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Isolation and Partial Characterization of Antimicrobial Proteins /Peptides from *Moringa Oleifera*

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ABSTARCT

Moringa oleifera is a worldwide distributed plant because of its number of pharmacological and nutritional benefits. In this study, protein extracts of *M. oleifera* leaves and flowers were prepared. Extracts were purified by dialysis and quality of the sample was assessed through SDS-PAGE. Antimicrobial and antioxidant activity of these extracts was determined by using disk diffusion method, Dpph (free radical scavenging assay) and enzymatic assay. The antimicrobial activity of *M. oleifera* protein extract of flowers and leaves were determined against multidrug–resistant bacteria e.g. Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* and *Salmonella typhi*. Extract results showed the maximum zone of inhibition against MRSA and minimum zone of inhibition against *E. coli* and *S. typhi* whereas protein extract of leaves did not show any antibacterial activity against these bacteria. MIC of flower extract was determined as 166µg/ml against MRSA whereas *E. coli* and *S. typhi* was recorded as 500µg/ml. Enzymatic activity (peroxidase) result showed that leaf and flower protein extract possess higher enzymatic activity at time of 10 minutes and lower at time of 20 and 30 minutes. Protein extract of leaf showed higher scavenging activity at time of 40 and 60 and lower at time of 20 whereas flower protein extract showed higher scavenging activity at time of 60 min and lower at time of 40 and 20. Present study showed that *M. oleifera* possess potent antimicrobial and antioxidant activity and it may be used as potential drug against multidrug-resistant bacteria.

Keywords: *Moringa oleifera*, protein extracts, antibacterial, antibiotic resistance, antioxidant

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INTRODUCTION

Moringa oleifera is the most worldwide distributed plant (1). It is associated to the family of Moringaceae. There are about 33 species of Moringaceae and among those 13 best known species are, M. *borziana*, M. *ovalifolia*, M. *arborea*, M. *drouhardi*, M. oleifera, M. *rivae*, M. *stenopetala*, M. *ruspoliana*, M. *hildebrandtii*, M. *pygmaea*, M. *longituba* and M. *concanesis*, M. *peregrina* (2).The height of this tree ranges from 5 to10m and it can be cultivated throughout the plains. They can survive in destitute soil and grow in the hot dry lands, and in the humid tropics. It has capability to tolerate a wide range of rainfall (1). M. oleifera is native of the Asia Minor, Africa, Arabia, Pakistan, India, Philippines, Central America, North, South America, Cambodia and Caribbean Islands. In Pakistan, it is called Sohnjana whereas in some other countries it is referred as "Horse radish" and "Drumstick" (1). It possesses medicinal properties and is also known for its nutritional benefits and environmental conservation etc. (2). The 44 known compounds have been isolated from this plant till now (3).

M. oleifera possesses highly valued proteins, mineral and fiber which plays an essential role in human nutritional consumption and is used for medicinal and nutritional purposes as it contains 10 times more vitamin A than carrots, 15 times more potassium than bananas, 9 times more protein than yogurt, 25 times more iron than spinach, 7 times more vitamin C than oranges and 17 times more calcium than milk (Daba, 2016). The various parts of M. oleifera possess numerous medicinal and

pharmacological properties like leaves are commonly used for treatment of hyperglycemia, asthma, flu, heartburn, diarrhea, pneumonia, reduce cholesterol and blood pressure. Leaves are also referred as antimicrobial, anticancer, antioxidant and antidiabetic agents. Seed powder is used in various treatments of hyperthyroidism, Crohn's disease, rheumatism, cramp, epilepsy gout, sexually transmitted diseases, and also contain anti-inflammatory, antimicrobial agents. Root bark of M. oleifera act as anticancer, anti-inflammatory and cardiac stimulant agent. Flower of M. oleifera act as hypocholesterolemia or anti-arthritic agents that can be used for treatment of liver, diarrhea, spleen problem and joint pain (1).

Many antibiotics and chemotherapeutic agents are being used to fight against infectious diseases. By the excessive use of drugs, bacteria strains are getting resistant to many drugs which is the growing issue. So, researchers found alternate therapies to fight against infectious diseases. In developing countries, the medicinal plants are being used for treatment of infectious diseases because of high cost of antibiotics. The antimicrobial protein/peptide possess more attention because the combating with the new generation of antibiotics against drug resistant bacteria is very authoritative. The most crucial advantage of antimicrobial protein/peptides is that in direct contrast to conventional antibiotics and they have many targets and several modes of action. So, resistance against such antibacterial protein/peptides is apparently difficult in comparison with existing antibiotics. The antimicrobial protein/peptide possessed by plant could be better than human because they contact with human pathogens to induce such resistance mechanism in them (4)

Antimicrobial peptides/protein are small conserved component part of innate immune response and they are found in all class of life like human, plants and animals (4). The molecular weight of antimicrobial protein/peptide is 4 to 50kDa that plays an important role in the defense system of the plant and also inhibits various bacterial and fungal infections. From the different parts of plant like flower, seed, leaves, roots and stems, the antimicrobial protein/peptide extracted that work against phytopathogens as well as pathogenic microorganisms such as parasites, bacteria, fungi, virus and neoplastic cells (3).

M. oleifera is used for food and the extracts of this plant possess various curative antimicrobial characteristics (5). M. oleifera have coagulative activities on antitumor, antiepileptic, anti-inflammatory, waste water, diuretic, antioxidants, antiulcer, antibacterial, antidiabetic, cholesterol lowering, antispasmodic, circulatory stimulant, hepatoprotective, antifungal and antipyretic activities and also contain different forms of micro or macro elements (6). After multi-drug resistance, the antimicrobial potential of medicinal plant is studied all over the world and some of studies have been reported that different parts of M. oleifera possesses number of antimicrobial activity against many pathogenic microorganisms (7).

M. oleifera also act as antioxidant (1). Antioxidants have potential to stabilize and deactivate free radicals which may slowly damage components of cell. Plants have an effective antioxidant defense system such as polyphenol oxidase (PPO), catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) enzymes. The body contains some complex antioxidants that depends on endogenous enzymatic and non-enzymatic antioxidants. These molecules act against free radicals that protect body tissues and some essential biomolecules from damaging. Some of studies have suggested that exposure of free radicals can be minimized by consumption of antioxidant rich food and it also increases the potential of body that will reduce the risk of health problems. Antioxidants such as ascorbic acid, polyphenolic, alpha-lipoic acid, vitamin A and thioredoxin etc. play an important role in treatment and prevention of diseases caused by oxidative damage (8). The purpose of this study is to determine considerable antimicrobial proteins/peptides from M. oleifera leaves and flowers that act against the multidrug–resistant bacteria. In addition, antioxidant potential of this plant is also determined.

MATERIAL AND METHODS

Plant Material

The fresh leaves and flowers of Moringa oleifera were collected from the region of Lahore, Punjab. The leaves and flowers were washed with distilled water then dried and saved into dirt free container.

Crude Preparation

The protein extracts of leaves and flowers were prepared by using of phosphate buffer solution and liquid nitrogen. After treating with liquid nitrogen, the protein extracts were kept in freezer at -20℃ for 45min for complete lysis of cell wall and this step was repeated twice. After making of protein extract, centrifugation was done at 6000rpm for 2 to 3 min., supernatant was collected and stored at 4℃.

Ammonium sulfate precipitation

Liquid nitrogen and phosphate buffer solution processed samples were treated with different concentration of ammonium sulfate. The first fraction of ammonium sulfate (10%) was made for flowers whereas for leaves, second concentration of (20%) was made. After dissolving protein into ammonium sulfate, centrifugation was done at 15000rpm for 15 to 20 min. at 4℃ to get protein pellet, which was dissolved in phosphate buffer solution and stored at 4℃ (9).

Dialysis

The protein pellet; dissolved in phosphate buffer solution, was poured into dialysis membrane and both sides of tube were clipped with dialysis clips. Dialysis tube was placed overnight in distilled water with magnetic stir on magnetic plate (10).

Quantification of Protein and SDS-PAGE

The concentration of protein content in sample was determined by protein dye binding method Bradford at 595nm by using albumin as standard (11). The protein extracts of M. oleifera were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis, using 12% gel by method of Laemmli (12).

Antimicrobial Activity

Antimicrobial activity of protein extracts of M. oleifera leaves and flowers was used against Methicillin-resistant Staphylococcus aureus (MRSA), Escherichia coli and Salmonella typhi by using disc diffusion assay. The strains of each bacterium were swabbed on Muller Hinton agar plates after adjusting the turbidity of inoculum with 0.5 McFarland's standard. Protein solution was loaded onto sterilized discs and incubated at 37℃ for 24 hrs. Then zone of inhibition was measured by vernier caliper and assay was performed in triplicates.

Minimum Inhibitory Concentration (MIC)

MIC of flower protein extract was measured by serial dilution method. The different concentration was prepared. The inoculum was prepared from nutrient broth then tubes were incubated at 37℃ for 24hrs and antibacterial activity was determined respectively (13).

Antioxidant Assay

2,2-diphenyl-1-picrylhydrazyl (Free radical scavenging activity)

The DPPH (Free radical scavenging assay) was used to determine the scavenging activity. In the solution, 0.001g of DPPH were dissolved in 20ml of methanol. 1ml DPPH solution was added in 1ml of plant extract. The test tubes were incubated for 20min at 25℃. The blank solution was prepared in the same way without adding plant extracts. At 517nm, absorbance of plant extracts was measured by spectrophotometer. The following formula was used to find scavenging activity (14).

%Radical Scavenging activity= (Absorbance Blank– Absorbance Sample) × 100 ÷ Absorbance Blank

Enzyme Assay

Peroxidase Assay

The pyrogallol was used as substrate to determine peroxidase activity. The solution contains, 100mM phosphate buffer solution (7.0 pH), 5mM Hydrogen peroxide and 500µl of enzyme solution. Then 100µl of distilled water, 300µl phosphate buffer solution, 300µl of pyrogallol 100µl of hydrogen peroxide solution, and 500 µl of extract was added. The use of hydrogen peroxide increased the oxidation of pyrogallol and at 420nm, its absorbance was measured on spectrophotometer (15).

RESULTS

Plant Material

The plant (M. oleifera) flowers were yellowish-white in color with sweet light fragrance and the leaves were dark in color with round–elliptic shape as shown in figure 1.

Sample Preparation

The flowers and leaves were treated with liquid nitrogen and phosphate buffer solution that formed homogenized mixture: flower formed milky and light brown mixture while leaves formed thick and dark brown mixture as shown in figure 1.

Dialysis, protein quantification and analysis

The dialyzed protein extracts of flower and leaves showed clear white solution. Total contents of protein were evaluated by Bradford Reagent. The results showed that flower extract contains 10µg/ml of protein**.** Protein profile of both leaves and flower protein extract were determined on SDS-PAGE gel. The bands of sample were compared with universal marker weight. The bands of protein extract were visualized on gel and recorded from 9 to 59 kDa.

Antibacterial Activity

Antibacterial activity was evaluated by disk diffusion method. The selected bacterial strains were used for this activity and the results are shown in table 1. The antibacterial activity of plant extracts is described in table 1. The protein extract of flower showed higher activity against the strain MRSA (13.5±1.32) and *E.* coli (14.3±0.57) and lower activity against the *S. typhi* (11.4±0.49) as shown in figure 2. However, protein extract of leaves doesn't show any antibacterial activity against the selected bacterial strains.

Minimum Inhibitory Concentration

MIC of protein extract of flower was recorded at 166µg/ml against MRSA whereas against the *E. coli* and *S. typhi* MIC was (500µg/ml).

2,2-diphenyl-1-picrylhydrazyl (scavenging assay)

Antioxidant activity of *M. oleifera* was evaluated by free radical scavenging assay.

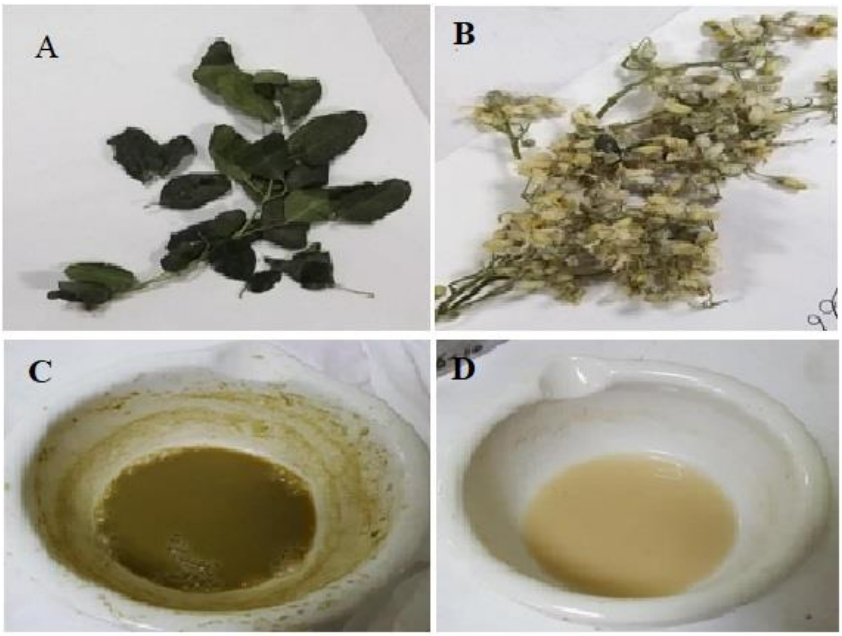

Figure1: *Moringa oleifera* (A) Leaves (B) Flower and (C) homogenized mixture of leaves and (D) homogenized mixture of flowers.

Table 1: Antimicrobial activity of protein extract of flowers and leaves against MRSA, *E.coli and S. typhi.*

Plant Extract	**Zone of inhibition in mm (mean ± standard deviation)**		
Flower	MRSA	*Escherichia coli*	*Salmonella typhi*
	13.5±1.32	14.3±0.57	11.4±0.49
Leave	0±	0±0	0±0

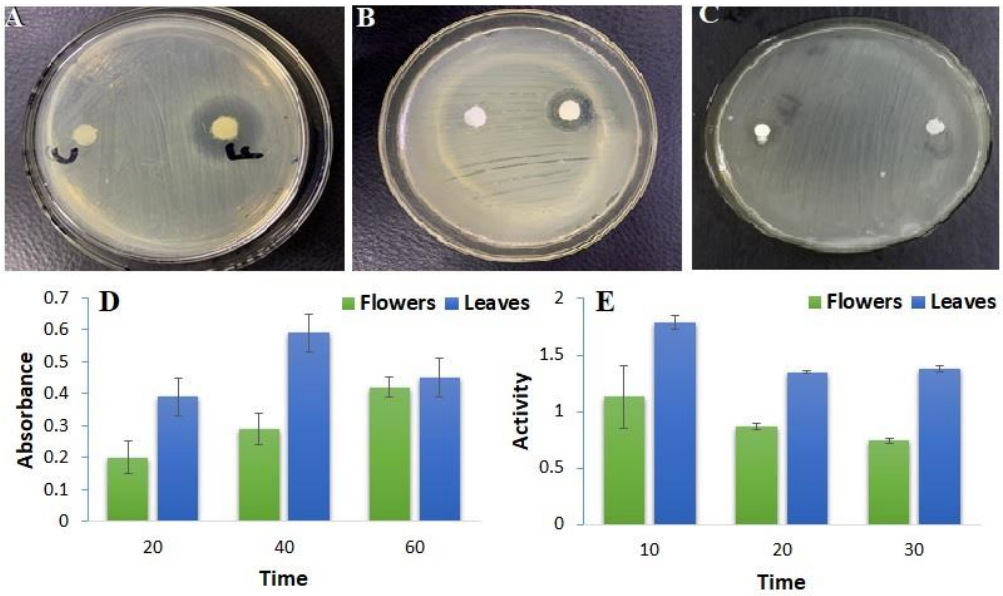

Figure 2: Antimicrobial activity of protein extarcts of *Moringa oleifera* against (A) MRSA (c:control; f:flower) (B) *E. coli* and (C) *S. typhi*. Determination of (E) DPPH free radical scavenging activity (E) Peroxidase activity of leaves and flowers of *M. oleifera*.

The protein extract of flower showed maximum antioxidant activity at time of 60 minutes i.e. **(**0.42±0.032) and minimum antioxidant activity at time of 20 and 40 minutes i.e. (0.243±0.051, 0.29±0.050) respectively. However, the protein extract of leave showed higher antioxidant activity at time of 40 and 60 minutes being (0.59±0.36, 0.45±0.40) but lower antioxidant activity at time of 20 minute (0.39±0.22). The data of DPPH free radical scavenging is shown in figure 2.

Enzyme Assay (peroxidase)

The protein extract of flower and leave showed higher enzymatic activity at time of 10 minute (1.13U/ml±0.28, 1.79U/ml±0.06) and they showed lower enzymatic activity at time of 20 and 30 minutes i.e. (0.867U/ml±0.03, 0.742U/ml±0.022, 1.35U/ml±0.006 and 1.38U/ml±0.03) as shown in figure 2.

DISCUSSION

Moringa oleifera is worldwide distributed plant and many of research reports has demonstrated that it contains number of nutritional and pharmacological properties and it also contains biological activity such as antimicrobial, anthelmintic, antifungal, antibacterial, anti–allergic activities and also possess some antioxidants (2).

In present work, antimicrobial proteins/peptides were extracted from leaves and flowers of *M. oleifera* by phosphate buffer extraction method. Different methods such as use of liquid nitrogen and phosphate buffer solution etc. are reported in previous studies (4).

In current study, the total concentration of proteins was evaluated by Bradford assay. While Lowery method can also be used for protein quantification as some previous study reported. (16). Lowery *et al*.,1951 illustrated that his method is sensitive and require simple procedure (17). While Kruger, 2009 suggested that the Bradford method is more simple, sensitive and faster as compared to Lowery method because it doesn't interfere with non-protein components of biological samples (18). After quantification, the crude protein extract of leave and flower of *M. oleifera* were loaded onto 12% of SDS-PAGE. A lower kDa of proteins has been observed from gel. As pervious study reported the lower kDa proteins range from seeds of millets (19), present study also showed lower than 100 kDa of protein bands of *M. oleifera* leaves and flower protein extracts. Dahot.,1988. reported that leaf extract of *M. oleifera* possess small protein/peptides which play an essential role in defense system of plant (16). Moyo *et al*.,2012 also demonstrated that peptides play an essential role in the plant defense system, as these peptides interact with anionic and fatty acids components of bacterial cell membrane that cause leakage of cytoplasmic contents and then peptides enter in the bacterial cell wall and activate autolytic enzymes (20).

The antimicrobial activity of different protein extract of plant (M. oleifera) was evaluated by disk diffusion assay against MRSA, E. coli and S. typhi. The results showed that flower protein extract showed maximum antimicrobial activity against MRSA and lower zone of inhibition against the E. coli and S. typhi. Previous studies have reported that flower methonalic extract showed higher antimicrobial activity against the E. coli and Salmonella spp (21). Another study has reported that leaf aqueous extract showed lower activity against the gram negative and gram positive bacteria (19).While some studies showed that leaf extract of M. oleifera have lower kDa protein peptide bands showing higher antimicrobial activity against the E. coli, K. pneumoniae, and S,aureus (16). Abalaka et al. showed least antimicrobial activity of leaf aqueous extract against E.coli, Salmonella spp, P. aeruginosa and S. aureus (19). Abalaka et al., 2012). Many of researchers have reported that either water extract showed least activity or no activity against the gram negative and gram positive bacteria, may be due to containing of such compounds like myriads that may act as antagonistically with all activities (20). These observations indicate that protein extraction method and resistance level of the pathogen influence the overall results of natural extracts. Gram +ve bacteria possess single layer of peptidoglycan which is permeable, while Gram –ve bacteria have thin layer of peptidoglycan on cell wall and outer membrane contains lipopolysaccharides which is impermeable and makes Gram +ve more sensitive than Gram- ve (20). Many pathogens show different levels of sensitivity or resistance to the extracts from different parts of the same plant based on the presence of protein/peptides and compounds that are murderous for pathogens. It is therefore, plants can be used for treatment of various infections caused by multidrug-resistant microorganism.

The MIC was determined by two-fold serial dilution to evaluate the MIC of extracted proteins. The results of current study reported that flower protein extract showed lower value of MIC against MRSA and higher values of MIC were recorded against *E. coli* and *S. typhi*. Previous study has been reported that gram positive bacteria showed higher MIC value then gram negative bacteria (23). As gram negative bacteria possess two layers of membranes (cell wall and outer membrane) which may have restricted the hydrophobic compounds interaction with cell membrane whereas gram- positive bacteria contain single layer of membrane through that hydrophobic compounds were easily damage the cell membrane (13, 24) .

Antioxidant activity was evaluated by DPPH (Free radical scavenging assay). The flavonoids and phenolic compounds transfer proton to DPPH that transform its color from purple to yellow (25). Result showed higher scavenging activity of flower extract at time of 40 minutes which shows that at it has high polarity and lower at time of 20 and 60 that shows lower polarity whereas leave shows higher scavenging activity at time of 40 and 60 and lower at time of 20. Arulmozhi and Wilson, 2015 reported higher scavenging activity of water extract of flower (26). Another study demonstrated that *M. oleifera* contains number of bioactive compounds such as carotenoids, alkaloids, flavonoids, tannins and vitamins etc. Many studies were conducted on different parts of *M. oleifera* that contains potent pharmacological properties e.g. leaves are being used for the treatment of diabetes, hypertension and typhoid fever whereas the other parts (seed, flower, roots) also possess potent bioactive compounds and biological activity against various infections (27). The antioxidant potential was determined by peroxidase assay. Results reported that leaf and flower crude protein extract showed higher activity at time of 10 and lower at time of 20 and 30. According to earlier reports, this plant has significant antioxidant capacity i.e., POD and catalase. It also showed remarkable wound healing activity which has great potential for development of plant-based product (27). Of note, extracts from different parts of *M. oleifera* possess potent bioactive compounds and show biological activity against the multi resistant microorganisms. Therefore, future studies may elucidate which proteins are specifically performing higher antimicrobial activity (26)

CONCLUSION

This study reveals that protein extract of flower showed higher antimicrobial activity against the MRSA and lower activity against *E. coli* and *S. typhi* whereas protein extract of leaves did not show antimicrobial activity against the tested microorganisms. The antioxidant activity of both extracts of flower and leave showed considerable enzymatic activity. Therefore, it was concluded that *M. oleifera* possess high potential pharmacological properties that can be used against the multi-resistant microorganisms and to develop novel drugs for many infections. More medicinal plant needs to be explored that possess potent antimicrobial activity against multi resistant microorganisms. Secondary metabolites and their characterization from different medicinal plant will be beneficial for the determination of active compounds that work against the various pathogens

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Disclaimer

We hereby declare that this research article is our own work. All texts either quoted directly or in paraphrased has been indicated by in-text citations. This work has not been submitted to any other examination authority.

Conflict of interest

There is no conflict of interest associated with this work.

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