doi.org/10.36721/PJPS.2022.35.4.SP.1159-1168.1

Interference of morintides on some virulence determinants of selected bacterial pathogens

Qudsia Jaffar[1], Uzma Qaisar[2], Muhammad Shahid[1], Nisar Ahmed[3] and Amer Jamil[1]*
[1]Department of Biochemistry, University of Agriculture Faisalabad, Pakistan
[2]School of Biological Sciences, University of the Punjab, Lahore, Pakistan
[3]Center of Agricultural Biochemistry and Biotechnology, University of Agriculture Faisalabad, Pakistan

Abstract: In this study, small antimicrobial peptides of <10 kDa (AMPs) from *Moringa oleifera* L were separated from crude protein by Ultra-15 Centrifugal filter devices and partially purified. The potency of morintides to interfere with the virulence determinants like biofilms, siderophores and elastase of selected bacteria was investigated by spectrophotometric method and Pseudomonas quinolone signal (PQS) by Thin-layer chromatography (TLC). GraphPad Prism 5.0 was used for statistical purpose. Assays were subjected to a two-way analysis of variance with Bonferroni as post-test. *Shigella flexneri, Klebsiella pneumoniae, Escherichia coli* and *Staphylococcus aureus*, showed a decrease in the attachment of cells to form biofilm in the presence of the morintides. While *Pseudomonas aeruginosa* and *Salmonella typhi* showed an increase in the attachment of cells to form biofilms. Morintides were very effective in the disruption of developed biofilms in cases of *S. aureus, E. coli, K. pneumoniae* from surgical wounds and *S. flexneri*, while others remained ineffective in the disruption of developed biofilms. Siderophore production was decreased by all bacterial strains under investigation in the presence of morintides except *P. aeruginosa*. The outcome of the research may have a significant contribution to drug discovery against antibiotic resistant biofilms of bacteria.

Keywords: Biofilms, elastase, *Moringa oleifera,* morintides, siderophores, virulence factors.

INTRODUCTION

Bacterial infection is the main reason for mortality, especially in developing countries, where wound damages are massively increasing day by day due to gunshot, bomb blasts, road *accidents* and earthquakes. Wounds provide a beneficial environment for bacteria to grow, nourish and colonize (Waheed *et al*., 2022). One major cause of increased resistance to wounds, either burn or surgical, is the development of biofilms which are bacterial communities attached to a solid surface and create a mesh-like appearance. The biofilms secrete several polymeric substances and are adherent to each other along with the solid surface (Onsare *et al*., 2014). Biofilms are a thousand times more resistant than commonly found bacterial strains. These biofilms cause several chronic infectious diseases i.e. kidney stones, lung infections and bacterial endocarditis and often get worse leading to the death of most of the patients and hence the survival rate is decreased (Khalid *et al*., 2018). *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae* and *Vibrio cholera* are notorious for the formation of biofilms on mucosal and epithelial surfaces and are lethal for patients suffering from chronic diseases (Hall-Stoodley and Stoodley, 2005).

Antimicrobial peptides (AMPs) are produced in various organisms as a defense against pathogens. The AMPs in plants generally have 10-50 amino acids with cationic charge, however, they differ significantly in structure and sequence. *Moringa oleifera* Lamark (Moringaceae) is a medicinal plant with almost all essential elements like minerals, amino acids, proteins, vitamins, growth factors and important ions like zinc, potassium and iron (Patil *et al*., 2022) and it has diverse bioactivities like antibiotic, antioxidant, anticarcinogenic, (Penalver *et al*., 2022 and Suhartono *et al.,* 2019). However, little work has been reported on AMPs from *M. oleifera.* Here we report AMPs designated as morintides isolated from *M. oleifera* and their effect on bacterial quorum sensing mediated virulence factors. The findings of this work may lead to broad applications of morintides in the therapeutic and pharmaceutical industries against the problems of devastating antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains

Pure growth of each bacterial species listed in table 1 was tested for their purity by specific biochemical tests. Bacterial cultures were streaked on Luria Bertani agar (LB) (1% NaCl, 1% tryptone, 1.5% agar 0.51% yeast extract). Culture plates were incubated at 37ºC for 16h and were stored at 4ºC. Overnight growth of the culture was adjusted to $1x10^8$ CFU/ml.

Sample collection and identification of plant materials

Leaves of *Moringa oleifera* L. were taken from the Botanical Garden of the University of the Punjab Lahore Pakistan. Identified and authenticated of the plant species

**Corresponding author:* e-mail: amerjamil@yahoo.com

was done by a taxonomist from Department of Botany, University of the Punjab Lahore.

Extraction of proteins and peptides from Moringa oleifera L

Fresh five kilogram leaves of *Moringa oleifera* L. were taken and washed with water, wrapped into aluminum foil, frozen into liquid nitrogen and then stored at -80°C till further analysis. Protein extraction was carried out with sodium phosphate buffer protocol with slight modifications (Khalid *et al*., 2018). Fresh cleaned leaves were ground in a pestle and mortar with liquid nitrogen to get 100 g fine powder. The fine powder was suspended in ice-cold 300ml of sodium phosphate buffer (15mM NaH_2PO_4, 10mM Na_2HPO_4, 2mM EDTA, 100mM KCl, 1mM PMSF, 1.5% PVPP, 2mM thiourea). The sample was vortexed, incubated stirred at 4ºC for overnight and centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was collected and filtered through Whatman filter paper 1 (pore size: 11 μm) at 4°C and then added to Amicon Ultra-15 Centrifugal filter devices, 10,000 Da MMCO (Millipore, UFC 9010024) (Thomas *et al*., 2017 and Khalid *et al*., 2018). 80% saturation was performed with ammonium sulphate for partial purification on the filtrate and the supernatant separately, and then centrifuged at 10,000 rpm at 4°C for 15 min. Pellets were washed thrice with extraction buffer, air-dried and stored at 4°C. Partially purified filtrate represented <10 kDa peptides designated as morintides and the supernatant represented partially purified total protein. Protein content was estimated using Bradford reagent on OPTIMA SP-300 spectrophotometer at 595 nm as described previously (Bradford, 1976).

SDS-PAGE analysis

Partially purified total protein (48μg) and partially purified morintides (11.2μg) were analyzed with SDS-PAGE on resolving gel (12%) and stacking gel (5%) (Laemmli, 1970).

Disc diffusion assay

The antimicrobial activity of morintides was determined against each bacterial strain by the disc diffusion method (Khalid *et al*., 2018). The sterilized 6 mm filter paper discs (GE Healthcare Life Sciences, Whatman Grade A CAT No. 2017-006) were placed on agar in Petri plates and 7 μg morintides were applied to each disc. 20μl distilled water and 100μg ampicillin were used as a negative and positive control, respectively. The Petri plates were incubated overnight at 37ºC. Zones of inhibition were measured using a zone reader. Assays were performed in triplicates.

Minimum inhibitory concentration (MIC)

MIC of each bacterial strain was calculated by microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI) in 24 well cell culture polystyrene plates (Wayne, 2018). Colony-forming units (CFUs) were calculated by the disc diffusion method. Different concentrations of morintides viz 0, 1.75, 3.5, 7 and 14μg were used to inhibit *S. aureus* growth. Concentrations of morintides used were 0, 7, 14, 28, 56 and 112μg against *K. pneumoniae, P. aeruginosa* and *S. typhi.* Concentrations of morintides used were 0, 3.5, 7, 14, 28 and 56μg against *S. flexneri* and *E. coli.* The lowest concentration of morintides at which bacterial growth was visibly inhibited is considered MIC.

Effect of Morintides on virulence determinants

The effect of morintides on virulence determinants was conducted through biofilms, siderophores, elastase and Pseudomonas quinolone signal assays.

Biofilm prevention potential of morintides

Biofilm prevention potential of morintides was explored against all bacterial strains (Khalid *et al*., 2018). Sterile round plastic coverslips (Thermonax^R plastic coverslips of 13 mm diameter) were developed by immersing in bacterial cultures in the presence or absence of morintides in 24 well-cell culture polystyrene plates. Each bacterial strain was studied at its respective MIC of morintides. Autoclaved water was used as the negative control. Biofilms were quantified by crystal violet stain (1%). Microscopic analysis of developed biofilms of each strain was done with a light microscope (Olympus UTR190, 1X83) at 20 X resolution to compare morintides treated and control biofilms. Inhibition of initial cell attachment potential to cover slips to form biofilms by bacterial species has been expressed as % and it was calculated by using the following formula.

$$\% \text{ inhibition} = 100 - \left[\left\{ \frac{\text{OD595 of test}}{\text{OD595 of negative control}} \right\} \times 100 \right]$$

Biofilms disruption potential of morintides

Biofilm disruption potential of morintides was explored against all bacterial strains (Khalid *et al*., 2018). Biofilms were developed for 16 h for each bacterial strain in the absence of morintides. After 16 h of development, each biofilm was taken out and transferred to another well of the cell culture plate. To 1ml autoclaved LB, morintides at MIC for each bacterial strain were added into each well of the plate. Autoclaved water was used as a negative control. Plates were placed in shaker incubator at 37ºC at 60 rpm for 24h, 30h and 48 h separately. After 24, 30 and 48h of incubation, growth of planktonic cells and biofilm quantification were observed after staining with crystal violet (1%).

Siderophore assay

Bacterial strains were grown in iron-limited M9 Minimal medium (0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.1% NH_4Cl, 0.05% NaCl). The media was autoclaved at 121 ºC for 15 min, cooled at 55 ºC, then 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2% Glucose (w/v) (For *P. aeruginosa* 0.2% glycerol

was added in the medium in place of 0.2% Glucose) were added as previously described (Miller, 1972). Siderophores production was measured using the previously described method with slight modifications (Ghosh *et al*., 2015). 1 ml overnight LB broth culture of each bacterial strain ($1x10^8$ CFU/ml) was centrifuged at 10,000 rpm for 5 min to get pellets which were dissolved in M9 minimal medium OD_{600} =0.02-0.04 to make inoculum. 1ml of inoculum was added to each well of the polystyrene plates. Morintides were added at MIC for each bacterial strain. Autoclaved water was used as a negative control. Plates were placed in a shaker incubator at 37ºC overnight. After 24h, 1ml inoculum from each well was centrifuged at 10,000 rpm for 5 min. 100μl Chrome azurol sulfonate (CAS) reagent was added to 900 μl supernatant. It was incubated at 37°C for 20 min. Absorbance was measured at 630 nm. 100µl CAS and 900 μl of uninoculated M9 minimal medium were used as reference. M9 minimal medium was used as blank. Siderophore content was calculated using the following formula.

$$\%\,\text{Siderophore units} = \left[\frac{(\text{Ar} - \text{As})}{\text{Ar}}\right] \times 100$$

Where:

A_r = Absorbance of reference at 630 nm, A_s = Absorbance of sample at 630 nm

Elastin Congo red assay

As only *P. aeruginosa* produces elastase enzyme, elastin Congo red assay was performed for *P. aeruginosa* MPAO1 as described previously (Alipour, 2010). 10mL LB broth was taken and inoculated with 100µL of overnight culture ($1x10^8$ CFU/mL). 1120µg morintides were added and kept in a shaker incubator at 37ºC for 12-14h. Autoclaved water was used as a negative control. 1mL culture was centrifuged at 6,000 *g* for 5 min. 100 µL supernatant was added in 2mL 10mM $NaHPO_4$ and 30 mg Congo red dye and placed in a shaker incubator at 37ºC for 14h. Then, it was centrifuged at 6,000 *g* and 1mL supernatant was taken and its absorbance was measured at 495 nm. $NaHPO_4$ buffer was taken as blank.

Pseudomonas Quinolone signal (PQS) detection

P. aeruginosa quinolone signal (PQS) molecules were detected through thin layer chromatography (TLC) as described previously (Qaisar *et al*., 2013). Overnight culture of *P. aeruginosa* MPAO1 ($1x10^8$ CFU/ml) was sub-cultured into LB broth to OD 0.02. 112 µg morintides/ml of LB broth were added and then placed at 37 °C in a shaking incubator for 16 h. Autoclaved water was used as a negative control. TLC plate was illuminated under UV light (365 nm) and photographed.

Thermal and proteolytic enzyme stability assay

To analyze the thermal stability of morintides, they were incubated at 50ºC, 70ºC and 100ºC for 30 min as described previously (Ebbensgaard *et al*., 2015). Unheated morintides were used as a negative control. To analyze the proteolytic stability of morintides, different concentrations of proteinase 'K' were incubated with morintides at ratios of (1: 100), (10: 100) and (15: 100) (w/w) as described previously (Ebbensgaard *et al*., 2015). 100mM Tris HCl buffer of pH = 7.5 was used to maintain the ratio. Then the samples were incubated for 30 min at 37ºC. Further incubation was done at 90ºC for 10 min to inactivate proteinase 'K'. Disc diffusion assay was performed against *S. aureus* ATCC 25923 to evaluate the remaining antibacterial activities by measuring zones of inhibition with a zone reader.

STATISTICAL ANALYSIS

Each assay was performed in triplicate runs for each bacterial strain. GraphPad Prism (GraphPad Software, Version no. 5.0) was used as described previously (Kruczek *et al*., 2014) for statistical purposes. Assays of biofilms formation, siderophore production and elastin Congo red were subjected to a two-way analysis of variance (ANOVA) with Bonferroni post-test. Assays of thermostability and protease stability were subjected to one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test. A comparison was done at 0.05 level of significance to see a significant difference among the control and different treatments.

RESULTS

SDS-PAGE analysis of peptides

SDS-PAGE analysis confirmed the protein nature of partially purified total proteins in lane 2 and one sharp isolated band in lane 3 depicted partially purified <10 kDa morintides from *M. oleifera* as shown in fig. 1.

Disc diffusion assay and minimum inhibitory concentration (MIC) of morintides

Antimicrobial susceptibility assays showed that all laboratory` strains and clinical isolates were sensitive to morintides. Morintides were most sensitive against *S. aureus* and *S. flexneri*, intermediary sensitive to *E. coli*, *K. pneumoniae* and *S. typhi* and less effective to *P. aeruginosa*. Laboratory strain of *S. aureus* showed growth inhibition even at low concentrations of morintides as compared to its clinical isolates of burn and surgical wounds. Bacterial growth was decreased by increasing the concentration of morintides for each bacterium. It was found that *P. aeruginosa* was resistant to the highest concentration of morintides (112μg) used in the investigation (table 2).

Biofilm inhibition potential of morintides

The prevention potential of morintides on initial bacterial cell attachment to form biofilms was compared with control. In the case of laboratory strains of bacteria, *S. aureus* showed a 4 X decrease in biofilm formation and a

Table 1: Bacterial strains used in the study

Source of Isolation	Bacterial Strains	Location
Laboratory strains	*Pseudomonas aeruginosa* MPAO1	University of Punjab
	Staphylococcus aureus ATCC 25923	University of Health Sciences, Pakistan
Burn wound pathogens	*Staphylococcus aureus*	Burn Center, Jinnah Hospital, Lahore.
	Klebsiella pneumoniae	
	Pseudomonas aeruginosa	
Surgical wound pathogens	*Staphylococcus aureus*	Laboratory of Microbiology, Allama Iqbal Medical College, Lahore.
	Klebsiella pneumoniae	
	Pseudomonas aeruginosa	
Other clinical isolates	*Salmonella typhi Escherichia coli*	University of Health Sciences Pakistan

Table 2: MIC values of morintides against different strains of bacteria

Groups	Bacterial specie	MIC (*u*g)
Laboratory strains	*Staphylococcus aureus* ATCC 25923	3.5
	Pseudomonas aeruginosa MPAO1	> 112
Burn wound pathogens	*Staphylococcus aureus*	7.0
	Klebsiella pneumoniae	112
	Pseudomonas aeruginosa	> 112
Surgical wound pathogens	*Staphylococcus aureus*	7.0
	Klebsiella pneumoniae	112
	Pseudomonas aeruginosa	> 112
Some other clinical isolates	*Escherichia coli*	28
	Shigella flexneri	28
	Salmonella typhi	56

5 X decrease in planktonic cells, while, there was a 2 X increase in biofilm formation and no effect on planktonic cells in case of *P. aeruginosa* in the presence of morintides. In the case of burn wound pathogens, *S. aureus* showed a 2 X decrease in biofilm formation as well as planktonic cells. *K. pneumoniae* also showed a 1.5 X decrease in biofilm formation as well as planktonic cells, while, there was a 2 X increase in biofilm formation and no effect on planktonic cells by *P. aeruginosa* was found in the presence of morintides.

In the case of surgical wound pathogens, *S. aureus* showed a 3 X decrease in biofilm formation as well as planktonic cells. Morintides caused a 2 X decrease in biofilm formation, as well as planktonic cells by *K. pneumoniae* while, a 2 X increase in biofilm formation was observed by *P. aeruginosa*. Among some other clinical isolates, *S. flexneri* showed a 3 X decrease in biofilm formation as well as a 2 X decrease in planktonic cells. In the case of *S. typhi*, there was a 2 X increase in biofilm formation and a 1.5 X decrease in planktonic cells. *E. coli* showed a 2 X decrease in biofilm formation as well as planktonic cells in the presence of morintides. Results of biofilm inhibition assays are shown in fig. 2 A, B, C and D. Inhibition of initial cell attachment to form biofilms has been expressed in percentage (fig. 3), which indicated that *S. aureus* suffered the most percent inhibition of initial cell attachment to form biofilms among all of them. Microscopic analysis has also confirmed these results of inhibition as compared to controls as shown in fig. 4 A, B, C and D.

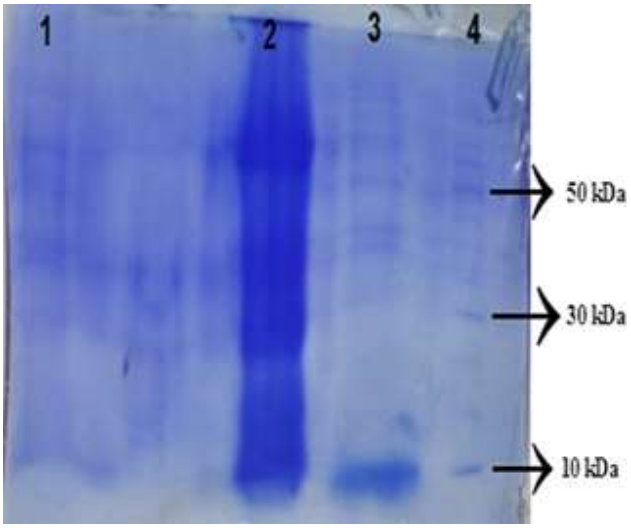

Fig. 1: SDS-PAGE analysis of protein and peptides extracted from *M. oleifera*. Lane 1 = Protein in crude extract, Lane 2 = Partially purified total protein, Lane 3 = Morintides, Lane 4 = Protein marker.

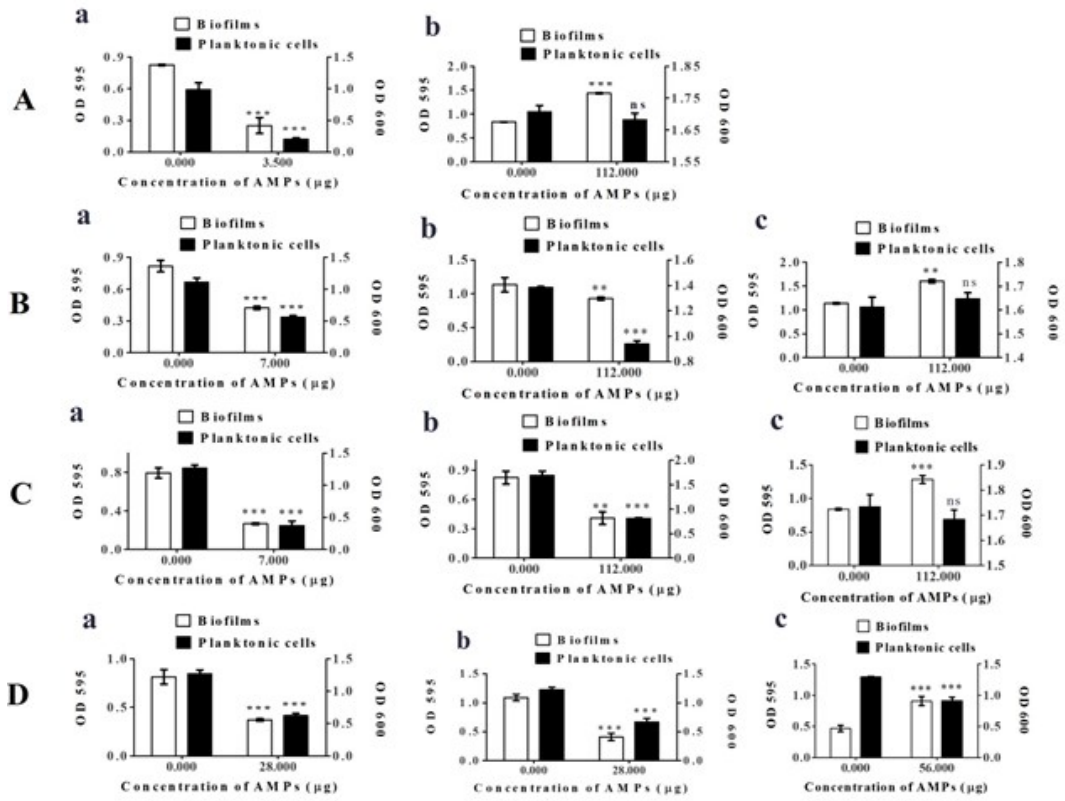

Fig. 2: Effect of morintides on biofilm formation of row (A) = laboratory strains of bacteria (a): *S. aureus* ATCC 25923 and (b): *P. aeruginosa* MPAO1, row (B) = Burn wound pathogens: (a) *S. aureus*, (b) *K. pneumoniae* and (c) *P. aeruginosa*, row (C) = Surgical wound pathogens: (a) *S. aureus*, (b) *K. pneumoniae* and (c) *P. aeruginosa*, row (D) = Some other clinical isolates of bacteria: (a) *E. coli,* (b) *S. flexneri* and (c) *S. typhi*. Bars represent values of mean ± SEM (n = 3). ***P<0.001 indicates significant difference from control. **P<0.01 indicates significant difference from control Non-significant (ns) difference from control indicates P>0.05.

Biofilm disruption potential of morintides

It was concluded that *S. aureus*, *K. pneumoniae* from the surgical wound, *E. coli* and *S. flexneri* showed significant disruption of biofilm cells as compared to control biofilms during 24h of morintides treatment. After disruption, the bacterial cells were killed by morintides shown by a decrease in planktonic cells. Biofilm cells of *K. pneumoniae* from burn wounds, *P. aeruginosa* and *S. typhi* showed no disruption effects during 24h of morintides treatment indicating that pre-developed biofilm cells of these bacteria were resistant against morintides. After 24h of incubation, there was a reduction in biofilm formation by all bacterial species in morintides treated samples and controls. At a later time point after 48 h of incubation, disruption in biofilm cells was more pronounced indicating that biofilm cells became weakened probably due to a decline in the nutrients in the culture medium. The biofilm disruption potential of morintides is shown in fig. 5 A, B, C and D.

Siderophore assay

It was observed that siderophore production was decreased by almost 2 X by all bacterial strains under investigation in the presence of morintides except *P. aeruginosa,* which showed no effect on siderophores production in the presence of morintides as shown in fig. 6 A, B, C and D.

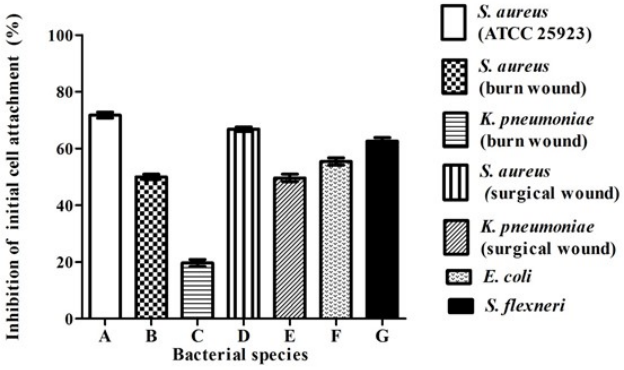

Fig. 3: % Inhibition of initial cell attachment of (A) *S. aureus* ATCC25923, (B) *S. aureus* from burn wound, (C) *K. pneumoniae* from burn wound, (D) *S. aureus* from the surgical wound, (E) *K. pneumoniae* from the surgical wound, (F) *E. coli* and (G) *S. flexneri* by morintides. Bars represent values of mean ± SEM (n = 3).

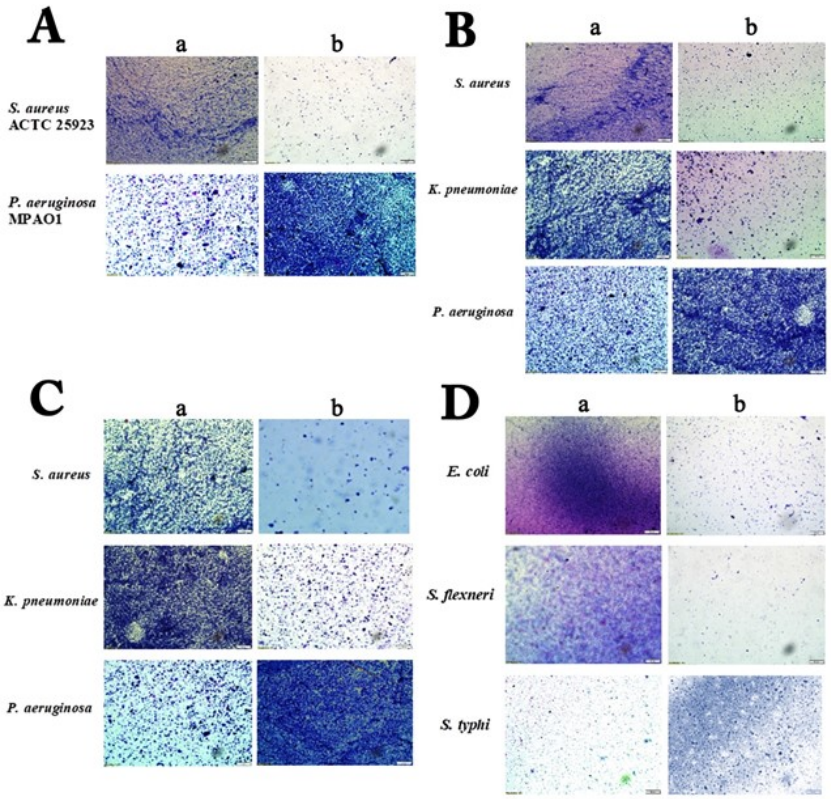

Fig. 4: Qualitative analysis of biofilms of (A) laboratory strains of bacteria, (B) burn wound pathogens, (C) surgical wound pathogens and (D) some other clinical isolates by light field microscope. Lane a = Control (morintides untreated biofilms), Lane b = morintides treated biofilms.

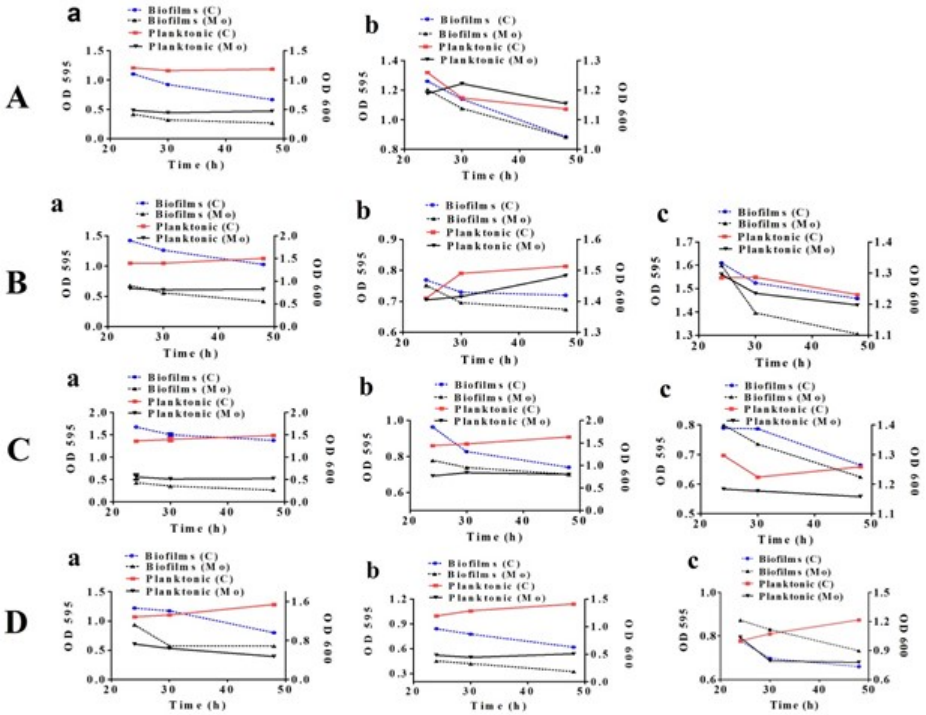

Fig. 5: Effect of morintides on pre-developed biofilms of row (A) = laboratory strains of bacteria (a): *S. aureus* ATCC 25923 and (b): *P. aeruginosa* MPAO1, row (B) = Burn wound pathogens: (a) *S. aureus*, (b) *K. pneumoniae* and (c) *P. aeruginosa*, row (C) = Surgical wound pathogens: (a) *S. aureus*, (b) *K. pneumoniae* and (c) *P. aeruginosa*, row (D) = Some other clinical isolates of bacteria: (a) *E. coli,* (b) *S. flexneri* and (c) *S. typhi*.

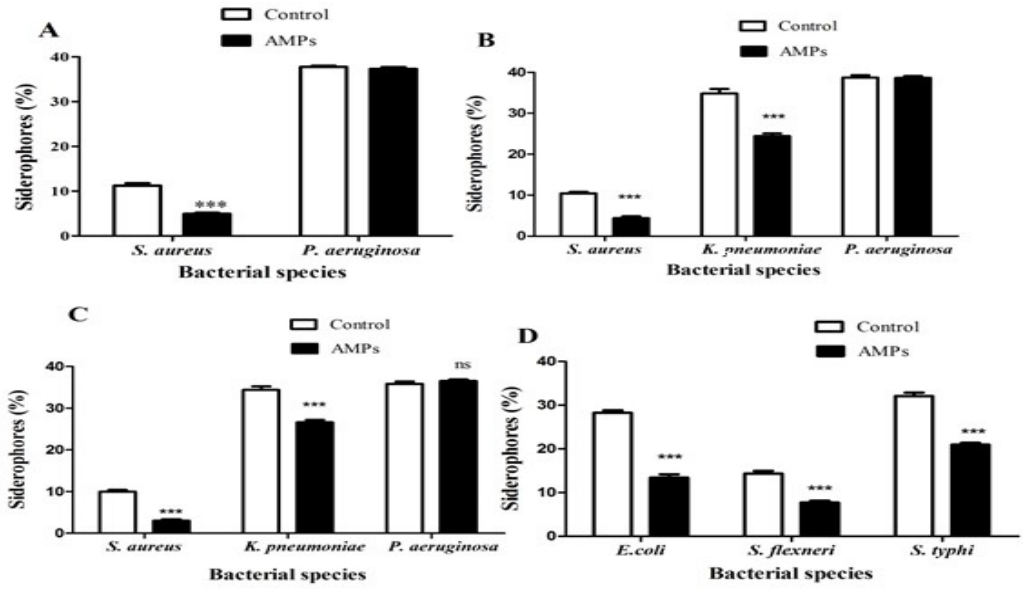

Fig. 6: Effect of morintides on siderophores production by (A) = laboratory strains of bacteria, (B) = burn wound pathogens, (C) = surgical wound pathogens and (D) = some other clinical isolates of bacteria. Bars represent values of mean ± SEM (n = 3). ***P<0.001 indicates significant difference from control. Non-significant (ns) difference from control indicates P>0.05.

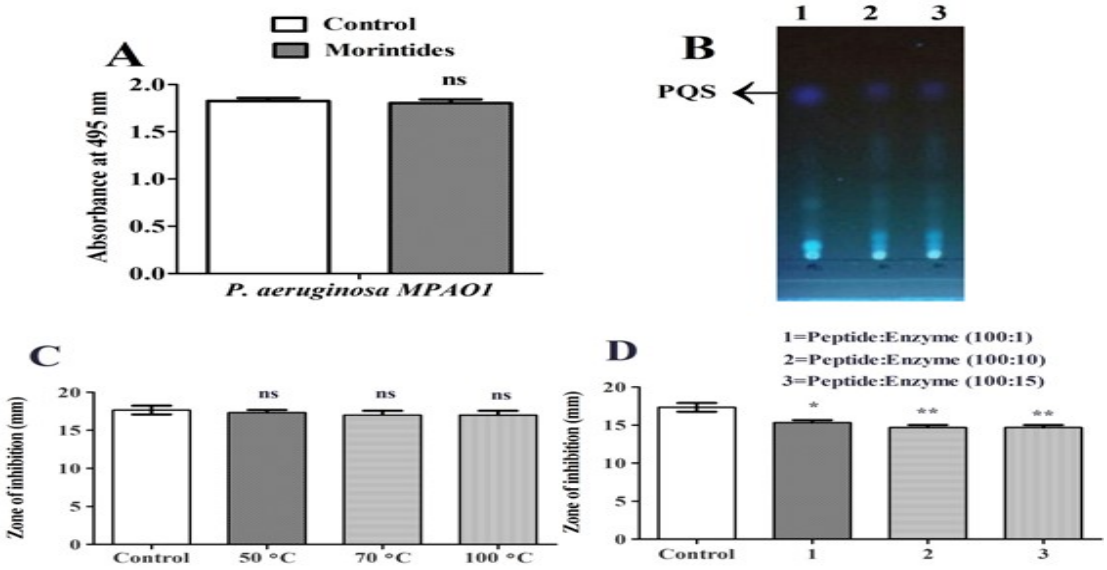

Fig. 7: (A) Effect of morintides on elastase production of *P. aeruginosa* MPAO1. Non-significant (ns) difference from control indicates P > 0.05. (B**)** Effect of morintides on PQS production in *P. aeruginosa* MPAO1 through TLC. Lane 1 = PQS standard, Lane 2 = Control strain of *P. aeruginosa* MPAO1 cultures grown in the absence of morintides. Lane 3 = *P. aeruginosa* MPAO1 cultures treated morintides, (C) Comparison of antibacterial activities of different heat-treated and non-heat treated morintides against *S. aureus*. Control = Heat untreated morintides. Non-significant (ns) difference from control indicates P-value >0.05. **(**D) Comparison of antibacterial activities of different concentrations of proteinase 'K' treated and non-treated morintides against *S aureus*. Bars represent values of mean ± SEM (n = 3). Control = Proteinase untreated morintides. **P<0.01 and *P<0.05 indicate significant difference from control.

Elastin Congo red assay and pseudomonas quinolone signal (PQS) detection

It was observed that morintides did not block the production of elastase and PQS in *P. aeruginosa* as shown in fig. 7 A and B.

Thermostability and Protease stability assays

Antibacterial activities of heat-treated morintides were not significantly reduced as compared to control (native morintides), which demonstrates that morintides were not affected as they were stable even at high temperatures up

to 100°C for 10 min. It was observed that about 85% of antibacterial activity of the morintides remained stable and only 15% activity of morintides was lost when subjected to proteinase 'K' digestion at (100: 1), which was further decreased by 20% by treating the peptide with proteinase 'K' (100: 10). No further decrease in activity was observed when peptides were treated at higher concentrations (100: 15). It means that morintides were sensitive up to some extent to proteinase 'K' digestion beyond which they resisted digestion and remained structurally intact (figs. 7 C and D).

DISCUSSION

When bacterial cell population density increases, bacterial cells release some signaling molecules into the environment for intercellular communication which are regulated through a circuit known as the quorum sensing (QS) system that controls the gene expression (Lamin *et al*., 2022). These signaling molecules are known as virulence determinants which are required by bacteria for their pathogenesis including biofilm formation, siderophores, elastase and pseudomonas quinolone signals production (PQS) (Suhartono *et al*., 2019). These QS signals of bacteria can be decreased or quenched by giving stress in the form of different chemicals or antibiotics to reduce the bacterial pathogenesis. In the present study, morintides were isolated from *Moringa oleifera* in search of novel drugs from natural sources to reduce the bacterial pathogenesis by quenching QS signals. In the present study, morintides were found to be sensitive to all bacterial strains under investigation except *P. aeruginosa*, which was resistant to the highest concentration of morintides (112µg) used in the investigation. It may be possible that the concentration of morintides investigated more than that may have growth inhibitory effects against *P. aeruginosa*. Chandrashekar *et al*. (2021) also used <3 kDa bioactive peptides from *M. oleifera* purified protein to inhibit the growth of *E. coli, S. flexneri, S. aureus, S. typhi* and *K. pneumoniae. M. oleifera* leaf extract in very high concentration was used in another study by Fadeyi *et al.* (2015) to inhibit the growth of *E. coli* and *S. aureus* as compared to morintides used in the present study. So, it was concluded that purified morintides from *M. oleifera* in the present study were much more effective as compared to leaf extract comprising a mixture of compounds from *M. oleifera*. In another study by Tavares *et al*. (2012), Pg AMPs were used in very high concentrations to inhibit the growth of *E. coli* and *S. aureus.*

Aqueous extracts from *Moringa peregrine* inhibited the formation of biofilms by *S. aureus, P. aeruginosa, K. pneumoniae, E. coli* and *S. typhi* in the previous investigations (Alhusnan LA and Alkahtani, 2016). In this study significant biofilm prevention potential was observed by morintides extracted from *M. oliefera* which indicated the fact that morintides might contain the properties which disrupt the quorum-sensing pathway leading to up or down regulation of biofilm-forming genes. Stronger anti-biofilm effects were observed by *S. aureus* and *K. pneumoniae* from surgical wound pathogens as compared to these bacterial strains from burn wound pathogens indicating that surgical wound pathogens were more sensitive than burn wound pathogens against morintides. A similar trend of results was also reported in a previous study in which surgical wound pathogens were found to be more sensitive than burn wound pathogens against morintides isolated from *Peganum hermala* (Khalid *et al*., 2018). MIC values against *S. aureus* resulted in this study were much low as compared to flavonoids isolated from *M. oleifera* in a previous study in which stronger anti-biofilm effects were observed with flavonoids extracted from *M. oleifera* against *S. aureus* and *P. aeruginosa* (Onsare and arora, 2014). It may be due to the much higher concentration of flavonoids used as compared to morintides in the present study. Some compounds in the ethanolic extract from *M. oleifera* were sensitive as against *Pseudomonas aeruginosa* in a previous study (Suhartono *et al*., 2019). Significant antibiofilm effects of some compounds present in methanol extract from *Mangifera indica* were also found against *Pseudomonas aeruginosa* in another investigation (Husain *et al*., 2017). The same reason for very high concentration or synergistic effect of antibiofilm compounds in methanol/ethanol extracts could be responsible here.

The results of inhibition of biofilms were encouraging. So, morintides were applied on pre-developed biofilms. Pre-developed biofilms of *S. aureus* species showed the highest sensitivity to morintides among all bacterial species under observation. It was observed that morintides were ineffective in disruption of developed biofilms in the case of *P. aeruginosa*, *K. pneumoniae* from burn wound and *S. typhi*. These results were in line with a previous investigation performed by Nidadavolu *et al*. (2012) in which the garlic extract was found to be only effective in the disruption of pre-developed biofilms of *S. aureus* (Gram-positive bacteria) but remained ineffective to disrupt the established biofilms formed by *K. pneumoniae* (Gram-negative bacteria). It might be possible that biofilms developed by Gram-negative bacteria were more resistant due to more self-protected substances released by these bacteria as compared Gram-positive bacteria. The resistance might have been observed due to the fact that the applied concentration of morintides was insufficient to disrupt the pre-developed biofilms or it might be possible that morintides could not diffuse the matrix of pre-developed biofilms, therefore, bacterial cells remained unexposed to morintides. It might be possible that higher concentration or more contact time with morintides was required to disrupt the developed biofilms of these bacterial species. The same kind of ineffective disruption of biofilms was observed by Simoes *et al*.

(2011), when disinfectants were used to treat pre-developed biofilms of *Pseudomonas fluorescens.* So, it was observed that disinfectants did not penetrate the matrix of biofilms and were left on the surface leaving the microbe untreatable for disinfectant. A previous study performed by Onsare and Arora (2014) also concluded that there was no single compound that could absolutely control biofilms. Results of biofilm disruption potential by morintides were somewhat encouraging relative to the level of inhibition. Hence, morintides can be used as broad-spectrum anti-biofilm agents. Morintides from the present study were very effective against siderophore inhibition by all bacterial strains under investigation except *P. aeruginosa.* So it was assumed that morintides might interfere with the regulation of the operon system for siderophores leading to their decreased production. However, inhibitory effects of $ZnSO_4$ and $CoCl_2$ on siderophores production were observed by *P.* and enhanced production of siderophore was found by *B. subtilis* at the same concentrations of $ZnSO_4$, $CoCl_2$ and streptomycin in another study performed by Erdem *et al*. (2016). It was observed that morintides did not affect siderophores and elastase production and PQS system of *P. aeruginosa* may be due to the low concentration of morintides investigated against *P. aeruginosa* or the presence of a versatile type of antibiotic-resistant mechanisms in *P. aeruginosa* as demonstrated in a previous study (Cummins *et al*., 2009). However, down-regulation of the genes involving elastase production from herbal extracts prepared from *Lagerstroemia speciosa* by *P. aeruginosa* was observed in a study performed by Singh *et al*. (2012) and up-regulation of PQS production by *P. aeruginosa* in the presence of 'Colistin' (an AMP) was found in the previous investigations of Cummins *et al.* (2009). Previous studies reported that peptides from *M. oleifera* were among the most stable biologically active peptides even at high temperatures and protease treatments, which justifies the results of the present study. Results of protease stability and proteinase digestion assay of the present study correlate with the findings in a previous study by Kini *et al*. (2017) in which >95% structural stability of a peptide (4.5 kDa) isolated from *M. oleifera* was maintained even when it was heated for 1 hour at 100°C and also when it was digested with pepsin and carboxypeptidase A at peptide to enzyme ratio (w/w) 20: 1 and 50: 1 respectively, for up to 6h. The same findings were observed in previous investigations of Cammue *et al*. (1992) that antifungal activities remained stable of peptides from *Mirabilis jalapa* when heated up to 100°C for 10 min even when digested with proteinase 'K'. However, our results were comparable to a previous study by Rehman and Khanum (2011) in which peptides isolated from *Pisum sativum* were completely inactivated when heated at 50°C or digested by proteinase 'K'. Resistance to protease treatments might be due to some specific type of peptides present in high concentrations which resist digestion to proteinase 'K'. For example, protease inhibitor types II and I were reported in Solanaceae (Chye *et al*., 2006) and peptidic proteinase inhibitors were found in Asteraceae, Fabaceae and Poaceae family of plants (Sarkar, 2009).

CONCLUSION

Our study shows the importance of the development of novel AMPs from natural as well as economic source. This was the first study in which the effect of morintides from *M. oleifera* was studied on biofilms which are much more resistant to antibiotics. Inhibition of attachment of cells to abiotic surfaces could be a first step combating highly resistant biofilms of these bacterial species. Our results demonstrate that morintides have high antibiofilm potential as compared to other already reported phytochemicals extracted from *M. oleifera.* It could be a promising futuristic antivirulent bioactive agent. Moreover, co-application of these peptides with existing antimicrobial agents can be a breakthrough in drug discovery against the antibiotic resistance menace. Further studies about molecular mechanism involving QS interruption by morintides are needed.

ACKNOWLEDGEMENTS

We are also very thankful to Higher Education Commission for providing fund for our project.

REFERENCES

Al-Husnan LA and Al-kahtani MDF (2016). Impact of Moringa aqueous extract on pathogenic bacteria and fungi *in vitro*. *Annals Agri. Sci*., **61**(I2): 247-250.

Alipour M, Suntres ZE, Lafrenie RM and Omri A (2010). Attenuation of *Pseudomonas aeruginosa* virulence factors and biofilms by co-encapsulation of bismuth-ethanedithiol with tobramycin in liposomes. *J. Antimicrob. Chemotherap*., **65**(4): 684-693.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., **7**(72): 248-254.

Cammue BPA, Bolle MFD, Terras FRG, Proost P, Damme JV, Reess SB, Vanderleydens J and Broekaert WF (1992). Isolation and characterization of a novel class of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. *The J. Biochem*., **267**(4): 2238-2233.

Chandrashekar S, Vijayakumar R, Chelliah R, Daliri EB, Madar IH, Sultan G, Rubab M, Elahi F, Yeon SJ and Oh DH (2021). *In vitro* and *in silico* screening and characterization of antimicrobial napin bioactive protein in *Brassica juncea* and *Moringa oleifera*. *Molecules*, **26**(7): 2080.

Chye ML, Sin SF, Xu ZF and Yeung EC (2006). Serine proteinase inhibitor proteins: Exogenous and endogenous functions. *In Vitro Cell Dev. Biol. Plant*., **42**(1): 100-108.

Cummins J, Reen FJ, Baysse C, Mooij MJ and O-Gara F (2009). Subinhibitory concentrations of the cationic antimicrobial peptide colistin include the pseudomonas quinolone signal in *Pseudomonas aeruginosa. Microbiol.,* **155**(9): 2826-2837.

Ebbensgaard A, Mordhorst H, Overgaard MT, Nielsen CG, Aarestrup FM and Hansen EB (2015). Comparative evaluation of the antimicrobial activity of different antimicrobial peptides against a range of pathogenic bacteria. *PLoS One.,* **10**(12): e0144611.

Erdem B, Dayangac A, Gunaydin M, Tulumoglu S and Yilmaz M (2016). Effect of heavy metals and antibiotics on siderophores producing bacterial isolates. *J. Acta Physica Polonica A*., **130**(1): 181-183.

Fadeyi A, Raheem RA, Bello MK, Njan AA, Olatunji LA, Afolabi SO and Olorundare OE (2015). Antibacterial activity of the crude extracts of *Moringa oleifera* leaf lam (Moringaceae). *Nig. J. Pharm. Sci.,* **14**(1): 1-8.

Ghosh SK, Pal S and Chakraborty N (2015). The qualitative and quantitative assay of siderophore production by some microorganisms and effect of different media on its production. *Int. J. Chem. Sci*., **13**(4): 1621-1629.

Hall-Stoodley L and Stoodley P (2005). Biofilm formation and dispersal and the transmission of human pathogens. *Trends Microbial*, **13**(1): 7-10.

Husain FM, Ahmad I, Al-Thulbiani AS, Abdulreesh HH, Alhazza IM and Aqil F (2017). Leaf extracts of *Mangifera indica* L. inhibit quorum sensing-regulated production of virulence factors and biofilm in test bacteria. *Front. Microbiol*., **24**(8): 727.

Khalid R, Jaffar Q, Tayyeb A and Qaisar U (2018). *Peganum hermala* (PhAMP) impede bacterial growth and biofilm formation in burn and surgical wound pathogens. *Pak. J. Pharm. Sci*., **31**(6): 2597-2605.

Kini SG, Wong KH, Tan WL, Xiao T and Tam JP (2017). Morintides: cargo-free chitin-binding peptides from *Moringa oleifera. BMC Plant. Biol*., **17**(68): 1-13.

Kruczek C, Qaisar U, Hamood JAC and Hamood AN (2014). Serum influences the expression of *Pseudomonas aeruginosa* quorum sensing genes and QS controlled virulence genes during early and late stages of growth. *Microbiol. Open*, **3**(1): 64-79.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*., **227**(5259): 680-685.

Lamin A, Kaksonen AH, Cole IS and Chen XB (2022). Quorum sensing inhibitors applications: A new prospect for mitigation of microbiologically influenced corrosion. *Bioelectrochemistry*, **145**(1): 108050.

Miller JH (1972). Experiments in molecular genetics. Cold Spring Harbor, 5th ed., Cold Spring Harbor Laboratory, New York, USA, pp.352-55.

Nidadavolu P, Amor W, Tran PL, Dertien J, Hamood JA and Hamood AN (2012). Garlic ointment inhibits biofilm formation by bacterial pathogens from burn wounds. *J. Med. Microbiol*., **61**(5): 662-671.

Onsare JG and Arora DS (2014). Antibiofilm potential of flavonoids extracted from *Moringa oleifera* seed coat against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans. J. Appl. Microbiol*., **118**(2): 313-325.

Patil S, Mohit BV, Marathe KR, Salunkhe NS, Marathe V and Patil VS (2022). Moringa tree, gift of nature: A review on nutritional and industrial potential. *Current Pharmacol. Rep*., 8(1): 262-280.

Penalver R, Martinez-Zamora L, Lorenzo JM, Ros G and Nieto G (2022). Nutitional and antioxidant properties of *Moringa oleifera* leaves in functional foods. *Foods*, **11**(8): 1107.

Qaisar U, Luo L, Haley CL, Brady SF, Carty NL, Hamood JAC and Hamood AN (2013). The PVC operon regulates the expression of *Pseudomonas aeruginosa* fimbrial chaperon/usher pathway (Cup) genes. *PloS One*, **8**(4): e62735.

Rehman S and Khanum A (2011). Isolation and characterization of peptide (s) from *Pisum sativum* having antimicrobial activity against various bacteria. *Pak. J. Botany*., **43**(6): 2971-2978.

Sarkar A (2009). Herbal Toxicology. Published by Discovery Publishing House. New Delhi, India, pp.278-81.

Simoes LC, Lemos M, Pereira AM, Abreu AC, Saavedra MJ and Simoes M (2011). Persister cells in a biofilm treated with a biocide. *Biofouling*, **27**(4): 403-411.

Singh BN, Singh HB, Singh A, Singh BR, Mishra A and Nautiyal CS (2012). *Lagerstroemia speciosa* fruit extract modulates quorum sensing-controlled virulence factor production and biofilm formation in *Pseudomonas aeruginosa*. *Microbiol*., **158**(2): 529-538.

Suhartono S, Ismail YS and Muhayya SR (2019). The interference of *Moringa oleifera* leaf extracts to modulate quorum sensing-facilitated virulence factors. *Biodiversitas*, **20**(10): 3000-3004.

Tavares LS, Rettore JV, Freitas RM, Porto WF, Duque APN, Singulani JL, Silva ON, Detoni ML, Vasconselos EG, Dias SC, Franco DL and Santos MO (2012). Antimicrobial activity of recombinant Pg-AMP1, a glycine-rich peptide from guava seed. *Peptides*, **37**(2): 294-300.

Thomas AS, Saravanakumar R and Gupta PV (2017) Evaluation of cytotoxic activity of protein extracts from the leaves of *Morinda pubescens* on human cancer cell lines. *Braz. J. Pharmacog*., **27**(1): 99-104.

Waheed M, Mehreen A, Ishtiaq S, Saleem M and Kanwal U (2022) Evaluation of *Conocarpus erectus* against multidrug resistant *Staphylococcus aureus*: Cell to animal body. *Pak. J. Pharm. Sci*., **35**(1): 276-280.

Wayne PA (2018). Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; M07-S15, 13th informational supplement. ISBN. 1-56238-836-3.