Short Communication

ANTIMICROBIAL ACTIVITY OF SMALL PROTEIN OF MORINGA OLEIFERA LEAVES

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SUMMARY: Three fractions from the leaves of Moringa oleifera were obtained on Sephadex G-25 column chromatography. Single bands of these fractions were detected on Polyacrylamide SDS gel electrophoresis. An antibacterial action of small protein/peptide was tested against E. coli Kl. aerogenes, KI. pneumoniae, S. aureus, and B. subtilis. Fraction P1, P2 and P3 showed strong inhibitory activity against E. coli, S. aureus and B subtilis but clear zone of inhibition was also noted against K1. aerogenes with peptide 1. Fraction P2 showed significant zone of inhibition against Aspergillus niger.

Key Words: Antibacterial, Antifungal, Moringa oleifera, Chromatography, Electrophoresis.

INTRODUCTION

Plants normally grow on different nature of soils which are extremely rich in microorganisms and infection remains a rare event. To keep out potential invaders, plants produce a wide range of selective antibacterial compounds either in a constitutive or an inducible manner (1). Among these compounds several low molecular weight proteins or peptides with antibacterial or antifungal activity have been isolated in recent years from various plants (2-4) and are believed to be involved in a defence mechanism against phytopathogenic fungi by inhibiting microorganisms growth through diverse molecular modes, such as binding to chitin or increasing the permeability of the fungal membranes or cell wall. Another strategy followed by plants to thwart invaders is based on the localized production of antimicrobial low molecular weight secondary metabolites known as phytoalexins (5,6). Moreover, the synthesis of many presumed defence related proteins are induced when plants are confronted with pathogens (7).

In this country, numerous studies have been carried out to extract various natural products for screening antimicrobial activity but attention has not been focused to isolate small proteins/peptides for antimicrobial activity from the plants of Pakistan. The present

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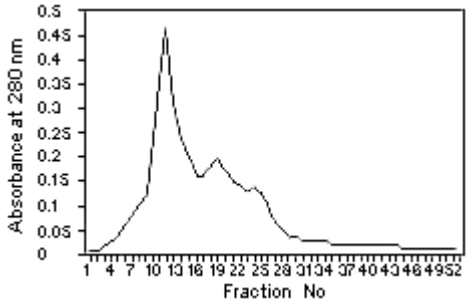
Figure 1: Separation of *Moringa oleifera* leaves extract on Sephadex G-25.



investigation represents a preliminary screening in an ongoing program on antimicrobial peptide/small protein from plants used medicinally in Pakistan. This is necessary because many natural antibiotics are photosensitizers, an example is hypericin which is significantly more active against the AID virus in light than dark (8). The present report is a first systematic attempt to isolate small proteins/peptides from the leaves of *Moringa oleifera* possessing antifungal and antibacterial activity.

MATERIALS AND METHODS

Materials

The leaves of *Moringa oleifera* were collected during June-July 1995, the plants from the employee residence University of Sindh, Jamshoro, Pakistan. Sephadex G-25 and Commassie blue R-250 were obtained from Sigma Chemicals USA. Acetic acid, diethyl ether, SDS, acrylamide-bisacryl amide, were purchased from E. Merck, Germany.

Preparation of soluble extract

Moringa oleifera leaves were dried at room temperature and were ground into fine powder with mortar and pestle. The dried powder of leaves (50 g) was defatted with 150 ml of diethyl ether at room temperature for 8 hours and filtered with Whatman no. 1. The residue was extracted with 33 ml cold distilled water and centrifuged (Kubota refrigerated centrifuge, Japan) at 6000 rpm. The supernatant was transferred to 100 ml volumetric flask and this procedure was repeated twice and volume was made upto the mark with distilled water. Small proteins/peptides were precipitated by the addition of two fold acetone. The precipitates were dissolved in sterilized distilled water and dialyzed over night.

Separation on sephadex G-25

The dialyzed sample was subject to Sephadex G-25 (1.5 x 137 cm) column and eluted with 0.2 M acetic acid. Fraction of 4.5 ml each was collected on fraction collector (Eyla Fraction Collector, Japan) at a flow rate of 48 ml/hr.

Determination of protein

The absorbance at 280 nm was measured to monitor the protein during chromatography separation. The protein content of water extract was measured by the method of Lowry *et. al.* (9) using bovine serum albumin as standard.

Electrophoresis

The homogeneity of pooled fractions were checked by 12% SDS polyacrylamide disc gel electrophoresis by applying 50 μl of denatured sample on the surface of the gel. Power supply was adjusted at 5 ma current per tube for 80 minutes. The gel was stained with Coomassie blue R 250 and destained with methanol : acetic acid : water (30 : 60 : 10 V/V) as reported by Davis (10), Hames and Rickwood (11).

Table 1: Protein content and antimicrobial activity of crude aqueous extract of *Moringa oleifera* leaves.

Protein content		0.80 mg/ml		
Antibacterial activity				
Escherichia coli	Klebsiella areogenes	Kl. pnoumoniae	S. aureus	Bacillus subtilis
Trace	+	-	+	+
Antifungal activity				
Aspergillus niger	Aspergillus fumigatus	Aspergillus flavus	Penicillium expansum	
-	Trace	-	-	

Table 2: Purification of small protein from *Moringa oleifera* leaves.

Purification steps	Volume ml	Total protein mg	% Yield
Crude	100.0	80.00	100.00
Dialyzed	10.0	48.57	60.71
Sephadex G-25		28.18	35.23
Fraction - I	67.5	14.63	18.29
Fraction - II	36.0	7.76	9.70
Farction - III	27.0	5.79	7.24

Antibacterial activity

The cultures of bacteria (*E. coli. KI. aerogenes. KI. pneumoniae. S. aureus and B. subtilis*) grown overnight at 37°C were used for testing the antibacterial activity from different fractions separated on Sephadex G-25 and aqueous extract of *Moringa oleifera* leaves. The antibacterial activity was checked by seed plate method as reported by Rasheed *et. al.* (12). In this technique meat extract nutrient medium containing 1.5% agar was adjusted to pH 7.0 and it was distributed in 40 ml quantity in screw capped bottles and sterilized. The bacterial culture was then added aseptically to the agar medium at 45°C, mixed well and poured immediately in sterilized petriplates. After hardening, wells were cut into agar and 100 μl of the *Moringa oleifera* leaves extract and fractions were placed in these wells. The plates were incubated at 37°C and observations were made after 24 to 72 hours.

Antifungal activity

Antifungal activity was tested against *Aspergillus niger, A. fumigatus, A. flavus and P. expansum*. The diffusion plate method was used to test *M. oleifera* leaves fractions with slight modification as reported by Terras *et. al.* (13). In this technique, 0.1 ml of the fungal spore suspension (grown for 3 days in 10 ml of nutrient Dextrose agar) was thoroughly mixed with 20 ml of melted Sabouraud dextrose agar and poured into sterilized petri plates. When the agar was set, 5 holes of 6 mm diameter bore were made on each of the seeded plate. These holes were filled with 100 μl of the testing sample. Experiments were performed in duplicate. The petri plates were incubated at 28°C for 7-8 days. All the culture plates were examined after 24-96 hours. The zone of inhibition produced by the plant fractions was compared with zone produced by the standard.

Determination of minimal inhibitory concentration (MIC)

The minimum inhibitory concentration was determined by serial dilution in the same type of agar, with concentration ranging from 5, 10, 25, 50, 75 and 100 μg/ml. The inoculum was prepared from fresh overnight broth culture in nutrient dextrose broth. Plates were incubated for 24 hours at 37°C and 96 hours at 28°C for antibacterial and antifungal activity respectively.

RESULTS

The results of protein content, antibacterial/antifungal activity from the water extract of *M. oleifera*

Figure 2: Electrophoresis pattern of crude and purified fractions of *Moringa oleifera* leaves.

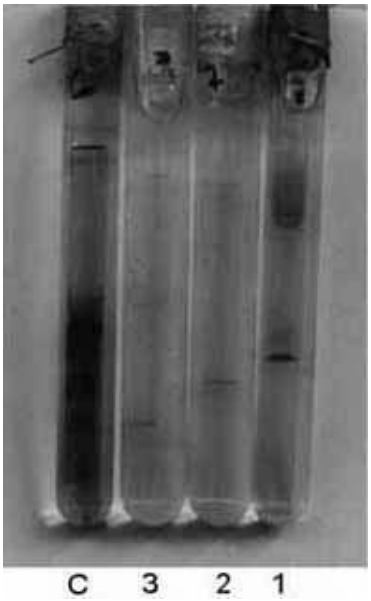


Table 3: Antimicrobial activity of small protein/peptides isolated and purified from *Moringa oleifera* leaves against bacteria and fungi.

Antibacterial activity					
Peak	Escherichia coli	Kilebsiella aerogenes	Klebsiella pneumoniae	Staphylococcus aureus	Bacillus subtilis
P - 1	+	++	-	+	+
P - 2	+	-	-	++	++
P - 3	++	-	-	++	++
Tarivid	Trace	-	-	++	+++
Enoxabid	++	-	+++	+++	++++

Antifungal activity				
Peak	Aspergillus fumigatus	Aspergillus niger	Aspergillus flavus	Penicillium expansum
P - 1	-	Trace	-	-
P - 2	-	+	-	-
P - 3	-	-	-	-
Nystatin	-	++	Trace	++
Griseofulvin	+	++++	++	+++

Negative = -
Positive = +

Trace = 2 mm inhibitory zone,
+ = 5 mm inhibitory zone,
++ = 10 mm inhibitory zone,
+++ = 15 mm inhibitory zone,
++++ = 20 mm inhibitory zone,

leaves are shown in Table 1. Separation of *M. oleifera* sample on Sephadex G-25 column chromatography showed three peaks (Figure 1). The small proteins/peptides associated with these optical density peaks were termed as P1, P2 and P3 in the order, in which they were eluted from the column and their protein content is shown in Table 2. The homogeneity of pooled fractions was checked by SDS polyacrylamide disc gel electrophoresis and was found homogeneous by showing single band (Figure 2). Antibacterial/antifungal activity of P1, P2, and P3 fractions are shown in Table 3. The minimum inhibitory concentration of isolated fractions against bacterial and fungal species is shown in Table 4.

DISCUSSION AND CONCLUSIONS

It is clearly noted that aqueous extract of *M. oleifera* leaves posses significant antimicrobial activity against gram positive and negative fungal species. Fractions P1, P2 and P3 were found active against *E.*

Table 4: Minimal inhibitory concentration of *Moringa oleifera* leaves fractions in the serial dilution method.

Test Organisms	MIC μg / ml		
	Fraction P - 1	P - 2	P - 3
Bacteria			
Escherichia coli	75	75	25 - 50
Klebsiella aerogenes	25 - 50	> 100	> 100
Klebsiella pneumoniae	> 100	> 100	> 100
Staphylococcus aureus	75	25 - 50	25 - 50
Bacillus subtilis	75	25 - 50	25 - 50
Fungi			
Aspergillus fumigatus	> 100	> 100	> 100
Aspergillus niger	75 - 100	75	> 100
Aspergillus flavus	> 100	> 100	> 100
Penicillium expansum	> 100	> 100	> 100

coli, S. aureus and *B. subtilis* but fraction P1 was found active by producing clear zones of inhibition against *Kl. aerogenes* as shown in Table 2. Moreover, fraction P2 was found moderately active against *A. niger*. Fractions P1, P2 and P3 were not found effective against the growth of *Kl. pneumoniae, A. fumigatus, A. flavus* and *P. expansum*. This observation provides strong circumstantial evidence that small proteins/peptides play an important role in plants of antimicrobial defence system (13). Probably, this study provides considerable scope in exploiting the local indigenous resources for isolation of antimicrobial peptides/small proteins. Further work is under progress in this Laboratory for the analysis of amino acid composition of active small protein/peptide and results will be reported in near future.

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