Research Article

COMPARATIVE EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF PROTEIN FROM MEDICINAL AND ECONOMICALLY IMPORTANT PLANTS

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ABSTRACT

Microbes are increasingly developing defensive mechanisms against known available antibiotics. Cationic protein shows wide range of activity against pathogenic microorganisms. In the present study, evaluation of antimicrobial activity of five various plants seed protein against various pathogenic microorganisms revealed that *Desmodium motorium* (Houtt.) Merr. was effective against four pathogenic microorganisms. Among the tested crude seed protein of *Desmodium motorium* (Houtt.) Merr. and *Barleria lupulina* L. showed maximum zone of inhibition against *Bacillus subtilis* MTCC 2393. The cationic crude protein has the greatest activity toward Gram-positive bacteria, including *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* MTCC 2393, *Corynebacterium sp.* MTCC 3080 with MIC's ranging between 135 to 210 µg/ml, therefore it can be concluded that *Desmodium motorium* (Houtt.) Merr. has great medical potential and further purification and structural elucidation of bioactive protein is required.

Keywords: Antimicrobial protein, Medicinal plant, Minimum Inhibitory Concentration, Cationic protein.

INTRODUCTION

The increasing antibiotic resistance exhibited among common pathogenic microorganisms encountered in wounds and other infections is a serious current public health issue, which will have a major impact on antimicrobial treatments in the future[1]. Plants are promising resources for finding the new antimicrobial agents compared to other sources[2]. Plants have attracted interest because they can defend themselves against microbial infection even though they do not have the adaptive immune response system present in higher vertebrates. To protect themselves from infection by pathogens, plants produce various compounds with antimicrobial peptides (AMP)[3]. Plant seeds are usually sown on a substrate that is extremely rich in microorganisms. Infection in seeds or seedling tissues normally occurs at relatively low frequency. It is believed that seed proteins that exhibit antimicrobial activity may participate in the protection of seeds against potential microbial invaders[4]. Many proteins with antifungal and/or antibacterial activity have already been detected in seeds. These proteins have been extensively studied because of their potential applications in the food industry as natural biopreservatives and in pharmaceuticals as antimicrobials[5,6].

Antimicrobial proteins and peptides have been isolated from seeds of various other plants. They are believed to play a role in plant defence because of their strong antimicrobial activity[7]. In this work we screened *Samanea saman (Jacq.) Merr.*, *Caesalpinia bonduc* (L.) Roxb., *Tamarindus indica* L., *Barleria lupulina* L., and *Desmodium motorium* (Houtt.) Merr. seed protein for the presence of inhibitory activity against pathogens. These plant species are common in India.

MATERIALS AND METHODS

Plant Materials

Mature seeds from *Samanea saman* (Jacq.) Merr. (*S. saman*), *Caesalpinia bonduc* (L.) Roxb. (*C. bonduc*), *Tamarindus indica* L. (*T. indica*), *Barleria lupulina* L. (*B. lupulina*), and *Desmodium motorium* (Houtt.) Merr. (*D. motorium*) were collected and the plant materials were identified by Botanical survey of India, Southern regional centre, Coimbatore and Kerala Forest Research Institute, Thrissur, India.

Microorganisms Tested

The following bacteria were used for determining the antibacterial activity: *Pseudomonas aeruginosa* ATCC 27853 (*Ps. aeruginosa*), *Enterococcus faecalis* ATCC 29212 (*Ent. faecalis*), *Staphylococcus aureus* ATCC 25923 (*Staph. aureus*), *Bacillus subtilis* MTCC 2393 (*B. subtilis*), *Salmonella enterica* typhimurium MTCC 98 (*Salm. enterica*), *Corynebacterium sp.* MTCC 3080 (*Coryne. sp.*), and *Raoultella planticola* MTCC 530 (*R. planticola*). The following fungal strains were used for determining antifungal activity: *Candida albicans* MTCC 3017 (*C. albicans*), *Botrytis cinerea* MTCC 2880 (*B. cinerea*), *Rhizopus microsporus* var. oligosporus MTCC 2785 (*R. microsporus*), *Mucor hiemalis* MTCC 1277 (*M. hiemalis*), *Fusarium oxysporum* MTCC 2087 (*F. oxysporum*).

Extraction of Protein

Cationic protein from mature seeds of each plant was extracted individually as previously described[8], with minor modifications. Mature seeds were washed in tap water and surface sterilized using 70% ethanol (15 seconds) and 0.1% mercuric chloride (3 minutes) (Hi-media, India) and rinsed thoroughly with sterile distilled

water. Ice cold extraction buffer (pH 5.4) (10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, 2 mM EDTA) and 1.5% (w/v) polyvinyl polypyrrolidone (PVPP, Hi-media) suspension was stirred for two hours at 4°C. Prior to use of the extraction buffer, thiourea (2 mM) and phenylmethylsulfonyl fluoride (PMSF dissolved in isopropanol, 1 mM) were added. Ten grams (10gm) of seeds were ground in a coffee mill, and the meal extracted with 20 ml of the extraction buffer for two hours at 4°C, under continuous stirring. Solid material was subsequently removed by centrifugation (20 minutes at 7,000g) and solid ammonium sulphate added to the supernatant until 30% relative saturation was reached. The precipitate was allowed to form overnight at 16°C under gentle stirring. After centrifugation (30 minutes at 7,000g) the supernatant was adjusted to 70% relative ammonium sulphate saturation. The final precipitate formed overnight at 16°C under gentle stirring, was collected by centrifugation (30 minutes at 7,000g) and the pellet redissolved in minimum amount of sterile distilled water. This suspension was clarified by centrifugation (30 minutes at 7,000g), and the pellet was extensively dialyzed against distilled water using dialysis tubing with a molecular mass cut-off of 1,000 Da. The Dialyzed crude protein pellet (P1) was used for further studies. The resulting supernatant was heated at 80°C for 15 minutes, in order to isolate heat tolerant protein. A final centrifugation (30 minutes at 7,000g) was performed to remove the coagulated material and the supernatant was extensively dialyzed against distilled water using dialysis tubing with a molecular mass cut-off of 1,000 Da. The resulting dialyzed crude protein (P2) was used for further studies.

Protein Estimation by Lowry Method

Protein was estimated by Lowry method[9]. 1 mg of dried protein pellet was dispersed in 1 ml of phosphate buffer (pH 7.8). Suitable aliquot (1ml) of extracted protein solution was taken. Protein estimation was done by Lowry's method. Bovine serum albumin (BSA) was used as standard. Absorbance was recorded at 660nm. From the standard curve, the amount of protein in the sample was determined.

Antifungal and Antibacterial Activity Assays

Antifungal activity was measured by radial diffusion method[10]. An agar plug containing mycelia of the test fungus was harvested from actively growing fungal plates and placed in the centre of Petri plate containing potato dextrose agar (PDA). The plates were incubated at 26 °C for two days in the dark until the mycelia colony reached a diameter of 3 cm. Sterile blank paper discs (Whatman No. 1; 6 mm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelia colony. An aliquot (20 µl) of the protein fractions were added to disk. Control discs were prepared by replacing the protein sample with the same volume of antifungal drug (Ketoconazole). The plates were incubated at 23 °C for 48 h until mycelia growth had enveloped the discs containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity.

The antibacterial activity was detected by agar disc diffusion assay[11] and tested against all indicated strains. The entire surface of the PDA plate was covered with the 0.5 McFarland suspension of the indicator strain, and the plate was air dried for 15 minutes before the sterile empty discs were laid on the surface. An aliquot of 20 µl protein fractions was added to disk on PDA plates. Control discs (Chloramphenicol) were prepared by replacing the protein sample with the same volume of antibiotic drug. Zones of inhibition (mm) were measured after incubation at 37°C for 24 h. Antifungal and antibacterial activity was performed in triplicate.

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) was determined according to DeGray *et al.*[12] To determine minimum inhibitory concentration of P1 and P2, spores of fungus were collected from potato dextrose agar (PDA) plates by flooding a 2-week-old culture with a solution of 0.01% (v/v) Tween-20 and rubbing the surface. The spore suspension was filtered through glass wool to remove mycelial fragments. The spore concentration (per milliliter) was determined using a hemocytometer. Bacteria were harvested from log phase-grown cultures, and the concentration were determined based on plating of serial dilutions of the cultures. Known amounts of pathogen (1,000 spores for fungi or 1,000 bacteria) were added to serial dilutions of P1 and P2, ranging from 0 to 256 µg/mL in individual wells of a 96-well microtiter plate. An equivalent amount of growth medium (PDB for bacteria and fungi) was added to each well, bringing the total volume to 50 µL per well. Plates were incubated overnight at 25°C with gentle shaking. The following day, wells were scored for the presence or absence of growth. The lowest concentration of P1 and P2, which inhibited all cell growth, were recorded as the minimum inhibitory concentration (microgram per millilitre) value. Simultaneously, Chloramphenicol and Ketoconazole, MIC values were also determined.

RESULTS

AMP isolated from plants seeds shown great promise in the fight with pathogenic microorganisms and therapeutic drug design. For this purpose, we investigate the presence of antimicrobial protein in the seeds of various plants. Our working hypothesis is that seeds contain various defence proteins. The purification of antimicrobial protein from plants seed was performed as method described by Terras *et al.*[8] The five plant seeds crude protein pellets (P1 and P2) were isolated. The Protein content was estimated by Lowry Method. Among P1 and P2 pellets, *T. indica* P1 pellet contain maximum 726 µg/ml protein whereas minimum protein 178µg/ml estimated in P2 of *B. lupulina* seed pellet (Fig 1).

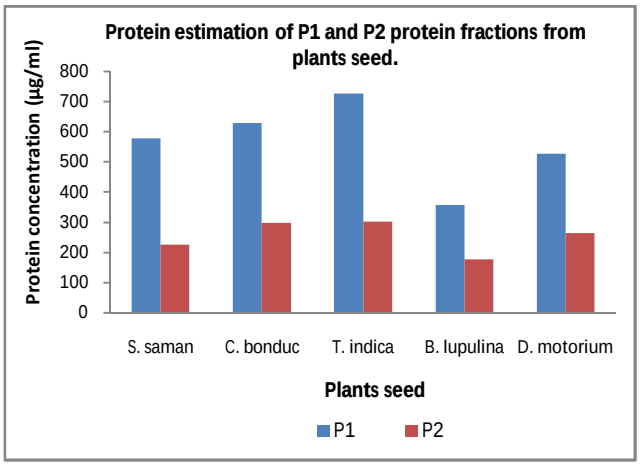

Figure 1: Estimation of protein fractions (P1 and P2) from plants seed.

The crude protein P1 and P2 from seeds of *T. indica*, *B. lupulina*, and *D. motorium* showed variable levels of activity against tested microorganisms (Table 1). P1 pellet of *T. indica* showed activity against *B. subtilis* and *Coryne. sp.* and *B. lupulina* crude protein P1 active against *Ent. faecalis* and *B. subtilis*. *D. motorium* protein pellet P1 showed activity against four microorganisms including *Ent. faecalis, B. subtilis, and Coryne. sp.*, simultaneously it was inhibited growth of the *B. cinerea*. *S. saman* and *C. bonduc* did not exhibit any inhibitory activity against microorganisms. The heat-stable protein fraction (P2) failed to inhibit the growth of the tested microorganisms. The crude protein was less active when compared to control Chloramphenicol and Ketoconazole.

The antimicrobial activity of the P1 and P2 protein fractions measured in terms of MIC is listed in Table 2. The minimum and maximum MIC value was recorded when P1 protein fraction from *D. motorium* and *B. lupulina* was used against *B. subtilis*. The P1 protein fraction from *D. motorium* showed 148 µg/ml MIC value.

Table 1: *In vitro* inhibition (mm) of various pathogens exhibited by the P1 and P2 protein fractions

		Ps. aeruginosa	*Ent. faecalis*	*Staph. Aureus*	*B. subtilis*	*Salm. enterica*	*Coryne. sp.*	*R. planticola*
S. saman	P1	-	-	-	-	-	-	-
	P2	-	-	-	-	-	-	-
C. bonduc	P1	-	-	-	-	-	-	-
	P2	-	-	-	-	-	-	-
T. indica	P1	-	-	-	15±0.44	-	9±0.6	-
	P2	-	-	-	-	-	-	-
B. lupulina	P1	-	10±1.15	-	8±1.5	-	-	-
	P2	-	-	-	-	-	-	-
D. motorium	P1	-	9±0.76	-	15±0.57	-	8±0.72	-
	P2	-	-	-	-	-	-	-
Control		22±0.20	30±0.44	24±0.57	20±0.60	27±0.41	30±0.58	24±0.57

Where "-" shows, inhibition not detected; All values were expressed in ± Standard Error Mean

Table 2: Minimum inhibitory concentration (µg/ml) of P1 and P2 protein fractions from plants seed.

	Ent. faecalis	*B. subtilis*	*Coryne. sp.*	*B. cinerea*
T. indica (P1)	-	158±1.5 µg/ml	162±µg/ml	-
B. lupulina (P1)	160±0.88 µg/ml	210±1.2 µg/ml	-	-
D. motorium (P1)	174±1.45 µg/ml	135±1.15 µg/ml	186±0.57 µg/ml	148±2.5 µg/ml
Control	10±0.60 µg/ml	15±0.29 µg/ml	8±0.33 µg/ml	2±0.5 µg/ml (Ketoconazole)

All values were expressed in ± Standard Error Mean

DISCUSSION

This report described the isolation and antimicrobial screening of plants protein, In last few decades several AMP have been found in various seeds and these protein were additionally linked to plant defence mechanisms[13-17]. When plants were wounded or attacked by pathogenic microorganisms, it triggers an array of potent defence mechanisms, one of which is to synthesize proteins, peptides and low molecular-weight compounds that have antimicrobial effects. Antimicrobial proteins and peptides are widely distributed in nature and are synthesized not only by plants but also by bacteria, insects, fungi and mammals[7]. Plant-pathogen interactions involve the generation of various elicitors, including glycoprotein, peptides and oligosaccharides, which are responsible for the induction of some defence responses[18]. Most pathogenesis related (PR) proteins have a damaging action on the structures of the parasite for example PR-1 and PR-5 family members interact with the plasma membrane whereas PR-2,3,4,8, and 11 families can attack the fungal cell wall [19,20].

Osborn in 1995[21] isolated and characterized five antifungal proteins from seeds of *Aesculus hippocastanum*, *C. ternatea*, *Dahlia merckii*, and *Heuchera sanguinea*. Those proteins shows homologous to plant defensins, previously isolated and characterized from radish seeds. These proteins have been shown to inhibit the growth of a wide range of fungi, and some Gram positive bacterium. Various classes of cysteine-rich peptides have been implicated in the resistance mechanism of plants against pathogens[22]. It is clear that these peptides play an important role in the protection of plants against microbial infection and thus could prove to be useful tools for the genetic engineering of fungal

resistance in transgenic plants[23, 24]. Most of the research work on cysteine-rich antimicrobial peptides is based on the assumption that they are involved in defence mechanisms of plants against phytopathogens[8, 25]. The antimicrobial activity of plant defensins was first reported from radish seeds and since then this class of peptides has been found in several plant species[8, 26]. Some Lipid Transfer Proteins have also been demonstrated to inhibit phytopathogens, such as *Pseudomonas solanacearum*, *Clavibacter michiganensis*, *Fusarium solani*, *Rhizoctonia solani, Trichoderma viride* and *Cercospora beticola*[24]. The cationic crude protein has the greatest activity toward Gram-positive bacteria, including *Ent. faecalis, B. subtilis*, and *Coryne. sp* with MIC's ranging between 135 to 210 µg/ml. These results were similar to the findings of Hussain *et al*.[27]

CONCLUSION

In conclusion, it was reveals that *T. indica*, *B. lupulina*, and *D. motorium* contains AMP which inhibits the growth of bacteria and a fungi as compared with growth on control medium. Further research is needed for purification of protein/peptide in order to determine the sequence and structure. However this study revel that *D. motorium* was good source for the isolation of AMP.

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