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FUNCTIONAL CHARACTERIZATION OF A POTENT ANTIMICROBIAL AND INSECTICIDAL CHITIN BINDING PROTEIN FROM SEEDS OF *IBERIS UMBELLATA* L.

AHSAN SAEED[1], ZAHRA RAFIQ[1], QAMAR SAEED[2], BINISH KHALIQ[3], ANWAR ULLAH[4], SOHAIB MEHMOOD[1], ZAHID ALI[5], MUHAMMAD YASIN ASHRAF[3] AND AHMED AKREM[1*]

[1]*Botany Division, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Pakistan*
[2]*Department of Entomology, Bahauddin Zakariya University, Multan, Pakistan*
[3]*Institute of Molecular Biology and Biotechnology, University of Lahore, Lahore, Pakistan*
[4]*Department of Biosciences, COMSATS University Islamabad, Islamabad 44000, Pakistan*
[5]*Plant Biotechnology & Molecular Pharming Lab, Department of Biosciences, COMSATS University Islamabad (CUI) Islamabad, Pakistan*
[*]*Corresponding author at: ahmedakrem@bzu.edu.pk; Tel: +923124659831.*

Abstract

Chitin-binding proteins belong to the 2S albumin family and are helped in the plant defense, especially against fungal pathogens. A chitin-binding protein, Iu-CBP, has been identified and characterized from *Iberis umbellata* seeds. The purified form of this protein showed approximately an 11 kDa band under non-reduced condition on SDS-PAGE. LC-MS/MS provided a single fragment of amino acid sequence of 23 residues (QAVQSAQQQQGQVGPQQVGHMYR). UniProtKB database showed 100% sequence similarity with *Moringa oleifera* chitin-binding protein (Mo-CBP$_3$-1) which classically contained two proteolytically matured α-helical chains linked by disulfide bonds along surfaced Arginines responsible for antimicrobial activity. Iu-CBP showed antimicrobial activity against bacterial pathogens i.e *Bacillus subtilis, Xanthomonas oryzae, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* at concentrations of 5.0 and 10.0 µg per disc. Similarly, a 20 µg/disc dose of Iu-CBP inhibited the mycelial growth of *Aspergillus flavus*. At a concentration of 3.0 mg/ml, Iu-CBP had a significant reduction in progeny population of *Sitophilus oryzae* and *Tribolium castaneum.* Chitin-binding proteins have historically been studied as a source of carbon and nitrogen in plants; more recently, their thermostability, antimicrobial and insecticidal properties, along with their water sterilizing qualities have attracted the interest of the scientific community.

Key words: *Iberis umbellata,* Chitin-binding protein, Antibacterial, Antifungal, Insecticidal.

Introduction

The plant kingdom has been providing a variety of medicinal compounds; plant-derived secondary metabolites and proteins; representing a large and diverse reserve of therapeutics. Generally, these compounds are constitutively produced and with the development of antimicrobial resistance, the demand for these natural products has increased dramatically (Bonev & Brown, 2019). Plant-derived compounds and proteins are often recommended as nutraceuticals of pharmaceutical-grade and also as standardized nutrients. Also, the purified compounds and proteins are prescribed as drugs. Chemists are using natural compounds as scaffold molecules to develop upcoming anti-inflammatory, anti-nociceptive and antimicrobial drugs (Nelson *et al.*, 2017; Ahmed *et al.*, 2018). Additionally, plant-derived proteins have become defensive molecules because of their inhibitory mechanisms against a wide range of pathogens including insects, bacteria, viruses and fungi (Ye *et al.*, 2001; Rubinstein *et al.*, 2004; Barrientos & Gronenborn, 2005). This is noteworthy because insects are responsible for annually 40% loss of global stored food product (Nelson *et al.*, 2017).

Storage proteins play an essential role in plant survival as they are integral for plant growth maintenance and defense mechanisms. Seed storage proteins are compact, globular and contain conserved cysteine residues (Menéndez-Arias *et al.*, 1987). They are retained in protein bodies of premature seeds and are then used as a nutrient source (i.e. amino acids and carbon skeletons) during germinating seedling. The 2S albumin, based on the sedimentation coefficient (Youle & Huang, 1981), is widely distributed throughout spermatophytes (Shewry *et al.*, 1995). The 2S albumins are belonging to the prolamin superfamily with chitin-binding proteins being one type of these 2S albumins (Breiteneder & Radauer, 2004). Such an example of chitin-binding protein is of *Moringa oleifera* isoform 3-1 (Mo-CBP$_3$-1) which has been reported as a thermostable, antimicrobial and water purifying agent (Ullah *et al.*, 2015). The chitin-binding proteins (CBPs) have been isolated from barley, potato, tobacco, tomato and many other plants (Friedrich *et al.*, 1991; Hejgaard *et al.*, 1992; Ponstein *et al.*, 1994; Koo *et al.*, 1998; Van Damme *et al.*, 1999). These proteins have been strong growth inhibition activity against *Fusarium culmorum*, *F. graminearum*, *Trichoderma herzianum* and *Botrytis cinerea* (Bormann *et al.*, 1999; Gifoni *et al*., 2012). Other plant-based antimicrobial proteins are classified by primary structure differences such as Thionins (De Caleya *et al.*, 1972), Defensins (Polya *et al.*, 1992; Terras *et al.*, 1992), LTPs (Neumann *et al.*, 1993; Neumann *et al.*, 1995), Hevein type peptides and knottin type proteins (Cammue *et al.*, 1992) having potent antimicrobial activities against humans and plant pathogens.

Iberis umbellata L. is commonly known as garden candytuft or globe candytuft. It is an annual and herbaceous flowering plant of genus *Iberis* belonging to the family *Brassicaceae*. Although it is generally found in Europe, from Spain to Greece, especially near the coasts but it also occurs in Northern America. The present study focuses on the identification and functional characterization of a CBP from seeds of *Iberis umbellata* (Iu-CBP). Biologically

active plant-based compounds provide a promising alternative to chemical drugs and pesticides.

Materials and Methods

Plant material: *Iberis umbellata* seeds were obtained from the Botanical Garden, Bahauddin Zakariya University, Multan, Pakistan and stored at 25 °C.

Isolation and purification of Iu-CBP: 10 g of *I. umbellata* seeds were ground in liquid nitrogen and mixed in 60 ml of 100 mM phosphate buffer (pH 7.0). The crude extract was centrifuged at 11,200 g for 15 min at 4°C. The resulting supernatant was separated from the pellet and filtered through a 0.8 µm pore size filter paper. The crude extract (50 ml) was treated with a 70% ammonium sulfate constant under cold conditions (4°C). The precipitate was re-dissolved in the same volume of extraction buffer and salt was removed through dialysis (Spectra/Por 3; Catalog no. 132724; MWCO 3 kDa) using the same buffer. The dialyzed sample was loaded onto the cation exchanger Hi-Trap SP FF column; equilibrated with 100 mM phosphate buffer (pH 7.0), at a flow rate of 0.5 ml/min and eluted with NaCl gradient 0–1 M in the same buffer. Pure sample was loaded on to a Hi-Load 16/60 Superdex 200pg column at a flow rate of 0.5 ml/min. A UV detector (280 nm) was used to record eluent absorbance. The fractions were analyzed by a 12% SDS-PAGE (Laemmli, 1970). The electrophoretic mobility of the sample and protein marker (Thermo Scientific™, 26610) was compared on SDS-PAGE. The gel was stained with the 0.25% (w/v) CBB R-250 (Sambrook *et al.*, 1989) in stainer solution (methanol/acetic acid/water solution) and de-stained with the same solvent without Coomassie dye. Protein quantification was done by Bradford assay (Bradford, 1976) using BSA protein as a standard.

LC-MS/MS data: Coomassie-stained protein bands were excised into 2 mm^3 small pieces and placed in a sterile micro-centrifuge tube. The proteins were reduced by treated with 10 mM DTT, the cysteine amino acids were amended with iodoacetamide and the protein in the gel was breakdown with trypsin. The gel pieces were repetitively removed and both extracts were desiccated by vacuum concentrator and re-dissolved in formic acid (0.1%). The LC-MS/MS data were performed with a nano-LC (nano ACQUITY, Manchester, UK) combined with ESI to a quadrupole orbitrap mass spectrometer (Orbitrap QExcactive, Thermo Scientific, Germany).

LC-MS raw data were further proceeded by using Proteome Discoverer 2.0 (Thermo Scientific, Germany). For the identification of protein, MS/MS spectra data were hunted via Sequest HT against the plant SwissProt database and a homemade contaminant database (298 entries). The findings were processed through different parameters like precursor mass tolerance was fixed up to 10 ppm while fragment mass tolerance was set up to 0.5 Da. In addition, a carbamidomethylation on cysteine residues was customary fixed alterations, oxidation of methionine residue was set as a changeable modification and two missed cleavages were allowed. All identifications were validated manually.

Antibacterial activity: The antibacterial potential of purified Iu-CBP was evaluated using the disc diffusion method (Boyle *et al.*, 1973). Various bacterial pathogens such as *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Xanthomonas oryzae* were tested. Antibiotic (Calamox, Bosch) 3.0 µg/disc was used as the positive control while 100 mM phosphate buffer (pH 7.0) was used as the negative control. All the bacterial strains were added in the culture medium and incubated at 37°C for 8-10 hours. Four sterile discs of 6 mm in diameter were placed at equidistance to each other. Two concentrations of Iu-CBP (5.0 & 10.0 µg/disc) were used against bacterial strains.

Antifungal activity: *Aspergillus flavus*; FCBP-PTF-862 was purchased from FCBP, University of the Punjab, Lahore, Pakistan. This fungal specie was kept at 4°C and then propagated on YPSA medium. Three discs (15 mm) of sterilized filter paper were placed in petri dishes spaced equally apart. The purified Iu-CBP (20 µg) was loaded onto the disc. Phosphate buffer (15 µl) was used as a negative control and Fungicide TOPSIN® 4.5 FL (10 µl) used as the positive control in fungal growth inhibition. A loopful of fungus was then placed in the center and incubated at 30°C. The fungal growth was observed after 48-72 hours of incubation.

Entomotoxic activity: For the insecticidal assay, in order to get homogenized populations, *Sitophilus oryzae* and *Tribolium castaneum* were obtained from warehouses of wheat and rice in Multan and reared in the laboratory. Infestation free wheat and rice grains were sterilized at 60ºC for 30 minutes and 5% brewer's yeast was added during cooling (Sial *et al.*, 2017). Culture jars were kept at 30 ± 5ºC and 60 ± 5% R.H (Jaleel *et al.*, 2015). Adults of *S. oryzae* sexed were separated by the size of snout and rostrum. The newly pupated *T. castaneum* larvae were retrieved and sexes were separated based on their abdominal morphological characteristics.

Three concentrations of purified Iu-CBP (1.0, 2.0 and 3.0 mg/ml) were prepared in 100 ml of 100 mM phosphate buffer (pH 7.0). All three concentrations were used for *S. oryzae* insecticidal activity assay (Gressent *et al.*, 2007). Three sets of 100 grams of rice were sprayed with the individual concentration of Iu-CBP and left undisturbed until the kernels were dried. Dried rice grains were divided into 5 replicates; 20 individuals of *S. oryzae* were released in each replication unit (Vassilakos & Athanassiou, 2012). Mortality was observed each 3rd day and dead individuals were removed after 9 days of the experiment. Afterward, the remaining adult individuals (Parent generation) allowed for the progeny production to check the efficacy of protein sample on the lifespan of rice weevil after F1 generation (35 days) and F2 generation (65 days) as well (Athanassiou *et al.*, 2010).

The same concentrations (1.0, 2.0, and 3.0 mg/ml) were mixed with 150 g of flour to form a compact dough (Gressent *et al.*, 2011). The dough was air-dried and ground into a fine powder which was then split into five 30 g sub-sets. Each replicate was assigned a pair of *T. castaneum.* After ten days, adults were put out from experiment and data was recorded weekly for life cycle

attributes i.e., larvae, pupae and F1 adults. The data were compared with the control to observe the effectiveness of purified Iu-CBP against *T. castaneum*. Insecticidal test data were evaluated through "Statistix 8.1" (Analytical Software, 2005) (Shahzad *et al.*, 2016). Time-mortality data were recorded on the base of dead *S. oryzae* and *T. castaneum* during the above-specified intervals. Insect progeny was observed by counting alive individuals within specified periods. The highest numbers of insects were observed in the control groups. All the data were subjected to one way ANOVA and means were separated by Tukey's HSD test with a level of significance of 0.05.

Results

Purification of Iu-CBP: One gram seeds of *I. umbellata* produced 200 mg of crude extract protein in phosphate buffer (100 mM; pH 7.0). Iu-CBP was pellet down by using 70% Ammonium sulfate precipitation and total water-soluble protein content was reduced to 140 mg. Cation exchange chromatography produced highly purified Iu-CBP fractions which were pooled together after SDS-PAGE analysis and yielded 15 mg protein which was further dialyzed and subjected to gel filtration column. The peak fractions (Fig. 1A) were loaded on 12% SDS-PAGE and lyophilized (~ 10 mg). Purification times and percentage recovery have been summarized in Table 1. Iu-CBP showed a highly pure ~11 kDa band in the absence of β-mercaptoethanol (Fig. 1B).

Table 1. Different purification steps are depicting the yield of Iu-CBP from one gram of seed powder.

Purification steps	Total protein (mg)	Purification (times)	Recovery (%)
Crude	200	1	100
Ammonium sulfate fractionation (70%)	140	1.42	70
Hi-Trap SP FF column	15	13.33	7.5
Hi-Load 16/60 Superdex	10	20	5

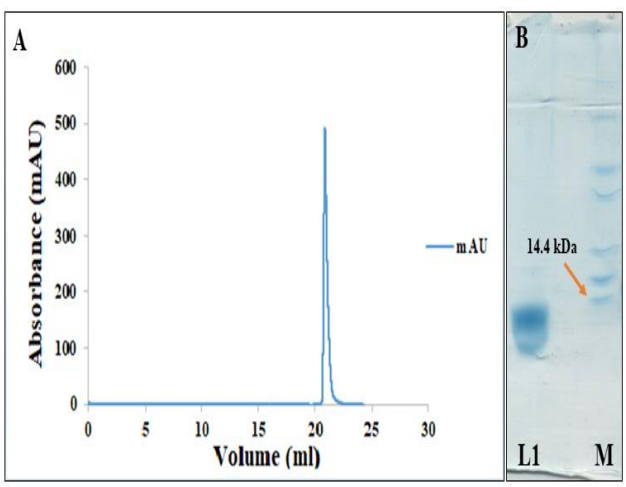

Fig. 1. SDS-PAGE analysis of the purified Iu-CBP. A is showing the gel filtration chromatogram while B is showing the corresponding purity of the pooled Iu-CBP fractions. Lane M is the standard protein ladder (Thermo Scientific™, 26610) while lane L1 is showing the Iu-CBP with a molecular weight of approximately 11 kDa.

Protein identification: Purified Iu-CBP, subjected to LC-MS/MS spectrometry, yielded a single fragment of 23 amino acids (QAVQSAQQQQGQVGPQQVGHMYR). The peptide sequence was a blast in UniProtKB database (Jain *et al.*, 2009) and showed 100% sequence homology/identity with the chitin-binding protein of *Moringa oleifera* (Mo-CBP$_3$-1) (UniProt ID: W5S2D2) (Fig. 2).

Catalytic Arginines of Mo-CBP$_3$-1: The crystallographic molecular structure of Mo-CBP$_3$-1 is showing the positions of Arg-104 (H3), Arg-111 & 116 (H4). These arginines are responsible for the antimicrobial activity of these chitin-binding proteins by producing porosity in the cell membranes of microbial pathogens. Additionally, other structural details are highlighted in cartoon ribbon model (Fig. 3).

Antimicrobial assay: After being tested for antibacterial activity against five pathogenic bacteria using the disc diffusion method, two concentrations of purified Iu-CBP (5.0 and 10.0 µg/disc) produced significant zones of inhibition against bacterial pathogens such as *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Xanthomonas oryzae*. No activity was observed on discs soaked with buffer (Fig. 4). Similarly, notable antifungal activity of Iu-CBP was observed against phytopathogenic *Aspergillus flavus* (Fig. 5). Iu-CBP (20 µg/disc) inhibited the mycelia growth of this fungal strain.

Insecticidal activity

Time mortality of *S. oryzae* exposed to an insecticidal protein: The insecticidal effects of Iu-CBP on *S. oryzae* were significant after the 3rd, 6th, and 9th days of treatment. The concentration of 3 mg/ml showed the most insecticidal activity during 3 to 9 days. However, for concentrations of 1 mg/ml and 2 mg/ml at day 3, insect responses were not differentiable from each other. 6th day readings showed a significant variance of mortality for all concentrations. 9th day data revealed the response of 2 mg/ml treatment was similar to the responses of 1 and 3 mg/ml treated insects. For all treatments, the insects displayed a significantly different response from that of control, where the mortality of individuals was minimal. Overall, time after treatment and mortality of individual insects are positively correlated (Fig. 6).

Iu-CBP efficacy against *S. oryzae* progeny: A decreasing trend of mean adult progeny production was observed at all three protein concentrations, while the control group (CT) showed comparatively high survival rates (Fig. 6). At days 35 (F1), the concentration of 3.0 mg/ml was the most effective in reducing *S. oryzae* mean progeny with the survival rate of 11.4 ± 1.3 in comparison to a control value of 46.6 ± 5.2. Similarly, the same trend was observed for F2 generation (65 days) exhibiting a survival rate of only 18.2 ± 1.2 in comparison to a control value of 81.2 ± 5.3; which strongly indicates the efficacy of Iu-CBP as an insecticide for *S. oryzae* (Fig. 7). However, F2 showed more resistance to all doses of Iu-CBP in comparison to F1 progeny.

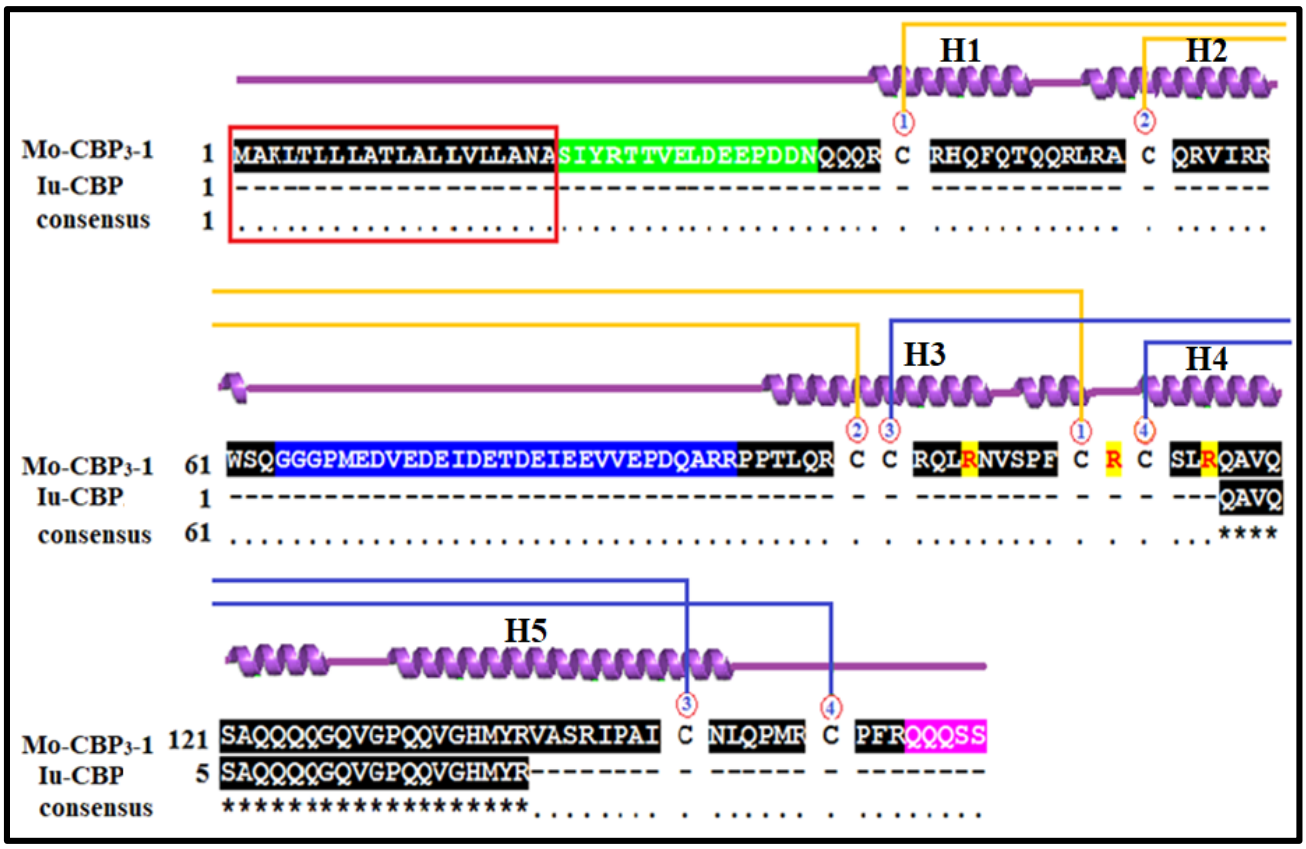

Fig. 2. Multiple sequence alignment of Iu-CBP with *Moringa oleifera*-Chitin Binding Protein (Mo-CBP$_3$-1). Identical amino-acid residues among two proteins are indicated by asterisks.

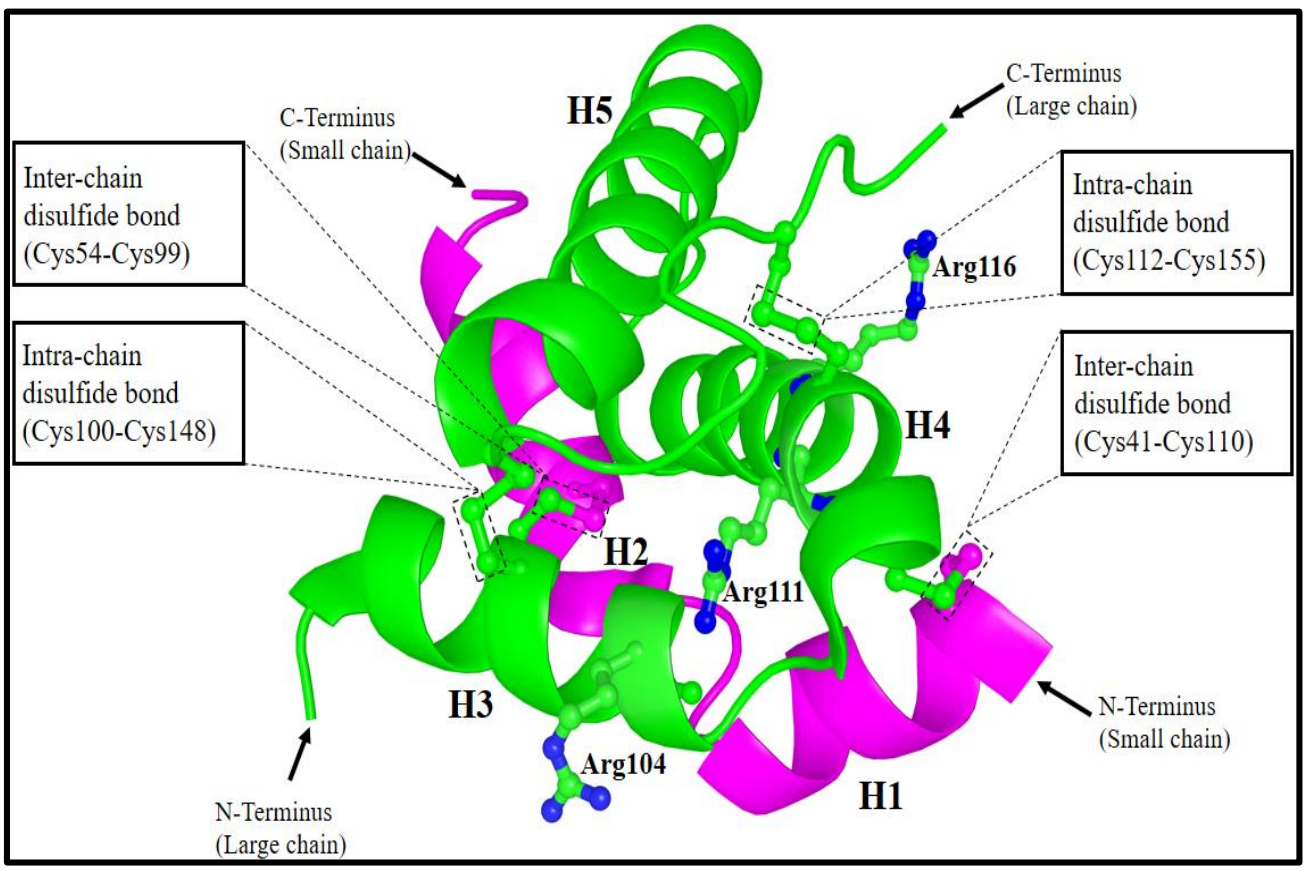

Fig. 3. Ribbon model of *Moringa oleifera* chitin-binding protein Mo-CBP$_3$-1 (PDB ID: 5DOM) depicting the positions of catalytic arginine residues.

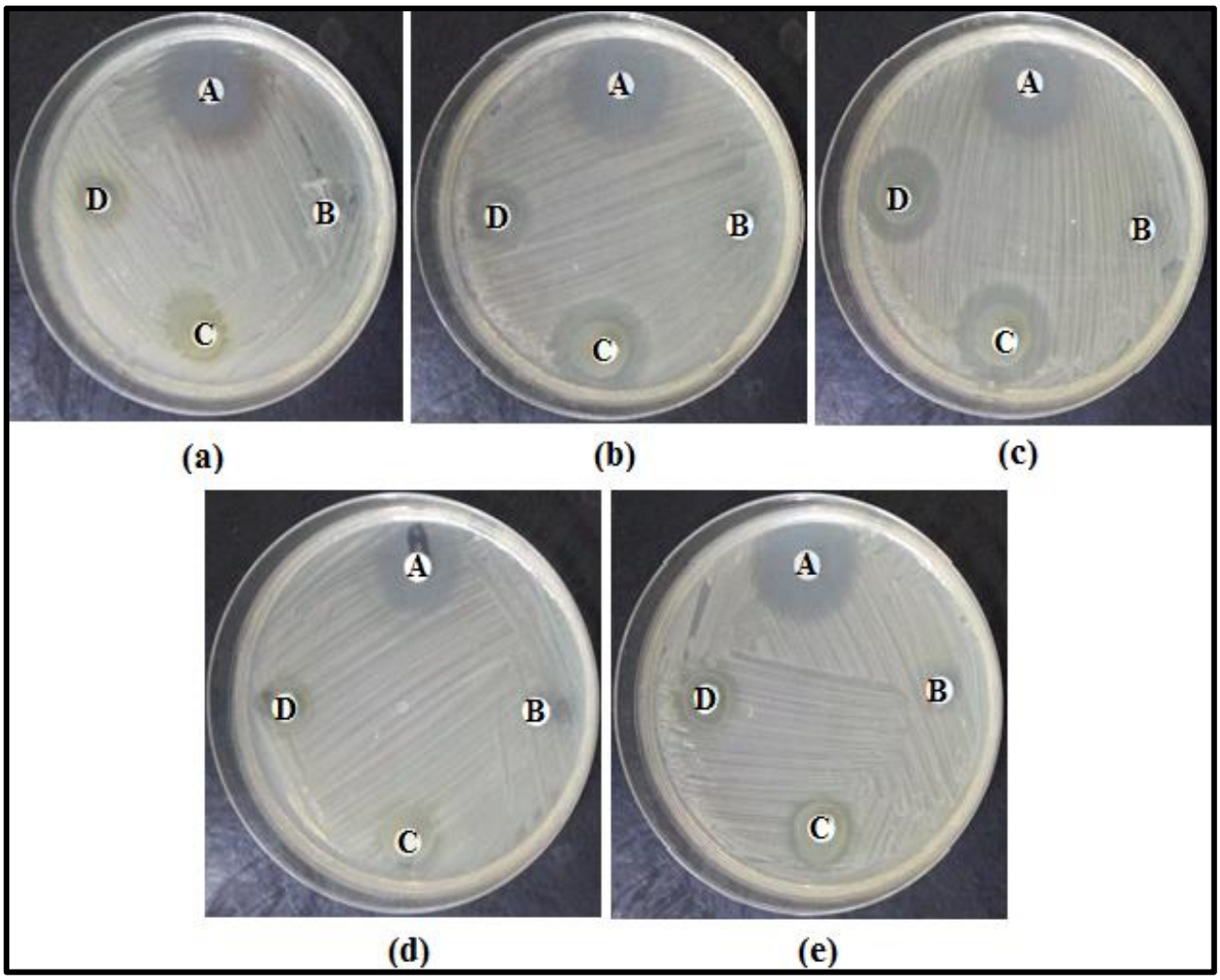

Fig. 4. Antibacterial activity of Iu-CBP against different pathogenic bacteria. (a) *Bacillus subtilis*, (b) *Escherichia coli*, (c) *Pseudomonas aeruginosa*, (d) *Staphylococcus aureus*, and (e) *Xanthomonas oryzae*.

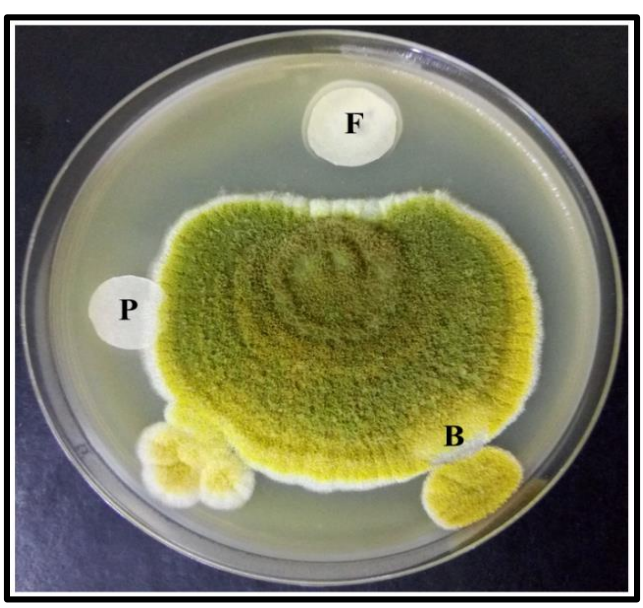

Fig. 5. Antifungal activity of Iu-CBP against *Aspergillus flavus*. Disc F was used as a positive control containing fungicide while disc B was used as a negative control. Disc P is indicating the Iu-CBP concentration of 20 µg/disc which successfully inhibited the mycelia growth.

Iu-CBP impact on life stages of *T. castaneum*: Iu-CBP, at a concentration of 3.0 mg/ml, significantly reduced the mean number of *T. castaneum* larvae with a population number of 7.8 ± 1.4 in comparison to the control population of 51.8 ± 2.4. Similarly, the pupal population was observed to be 5.4 ± 0.7 at the 3.0 mg level dose, 8.8 ± 1.3 at the 2.0 mg concentration and 9.4 ± 1.6 at 1.0 mg/ml. The highest mean number of pupae was observed in the control group (35.6 ± 1.6) (Fig. 8). The equal ratio of both sexes pupae was examined at the 3.0 mg dose and this dosage also resulted in the fewest pupae. The significant declivity was detected in the mean population number of adults at the maximum dose of 3.0 mg (2.8 ± 0.7), followed by 2.0 mg (5.4 ± 0.7). The greatest numbers of *T. castaneum* were observed throughout all stages in the control group (Fig. 8). A minimal variance was obtained in the mean number of males and females at the 3.0 mg/ml dose, 2.4 ± 0.4 (male), and 2.6 ± 0.7 (female).

Discussion

The emergence of antibiotic-resistant super microorganisms and large annual losses of stored commodities due to insects are major concerns of the World Health Organization (Costa, 2015). Plant-based natural compounds are the targeted candidates of

antimicrobial and insecticidal agents (Hayashi *et al.*, 2013). This study identified 11 kDa 2S albumin (Iu-CBP) from seeds of *I. umbellata*. LC-MS/MS residual data show a 100% sequence homology with 2S albumin of *Moringa oleifera* (Mo-CBP_3-1). Multiple sequence alignment between the two proteins indicates that prepropeptide Mo-CBP3-1 (ppMo-CBP3-1) possesses 163 residues and goes through extensive post-translational modifications to form the active mature protein (Fig. 2). During the maturation process, four fragments are cleaved from the primary sequence. These fragments include a signal peptide (20 residues), an N-terminal pro-sequence (16 residues), a linker peptide joining the two chains (29 residues) and a 5 residual C-terminus section of the heavy chain. The light chain contains 2 cysteine residues (Cys41 and Cys54) while the heavy chain contains 6 (Cys99, Cys100, Cys110, Cys112, Cys148, and Cys155) and the two chains are joined via two inter-chain disulfide linkages (Cys41-Cys110 and Cys54-Cys99). There are two intra-chain linkages (between Cys100-Cys148 and Cys112-Cys155) only present in the heavy chain (Figs. 2 & 3). The light chain contains 26 residues of pp Mo-CBP_3-1 (37 to 63 amino acids); the heavy chain consists of 66 amino acids of pp Mo-CBP_3-1 (amino acids 93 to 158) (Ullah *et al.*, 2015). In the absence of β-mercaptoethanol, the protein's apparent molecular mass is ~11 kDa, but under reducing conditions, this protein gives two bands with molecular weight of 3.5 and 7.5 kDa for light and heavy chains respectively. There are five helices which are connected by short loops.

It is important to mention that purified Iu-CBP is a mixture of different isoforms that were used together for further functional characterization of this potent protein against different pathogens. Along bacterial pathogens, plant pathogenic fungi has already been reported to produce tremendous losses to agriculturally important crops by root deterioration which leads to great loss of Pakistan economy (Dawar *et al*., 2020). The purified Iu-CBP is a potential candidate for being an antimicrobial and insecticidal agent. It is exhibited substantial inhibition of both bacterial and fungal pathogens. Iu-CBP produced strong inhibition zones against all tested bacterial species at concentrations of 5.0 and 10.0 µg/disc. Similarly, the 20 µg/disc concentration of Iu-CBP showed the potential for inhibition of fungal mycelial growth. Within the last decade, 2S albumins have been reported to exhibit strong antimicrobial activity. A novel 2S albumin, SiAMP, isolated from *Sesamum indicum* kernels, was tested against several bacterial and fungal species (Maria-Neto *et al.*, 2011). It was found that these proteins damage the bacterial cell by exposing its cations to the negatively charged host cell membrane. The ionic interactions disturb the permeability of the plasma membrane and cause the death of the bacterial cell (Maria-Neto *et al.*, 2011). The arginine residues (Arg104, Arg111, and Arg116; Figs. 2 & 3) of Mo-CBP3-1 are considered crucial for its antibacterial properties (Katre *et al.*, 2008). Additionally, Iu-CBP significantly repressed the mycelial growth of *Aspergillus flavus* when compared with the control (Fig. 4). The chitin-binding proteins are rich with positively charged residues (like arginine), could interfere with the negatively charged components of cell membrane which cause the disarrangement of the membrane and leads toward cell lysis (Choi & Lee, 2014; Ullah *et al*., 2015). Chitin-binding isoforms (Mo-CBP_2, Mo-CBP_3, and Mo-CBP_4) inhibited the mycelial growth of *Candida tropicalis, C. krusei*, *C. albicans* and *C. parapsilosis* by disrupting the integrity of their plasma membranes (Wang *et al.*, 2015).This disruption was accompanied by pore formation, the depolarization of the lipid domain organization and interference of the electrochemical balance leading to cell death (Lee & Lee, 2015).

Entomo-poisonous plant particles have the potential to be recognized as bio-pesticide sprays (Bednarek & Osbourn, 2009). Furthermore, insecticidal efficacy of Iu-CBP was measured against the most harmful pests of stored grains, *S. oryzae* and *T. castaneum*. Iu-CBP showed promising results in the decline the mean values of *S. oryzae* and *T. castaneum*. The numbers of larvae and pupae population of *T. castaneum* were highly reduced when exposed on Iu-CBP mixed flour and, thus, did not complete metamorphosis to adults. These results strongly agree with the previously reported studies on other stored grain insect pests (Gressent *et al.*, 2003; Gressent *et al.*, 2007; Rahioui *et al.*, 2007; Muench *et al.*, 2014). *Pisum sativum* albumin PA1b was found to be a potent bio-pesticide against cereal weevils (Higgins *et al.*, 1986; Gressent *et al.*, 2011). PA1b demonstrated its entomotoxic activity by binding to the "e" and "c" subunits of insect vacuolar ATPase (Chouabe *et al.*, 2011; Muench *et al.*, 2014). Iu-CBP delayed the insect's life stages in a similar fashion to what was found by Rahioui *et al*., (2014) (Rahioui *et al.*, 2014), which observed 100% of larval mortality and delayed in development of *D. melanogaster*.

Conclusions

The investigation and characterization of such kind of molecules may help towards the control of human diseases and plant pathogens. Iu-CBP is a low molecular weight and thermostable protein with strong activity against microbial pathogens and stored grain insect pests. The potential of these findings could be used in the development of Iu-CBP based nano-formulations against agricultural insect pests and will be opening the means in insect pest management strategies, effectively.

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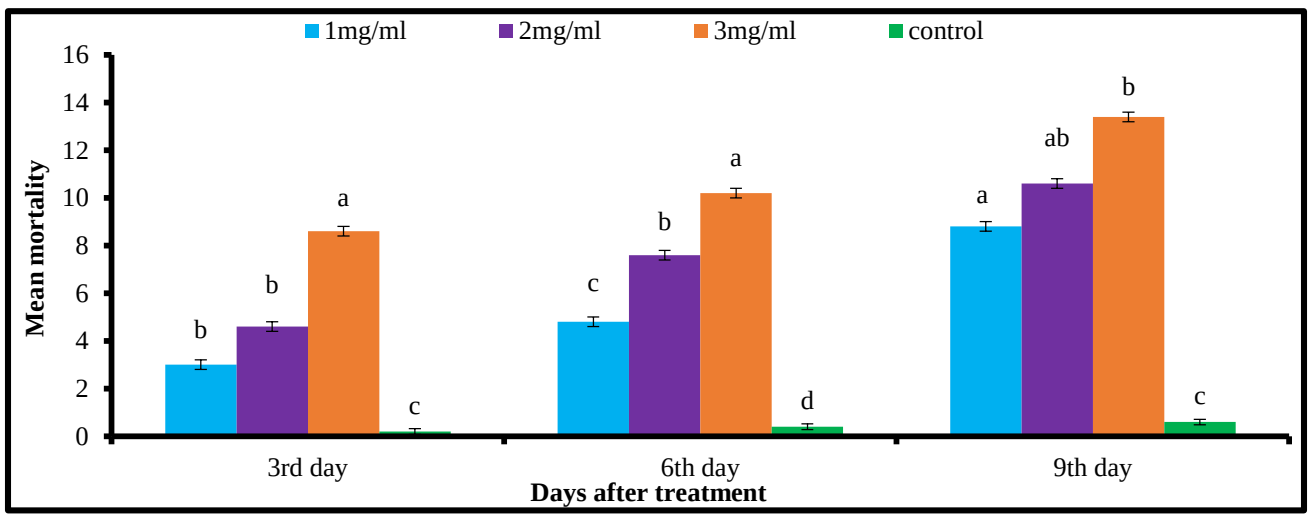

Fig. 6. Effect of Iu-CBP on time mortality of *S. oryzae*. Comparative mean mortality (±SE) of *S. oryzae* and the control set is presented with significant mortality of the insect in response to treatments.

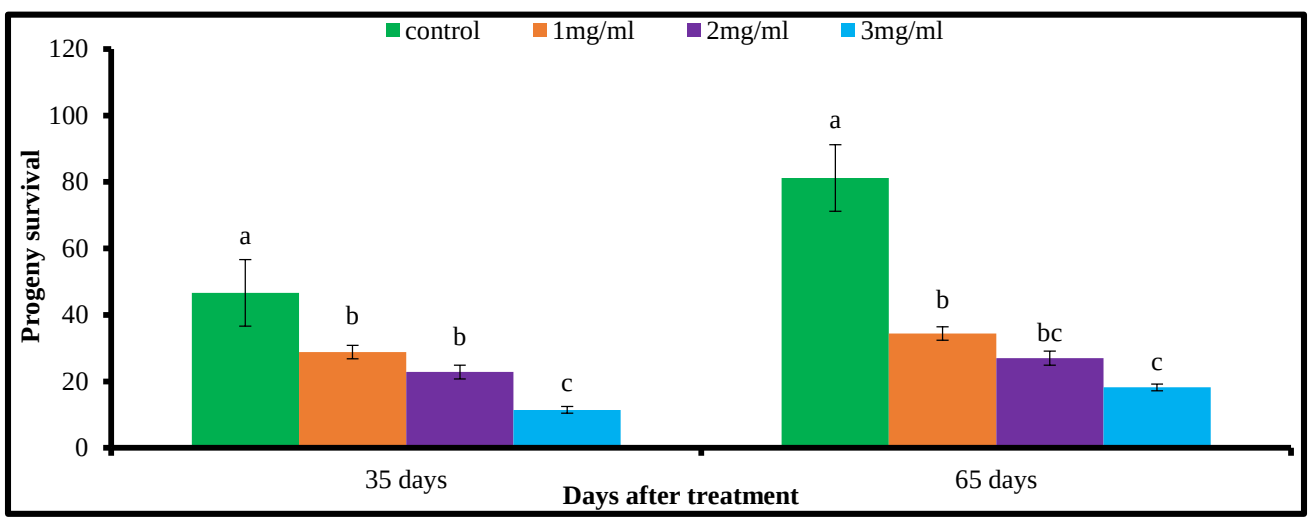

Fig. 7. Effect of Iu-CBP on two progenies of *S. oryzae* after 35 (F1) and 65 (F2) days. Comparative analysis with the control group indicates the efficacy of protein doses. Significantly, quite reduced progeny (F1) was detected at the highest dose (3.0 mg/ml) with maximum survival in control. Interestingly, F2 progeny exhibited significant mortality in comparison to control. However, a decline was observed in F2 mortality on all Iu-CBP treatments in comparison to the F1 generation.

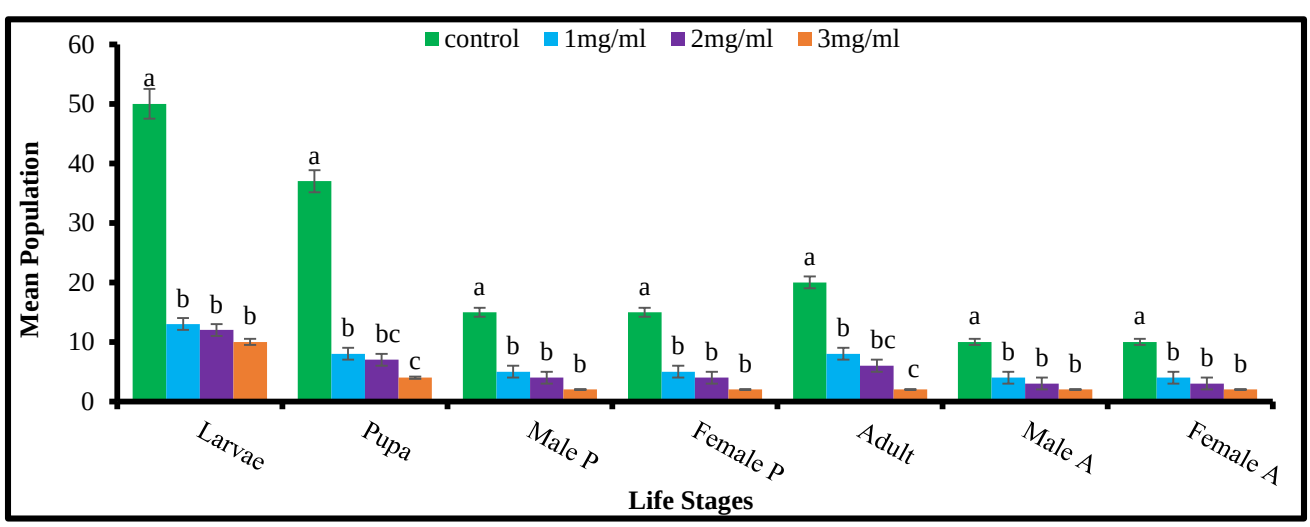

Fig. 8. Effect of Iu-CBP on the life stages of *T. castaneum*. Minimal mean population ± SE was detected significantly ($p<0.001$) reduced at 3.0 mg/ml dose with larvae, pupae, and adults when compared to the control set (CT).

References

Ahmed, M., M.A. Qadir, A. Hameed, M. Imran and M. Muddassar. 2018. Screening of curcumin-derived isoxazole, pyrazoles, and pyrimidines for their anti-inflammatory, antinociceptive, and cyclooxygenase-2 inhibition. *Chem. Biol.*, 91: 338-343.

Athanassiou, C.G., F.H. Arthur and J.E. Throne. 2010. Effects of short exposures to spinosad-treated wheat or maize on four stored-grain insects. *J. Econ. Entomol*., 103: 197-202.

Barrientos, L.G. and A.M. Gronenborn. 2005. The highly specific carbohydrate-binding protein cyanovirin-N: structure, anti-HIV/Ebola activity and possibilities for therapy. *Mini-Rev. Med. Chem.*, 5: 21-31.

Bednarek, P. and A. Osbourn. 2009. Plant-microbe interactions: chemical diversity in plant defense. *Sci.*, 324: 746-748.

Bonev, B. and N. Brown, 2019. *Bacterial resistance to antibiotics: from molecules to man* (Ed.) John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, USA.

Bormann, C., D. Baier, I. Hörr, C. Raps, J. Berger, G. Jung and H. Schwarz. 1999. Characterization of a novel, antifungal, chitin-binding protein from *Streptomyces tendae* Tu901 that interferes with growth polarity. *J Bacteriol.,* 181: 7421-7429.

Boyle, V.J., M.E. Fancher and R.W. Ross. 1973. Rapid, modified Kirby-Bauer susceptibility test with single, high-concentration antimicrobial disks. *Antimicrob. Agents Chemother.*, 3: 418-424.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* 72: 248-254.

Breiteneder, H. and C. Radauer. 2004. A classification of plant food allergens. *J. Allerg. Clin. Immunol*., 113: 821-830.

Cammue, B., M. De Bolle, F. Terras, P. Proost, J. Van Damme, S.B. Rees, J. Vanderleyden and W.F. Broekaert. 1992. Isolation and characterization of a novel class of plant antimicrobial peptides form *Mirabilis jalapa* L. seeds. *J. Biol. Chem*., 267: 2228-2233.

Choi, H. and D.G. Lee. 2014. Antifungal activity and pore-forming mechanism of astacidin 1 against *Candida albicans*. *Biochimie*., 105: 58-63.

Chouabe, C., V. Eyraud, P. Da Silva, I. Rahioui, C. Royer, C. Soulage, R. Bonvallet, M. Huss and F. Gressent. 2011. New mode of action for a knottin bioinsecticide: Pea Albumin 1 subunit b (PA1b) is the first peptidic inhibitor of V-ATPase. *J. Biol. Chem*., jbc. M111. 281055.

Costa, S.J. 2015. Taking it to scale: Post-Harvest Loss Eradication in Uganda 2014–2015. UN World Food Programme, Kampala, Uganda.

Dawar, S., A. Hanif and R. Siddique. 2020. Management of root rot fungi by *Grewia asiatica* L. Leaves and on the growth of crop plants. *Pak. J. Bot.*, 52: 469-476.

De Caleya, R.F., B. Gonzalez-Pascual, F. García-Olmedo and P. Carbonero. 1972. Susceptibility of phytopathogenic bacteria to wheat purothionins *In vitro*. *Appl. Environ. Microbiol.*, 23: 998-1000.

Friedrich, L., M. Moyer, E. Ward and J. Ryals. 1991. Pathogenesis-related protein 4 is structurally homologous to the carboxy-terminal domains of hevein, Win-1 and Win-2. *Mol. & General Gen.,* 230: 113-119.

Gifoni, J.M., J.T. Oliveira, H.D. Oliveira, A.B. Batista, M.L. Pereira, A.S. Gomes, H.P. Oliveira, T.B. Grangeiro and I.M. Vasconcelos. 2012. A novel chitin-binding protein from *Moringa oleifera* seed with potential for plant disease control. *J. Pept. Sci*., 98: 406-415.

Gressent, F., P. Da Silva, V. Eyraud, L. Karaki and C. Royer. 2011. Pea Albumin 1 subunit b (PA1b), a promising bioinsecticide of plant origin. *Toxins*, 3: 1502-1517.

Gressent, F., G. Duport, I. Rahioui, Y. Pauchet, P. Bolland, O. Specty and Y. Rahbe. 2007. Biological activity and binding site characteristics of the PA1b entomotoxin on insects from different orders. *J. Ins. Sci*., 7: 12.

Gressent, F., I. Rahioui and Y. Rahbé. 2003. Characterization of a high-affinity binding site for the pea albumin 1b entomotoxin in the weevil *Sitophilus*. *Eur. J. Biochem*., 270: 2429-2435.

Hayashi, M.A.F., F.C. Bizerra and P.I. Da Silva Junior. 2013. Antimicrobial compounds from natural sources. *Front. Research Foundation,* 1: 33-43

Hejgaard, J., S. Jacobsen, S.E. Bjørn and K.M. Kragh. 1992. Antifungal activity of chitin-binding PR-4 type proteins from barley grain and stressed leaf. *Febs Letters*, 307: 389-392.

Higgins, T., P. Chandler, P. Randall, D. Spencer, L. Beach, R. Blagrove, A. Kortt and A. Inglis. 1986. Gene structure, protein structure, and regulation of the synthesis of a sulfur-rich protein in pea seeds. *J. Biol. Chem.*, 261: 11124-11130.

Jain, E., A. Bairoch, S. Duvaud, I. Phan, N. Redaschi, B.E. Suzek, M.J. Martin, P. McGarvey and E. Gasteiger. 2009. Infrastructure for the life sciences: design and implementation of the UniProt website. *BMC Bioinform.*, 10: 136.

Jaleel, W., Q. Saeed, S. Saeed, T. Ansari, M.N. Naqqash, N. Iqbal and U. Sial. 2015. Efficacy and time mortality of *Tribolium castaneum* (Herbst)(*Coleoptera*: *Tenebrionidae*) by some essential oils through contact and fumigant methods. *Appl. Sci. & Business Econo.*, 2: 1-7.

Katre, U.V., C. Suresh, M.I. Khan and S.M. Gaikwad. 2008. Structure–activity relationship of a hemagglutinin from *Moringa oleifera* seeds. *Int. J. Biol. Macromol.*, 42: 203-207.

Koo, J.C., S.Y. Lee, H.J. Chun, Y.H. Cheong, J.S. Choi, S.-i. Kawabata, M. Miyagi, S. Tsunasawa, K.S. Ha and D.W. Bae. 1998. Two hevein homologs isolated from the seed of *Pharbitis nil* L. exhibit potent antifungal activity. *BBA-Proteins Prot.,* 1382: 80-90.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680.

Lee, W. and D.G. Lee. 2015. Fungicidal mechanisms of the antimicrobial peptide Bac8c. *BBA-Biomembranes*, 1848: 673-679.

Maria-Neto, S., R.V. Honorato, F.T. Costa, R.G. Almeida, D.S. Amaro, J.T. Oliveira, I.M. Vasconcelos and O.L. Franco. 2011. Bactericidal activity identified in 2S albumin from sesame seeds and in silico studies of structure–function relations. *The Prot. J.*, 30: 340-350.

Menéndez-Arias, L., R.I. Monsalve, J. Gavilanes and R. Rodríguez. 1987. Molecular and spectroscopic characterisation of a low molecular weight seed storage protein from yellow mustard (*Sinapis alba* L.). *Int. J. Biochem.*, 19: 899-907.

Muench, S.P., S. Rawson, V. Eyraud, A.F. Delmas, P. Da Silva, C. Phillips, J. Trinick, M.A. Harrison, F. Gressent and M. Huss. 2014. PA1b inhibitor binding to subunits c and e of the vacuolar ATPase reveals its insecticidal mechanism. *J. Biol. Chem.*, 289: 16399-16408.

Nelson, K.M., J.L. Dahlin, J. Bisson, J. Graham, G.F. Pauli and M.A. Walters. 2017. The essential medicinal chemistry of curcumin: miniperspective. *J. Med. Chem.*, 60: 1620-1637.

Neumann, G.M., R. Condron, B. Svensson and G.M. Polya. 1993. Phosphorylation of barley and wheat phospholipid transfer proteins by wheat calcium-dependent protein kinase. *Plant Sci.*, 92: 159-167.

Neumann, G.M., R. Condron, I. Thomas and G.M. Polya. 1995. Purification, characterization and sequencing of a family of *Petunia* petal lipid transfer proteins phosphorylated by plant calcium-dependent protein kinase. *Plant Sci.*, 107: 129-145.

Polya, G.M., S. Chandra, R. Chung, G.M. Neumann and P.B. Höj. 1992. Purification and characterization of wheat and pine small basic protein substrates for plant calcium-dependent protein kinase. *BBA-Prot. Struc. & Mol. Enzymol.*, 1120: 273-280.

Ponstein, A.S., S.A. Bres-Vloemans, M.B. Sela-Buurlage, P.J. van den Elzen, L.S. Melchers and B.J. Cornelissen. 1994. A novel pathogen-and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. *Plant Physiol.*, 104: 109-118.

Rahioui, I., V. Eyraud, L. Karaki, F. Sasse, M. Carre-Pierrat, A. Qin, M.H. Zheng, S. Toepfer, C. Sivignon and C. Royer. 2014. Host range of the potential biopesticide Pea Albumin 1b (PA1b) is limited to insects. *Toxicon*, 89: 67-76.

Rahioui, I., C. Laugier, S. Balmand, P. Da Silva, Y. Rahbe and F. Gressent. 2007. Toxicity, binding and internalization of the pea-A1b entomotoxin in Sf9 cells. *Biochimie*, 89: 1539-1543.

Rubinstein, N., J.M. Ilarregui, M.A. Toscano and G.A. Rabinovich. 2004. The role of galectins in the initiation, amplification and resolution of the inflammatory response. *Tissue Antigens*, 64: 1-12.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. *Molecular cloning: a laboratory manual* Cold spring harbor laboratory press.

Shahzad, M.Q., Z.U. Abdin, S.K. Abbas, M. Tahir and F. Hussain. 2016. Parasitic effects of solitary endoparasitoid, *Aenasius bambawalei* Hayat (*Hymenoptera*: *Encyrtidae*) on cotton mealybug, *Phenacoccus solenopsis* Tinsley (*Hemiptera*: *Pseudococcidae*). *Adv. Ent.*, 4: 90.

Shewry, P.R., J.A. Napier and A.S. Tatham. 1995. Seed storage proteins: structures and biosynthesis. *The Plant Cell*, 7: 945.

Sial, M., Q. Saeed, S. Rahman and M. Qayyum. 2017. Upshot of food add-ons on the life history and development of *Tribolium castaneum* (Herbst)(*Coleoptera*: *Tenebrionidae*). *Afr. Ent.*, 25: 37-41.

Terras, F., H. Schoofs, M. De Bolle, F. Van Leuven, S.B. Rees, J. Vanderleyden, B. Cammue and W.F. Broekaert. 1992. Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J. Biol. Chem.*, 267: 15301-15309.

Ullah, A., R.B. Mariutti, R. Masood, I.P. Caruso, G.H.G. Costa, C.M. de Freita, C.R. Santos, L.M. Zanphorlin, M.J.R. Mutton and M.T. Murakami. 2015. Crystal structure of mature 2S albumin from *Moringa oleifera* seeds. *Biochem. Biophys. Res. Comm.*, 468: 365-371.

Van Damme, E.J., D. Charels, S. Roy, K. Tierens, A. Barre, J.C. Martins, P. Rougé, F. Van Leuven, M. Does and W.J. Peumans. 1999. A gene encoding a hevein-like protein from elderberry fruits is homologous to PR-4 and class V chitinase genes. *Plant Physiol.*, 119: 1547-1556.

Vassilakos, T.N. and C.G. Athanassiou. 2012. Effect of short exposures to spinetoram against three stored-product beetle species. *J. Econ. Entomol.,* 105: 1088-1094.

Wang, K., W. Dang, J. Xie, R. Zhu, M. Sun, F. Jia, Y. Zhao, X. An, S. Qiu and X. Li. 2015. Antimicrobial peptide protonectin disturbs the membrane integrity and induces ROS production in yeast cells. *BBA-Biomembranes*, 1848: 2365-2373.

Ye, X., T. Ng, P.W. Tsang and J. Wang. 2001. Isolation of a homodimeric lectin with antifungal and antiviral activities from red kidney bean (*Phaseolus vulgaris*) seeds. *J. Plant Chem.,* 20: 367-375.

Youle, R.J. and A.H. Huang. 1981. Occurrence of low molecular weight and high cysteine containing albumin storage proteins in oilseeds of diverse species. *Amer. J. Bot.,* 68: 44-48.

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