in the reaction mixtures and boiled for 10 minutes. Afterwards Optical densities were recorded by UV-Visible spectrophotometer at 600nm against blanks.

Inhibition assay

Preparation of Neem extract

0.02 grams of Neem leaves powder was weighed and mixed in 20ml of 50% methanol. The mixture was filtered and the extract having final concentration of 2mg/ml was stored. Range of concentration 0.2-2 mg/ml was prepared from the stock solution of neem extract. 200µl of crude enzyme extract of head, 200µl of buffer (pH 5) and 400µl of 1% CMC, in the series of test tubes was added. The same procedure was repeated for the extract of body. Negative controls were prepared by adding distilled water instead of crude extract enzyme. Positive controls were prepared by adding 200ul of crude enzyme extract, 400µl of 1% CMC, 200µl of buffer (pH 5), and 200µl of distilled water. The reaction mixtures were incubated at 50°C overnight. After incubation DNS assay was done as described earlier. Optical densities were measured at 600nm against blanks by UV-Visible spectrophotometer.

Range of different volumes 20ul to 200ul from the stock solution of saponins and were prepared. 200µl of crude enzyme extract of head, 200µl of buffer (pH 5) and 400µl of 1% CMC was added in the series of test tubes. The same procedure was repeated for the extract of body. Negative controls were prepared by adding distilled water instead of crude extract enzyme. Positive control was prepared by adding 200µl of crude enzyme extract, 400µl of 1% CMC, 200µl of buffer (pH 5), and 200µl of distilled water. The reaction mixtures were incubated at 50°C overnight. DNS assay [19] was done with all reaction mixtures and optical densities were recorded at 600nm against blanks.

Computational Analysis

Predicted sequence of *Apis dorsata* cellulase was taken from NCBI.

The cellulase sequence *of Apis dorsata* was uploaded to the online server for prediction of secondary and tertiary structure based upon the molecular modeling in form of PDB file. For this purpose Swiss-Model was used. Docking server was used for the docking analysis [20]. For the energy minimization of ligand molecule (Saponin 2), the MMFF94 force field [21] was used. Gasteiger partial charges were added to the ligand atoms. Rotatable bonds were defined and non-polar hydrogens were merged. Docking calculations were carried out on cellulase protein model of *Apis dorsata*. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the help of AutoDock tools. Affinity (grid) maps of 20×20×20 Å grid points and 0.375 Å spacing were generated using the Autogrid program [22]. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and electrostatic terms respectively. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method [22]. Initial position, orientation, and torsions of the ligand molecules were set randomly. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

RESULTS AND DISCUSSION

Neem extract was prepared from Neem leaves powder which was used to study the inhibitory effects of neem derived compounds on Cellulase activity from crude extract of head. The graph was plotted using percentage activity of Cellulase and concentration of neem extract which showed the inhibitory effect of neem derived compounds increases with the increase in concentration.

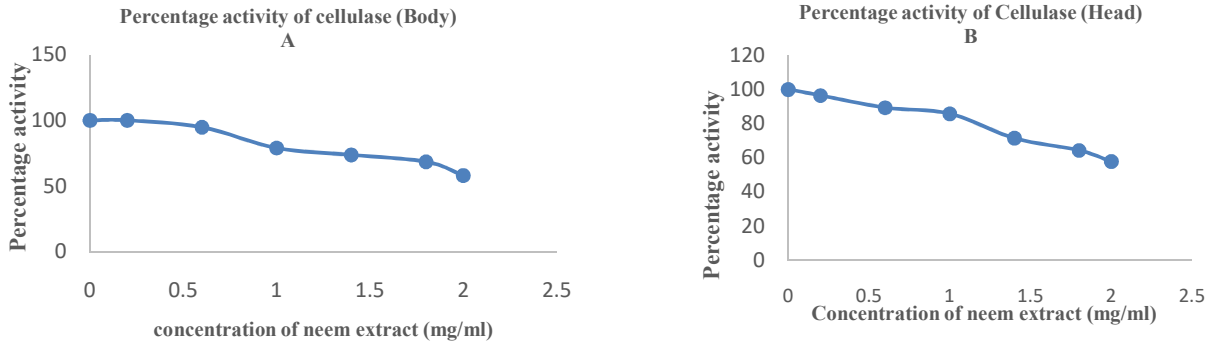

Fig 1: (A) Percentage activity of salivary gland Cellulase (B) Percentage activity of gut Cellulase in presence of Neem extract.

Purified saponins were used to study the effective volume for inhibitory effect on Cellulase from enzyme extract of head. The percentage activity was recorded by Dinitrosalicylic acid assay and graph was plotted that showed the maximum inhibition on 200µl of saponins.

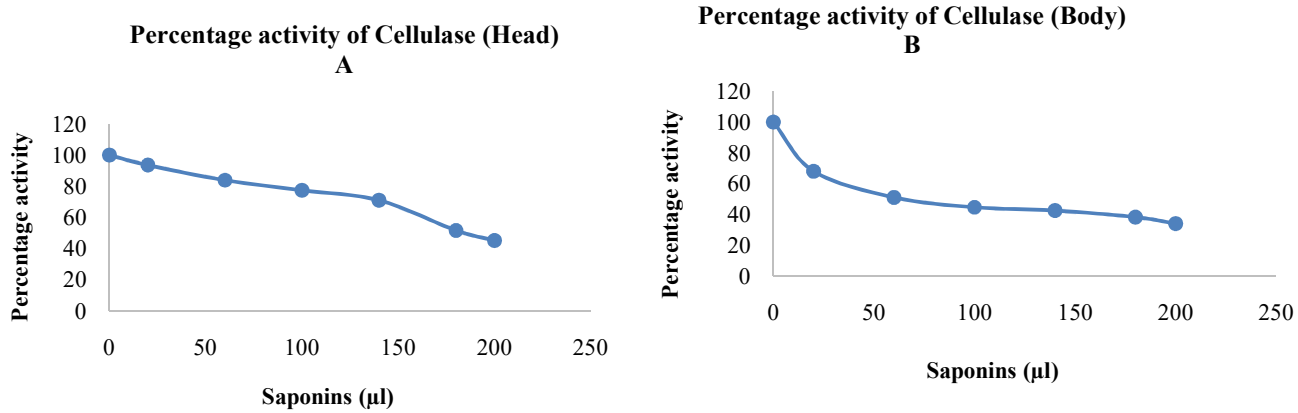

Fig 2: (A) Percentage activity of salivary gland Cellulase (B) Percentage activity of gut Cellulase in presence of Neem derived Saponins.

Surface active glycosides that naturally occur in certain plants species, animals and microorganisms are known as Saponins. Plants in sufficient, but lower amount in the marine animals and some bacteria are able to produce Saponins. Saponins are named because of their soap like properties. Usually Saponins consist of sugar moiety that contains glucose, xylose, glucuronic acid, galactose, and rhamnose or methyl pentose [23]. A glyosidic linkage is present between the sugar moiety and hydrophobic aglycone that may be terpenoid or steroid. The aglycone could have one or more carbon unsaturated bonds. Saponins are characterized by structurally diverse class of compounds that occur in many plant species and their skeleton is derived of precursor called oxideosqualene consist of 30 carbon to which a glycosyl group is attached. A diverse range of properties are exhibited by Saponins which include sweetness and bitterness [24], emulsifying and foam formation [25], pharmacological and medicinal [26], insecticidal, antimicrobial, haemolytical and furthermore molluscicidal activities. Wide range of applications of Saponins are found in the confectionery and beverages, in cosmetics and in the pharmaceutical products [27].

A comparative graph was drawn to access the differential percentage inhibition of neem extract and purified Saponins on both Head and Body crude extract containing cellulases.

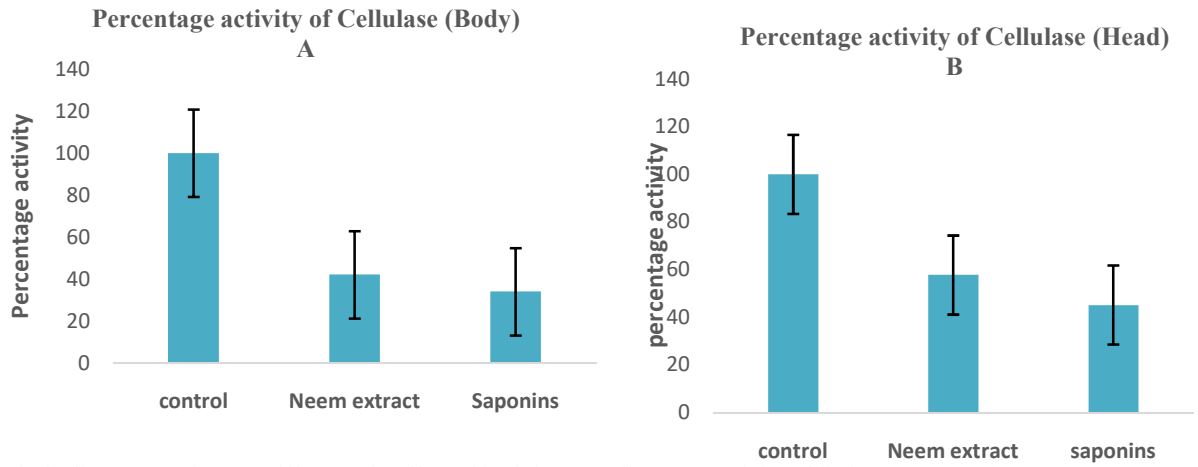

Fig 3: Graph showing the difference in effect of inhibitors on Cellulase activity of Salivary glands (A) and gut (B). The control shows maximum activity while Cellulase activity is less when treated with neem extract and Saponins

The cellulase sequence of Apis dorsata was searched by the accession number **XP_006610535** and contains **485** amino acids. The predicted sequence was used to find out the active site residues by Scan prosite tool of Expasy server and signature pattern was found to be from Glycosyl Hydrolase Family 9.

MKRVVGGKSSMDSNMFVTCVTTLVIT
SIALIGTINANPPYYVKPIEDENDYARV
LELSLLFYEAQRSGKLPENNRIPWRGD
SALEDRGLNGEDLTGGYYDAGDFVKF
GFTMASTTTLLAWGAVSWPEAYNAA
GQLDELRKAIKWATDYFIKCHVSEYV
FYGQVGDFSLDHTFWGRPEELNTTRP
AYKIDPDHPGSDLAGETAAALAASSIV
FRNYNPEYSANCLKHAKELYKFANKY
RGLYHEAIRGAAQYYESTDYGDELAW
AAVWLFKATNDTMYLEEAEHHYQHF
HLKERPNEFFYNKKVAGVQVLLAQMT
GQPEYQNAARAFCDFSVYQQKRTPKG
LLYIDKFGTLCHAANVAFVCLEAADSS
GIGDSQKYREFAEQQIYYMLGGGGRS
YVVGWGRNPPKQPHHAASSCPDRPAI
CGWSEFDKDAPNPQILYGALVSGPDE
ADKFHDHREDYVYTEVTLDYNAGFTS
ALAGLLQLRVKSTT

The highlighted area shows the active site residues of *Apis dorsata* cellulase. The Molecular model of *Apis dorsata* Cellulase was designed by using Swiss-Model and viewed by PyMOL viewer. The Molecular model showed that 16 helices, 2 beta sheets, 19 loops and 6 turns are present in cellulase protein structure.

The Cellulase enzyme of *Apis dorsata* was docked with Saponin molecule by using Docking server and it showed that Saponins are able to inhibit the Cellulase by binding its residues.

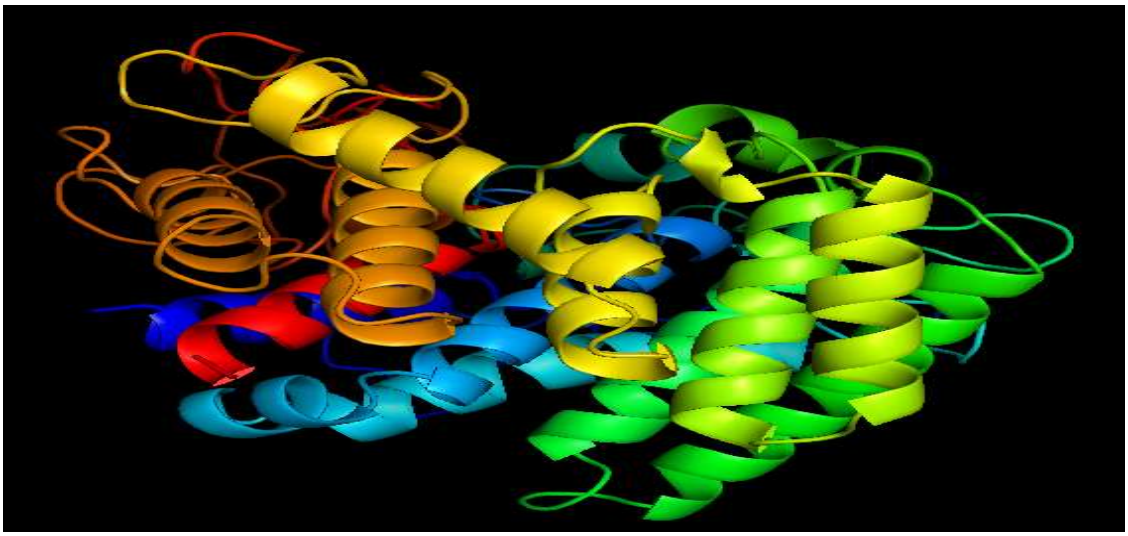

Fig 4: Homology Model of *Apis dorsata* Cellulase designed by Swiss-Model

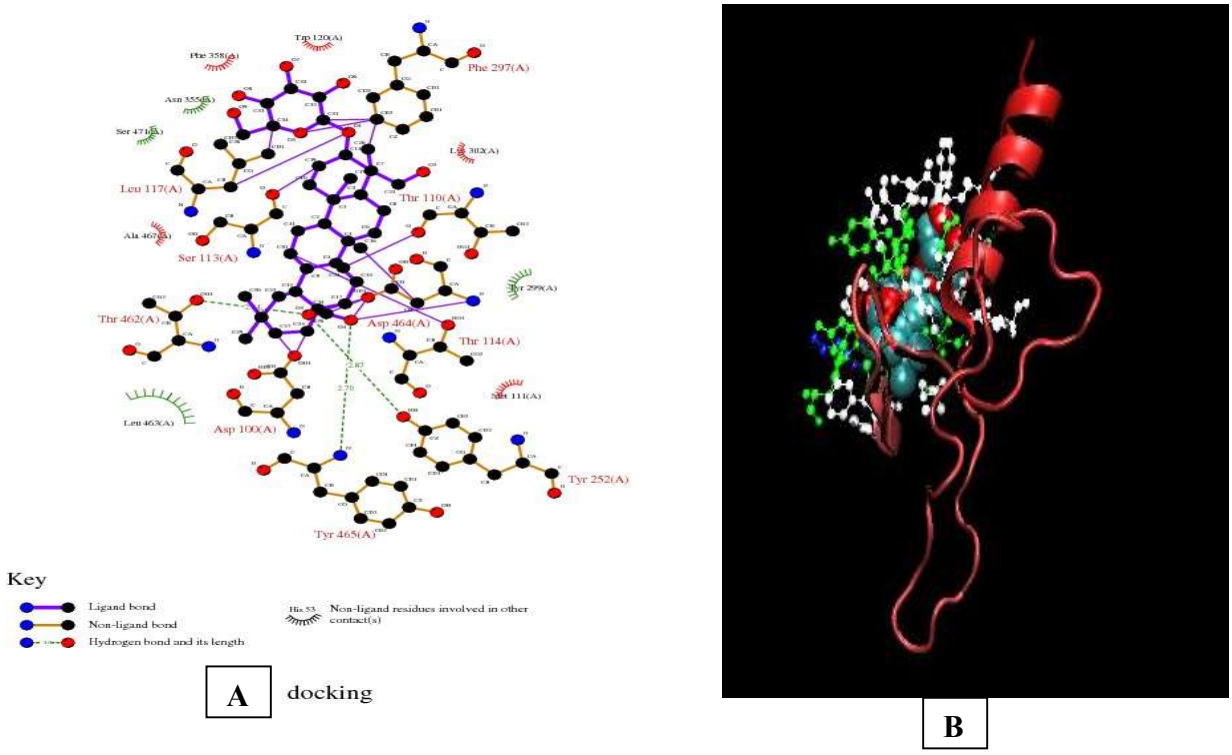

Fig 5: (A) Interactions of Saponin (ligand) to residues of Cellulase. (B) Docking of Saponin to *Apis dorsata* Cellulase. [28]

The binding energy and interaction surface of *Apis dorsata* and saponin molecule was found to be +4.76e+03 kcal/mol and 841.281 respectively. The docking studies showed that O3 and O4 of saponin molecule make hydrogen bonds with TYR252, THR462 and ASP464, TYR462 respectively. Polar interactions are

established between H3 and H4 of saponin molecule to SER471 of Cellulase residue, while ASP100 interacts with O3 and O4. MET111 interacts by hydrophobic forces with C24, C29, C30 whereas LEU117 interacts with C10, C15, C19, C31, C32, and C33 of saponin molecule. Cation-pi interactions are present between TRP120 to H3, H2 PHE297 to H1, H2 and PHE358 to H2, H3, H4 and H5 of saponin molecule. These many kinds of interactions between different residues of cellulase and atoms of saponin results in inhibition of cellulase activity.

Mercury, silver, copper, chromium, lead and zinc salts at about 10^{-3} are reported to inhibit [29]. Organic molecules quaternary ammonium salts or other detergents are also inhibitors of Cellulase [30]. Cellulases is reported to be inhibited by Cysteine, glutathione, cyanide and sodium sulfate [29,31] . Oxidizing agents can be potent inhibitors too. At 10^{-4} Halogens and compounds that release active halogens such as hypochlorite, dicholoromelamine, and tetra glycine potassium periodide may be active. A number of substituted phenols such as chlorophenols, saligenin, orthophenyl phenol and chlorophenyl phenols are known to be slightly active against certain fungal cellulases but Phenol itself is not an inhibitor of cellulase (Mandels and Reese 1963). Number of phenolic compounds like chlorophenols, tannin, and pinosylving monomethyl ether are reported to inhibit Cellulases of pest (Lyr, 1961). Neem derived compounds are potential inhibitors against cellulases present in various insects [32]. As our study also showed that purified saponins derived from Neem is able to inhibit the Honeybee Celulases.

The structure of saponins could be the possible reason for inhibition as they have terpenoids with attached sugar moiety in chair confirmation. Glucose moiety of cellulose molecule binds to cellulose hydrolyzing enzymes. Due to the structural identity, glycosidic compounds may inhibit the enzyme. It is proposed that carbohydrates could form hydrogen bonds with relative amino acid residues and thus it fits into the active/binding site of the enzyme. It is further reported that during catalysis of cellulose the sugar residue bound to catalytic site of cellulase undergoes a distortion and renders confirmation of planar half chair [32]. As discussed earlier, Honeybees are important pollinators but although neem based components are able to inhibit the cellulase activity in Honeybee as also reported earlier by Sami, and Shakoori,

(2007), it still forms a hive on neem tree. The possible explanation for this is Honeybee depends upon nectar and pollen which are not high in cellulose. Because of high structural similarity to the termite GHF9 gene, it was suggested that honey bee cellulase functions as an endo-cellulase. The wall of pollen grains can be subdivided to two layers and inner wall is mainly composed of cellulose [33]. Thus, to digest the cellulose wall to absorb all the nutrients from the pollen and to digest cellulose contaminants in the nectar, Cellulase may be needed. So, inner wall of pollen grain might be digested by Honeybees. Another proposal regarding presence of Cellulase is that it may function in digestion of other plant material containing cellulose to produce propolis.

The rate of Carbohydrate metabolism in bees seems to be mainly triggered by the signal of Glucose concentration in diet [34]. Enzymes present in alimentary canal, involved in digestion of carbohydrates are mainly: α-amylase that hydrolyses starch contained in pollen [35] and β-glucosidase [36] are secreted from the of honeybee workers. Sucrase present in hypo pharyngeal glands is highly active that degrades sucrose present in nectar to glucose and fructose. Sucrase is 50% of all the proteins that may comprise of this enzyme [37]. Another perspective to conduct this study was to gain an insight to effectively use neem derived inhibitors to control infectious agents of Honeybee. Anjum, et al reported Neem and Barbaka (*Vitex trifolia*) to be effective against gut bacteria and ectoparasitic mite *Varroa*. Neem extract showed intermediate activity against *Bacillus subtilis* and *Staphylococcus hominis.* This study concluded that Barbaka and neem extracts have antibacterial and matricidal activity and are reasonably safe [18] *Varroa jacobsoni* and *Acarapis woodi* are reported to be controlled by using neem oil, but further studies need to be done in order to devise a strategy to control honeybee mites effectively.

CONCLUSION

Neem derived saponins are potent Cellulase inhibitors, also inhibits the Honeybee Cellulases. As Cellulase is not chief digestive enzyme of honeybee thus it could be used to control crop pests and honeybee mites. Saponins mimic the structural similarity of glucose and competitively inhibit the Cellulase active site. So when substrate (Glucose) level rises in diet, it makes Saponin molecule to fall off and binds to active site itself. Further detailed experiments should be conducted to study

the effect of Neem derived Saponins on other enzymes of Honeybee that would possibly lead to efficient strategy to control crop pests and Honeybee infections.

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