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APPRAISAL AND PERCEPTIVITY OF PROTEINS PURIFIED FROM *Zygogramma bicolorata* (COLEOPTERA: CHRYSOMELIDAE) AGAINST PATHOGENS ENGENDERING MYCOTIC INFECTION

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ABSTRACT

The multidrug resistivity (MDR) of opportunistic microorganisms is still a major concern which requires the development of a new efficient drug with fewer side effects that can be added to the antifungal armamentarium. This study is the first to report the antifungal nature of proteins purified from the hemolymph of *Zygogramma bicolorata,* immunized with *Micrococcus luteus* (MTCC 106) and *Escherichia coli* (MTCC 443). Immunized hemolymph was assayed for antimicrobial sensitivity against pathogens, *Candida albicans* (ITCC 4718) and *Aspergillus niger* (ITCC 5479) by disc diffusion method. The crude extract of hemolymph was separated and purified into fractions by low-pressure size exclusion chromatography using a Sephadex G-25 column. Protein estimation for crude fraction was performed by Lowry's method followed by the assessment of antifungal activity. The study revealed that four fractions (fraction 6, 9, 11 and 15) out of 30 fractions exhibited activity against the test organisms and accommodated high and low molecular weight proteins/peptides of 120, 100, 90 and 66 kDa and two relatively low molecular weight polypeptides (36 and 18 kDa) respectively. Being MDR in dire matter, the efficacy of the drug is momentous.

Key words: Antimicrobial peptides, *Aspergillus niger*, *Candida albicans*, hemolymph, SDS-PAGE

INTRODUCTION

Hemolymph is a body fluid cognate to the blood in vertebrates in an open circulatory system. Insects are regularly and frequently exposed to pathogenic and opportunistic microorganisms. Insects lack adaptive immune system but an innate immune system composed of cellular and systemic response produces several antimicrobial peptides which disrupt pathogens thus act as host defense peptides (Xia *et al*., 2017). Antimicrobial peptides are produced in hemolymph as a result of response against infection or sepsis in insect body as it contains a high level of free amino acids. The fungal infection kills around 1.3 million people globally (Bongomin *et al*., 2017). More than 5000 peptides comprising of linear or cyclic, hydrophobic or amphipathic structures have been identified with antifungal properties (Zhao *et al*., 2013). Despite such success in antimicrobial development, the inexorable ongoing emergence of resistance worldwide continues to spur the

search for novel anti-infective to replace and/or supplement conventional antibiotics. Their cytotoxicity may involve binding to and disruption of membrane, membrane penetration and interaction with mitochondria or pore formation (Shai, 1995; Helmerhorst *et al*., 1999).

The beauty of anti-microbial peptides (AMPs) lies in their linear α-helical structure made up of hydrophobic and cationic amino acids clusters which are amphipathic in nature. Identification and characterization of their structure, function or sequence will contribute to better understanding of immune mechanism of insects against pathogens and enactment of these substances in biopharma's will lead to disease eradication (Li *et al*., 2006). Various researches have focused on the induction, separation and purification of hemolymph of insects. Cytrynska *et al*. (2007) characterized eight peptides from the hemolymph of immune-challenged greater wax moth, *Galleria mellonella* (Gm) larvae and reported that Gm defensin-like peptide significantly inhibited fungal and bacterial growth at a concentration of 2.9 and 1.9 µM, respectively. The antifungal activities of peptides purified from hemolymph of *Musca domestica* (housefly) have been reported and peptides identified at molecular level (Fu *et al*., 2009). However, perusal of literature reveals that no work has so far been done on the isolation, purification and identification of antimicrobial peptides from *Zygogramma bicolorata* Pallister. *Z. bicolorata*, referred to as 'Mexican beetles' due to its native origin of Mexico, belongs to class Insecta and family Chrysomelidae. These were introduced as biological control agent against noxious weed *Parthenium hysterophorus* so are also referred to as 'Parthenium beetle'. The life cycle of a beetle is about 6-8 weeks. This exotic beetle feeds only on parthenium fresh leaves and soft tissues of flower (Gupta *et al*., 2016).

The test organisms, *Aspergillus niger* (ITCC 5479) and *Candida albicans* (ITCC 4718), used in the present study were both opportunistic fungi responsible for various diseases, specifically causing otomycosis and candidiasis, respectively. *A. niger* induces phagocytosis of leukocytes, pulmonary aspergillosis, otomycosis and various kinds of infection in immune-compromised patients (Araiza *et al*., 2006). Out of more than 20 species of *Candida*, *C. albicans* stands fourth in causing bloodstream infections in India. It can cause superficial mycoses, invasive mucosal infections and disseminated systemic disease, particularly in AIDS patients, transplant recipients (Chander *et al*., 2013). The alarming emergence of drug-resistance of these fungal diseases demands the urgency of development of new, effective and safe anti-fungicides to combat the diseases. Keeping all these views in mind, polypeptide and proteins were purified from the hemolymph of *Z. bicolorata* and their antifungal activity determined using different assays.

MATERIALS AND METHODS

For present study, active culture of human pathogenic fungi *Aspergillus niger* (ITCC 5479) causing aspergillosis and *Candida albicans* (ITCC 4718) causing candidiasis were procured from the Division of Plant Pathology, Indian Agriculture Research Institute (IARI), New Delhi, India. The strains were sub-cultured and maintained on potato dextrose agar medium [Hi-media, Mumbai] at 37ºC. For growth and sporulation, *A. niger* was incubated for 48 h while *C. albicans* was incubated for 24 h (Tiwari *et al*., 2018). For immune-challenging experiments, two pathogenic bacteria *Micrococcus luteus* (MTCC 106) and *Escherichia coli* (MTCC 443) were procured from Microbial Type Culture Collection and Gene Bank, Chandigarh, India. *M. luteus* was maintained and sub-cultured in nutrient agar medium at 30ºC and *E. coli* in trypticase soy agar medium at 37ºC (Lamberty *et al*., 2001).

The Mexican beetle (*Zygogramma bicolorata*) was procured from National Bureau of Agricultural Insect Resources (NBAIR) Bengaluru, India. The beetles were reared under laboratory conditions (12/12 h light/ dark cycle) in insectary room of Department of Biochemistry, Pt. J.N.M. Medical College, Raipur, Chhattisgarh (India) in four closed baskets separated for adults, newly reared adults, larvae and eggs. *Z. bicolorata* is as a bio-control agent against an obnoxious weed

Parthenium hysterophorus (Fig. 1). Fresh leaves and flowers of parthenium were collected from the nearby area and fed to insectsevery alternate day. The young adults were used throughout the study (Jain *et al*., 2017).

For immunization-related experiments, heat-killed preparation of pathogenic bacteria *M. luteus* (MTCC 106) and *E. coli* (MTCC 443) were used (Lemaitre *et al*., 1997). For immunization, two groups of parthenium beetles of 500 each were made with one group challenged by injecting heat-killed bacterial mixture of pathogenic *M. luteus* and *E. coli* (1:1) to induce an immune response and the other group was kept non-immunized. For immune challenging part, the dorsal abdominal cavity of adult beetles was pierced with a needle dipped in a heat-killed mixture of bacteria. Pricking and infection caused the injury that by itself triggers immune response. The hemolymph was collected 24 h after immunization by pricking the thoracic region of beetle in an Eppendorf tube containing protease inhibitor cocktail with the pipette and stored at -20°C until use (Haselton and Fridell, 2011).

The immunized sample prior to loading was dissolved in 1.5 M tris-HCl buffer (pH 8.8) in 1:1 ratio and centrifuged to remove insoluble matter (Janson and Rydén, 1998). The protein concentration in hemolymph was estimated by Lowry's method using bovine serum albumin (BSA) as standard (1 mg mL^{-1}) [Hi-media, Mumbai, India] (Lowry *et al*., 1951). The absorbance was measured at 660 nm using UV-visible double beam spectrophotometer [ELICO, Hyderabad, India].

The re-dissolved immune hemolymph was subjected to 1^{st} step purification using a low-pressure size exclusion chromatography (SEC) on Sephadex G-25 in a glass column. Column size was 150 mm × 20 mm. The mobile phase was 0.75 M tris-HCl and the stationary phase was Sephadex G-25 gel. With a flow rate of 1.0 mL min^{-1} at the protein content of 1.2 mg mL^{-1}, the sample was injected in the column. At first the calibration and standardization were done by BSA and then the filtered immunized sample (1 mL volume) was loaded in column and eluted isoelectically using 0.75 M tris-HCl, pH 8.8 as elution buffer. After injecting filtered sample, each fraction was collected in 1.5 mL Eppendorf tubes separately (Hagel, 1993; Laurent, 1993). The protein concentration of each fraction was estimated by using BSA as standard (Lowry *et al*., 1951).

Antifungal susceptibility of purified *Z. bicolorata* proteins against pathogenic fungi *A. niger* (ITCC 5479) and *C. albicans* (ITCC 4718) was examined by disc diffusion method of Kirby-Bauer (Bauer *et al*., 1966). The 25 µL immunized and non-immunized crude hemolymph, as well as each fraction along with control, were loaded on each disc and incubated at 4ºC for 1 h and then incubated at 37ºC for 24 h. Dimethyl sulphoxide (DMSO) [Hi-media, Mumbai, India] was used as a negative control and ketoconazole as the positive control (1 mg mL^{-1}) (Cytryńska *et al*., 2007).

For molecular identification and characterization of peptide/proteins fractions, the purified protein was resolved on 12% w/v standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Bio-Rad, Singapore] method followed by silver staining and approximate molecular weight was determined (Laemmli, 1970). On preparation of 12% gels of 150 × 120 × 1.5 mm dimension, the fractions with significant antimicrobial activity, core protein along with pre-stained protein marker [Hi-media Mumbai, India] were electrophoresed at 100 V or 30 mA per gel. For analysis of relative molecular weight, gel was stained by silver staining method (Shevchenko *et al*., 1996).

The data obtained was analyzed using SPSS version 16.0. All the tests were performed in triplicates and for antimicrobial activity, arithmetic mean values along with standard deviation were considered for data analysis.

RESULTS AND DISCUSSION

The cells of insects produce several antimicrobial substances in defense against pathogens as a result of humoral immune response that acts as an autogenous antibiotic. It is generally assumed

that every insect species has some specified sort of peptides synthesized in response to non-self-recognition (Cytryńska *et al*., 2007). It is interesting to screen and characterize novel antimicrobial proteins (AMPs) as their being efficient encroacher, able evaders of antimicrobial resistance and potent therapeutics (Lehrer and Ganz, 1999; Fu *et al*., 2009). Till date, enormous AMPs have been found in different species, and it is certain that there are still abundant fungal infections that need to be treated and immeasurable antimicrobial compounds yet to be discovered. To assess antifungal susceptibility of immunized and non-immunized hemolymph isolated from *Z. bicolorata* against two pathogenic fungi *A. niger* (ITCC 5479) and *C. albicans* (ITCC 4718), disc diffusion assay was done. The marked inhibition activity was recorded against immunized sample formed from both *A. niger* and *C. albicans* (zone of inhibition, 30 and 8 mm, respectively) while negative control (DMSO) depicted no antifungal action (Fig. 2). The non-immunized hemolymph didn't exhibit any significant inhibition. Therefore, further studies were carried out in immunized samples only which showed promising results. This study established that *Z. bicolorata* produces a set of antimicrobial peptides on challenging their immunity, the same was observed with *Drosophila* (Lemaitre *et al*., 1997). In contrast, the studies on arthropod and termite insects have reported no change in antimicrobial concentration with unchallenged immunity and established that septic injury is enough to cause immune response (Destoumieux *et al*., 1997; Lamberty *et al*., 2001). Innate immunity responds promptly in vertebrates, invertebrates and plants against bacteria and fungi and releases antimicrobial peptides. The septic injury and infections trigger hasty synthesis of these peptides by blood cells and fat bodies and release them into hemolymph of insects (Lamberty *et al*., 2001).

Table 1: Protein contents in isolated fractions (done by Lowry's method)

Fraction No.	Protein contents (mg mL^{-1})
Control	0.07 ± 0.00
1	0.05 ± 0.00
2	0.07 ± 0.00
3	0.07 ±0.01
4	0.08 ± 0.001
5	1.57 ± 0.02
6	1.90 ± 0.03
7	1.76 ± 0.10
8	2.34 ± 0.07
9	2.52 ± 0.06
10	2.53 ± 0.00
11	3.04 ± 0.10
12	3.47 ± 0.20
13	4.28 ±0.10
14	2.85 ± 0.09
15	2.14 ± 0.08
16	1.76 ± 0.08
17	1.67 ± 0.07
18	1.85 ± 0.07
19	2.52 ± 0.08
20	2.80 ± 0.80
21	1.57 ± 0.07
22	1.19 ± 0.70
23	0.67 ± 0.90
24	0.52 ± 0.08
25	0.47 ± 0.06
26	0.42 ± 0.05
27	0.47 ± 0.06
28	0.34 ± 0.04
29	0.34 ± 0.05
30	0.34± 0.05

The immunized sample was initially dissolved in tris-HCl and protein estimation was done. BSA was used as standard and in reference to the protein content of crude hemolymph was found to be 12.50 mg mL^{-1}.

The 1st step of purification-chromatographic fractionation and purification profile of immunized hemolymph extract on a low-pressure size-exclusion chromatography with Sephadex G-25 was performed which allowed effective separation of 30 fractions containing 120-18 kDa molecular masses proteins/peptides. These fractions were implied on quantity estimation of proteins (Table 1). The high protein concentrations were found in fraction No. 11, 12, 13, 14 and 20.

The obtained fractions were subjected to study the antifungal activity against two fungi (*A. niger* ITCC 5479 and *C. albicans* ITCC 4718). During protein estimation, the fraction No. 1, 2, 3 and 4 exhibited similar OD as that of control so were ignored while antifungal activity test was done for fraction No. 5. Zones of inhibition against *A. niger* for fraction No. 6, 9, 11 and positive control were 11.0 ± 2.82, 19.5 ± 0.707, 21.0 ± 1.41 and 9.2 ± 0.21 mm, respectively, and in case of *C. albicans*, the fraction No. 6, 9, 11, 15 and positive control were of 9.0 ± 0.301, 7.0 ± 0.01, 7.0 ± 0.007, 11.5 ± 0.71 and 7.0 ± 0.001 mm, respectively. On performing student's t-test, the fraction No. 6, 9 and 11 showed significant antifungal activity against both the pathogenic organisms (Fig. 3 and 4) with p-value ≤ 0.0001 in all cases, except for fraction No. 15 as it was ineffective in other organism (Table 2). Except these, all the fractions were inactive against both the

Table 2: Efficacy of different fractions against pathogenic fungi (*C. albicans* & *A. niger*)

Fraction No.	Zone of inhibition (dia. in mm)		p- value
	C. albicans	*A. niger*	
Fraction-6	9.0 ± 0.30	11.0 ± 2.82	0.001
Fraction-9	7.0 ± 0.01	19.5 ± 0.71	<0.001
Fraction-11	7.0 ± 0.007	21.0 ± 1.41	<0.001
Fraction-15	11.5 ± 0.71	Ineffective	-
Positive control	7.0 ± 0.001	9.2 ± 0.21	<0.0001

*- Not defined

Fig. 1: *Z. bicolorata* feeding on *P. hysterophorus*

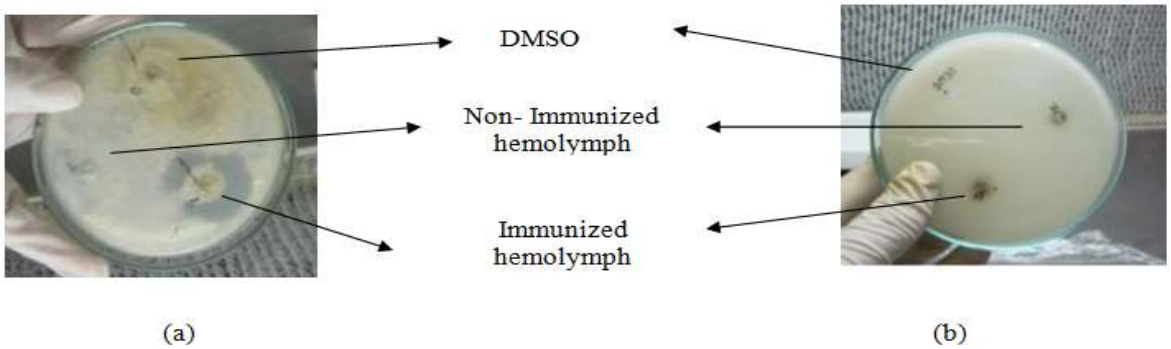

Fig. 2: (a) Showing zone of inhibition against *Aspergillus niger* (b) Showing zone of inhibition against *Candida albicans*

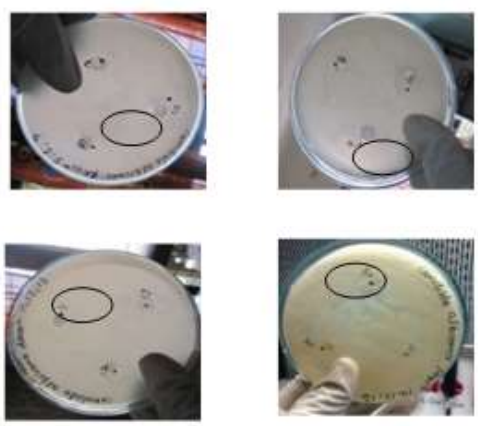

Fig. 3: Zone of inhibition (encircled portions) of different factions (6, 9, 11 & 15) against *C. albicans* on PDA incubated at 37°C for 24 h

Fig. 4: The zone of inhibition (encircled portions) of different factions (fractions 6, 9 & 11) against *A. niger* on PDA incubated at 37°C for 48 h.

strains or can be said ineffective i.e. all the mean values were zero. Fig. 5 represents the concentration of protein in each fraction along with the fractions showing highest resistivity against test fungal strains. The antifungal activity of fractions against *A. niger* and *C. albicans* performed by disc diffusion method revealed that among both the organisms fraction No. 11 showed highest activity in *A. niger* and fraction No. 15 in *C. albicans* which seemed ineffective in *A. niger.*

For further purification by SDS-PAGE electrophoresis, fraction No. 6, 9, 11 and 15 were chosen. Migration of fraction No. 6, 9, 11, 15 and core protein along with protein marker confirmed that all the fractions belonged to high as well as low-molecular weight proteins. The four fractions produced six proteins; four high-molecular weight (120, 100, 90 and 66 kDa) and two relatively low molecular weight polypeptides (36 and 18 kDa) along with the maker protein as per Fig. 6. Fraction 9, 11 and 15 contain all the six proteins whereas fraction 6 has rest five, except 100 kDa.

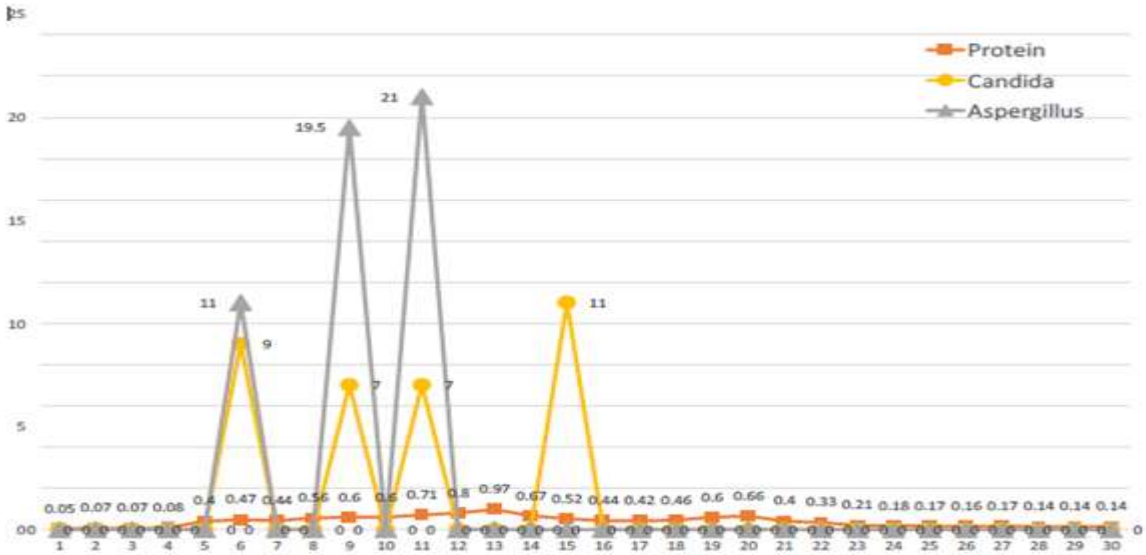

Fig. 5: SEC chromatogram of biological sample showing the protein content of each fraction along with the fractions exhibiting the antifungal activity in both the organisms

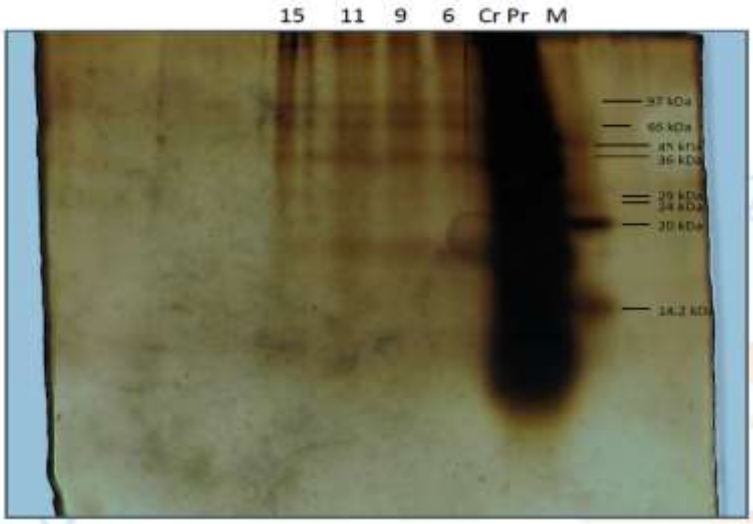

Fig. 6: 12% SDS-PAGE gel with silver staining of fraction No. 6, 9, 11, 15 and core protein along with the protein marker

Protein contents were very low for each fraction; therefore, it was not specified that for every faction (Fig. 6). The higher molecular weight proteins along with the lower peptides were also noted in the hemolymph of various other species of insects like *Galleria mellonela*, termite insect, *Drosophila* and other species of Insecta (Lamberty *et al*., 2001; Cytryńska *et al*., 2007).

Induction is a habitual process in various insect species (Cociancich *et al*., 1994). This is the first report of isolation and characterization of antifungal proteins from *Z. bicolorata* (Coleoptera). Based on the molecular weight of the proteins and small peptide fractions identified and isolated from the hemolymph, they probably comprised part of defense peptides. Among the 30 isolated proteins, 5 proteins/peptides were purified by size exclusion chromatography and SDS-PAGE to homogeneity and their molecular weight and antifungal activity against the two pathogenic fungi determined. As the purified peptides/proteins are chiefly active against the fungi; it suggests that the antifungal activity of it is related to the cell wall of fungi.

The multidrug resistance of opportunistic microorganisms is still a major concern and focus is on to develop a more efficient drug with lesser side effects. The present study depicted the antifungal nature and sensitivity of proteins and polypeptides purified from immunized hemolymph of *Z. bicolorata* against two pathogenic fungi *C. albicans* and *A. niger* causing candidiasis and otomycosis, respectively. However, the finding opens new avenues for future studies in the evolution of essential proteins or drugs against fungal infections. There are many advantages of the antimicrobial peptides *viz*., broad spectrum range, selectivity, fast killing and lack of resistance developed (Matsuzaki, 1999). The present study is a preliminary one and there is need for further characterization, identification and sequencing of protein fractions and also the mode of action and pathway behind the antifungal activity in hemolymph needs elucidation.

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Conflict of Interest: The authors declare that they do not have any conflict of interest.

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