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Purification of Bioactive Peptides from Edible Plants and Their Antibacterial Properties Against Food Borne Pathogens

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IM conceived idea and supervised. TM produce results after performing experimental work. SN draft and review. HM produced graphical presentation and statistical analysis. BM analyzed the data. MS supervised and review the project. All authors read and approved the final paper for publication.

Key words
Antimicrobial peptides, Edible plants, MIC, MBC, Food pathogens, Food preservation

ABSTRACT

To increase the shelf life of foodstuff and its preservation, using antimicrobial agents from natural sources is a promising approach. Antimicrobial peptides can combat pathogenic microorganisms either by killing them or inhibiting their growth. The objective is to investigate the antibacterial activity of plant peptides against food poisoning bacteria and to purify these peptides to find their application in the preservation of food. Fresh disease free leaves of selected plants after washing were powdered and proteins were extracted with protein extraction buffer (PBS) and Triton X 100. The proteins were precipitated with 85% ammonium sulfate solution, dialyzed, and purified by gel filtration chromatography. Two food spoiling bacterial strains i.e., *Bacillus cereus and Escherichia coli* were used for testing antimicrobial activity. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of purified peptides were determined and their effect on food material was checked. Inhibition activity of plant protein extracts from *Curcuma longa*, *Cycas revoluta, Punica granatum,* and *Moringa oleifera* exhibited strong antibacterial activity against *B. cereus* and *E. coli*. After gel filtration chromatography, peptide fractions with high biological activity were analyzed by SDS-PAGE and peptide fractions appeared as a single band with molecular weight < 15kDa. The results of MIC with bacterial suspension of 1.9×10^7 for *B. cereus* and 1.42×10^7 for *E. coli* CFU/ml revealed that *Moringa oleifera* peptide is the most effective antibacterial agent with MIC of 0.7µg/µl, and peptides exhibited same bactericidal activity against both microbes at MBC of 20µg. Temperature and pH sensitive studies showed that purified bioactive peptides were equally effective up to 35°C and pH 7.4 at 20µg concentration. Inactivation of peptides with trypsin revealed the proteinaceous nature of purified peptides. The application of these plant peptides as food preservative on peach slices suggested that these peptides could be utilized as natural alternate antimicrobial agents for food safety and preservation.

INTRODUCTION

Antibacterial substances are the class of elements that have potential to combat with pathogenic bacteria and reduce their pathogenicity by decreasing their metabolic activity (Eve, 2020). Different antimicrobial proteins/peptides have been isolated from microorganisms and also produced chemically or through fermentation process (Cowan, 1999). For centuries, a large number of plants and their extracts have been used in traditional medicines against bacterial infections (Okoli and Iroegbu, 2004). The medicinal plants are important due to the efficacy and safe properties of their constituents (Nascimento *et al*., 2000) and have been utilized for the treatment of transmittable diseases linked to antibiotic resistant strains.

Plant antibacterial peptides (ABPs) play an important role in the plant defense systems against pathogenic microbes. Some ABPs exhibit their specific activity towards Gram-negative or Gram-positive bacteria, however mostly active against both types (Barbosa *et al*., 2011). Chai *et al*. (2019) have reported plant ABPs which are found to be effective against food borne and food spoiling bacteria. The plant ABPs have been categorized into various groups named defensins, thionins (Chai *et al*., 2019), knottin-like

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proteins, and cyclotides (Flores *et al*., 2002) depending on their different properties (Games *et al*., 2016). Some promising mechanisms of action of ABPs against bacteria include membrane interruption and growth reticence (Nawrot *et al*., 2014). These peptides can be obtained from all the parts of plants which exhibit significant antibacterial activity against pathogenic bacteria (Jabeen and Khanum, 2017).

Food safety is a worldwide public health issue and various antimicrobials are commonly used to inhibit bacterial growth in different foodstuffs for decades (Kim *et al*., 2004). Many studies have also reported the use of plant extracts as antimicrobials in foods and soft drinks (Gulmez *et al*., 2006). The toxicity effects of chemical additives on public health have produced increasing interest in natural food preservatives (Suarez *et al*., 2003). ABPs are harmless and maintain the quality of food products during storage without compromising nutritional quality (Wang *et al*., 2011). Moreover, drug toxicity and hostile side effects of antibiotics have encouraged the use of plant antibacterial agents (Salas *et al*., 2015). The present study investigated the antibacterial activity of plant peptides from *Curcuma longa* (curcumin), *Cycas revoluta* (sago palm), *Moringa oleifera* (moringa), and *Punica granatum* (pomegranate) against food poisoning *B. cereus* and *E. coli* bacteria. The aim was also to purify these plant ABPs and to find their application in food preservation.

MATERIALS AND METHODS

Plant collection and microorganisms

Fresh disease-free leaves of twenty plants (Supplementary Table S1) were collected from the Botanical Garden of the University of the Punjab, Lahore, Pakistan. The leaves were washed with distilled water and finely powdered using liquid nitrogen.

Two food-spoiling bacterial strains i.e. *Bacillus cereus* and *Escherichia coli* used for testing antimicrobial activity were obtained from the culture bank of the University. Bacterial stock cultures were preserved at 4°C on LB agar slants.

Extraction of ABPs and antibacterial assay

0.2g of powdered leaves was taken in a test tube containing 0.25ml protein extraction buffer (PBS) and 0.5% Triton X 100. The mixture was vortexed for 30 sec and then centrifuged at 10,000 rpm at 4°C for 10 min. The clear supernatant was used for the detection of antibacterial activity (Zarei *et al*., 2011).

For sub-culturing, colonies of both strains were grown overnight at 37°C in Mueller Hinton (MH) (Oxoid Limited) broth in a shaking incubator. Optical density (OD) was measured at 600nm and diluted to maintain a viable cell count of 9×10^7 CFU/ml (10). The antibacterial activity of plant protein extract was estimated in triplicate against *B. cereus* and *E. coli* using agar well diffusion method. Agar plates were seeded with fresh culture under sterile conditions and 6 mm deep wells were made. The wells were loaded with 20µg proteins of each plant extract taking PBS as negative control (-C) and ampicillin as positive control (+C). The plates were incubated at 37°C for 24 h and checked for inhibition zones. Their diameters were measured in mm.

Peptide purification

Plant proteins showing high antibacterial activity were precipitated with 85% ammonium sulfate saturation. The solution was centrifuged at 10,000 rpm for 15 min at 4°C and the pellet containing precipitated protein was dissolved in PBS (pH 7.4). Both supernatant and dissolved ammonium sulfate pellet were dialyzed against the same buffer at 4°C using dialyzing tube with MWCO 6-8 kDa (Millipore Sigma). The antibacterial activity of the supernatant and the dissolved pellet was checked against both bacteria. Protein contents were assessed in crude extract, supernatant, and dissolved pellet by Bradford method (Bradford, 1976).

The dialyzed protein solution was subjected to gel filtration chromatography by applying the extract to a Sephadex G-75 column (Sigma Chemical Co.). 0.02M phosphate buffer (pH 7.0) was used as an elution buffer and the flow rate was maintained at 1ml/minute. 50 fractions (0.5 ml each) of plant peptides were collected and assayed for protein contents. Phytochemical analyses were performed to detect the presence of flavonoids, terpenoids, and alkaloids in crude extract, and purified preparations.

Homogeneity and molecular weight of purified peptides was determined by SDS-PAGE (Laemmli, 1970). After electrophoresis, gel was stained with 0.01% coomassie brilliant blue R-250 dye solution containing 30% methanol and 10% glacial acetic in distilled water. Excess dye was removed by destaining the gel with 30% methanol and 10% glacial acetic acid in distilled water.

Phytochemical analysis

Preliminary phytochemical analyses of the crude extracts and purified preparations were carried out for the existence of phytochemicals using the procedures described by Khan *et al*. (2011) and Wadood *et al*. (2013). For detection of flavonoids 5ml of 10% ammonia solution was added in a 10ml aqueous solution of test sample and filtered through Whatmann filter paper No.1. The appearance of yellow color after addition of 2ml of concentrated sulphuric acid confirmed the presence of

flavonoids (Khan *et al*., 2011). For detection of terpenoids 1ml of the chloroform and 2ml of concentrated sulphuric acid were added in 2 ml test sample extracted with methanol and filtered using Whatmann filter paper No.1. Formation of reddish brown color confirmed the presence of terpenoids (Wadood *et al*., 2013). For detection of alkaloids 5ml of 2% HCl was added in a 3ml test sample was prepared in hexane and filtered through Whatmann filter paper, and test tubes were heated. The mixture was filtered again, few drops of picric acid were added. The appearance of orange-red color indicated the presence of alkaloids (Wadood *et al*., 2013).

Antibacterial assay of purified peptide

Purified ABPs were used to determine their MIC using agar well method and their efficiency was measured in controlling food spoiling bacterial strains (Nawrot *et al*., 2014). Different concentrations of peptide ranging 0.5 to 20.0µg were prepared in PBS buffer (pH 7.4) separately, filtered through Millipore filter and their requisite amount was loaded on sterilized agar well plates. The plates prepared in triplicates were kept for 30 min at room temperature and then incubated at 37°C for 24 h. Diameter of inhibition zones was measured in mm.

The lowest concentration of purified antimicrobial peptide where no microbial growth appeared after 24 h was considered as minimum bactericidal concentration (MBC) (Nawrot *et al*., 2014). Cells were taken from inhibition zones of MIC plates and sub-cultured on sterile trypton soya agar (TSA) (Oxoid Limited) plates. Plates were incubated at 37°C for 24 h and examined bacterial growth.

Effect of temperature, pH and protease on antibacterial activity

Effect of temperature was assessed on purified ABPs at various temperatures ranging 30-45°C by incubating known amount of peptide for 30 min and then bioassayed. The influence of pH on antibacterial activity of isolated peptide was determined in buffer solutions of pH ranging 5.4-8.0 at room temperature for 30 min. Impact of pepsin on antimicrobial activity of peptide was evaluated by incubating 100µl reaction mixture containing 4U (1U/µl) of pepsin in 0.02M Tris–HCl, pH 7.4 and incubated at room temperature for 30 min. Enzyme was deactivated by heating for 5 min and activity before and after heating was checked by agar well method.

Antibacterial activity on peach slices

Purified peptide exhibiting high antibacterial activity was used to find its potential in food preservation (Wang *et al*., 2004). Fresh peach was cut into slices, washed with 70% ethanol and rinsed with deionized water. Sterile slices were treated with purified peptide and kept at room temperature for 30 min. The fruit slices were then inoculated with 100µl of culture medium containing pathogenic *B. cereus* and *E. coli* strains maintaining 7-9×10^7 CFU/ml. Peach slices without peptide were used as control. Samples were then incubated at 37°C for 24 h and cell counts were determined by counting colonies.

Statistical analysis

Data obtained was represented as arithmetic mean ± SD using SAS system version 9.1.3 (Cary, NC). $P < 0.05$ was considered significant. One-way analysis of variance (ANOVA) and Tukey`s HSD tests were used to find significant differences among mean treatments using SPSS statistical software package (SPSS, version 23.0, USA).

RESULTS

Antibacterial activity in various plants

Amongst various plant protein/peptide extracts tested for their antibacterial activity (Supplementary Table S1), four plant extracts; *Curcuma longa*, *Cycas revoluta*, *Punica granatum* and *Moringa oleifera* showed promising results by displaying antibacterial activity on LB agar against *B. cereus* and *E. coli*. Highest antimicrobial activity was observed in protein extract of *M. oleifera* with zone of inhibition (ZOI) of 21±0.11mm while comparatively least activity was found in *P. granatum* extract (ZOI 16±0.10mm) against *B. cereus* (Table I). Protein extract of *C. longa* was found to be more effective against *E. coli* with ZOI 18±0.12mm while *C. revolute* extract showed minimum activity (ZOI 16±0.14mm) against *E. coli*.

Table I. Zone of inhibition (mm) of crude (C) and precipitated plant protein extracts.

Plant sample	ZOI against *B. cereus* (mm)		ZOI against *E. coli* (mm)	
	C	P	C	P
C. longa	20±0.13^b	20±0.12^b	18±0.12^a	20±0.11^b
C. revoluta	18±0.12^c	18±0.11^c	16±0.14^c	19±0.12^b
P. granatum	16±0.10^d	17±0.12^d	17±0.16^b	17±0.14^d
M. oleifera	21±0.11^a	23±0.10^a	17±0.13^b	18±0.16^c

Superscript letters (a-d) indicate means which are significantly ($p < 0.05$) different.

Table I also shows antibacterial activity of purified plant protein extracts. Plant protein extracts were precipitated with 85% ammonium sulphate saturation and pellets obtained were subjected to antibacterial assay and found to be active against test microbes as indicated by their inhibition zones. The dissolved ammonium

sulfate precipitates of *M. oleifera* exhibited the highest antimicrobial activity (23±0.10mm) and least with *P. granatum* (17±0.12mm) against *B. cereus* (Table I). Against *E. coli*, dissolved ammonium sulfate precipitates of *C. longa* exhibited strong inhibitory activity (20±0.11mm).

Precipitated protein extracts were subjected to gel filtration chromatography using Sephadex G-75 column. Elution profiles of samples with 0.02 M phosphate buffer (pH 7.0) are shown in Figure 1. Active fractions exhibiting antibacterial activity against test microorganisms were pooled and resolved on 12% SDS-PAGE gel. Single band of each preparation was obtained and molecular weight of purified peptide of *C. longa, C. revolute, P. granatum and M. oleifera* was found to be 12kDa, 14kDa, 10kDa and 8kDa, respectively (Fig. 2).

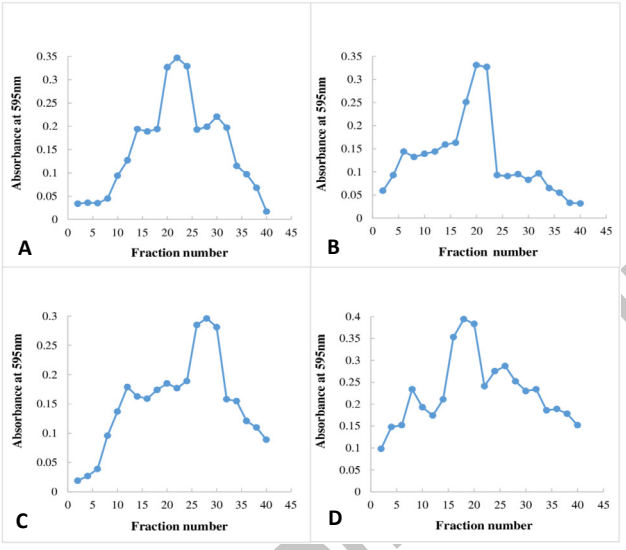

Fig. 1. Elution profile of (A) *Curcuma longa*, (B) *Cycas revolute,* (C) *Punica granatum* and (D) *Moringa oleifera* after GFC using Sephadex G-100.

Phytochemical analysis of plant extract

In order was to ensure that antibacterial activity exhibited in purified fractions was solely due to peptides, fractions were assayed for alkaloid, terpenoid and flavonoid. Phytochemical screening showed that plants under study were the source of these phytochemicals. Color reactions used for the detection of phytochemicals in these plant crude extracts were positive while the respective purified preparations produced no such coloration.

Antibacterial activity of ABP against foodborne microorganisms

Colony forming units (CFU/ml) were maintained to be 1.9×10^7 for *B. cereus* and 1.42×10^7 for *E. coli*. The results of *B. cereus* and *E. coli* growth at increasing concentration of purified peptides (0.7 to 20.0µg) indicated that both strains were sensitive with same MIC of 2.5µg for *C. longa*, *C. revoluta*, and *P. granatum* while microbial growth was inhibited at MIC of 0.7µg for *M. oleifera* peptide therefore, Moringa was the most effective antibacterial agent ($p < 0.05$) (Table II).

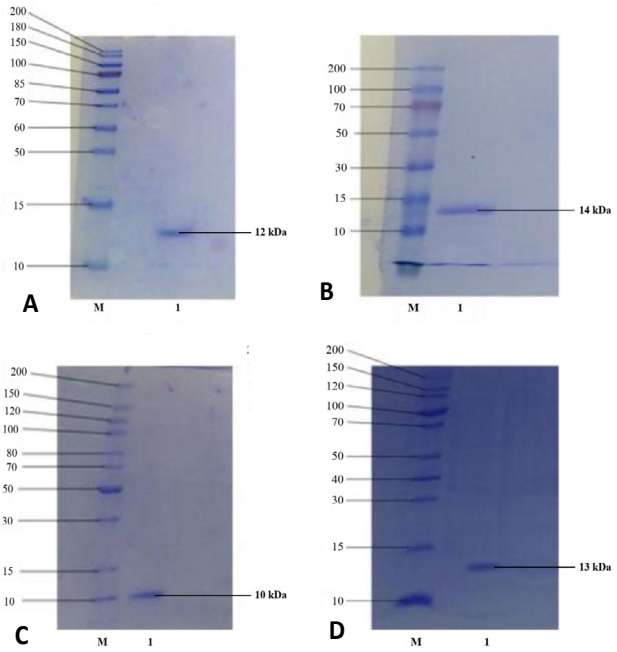

Fig. 2. SDS PAGE analysis of antibacterial peptides purified from (A) *C. longa*; (B) *C. revolute*; (C) *P. granatum* and (D) *M. oleifera*. M: Molecular weight markers; Lane 1: Purified peptides after gel filtration chromatography.

Purified peptides also displayed equal potential against both microbes at an MBC of 20µg. In case of *M. oleifera*, very small bacterial growth appeared in TSA plate inoculated with colonies from MIC plates at 15µg concentration against *B. cereus* while *E. coli* showed more sensitivity with *C. longa* with MBC of 15µg.

Effect of temperature on antibacterial activity of purified peptides

Temperature impacts greatly on biological activity and stability of proteins. In our study, at 30 °C *M. oleifera* crude protein exhibited significantly high ($p<0.05$) antibacterial activity (21±0.11mm) against *B. cereus* compared to other proteins. However, with increase in temperature, there was significant decrease ($p<0.05$) in antibacterial activity of *M. oleifera* and at 45°C, only 10±0.11mm inhibition was observed (Table III).

For *E. coli, M. oleifera* and *C. longa* showed

significantly high ($p<0.05$) antibacterial activity against *E. coli*. At 30°C there was no significant difference ($p>0.05$) in antibacterial activity of *C. longa* (19±0.11) and *M. oleifera* (19±0.12) however, activity decreased significantly ($p<0.05$) with increase in temperature (Table III).

Table II. MIC values of purified peptides against *B. cereus* and *E. coli*

Concentrations (µg/µl)	Inhibition zones (mm)			
	C. longa	*C. revolute*	*P. granatum*	*M. oleifera*
B. cereus				
0.5	0^{f}	0^{f}	0^{f}	0^{h}
0.7	0^{fB}	0^{fB}	0^{fB}	$7±0.3^{gA}$
1.25	0^{fB}	0^{fB}	0^{fB}	$11±0.62^{fA}$
2.5	$10±0.26^{eB}$	$7±0.28^{eC}$	$10±0.36^{eB}$	$15±0.1^{eA}$
5	$15±0.3^{dB}$	$11±0.35^{dD}$	$14±0.15^{dC}$	$20±0.1^{dA}$
10	$16±0.17^{cC}$	$14±0.15^{cD}$	$18±0.1^{cB}$	$22±0.26^{cA}$
15	$19±0.4^{bC}$	$17±0.1^{bD}$	$20±0.17^{bB}$	$23±0.23^{bA}$
20	$21±0.1^{aC}$	$20±0.5^{aD}$	$22±0.2^{aB}$	$25±0.3^{aA}$
E. coli				
0.5	0^{f}	0^{f}	0^{f}	0^{h}
0.7	0^{fB}	0^{fB}	0^{fB}	$10±0.26^{gA}$
1.25	0^{fB}	0^{fB}	0^{fB}	$15±0.17^{fA}$
2.5	$12±0.1^{eB}$	$10±0.3^{eC}$	$12±0.45^{eB}$	$18±0.1^{eA}$
5	$15±0.3^{dC}$	$14±0.34^{dD}$	$16±0.17^{dB}$	$20±0.1^{dA}$
10	$17±0.2^{cC}$	$16±0.17^{cD}$	$18±0.36^{cB}$	$21±0.26^{cA}$
15	$18±0.5^{bC}$	$18±0.2^{bC}$	$19±0.5^{bB}$	$23±0.4^{bA}$
20	$20±0.1^{aC}$	$19±0.43^{aD}$	$21±0.1^{aB}$	$24±0.45^{aA}$

Small superscript letters ($^{a-h}$) within a column (for each test bacteria) indicate means which are significantly different ($p < 0.05$), whereas capital superscript letters ($^{A-D}$) within a row (for each test bacteria) indicate significantly different mean observations.

Table III. Effect of temperature on antimicrobial activity (diameter of inhibition zone in mm) of crude plant protein extract against *B. cereus* and *E. coli.*

Sample	30°C	35°C	40°C	45°C
B. cereus				
M. oleifera	$21±0.11^{aA}$	$15±0.10^{aB}$	$15±0.10^{aB}$	$10±0.11^{aC}$
C. revoluta	$17±0.12^{dA}$	$12±0.14^{bB}$	^{bC} _	^{bC} _
P. granatum	$18±0.13^{cA}$	$10±0.16^{cB}$	^{bC} _	^{bC} _
C. longa	$19±0.14^{bA}$	^{dB} _	^{bB} _	^{bB} _
E. coli				
M. oleifera	$19±0.12^{aA}$	$16±0.14^{aB}$	$15±0.15^{aC}$	$10±0.12^{aD}$
C. revoluta	$17±0.14^{cA}$	$14±0.10^{bB}$	^{bC} _	^{bC} _
P. granatum	$18±0.13^{bA}$	$11±0.13^{cB}$	^{bC} _	^{bC} _
C. longa	$19±0.11^{aA}$	^{dB} _	^{bB} _	^{bB} _

Small superscript letters ($^{a-c}$) within column indicate means which are significantly different ($p < 0.05$), whereas capital superscript letters ($^{A-C}$) indicate significantly different mean observations.

Effect of pH on antibacterial activity of purified peptide

Literature shows that pH variation may alter peptide structural alignment thereby increasing/decreasing the antibacterial activity. In this study, all proteins showed significantly high ($p<0.05$) activity at pH 7.4 against *E. coli* and *B. cereus*, in comparison to other pH treatments (Table IV). At pH 5.4, all plant proteins showed significantly lower ($p<0.05$) activity which significantly increased ($p<0.05$) with increase in pH up to 7.4. Further increase in pH resulted in considerably decrease in their antibacterial potential as revealed by their inhibition zones.

Table IV. Effect of pH on antimicrobial activity (diameter of inhibition zone in mm) of crude plant protein extract against *B. cereus* and *E. coli*

Samples	pH 5.4	pH 6.4	pH 6.8	pH 7.4	pH 8.0
B. cereus					
C. longa	$6±0.13^{eE}$	$11±0.13^{bC}$	$14±0.13^{cB}$	$18±0.13^{bA}$	$9±0.10^{aD}$
C. revoluta	$6±0.16^{eE}$	$9±0.16^{cC}$	$12±0.16^{dB}$	$16±0.16^{dA}$	$8±0.14^{bD}$
P. granatum	$8±0.13^{bD}$	$11±0.13^{bC}$	$15±0.13^{bB}$	$17±0.12^{cA}$	$7±0.03^{cE}$
M. oleifera	$10±0.13^{aD}$	$13±0.12^{aC}$	$16±0.14^{aB}$	$19±0.11^{aA}$	$9±0.15^{aE}$
E. coli					
C. longa	$8±0.21^{aD}$	$12±0.21^{aC}$	$15±0.21^{aB}$	$19±0.21^{aA}$	$7±0.09^{bF}$
C. revoluta	$8±0.11^{aD}$	$11±0.11^{bC}$	$15±0.11^{aB}$	$19±0.11^{aA}$	$8±0.11^{aD}$
P. granatum	$6±0.13^{bD}$	$10±0.23^{cC}$	$13±0.16^{bB}$	$18±0.14^{bA}$	$6±0.14^{cD}$
M. oleifera	$6±0.11^{bE}$	$9±0.12^{dC}$	$12±0.10^{cB}$	$17±0.13^{cA}$	$7±0.10^{bD}$

Small superscript letters ($^{a-d}$) within column indicate means which are significantly different ($p < 0.05$), whereas capital superscript letters ($^{A-E}$) indicate significantly different mean observations.

Effect of proteolytic enzyme on antibacterial activity of purified peptide

To confirm antibacterial activity of purified peptides against test microbes is solely due to their proteinaceous origin, peptides were checked for their sensitivity to pepsin and residual activity was determined. No inhibition zones were observed when peptides were treated with pepsin with or without heating establishing that antibacterial activity of peptides was exclusively due to their protein nature and lost as result of their hydrolysis by pepsin.

Antibacterial assay on peach slices

We also evaluated the potential of purified peptides as food preservative agent on peach slices inoculated with *B. cereus* and *E. coli* for 48 h using 25µg protein concentrations. Slices without peptide, taken as control, became rotten as a result of bacterial growth while samples treated with peptides greatly reduced microbial count and remained fresh even after 48 h (Fig. 3).

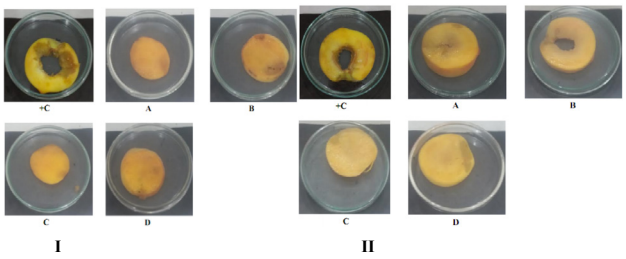

Fig. 3. Inhibition of *B. cereus* (I) and *E. coli* (II) growth on peach slices by antibacterial peptide purified from *C. longa* (A); *C. revolute* (B), *P. granatum* (C); *M. oleifera* (D) after 48 h using 25µg concentrations. +C indicates positive control without purified peptide.

DISCUSSION

People have been using different plants and natural products effectively as traditional therapeutics against pathogens for treatment of various diseases. Multiple-drug resistance and side effects of antibiotics has urged to find non-toxic natural antibacterial agents that can inhibit growth or kill bacteria causing different diseases (Dabur *et al*., 2007). These plant constituents are important due to their less toxicity, greater activity against antibiotic resistant pathogens and relative economic efficiency (Kumar *et al*., 2006; Kim *et al*., 2009).

Present study described the isolation of ABPs from different plants which are the part of their natural defense system and have biotechnological and pharmaceutical applications (Kumar *et al*., 2006; Li *et al*., 2021). Many medicinal plants and herbs producing antibacterial agents are reported and their potential against different microbes are studied (Nielsen *et al*., 2012; Marasini *et al*., 2015; Al-Akeel *et al*., 2017; Hansen *et al*., 2020). In present study, maximum activity was observed in protein extract of *M. oleifera* against *B. cereus* while *C. longa* extract exhibited highest activity against *E. coli*.

Studies relating to catalytic activities, kinetics, and response to different regulators can be conducted with purified peptides. In this study, dissolved ammonium sulfate precipitates of plant extracts displayed enhanced bioactivity against test microorganisms as compared to their crude extracts. The ABPs were also checked in the supernatants obtained after ammonium sulphate precipitation, however no inhibition activities were observed. After gel chromatography of precipitated proteins, molecular weight of purified peptides of *C. longa, C. revolute, P. granatum and M. oleifera* on SDS-PAGE was estimated as 12kDa, 14kDa, 10kDa and 8kDa, respectively. Researchers have also reported low molecular weight ABPs similar to our study like 10kDa peptide from *Momordica charantia* (Jabeen and Khanum, 2017) and 9.03kDa from *Phaseolus mungo* (Wang *et al*., 2004). Interestingly, a very low molecular mass entomocin peptide (4.8kDa) as compared to our peptides is reported in *B. thuringiensis* by Cherif *et al*. (2008), on the other hand, high molecular weight peptides are also reported such as in the leaves of *Trianthema portulacastrum* (>20kDa) by Samriti and Biswas (2019). Still, literature has reported ABPs showing diversity in molecular weight as low as 2kDa described by Sharma *et al*. (2011).

Different bioactive compounds produced in plants due to primary/secondary metabolism are called phytochemicals (Mendoza and Silva, 2018). These are also beneficial against various microbial diseases due to their antimicrobial activities (Huang *et al*., 2016). Therefore, to make sure regarding the biological activity in purified preparations exclusively due to peptides, phytochemicals were investigated in the study. Crude extracts produced color reactions for phytochemicals however, purified peptides with same screening procedures, and did not reveal their presence suggesting that biological activity was due to peptides in the purified preparations.

MIC and MBC of plant extracts were assessed for their bacteriostatic and bactericidal properties. The studies depicted that *B. cereus* and *E. coli* were sensitive with same MIC of 2.5µg for *C. longa*, *C. revoluta*, and *P. granatum* however, the strongest antibacterial was observed for peptide from *M. oleifera.* MBC was established by absence of bacterial growth of both strains streaked form their inhibition zone according to their lowermost MIC values. Interestingly, all these plant peptides exhibited same bactericidal activity against both bacteria. Mostafa *et al*. (2018) has also reported the effectiveness of *P. granatum* and *S. aromaticum* ethanolic extracts against food pathogenic bacteria like *S. aureus* and *P. aeruginosa* with MIC's ranging 2.5-5.0mg/ml. In another study, amongst traditional plants evaluated by Marasini *et al*. (2015) for their antibacterial action, *C. longa, C. camphora,* and *C. orchioides* extracts displayed inhibition activity of MIC <100µg/ml. A peptide, turgencin reported by Hansen *et al*. (2020), exhibited strong antimicrobial potential against gram-negative and gram-positive bacteria with MIC of 0.4µM. Variance in MIC of different plant is reported by researchers and according to Mostafa *et al*. (2018), such differences might be due to adaptation of various extraction methods, composition, nature of their constituents and test strains.

Temperature dependent studies showed that temperature revealed a variable impact on antimicrobial activity of peptide on test bacteria. *M. oleifera* peptide displayed considerably high ($p<0.05$) antibacterial activity against *B. cereus* at 30 °C, for *E. coli,* both *M. oleifera*

and *C. longa* peptides showed substantial high ($p<0.05$) activity at the same temperature. Research findings have reported high optimum temperature like *Momordica charantia* exhibited considerable activity at 50°C (10) and *S. cerevisiae* even showed significant activity up to 90°C (Thyab *et al*., 2020).

Physicochemical properties of proteins largely depend on their specific native conformation. A large change in pH may disturb protein structure and then affect their pH dependent properties i.e., antimicrobial activity in our case. All peptides exhibited lower activity at pH 5.4 which then significantly enhanced when pH increased from 5.4 to 7.4. A narrow pH rang is also reported for peptide from *Momordica charantia* i.e., 5.0-7.0 with maximum activity at pH 7.0 against *S. aureus* and *E. coli*. Contrarily, Baindara *et al*. (2016) have reported a wide pH range of 2.0-12.0 for penisin peptide from *Paenibacillus* sp.

In our study to check that antibacterial activity of peptides against microbes was due to their proteinaceous nature, their sensitivity to pepsin was determined. Comparable results are also observed previously by Cherif *et al*. (2008) where inhibition activity of entomocin peptide was completely lost after treatment with proteinase K revealing its protein nature.

Different plant ABPs possess competency to kill microbes or retard their growth against microorganisms that show resistance against traditional chemicals/ antibiotics (Seydim and Sarikus, 2006). In food processing, plant peptides are found to be very effective as food preservatives and capable to increase the shelf life of foodstuff by protecting from food spoiling bacteria (Joshi *et al*., 2018). Their application as biopreservative was evaluated on peach and found potentially effective in controlling the bacterial growth on peptide treated peach samples. Our results on food preservation are in agreement with previous studies which reported considerable potential of the peptides used in preserving minced meet and green olives, respectively (Upendra *et al*., 2016; Jabeen and Khanum, 2017).

CONCLUSION

Plants are useful for the extraction of ABPs because they are rich in proteins, easily available and cost effective. Among selected plant peptides, *M. oleifera* peptide was found to be the most effective antibacterial agent. This study can be exploited to use these plant peptides as efficient and less toxic food preservatives against different food-borne pathogens. Their role as natural alternative antimicrobial agents is valuable that evade health hazards of chemical antimicrobial substances. In future, synergy of these peptides with other compounds could be assessed to enhance their antibacterial activity and find their potential in health and food industry.

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Supplementary material

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Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

Purification of Bioactive Peptides from Edible Plants and Their Antibacterial Properties Against Food Borne Pathogens

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Supplementary Table S1. Taxonomic classification and medicinal uses of levels of selected plants.

Scientific name	Common name	Family	Genus/ specie	Medicinal uses
Alstonia scholaris	Blackboard tree	Apocynaceae	*Alstonia scholaris*	Pharmacological drugs, anticancer, dietary fiber source
Bauhinia variegate	Kachnar	Caesalpiniaceae	*Bauhinia variegate*	Antibacterial, antifungal, swelling reducing, thyroid hormone regulating
Cestrum nocturnum	Raat ki Rani	Solaceae	*Cestrum nocturnum*	Treatment of epilepsy, antibacterial
Cordia myxa	Lasura	Boraginaceae	*Cordia myxa*	Diuretics, demulcents, stomach aches and coughs
Curcuma longa	Haldi	Zingiberaceae	*Curcuma longa*	Antibacterial, strong healing agent, anti-inflammatory agaent
Cycas revoluta	Sago palm	Asparagaceae	*Chlorophytum acutum*	Immunity booster
Ficus benjamina	Black fig	Moraceae	*Ficus benjamina*	Source of fibre
Ficus carica	Fig	Moraceae	*Ficus carica*	Anti-inflammatory, antioxidant, good remedy for insect bites and stings, digestion promoter and cure for ulcer
Ficus religiosa	Sacred fig	Moraceae	*Ficus religiosa*	Traditional medicine to cure asthma, epilepsy, sexual disorders, gastric problems, inflammatory infectious disorders, diarrhea
Foeniculum vulgare	Fennel	Apiaceae	*Foeniculum vulgare*	Antifungal, antibacterial, antioxidant, antithrombotic
Moringa oleifera	Sohanjana	Moringaceae	*Moringa oleifera*	Antioxidant, reduce inflammation, lower cholesterol
Murraya koenigii	Curry tree	Rutaceae	*Murraya koenigii*	Herb containing anti-disease properties used as traditional medicine
Phaseolus vulgaris	Beans	Fabaceae	*Phaseolus vulgaris*	Anti-diabetic, anti-obese, starch blockers, have weight loss properties

Table continued on next pages.....

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0030-9923/2025/0001-0001 $ 9.00/0

Scientific name	Common name	Family	Genus/ specie	Medicinal uses
Plumbago capensis	Plumbago	Plumbaginaceae	*Plumbago capensis*	Treat lead poisoining, wound healing
Punica granatum	Pomegranate	Lythraceae	*Punica granatum*	Antibacterial, antifungal, swelling reducing, thyroid hormone regulating
Ravenala madagas-cariensis	Traveller's palm	Strelitziaceae	Ravenala madagas-cariensis	Antiseptic, contain healthy sterols such as stigma sterols, rich in sugar
Reseda odorata	Mignonette	Resedaceae	*Reseda odorata*	Herb containing anti-disease properties used as traditional medicine
Tamarindus indica	Tamarind	Apiaceae	*Tamarindus indica*	Antifungal, antibacterial, antioxidant, antithrombotic
Terminalia chebula	Harar	Combretaceae	*Terminalia chebula*	Antitussive, cardiotonic, homeostatic, diuretic, laxative, used to treat kidney disorders,
Thuja occidentalis	White-cedar	Cupressaceae	*Thuja occidentalis*	Disinfectants, insecticide, relieve constipation and headache, anti-fungal