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ANTIMICROBIAL PROTEINS/PEPTIDES ISOLATED FROM TWO CULTIVARS OF BOUGAINVILLEA

Samriti [1], Sukesh [1], Rajesh Biswas [2] and Kakoli Biswas [*1]

Department of Biotechnology [1], DAV College, Sector- 10, Chandigarh - 160010, Punjab, India.
Department of Zoology [2], Government Home Science College, Sector-10, Chandigarh - 160010, Punjab, India.

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Correspondence to Author:
Dr. Kakoli Biswas

Associate Professor,
Department of Biotechnology,
DAV College, Sector- 10, Chandigarh - 160010, Punjab, India.

E-mail: kakolibiswas14@gmail.com

ABSTRACT: The rapid increase in multi-drug resistance (MDR) infections present a challenge in the development of therapies against them. Antimicrobial peptides (AMPs) can be the answer to this challenge. AMPs play an important role in host defense mechanisms. Plants are the precious source of natural antimicrobial molecules, including antimicrobial peptides known as Plant Antimicrobial Peptides (PAMPs). The present research work was aimed to isolate antimicrobial proteins or peptides from the leaf and flower extract of *Bougainvillea* 'Texas King' and *Bougainvillea* 'Shubra' cultivars. Protein pellets obtained after 50% and 25% ammonium sulfate precipitation of *B. '*Shubra' leaf and flower extract showed maximum total protein of 311 µg/ml and 798 µg/ml respectively and 25% and 50% protein pellet of *B.* 'Texas King' leaf and flower extract showed maximum total protein of 529 µg/ml and 904 µg/ml respectively. Different protein pellets and supernatant were screened for antimicrobial activity against selected microorganisms. The protein samples having antimicrobial activity were electrophoresed on 12% Tricine SDS-PAGE to estimate the molecular masses of the antimicrobial protein/peptides. Molecular weight of antimicrobial proteins/ peptides from the leaves of *Bougainvillea* 'Texas King' and *Bougainvillea* 'Shubra' cultivars ranged between 14.1 to 72.4kDa. However, antimicrobial proteins/ peptides from *Bougainvillea* 'Texas King' and *Bougainvillea* 'Shubra' cultivars flowers ranged between 4.6 to 23.4 kDa. Above preliminary studies on *B. '*Texas King' and *B. '*Shubra' cultivars represents strong future prospects of antimicrobial proteins/peptides in therapeutics for the treatment of the diseases caused by *C. albicans*, *S. aureus*, and *B. licheniformis*.

INTRODUCTION: The rapid increase in multi-drug resistance (MDR) organisms poses a serious threat to public health. MDR infections presented a challenge in the development of new drug moieties against them. The paucity of new drugs to overcome the situation is adding to the complexity of the challenge. However, ongoing research on antimicrobial peptides (AMPs) can provide some answers to this situation. Antimicrobial peptides (AMP) play an important role in the host defense mechanism. All living organisms, from microorganisms to plants and animals, have an active mechanism to defend themselves against pathogen attack. Peptides are present in a high amount in living organisms. They play an important role in the ancient mechanism of innate immunity by providing the first line of defense against pathogens [1]. Antimicrobial peptides consist of short sequence peptides ranging from 10-50 amino acid residues (2-9 kDa) positively charged, with more hydrophobic amino acid [2].

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There are large numbers of plants, which have several medicinal properties. Many of them have antimicrobial compounds, plant antimicrobial peptides (PAMPs) are one of them. These PAMPs act as an effective weapon against broad species of microbes [3]. AMPs kill microorganisms by plasma membrane depolarization, ions and metabolite leakage, respiratory process interruption [4]. Plant AMPs are classified into six different families based on their sequence similarities, such as cystine motif, disulfide bond pattern, which help in the determination of their bond pattern [5]. These families are Thionins, Defensins, Lipid transfer protein, Snakin, Cyclotides, and Hevein like proteins [3]. PAMPs exert their action at various levels, which include membrane solubilization, depolarization, metabolite leakage, bipolar synthesis, respiratory process disruption [4]. PAMPs interfere with the DNA, RNA, and protein synthesis; therefore can be used to prevent the development of resistance in bacteria.

Certain important characteristics like size, cationic charge, amino acid composition are responsible for their antimicrobial property. The alignment of the peptide within bacterial phospholipid membrane confers specificity to the peptide. Various hypotheses have been given by different workers for mechanism of action of antimicrobial peptide. Different models were proposed for AMP mechanism namely "Carpet like" (membrane destruction / solubilization), "Barrel-stave" (trans-membrane pore formation) and "toroid" (worm hole) model [6, 7].

Plant AMPs have a strong potential to work against the pathogenic microorganisms [8]. Compared to plants' vast biodiversity, only a miniscule percentage of plants have been explored for their potential as possessing antimicrobial properties through AMPs [9]. Although significant literature is available on the antimicrobial properties of the medicinal plants, they have not been explored for their benefits for clinical use [10]. There are few reports on antiviral proteins from *Bougainvillea spectabilis* and *Bougainvillea xbuttiana* [11-14]. These antiviral proteins were not used against human pathogens. However, there is no report on isolation of PAMPs from *Bougainvillea* 'Texas King' and B*ougainvillea* 'Shubra' cultivars, which can be used against human pathogenic microorganisms.

This information encouraged us to conduct present research on two cultivars of *Bougainvillea,* namely *B.* 'Shubra' and *B.* 'Texas King' (www.Bougain villeas.com.Inc). The objective of the present study was to determine the antimicrobial activity of proteins/peptides from leaves and flowers of two *Bougainvillea* cultivars.

MATERIALS AND METHODS:

Material:

Medicinal Plant: Leaves and flowers of *B.* 'Texas King' and *B.* 'Shubra' cultivars were collected from the fields of Chandigarh. These cultivars were correctly identified and verified by the Department of Floriculture, Dr. Y. S Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh, India.

Microorganisms: The microorganisms used in this study are *Bacillus subtilis, Candida albicans, Escherichia coli,* and *Staphylococcus aureus* procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Fungi and bacteria were subcultured at regular intervals on Yeast extract peptone dextrose media (YEPD) and Nutrient agar (NA) respectively and ampoules were preserved as 10% glycerol stock stored at -80°C.

Preparation of Crude Extract: Leaves and flowers of *B.* 'Texas King' and *B.* 'Shubra' cultivars were freed from dirt by washing with tap water, followed by distilled water. The moisture content was removed by drying at 37°C. Dried leaves were ground using mortar pestle, maintaining the temperature at 4°C by keeping in ice. Antimicrobial proteins and peptides were extracted using phosphate buffer saline (PBS, pH 7.2). The leaf and flower powder of *B.* 'Texas King' and *B.* 'Shubra' cultivars were added in PBS buffer and frozen at -20°C, followed by thawing at 4°C. These steps were repeated thrice. Freeze-thawing treatment was carried out for 3-4 days for better extraction of proteins. The leaf extract was centrifuged at 10,000 RPM at 4°C for 30 min. The supernatant was filtered using Whatman filter paper to remove debris and then stored the filtrate at 4°C for further use [15].

Ammonium Sulfate Precipitation: Crude extract obtained from the above step containing soluble

proteins was treated with 25%, 50%, 75%, 90%, and 95% ammonium sulfate precipitation cuts while maintaining at 4°C until the salt dissolved completely, followed by centrifugation at 10,000 RPM for 30 min. Precipitated protein pellets were dissolved in PBS buffer and stored at -20°C for further use [16].

Protein Quantification: Bradford method was used to determine the protein content present in different protein pellet and supernatant obtained from ammonium sulfate precipitation after dissolving in PBS buffer (pH 7.2) [17]. Bovine serum albumin (BSA) solution of 1mg/ml concentration was used as the standard.

Agar Well Diffusion Assay: The antimicrobial activity of protein pellet and supernatant were determined by agar well diffusion assay [18]. For antifungal and antibacterial activity, YEPD and NA media were used respectively. A lawn was prepared with activated culture using sterile cotton swabs. Wells of 8 mm diameter were created using the backside of sterile autopipette tip, in which different volumes of protein pellet and supernatant were added **Table 1**. Petri plates were incubated at 27 °C and 37 °C for fungal and bacterial culture, respectively for 24 h. Antimicrobial activity was also carried out with the positive and negative control sample. Positive control samples used were, Fluconazole (70 µg/ml) against *C. albicans*, Chloramphenicol (50 µg/ml) against *B. subtilis* and Ampicillin (60 µg/ml) against *E. coli* and *S. aureus* were used. PBS buffer was used as a negative control. A clear zone of inhibition was observed around the wells having extracts with antimicrobial protein or peptides. The zone of inhibition was measured in millimetres.

TABLE 1: DIFFERENT VOLUME OF PROTEIN SAMPLES USED IN AGAR WELL DIFFUSION METHOD

Samples	Volume of each sample added in different wells (µl)
Dissolved Protein Pellets and Supernatant	100 50

Calculation for the Zone of Inhibition:

Zone of inhibition (mm) = Radius of clear zone from the center of well (mm) – Radius of well (mm)

Tricine-Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Tricine SDS-PAGE): The protein pellets and supernatant having anti-microbial activity were run on 12% Tricine SDS-PAGE gel [19]. During electrophoresis, the initial voltage was 50V and as the sample reached separating gel voltage was increased to 120V. The gel was run until the tracking dye reached 1cm from the bottom of the gel. The gel was dipped in the fixative solution (50% methanol + 10% Acetic acid + 100mM Ammonium acetate) to fix the bands for 30 min. The gel was stained with Coomassie Brilliant Blue G-250 dye for 3-4 hrs or overnight and then destained with 10% Glacial acetic acid (V/V).

Determination of the Molecular Weight of Unknown Samples:

Rm value = Distance migrated by protein (mm) / Distance migrated by tracking dye (mm)

The approximate molecular weight of bands present in different protein pellet and supernatant having antimicrobial activity was determined by interpolating and extrapolating the relative mobility (Rm) on the standard graph plotted between Rm and log molecular weight (Log MW) of the known standard protein ladder. Standard protein ladder comprising of four (26.6kDa, 17.0kDa, 14.2kDa, and 6.5kDa) molecular weight proteins was procured from Sigma, USA. The molecular weight of the unknown protein was interpolated from the standard graph. Different standard graphs were plotted depending on the dye front values of each SDS-PAGE gel.

RESULTS AND DISCUSSION: The crude extract of *B.* 'Texas King' and *B.* 'Shubra' leaves and flowers obtained after ammonium sulfate precipitation were screened for antimicrobial activity. The antimicrobial activity was determined in terms of the zone of inhibition. The protein pellets showing antimicrobial activity were subjected to Tricine SDS-PAGE to determine their molecular weight.

Protein Determination using Bradford Method: The protein concentration in leaf samples of *B.* 'Texas King' cultivar ranged between 270µg/ml and 529 µg/ml. Maximum total protein concentration (529 µg/ml) was found in 25% protein pellet of *B.* 'Texas King' leaf extract, whereas supernatant was found to have minimum protein concentration (270 µg/ml).

The protein concentration in pellets and supernatant of *B.* 'Texas King' flower extract ranged between 186 µg/ml and 904 µg/ml. The maximum total protein concentration of 904 µg/ml was found in 50% protein pellet of *B.* 'Texas King' flower extract, whereas 95% protein pellet was found to have a minimum protein concentration of 186 µg/ml. The protein concentration in the leaf samples of *B.* 'Shubra' cultivar ranged between 159 µg/ml and 311 µg/ml. Maximum total protein (311 µg/ml) was found in 50% protein pellet of *B.* 'Shubra' leaf extract, whereas 95% protein pellet showed minimum protein concentration of 159 µg/ml. The protein concentration in pellets and supernatant of *B.* 'Shubra' flower extract ranged between 798 µg/ml and 58 µg/ml. Maximum total protein pellet concentration (798 µg/ml) was found in 25% protein pellet of *B.* 'Shubra' flower extract, whereas supernatant was found to have minimum protein concentration (58µg/ml) **Table 2**; **Fig. 1**.

TABLE 2: TOTAL PROTEIN CONCENTRATION IN LEAF AND FLOWER EXTRACT OF *B.* 'TEXAS KING' AND *B.* 'SHUBRA' CULTIVARS

S. no.	Sample	Protein concentration (µg/ml)			
		B. 'Texas King'		*B.* 'Shubra'	
		Leaf extract	Flowers extract	Leaf extract	Flowers extract
1	25% Pellet	529	794	292	798
2	50% Pellet	517	904	311	671
3	75% Pellet	502	712	293	668
4	90% Pellet	503	525	303	429
5	95% Pellet	274	186	159	205
6	Supernatant	270	243	189	053

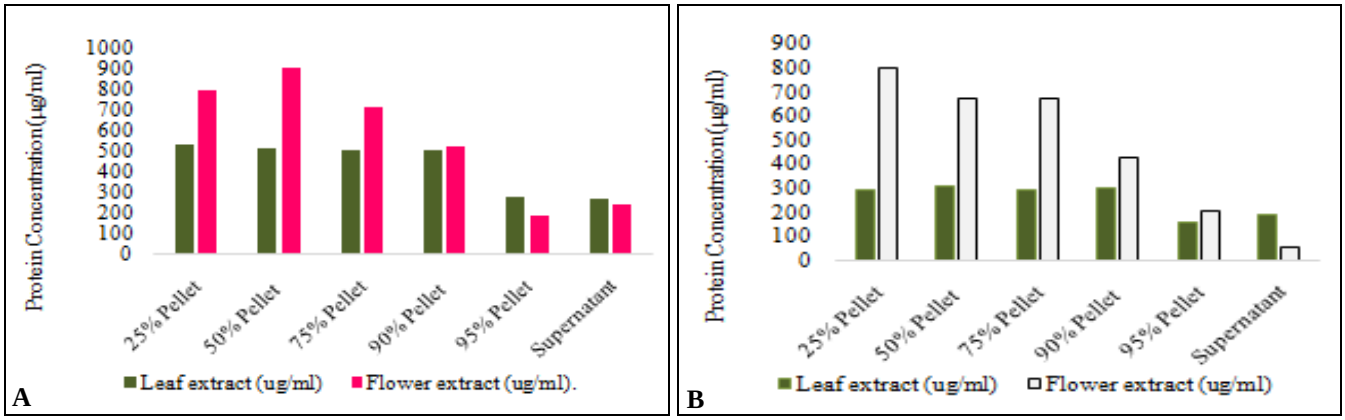

FIG 1: GRAPHICAL REPRESENTATION OF AMOUNT OF PROTEIN PRESENT IN LEAF AND FLOWER EXTRACT OF (A.) *B.* 'TEXAS KING' CULTIVAR (B.) *B.* 'SHUBRA' CULTIVAR

Antimicrobial Activity of Protein Pellet and Supernatant: Proteins and peptides extracted from leaf and flower of *B.* 'Texas King' extract and *B.* 'Shubra' extract were screened for antimicrobial activity using the agar well diffusion method. Antimicrobial activity was carried out against selected Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus licheniformis*), Gram-negative bacteria (*Escherichia coli*), and fungi (*Candida albicans*) **Table 3**; **Fig. 2**.

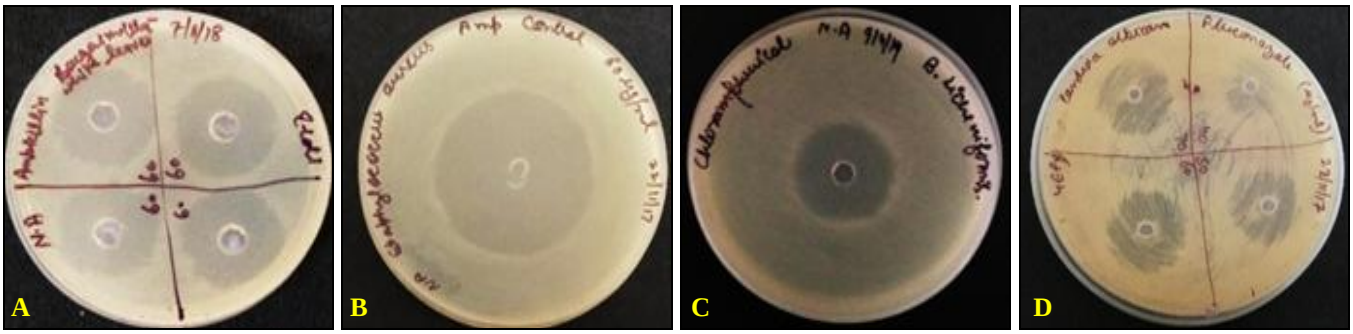

FIG. 2: ANTIMICROBIAL ACTIVITY OF POSITIVE CONTROLS AMPICILLIN AGAINST (A.) *E. COLI* (B.) *S. AUREUS* (C). CHLORAMPHENICOL AGAINST *B. LICHENIFORMIS* (D). FLUCONAZOLE AGAINST *C. ALBICANS*

TABLE 3: ANTIMICROBIAL ACTIVITY OF ANTIBIOTICS AGAINST VARIOUS MICROORGANISMS

S. no.	Antibiotic	Microorganism	Working conc. (µg/ml)	Zone of inhibition in millimetres (mm)
1	Ampicillin	*Escherichia coli*	60	12
		Staphylococcus aureus		21
2	Chloramphenicol	*Bacillus licheniformis*	50	12
3	Fluconazole	*Candida albicans*	70	10

The antimicrobial activity of different protein pellets and supernatant indicated that *B.* 'Texas King' leaf extract had antimicrobial activity against selected gram-positive bacteria (*B. licheniformis*) and fungi (*C. albicans*). No activity was observed against gram-positive bacteria (*S. aureus*) and gram-negative bacteria (*E. coli*). The extent of antimicrobial activity was depicted by the zone of inhibition. In leaf extract, the zone of inhibition ranged between minimum of 2.5 mm with 25% protein pellet to 8.0 mm with supernatant against *B. licheniformis*. Whereas, a minimum of 3.0mm zone of clearance with 25% protein pellet to a maximum of 8.0 mm with 75% protein pellet was observed against *C. albicans* **Table 4**, **Fig. 3**. The different protein pellets and supernatant of *B.* 'Texas King' flower extract had antimicrobial activity against one of the two gram-positive bacteria *(S. aureus)* whereas no activity was recorded against *B. licheniformis*, gram-negative bacteria (*E. coli*), and fungi (*C. albicans).* In flower, extract the zone of inhibition ranged from 2.0 mm (with 50% protein pellet) to 5.5 mm (with supernatant) against *S. aureus* **Table 5**, **Fig. 3**.

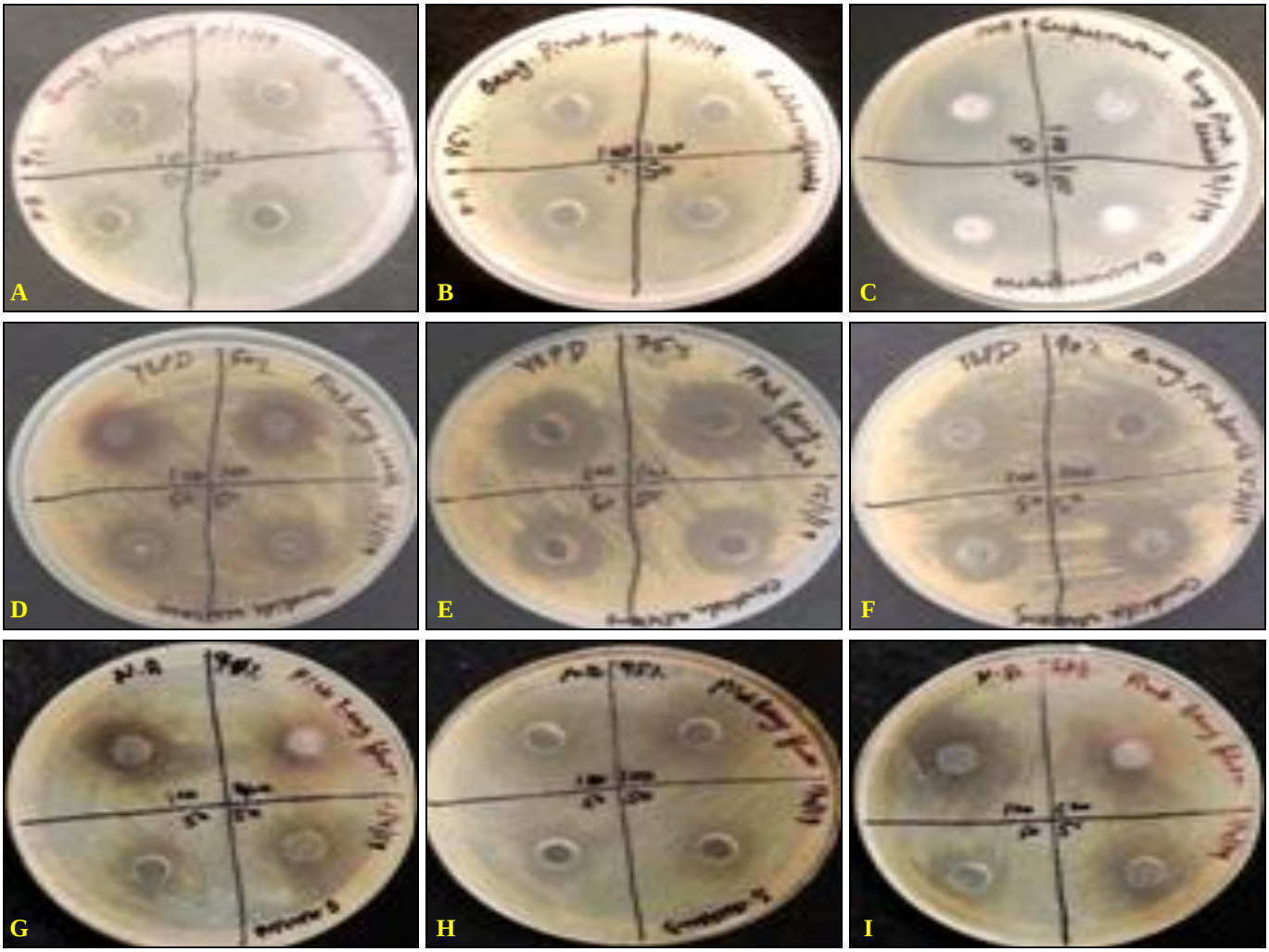

FIG. 3: ANTIMICROBIAL PEPTIDES SHOWING ANTIMICROBIAL ACTIVITY AGAINST SELECTED MICROORGANISMS, (A, B, C) 90%, 95% AND SUPERNATANT OF *B.* 'TEXAS KING'CULTIVAR LEAF EXTRACT SHOWING ANTIMICROBIAL ACTIVITY AGAINST *B. LICHENIFORMIS*. (D, E, F) 50%, 75% AND 95% PROTEIN PELLETS OF *B. 'TEXAS* KING' SHOWING ANTIMICROBIAL ACTIVITY AGAINST *C. ALBICANS*. (G, H, I) 90%, 95% AND SUPERNATANT OF *B.* 'TEXAS KING' CULTIVAR FLOWER EXTRACT SHOWING ACTIVITY AGAINST *S. AUREUS*

TABLE 4: ANTIMICROBIAL ACTIVITY OF DIFFERENT PELLETS OF *B.* 'TEXAS KING' CULTIVAR LEAF EXTRACT AGAINST SELECTED MICROORGANISMS

S. no.	Samples	Zone of inhibition in millimetres (mm) with different volumes of plant extract for Antimicrobial activity against micro-organisms							
		100(µl)	50(µl)	100(µl)	50(µl)	100(µl)	50(µl)	100(µl)	50(µl)
		S. aureus		*B. licheniformis*		*E. coli*		*C. albicans*	
1	25% Pellet	--	--	2.5	2.0	--	--	3.0	3.0
2	50% Pellet	--	--	5.0	3.0	--	--	4.5	3.0
3	75% Pellet	--	--	4.0	2.5	--	--	8.0	6.5
4	90% Pellet	--	--	7.0	6.0	--	--	6.5	6.5
5	95% Pellet	--	--	7.0	8.5	--	--	--	--
6	Supernatant	--	--	8.0	6.5	--	--	--	--

TABLE 5: ANTIMICROBIAL ACTIVITY OF DIFFERENT PELLETS OF *B.* 'TEXAS KING' CULTIVAR FLOWER EXTRACT AGAINST SELECTED MICROORGANISMS

S. no.	Samples	Zone of inhibition in millimetres (mm) with different volumes of plant extract for Antimicrobial activity against micro-organisms							
		100(µl)	50(µl)	100(µl)	50(µl)	100(µl)	50(µl)	100(µl)	50(µl)
		S. aureus		*B. licheniformis*		*E. coli*		*C. albicans*	
1	25% Pellet	--	--	--	--	--	--	--	--
2	50% Pellet	--	2.0	--	--	--	--	--	--
3	75% Pellet	2.5	3.0	--	--	--	--	--	--
4	90% Pellet	4.0	5.0	--	--	--	--	--	--
5	95% Pellet	2.5	3.0	--	--	--	--	--	--
6	Supernatant	4.5	5.5	--	--	--	--	--	--

The *B.* 'Subhra' cultivar leaf extract of different percent protein pellet and supernatant, when subjected to antimicrobial activity, showed strong activity against selected gram-positive bacteria (*S. aureus*) and fungi (*C. albicans*). No activity was observed against *B. licheniformis* and *E. coli*. The leaf extract showed a zone of inhibition which ranged from 3.5 mm (with 50% protein pellet) to 7.0 mm (with 75% protein pellet) against *S. aureus*, and it was found to be 3.0 mm and 7.0 mm with 90% and 75% protein pellet respectively against *C. albicans* **Table 6**, **Fig. 4**.

TABLE 6: ANTIMICROBIAL ACTIVITY OF DIFFERENT PELLETS OF *B.* 'SHUBRA' CULTIVAR LEAF EXTRACT AGAINST SELECTED MICROORGANISMS

S. no.	Samples	Zone of inhibition in millimeters (mm) with different volumes of plant extract for Antimicrobial activity against micro-organisms							
		100(µl)	50(µl)	100(µl)	50(µl)	100(µl)	50(µl)	100(µl)	50(µl)
		S. aureus		*B. licheniformis*		*E. coli*		*C. albicans*	
1	25% Pellet	--	--	--	--	--	--	--	--
2	50% Pellet	4.0	3.5	--	--	--	--	3.5	4.5
3	75% Pellet	7.0	6.5	--	--	--	--	7.0	6.5
4	90% Pellet	5.0	4.5	--	--	--	--	4.0	3.0
5	95% Pellet	--	--	--	--	--	--	--	--
6	Supernatant	--	--	--	--	--	--	--	--

TABLE 7: ANTIMICROBIAL ACTIVITY OF DIFFERENT PELLETS OF *B.* 'SHUBRA' CULTIVAR FLOWER EXTRACT AGAINST SELECTED MICROORGANISMS

S. no.	Samples	Zone of inhibition in millimetres (mm) with different volumes of plant extract for Antimicrobial activity against micro-organisms							
		100(µl)	50(µl)	100(µl)	50(µl)	100(µl)	50(µl)	100(µl)	50(µl)
		S. aureus		*B. licheniformis*		*E. coli*		*C. albicans*	
1	25% Pellet	--	--	--	--	--	--	3.0	2.0
2	50% Pellet	--	--	--	--	--	--	4.0	2.5
3	75% Pellet	--	--	--	--	--	--	6.0	5.0
4	90% Pellet	--	--	--	--	--	--	--	--
5	95% Pellet	--	--	--	--	--	--	--	--
6	Supernatant	--	--	--	--	--	--	--	--

The antimicrobial activity of different protein pellets and supernatant of *B.* 'Shubra' flower extract had activity against *C. albicans*, whereas no activity was observed against *B. licheniformis, S. aureus*, and *E. coli*. In flower extract of *B.* 'Shubra', the zone of inhibition ranged between 2.0 mm (with 25% protein pellet) to 6.0 mm (75% protein pellet) against *C. albicans* **Table 7**, **Fig. 4**.

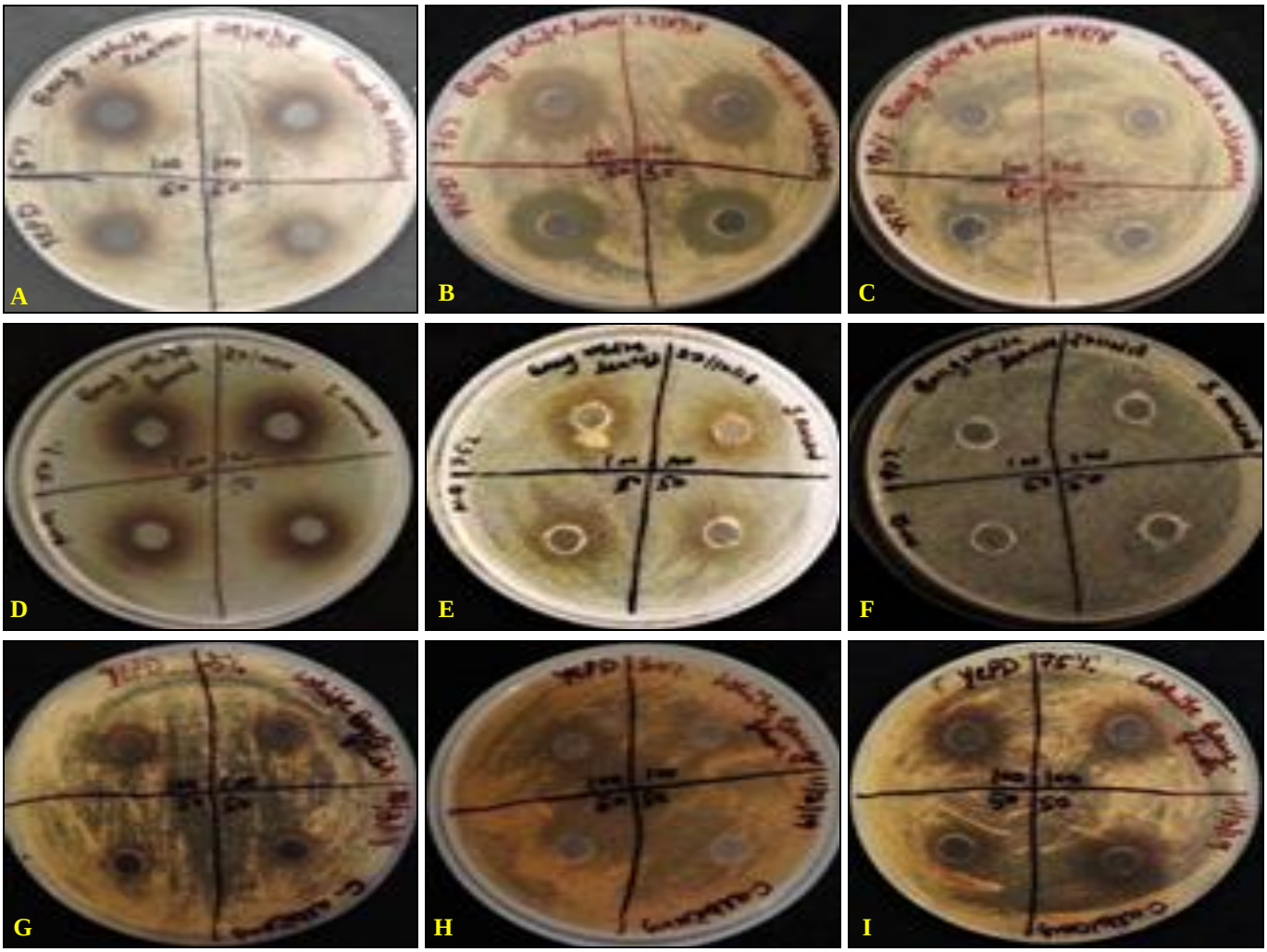

FIG. 4: ANTIMICROBIAL PEPTIDES SHOWING ANTIMICROBIAL ACTIVITY AGAINST SELECTED MICROORGANISMS, (A, B, C) 50%, 75% AND 90% PROTEIN PELLET OF *B.* 'SHUBRA' CULTIVAR LEAF EXTRACT SHOWING ANTIMICROBIAL ACTIVITY AGAINST *C. ALBICANS*. (D, E, F) 50%, 75% AND 90% PROTEIN PELLETS OF *B.* 'SHUBRA' CULTIVAR SHOWING ANTIMICROBIAL ACTIVITY AGAINST *S. AUREUS*. (G, H, I) 25%, 50% AND 75% PROTEIN PELLET OF *B.* 'SHUBRA' CULTIVAR FLOWER EXTRACT SHOWING ANTIMICROBIAL ACTIVITY AGAINST *C. ALBICANS*

The leaf extract of both the *Bougainvillea* cultivars showed antimicrobial activity against fungi (*C. albicans*), whereas no activity was observed against gram-negative bacteria (*E. coli*) with either leaf or flower extract of both the cultivars. However, antimicrobial activity was found against *B. licheniformis* and *S. aureus* with leaf extract of *B.* 'Texas King' and *B.* 'Shubra' cultivars, respectively. The flower extract of *B.* 'Texas King' and *B.* 'Shubra' showed antimicrobial activity against *S. aureus* and *C. albicans* respectively. Since, *B.* 'Texas King' and *B.* 'Shubra' cultivars has not been explored for antimicrobial proteins/ peptides till date, the comparison was made with the available literature on antimicrobial proteins/ peptides of plants belonging to the Nyctaginaceae family. Cammue *et al.*, (1992) isolated two AMPs (Mj-AMP1 and Mj-AMP2)from the seeds of *Mirabilis jalapa* L. Mj-AMP1 and Mj-AMP2 peptides comprising of 37 and 36 residues respectively [20]. These were basic in nature and consist of three disulfide bridges. These peptides show broad-spectrum antifungal activity and anti-gram-positive bacterial activity, whereas no activity was observed against gram-negative bacteria and cultured human cells. Similarly, in the present

study no antimicrobial activity was found against gram-negative bacteria.

The negative result obtained may be due to various reasons such as absence of action against these microbes, due to loss of its activity in the presence of these microorganisms and low concentration of an active protein [15]. Omptins are the proteases present in the outer membrane of gram-negative bacteria like *E. coli*. These proteases interact with lipopolysaccharide of the membrane. The bacterial proteases have the ability to convert active AMPs into inactive fragments that make the host bacteria resistant to this active AMPs [21-24]. Isolated peptides from *B.* 'Texas King' and *B.* 'Shubra' cultivars leaf and flower extract showed no antimicrobial activity with *E. coli* and this might be due to these proteases like molecules present on the membrane, which convert active AMPs to inactive molecules. The absence of action may be also due to the non-availability of specific membrane receptor protein on the surface of microbes for these antimicrobial proteins. Another probable explanation may be that after binding to the receptor its active site gets modified, thereby loss in its activity.

Tricine SDS-PAGE: The protein samples having antimicrobial activity were subjected to 12% Tricine SDS-PAGE to estimate the molecular masses of the antimicrobial protein/peptides. Different bands were observed in 25%, 50%, 75%, 90% and 95% saturated protein pellets of leaf and flower extract.

Different protein samples of *B.* 'Texas King' cultivar leaf extract showed multiple bands on Tricine SDS-PAGE. The molecular weight of proteins was calculated using a standard graph obtained from a protein marker. Three bands were observed in 25%, 75%, and 90%protein pellet ranging from 15.66kDa to 43.35kDa, whereas two bands were observed in each of the 50%, 95%, and supernatant of *B.* 'Texas King' protein sample ranging between 17.06kDa to 45.28 kDa **Table 8**, **Fig. 5**. In flower extract of *B.* 'Texas King', three bands of molecular size ranged between 5.11kDa to 22.69kDa were observed in each of the 25% and 75% protein pellet. Two bands of molecular weight 6.16kDa and 20.79kD were found in 50% protein pellet, and one band of 19.58kDa and 23.49kDa was observed in 90% and 95% protein pellet, respectively **Table 8**; **Fig. 6**.

Number of bands with different molecular weights was observed in both leaf and flower extract of *B.* 'Shubra' cultivar. In leaf, extract one band each of molecular weight 72.4kDa in 25% and 66kDa in the supernatant was observed. Whereas, two bands of 36.3kDa and 58.88kDa were observed in 50% protein pellet, and three bands ranging between 14.12kDa to 66.06kDa were observed in both the 75% and 95% protein pellet. In 90% protein pellet, four bands of molecular weight ranging between 26.30kDa to 66.06kDa were observed **Table 9**, **Fig. 7**. In flower extract, two bands of molecular size ranging between 4.64kDa to 22.13kDa were observed in both the 25% and 75% protein pellet. Three bands of molecular weight 4.89kDa, 7.85kDa, 22.13kDa were observed in 50% protein pellet, and one band of 19.5 kDa was found in 90% protein pellet **Table 9, Fig. 6**.

TABLE 8: APPROXIMATE MOLECULAR WEIGHT OF *B.* 'TEXAS KING' CULTIVAR PROTEIN SAMPLE

S. no.	Sample	Leaf extract			Flower extract		
		Relative mobility	Number of bands	Molecular weight (kDa)	Relative mobility	Number of bands	Molecular weight (kDa)
1	25% protein pellet	0.254	3	33.65	0.108	3	22.69
		0.454		21.08	0.243		17.25
		0.581		15.66	0.843		5.11
2	50% protein pellet	0.254	2	33.65	0.151	2	20.79
		0.545		17.06	0.751		6.16
3	75% protein pellet	0.163	3	41.59	0.451	3	11.32
		0.309		29.58	0.181		19.58
		0.400		23.93	0.843		5.11
4	90% proetin pellet	0.145	3	43.35	0.181	1	19.58
		0.272		32.28			
		0.545		17.06			
5	95% protein pellet	0.127	2	45.28	0.091	1	23.49
		0.509		18.53			
6	Supernatant	0.327	2	28.37	---	---	---
		0.527		17.78			

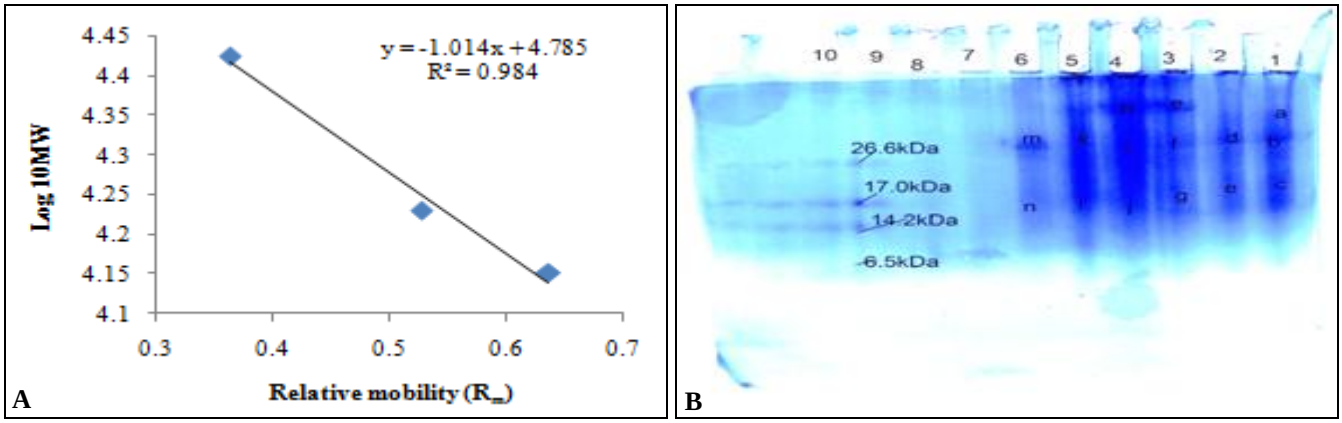

FIG. 5: (A) STANDARD GRAPH FOR ESTIMATION OF MOLECULAR WEIGHT OF ANTIMICROBIAL PROTEIN/ PEPTIDES IN LEAF EXTRACT OF *B.* 'TEXAS KING' CULTIVAR. (B) TRICINE SDS-PAGE ANALYSES OF DIFFERENT PROTEIN SAMPLES OF B. 'TEXAS KING' LEAF EXTRACT (LANE 1 TO 6). LANE 1- 25%, LANE 2- 50%, LANE 3- 75%, LANE 4- 90%, LANE 5- 95%, LANE 6- SUPERNATANT, LANE 9- LADDER, A TO N REPRESENT DIFFERENT PROTEINS BANDS IN 25%, 50%, 75%, 90%, 95% AND IN SUPERNATANT OF LEAF EXTRACT

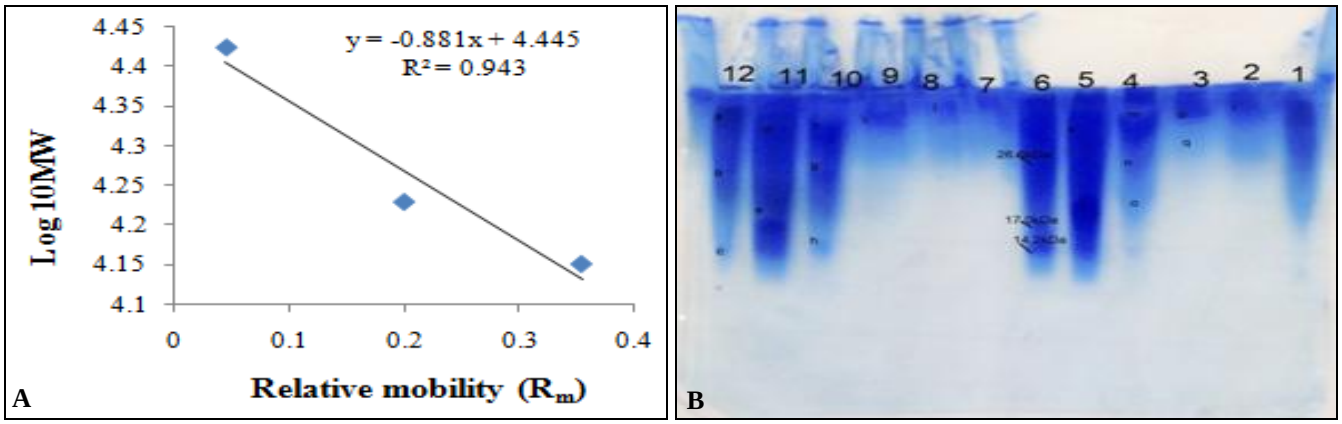

FIG. 6: (A) STANDARD GRAPH FOR ESTIMATION OF MOLECULAR WEIGHT OF ANTIMICROBIAL PROTEIN/ PEPTIDES IN FLOWERS EXTRACT OF *B.* 'TEXAS KING' AND *B.* 'SHUBRA' CULTIVARS. (B) TRICINE SDS-PAGE ANALYSIS OF DIFFERENT PROTEIN SAMPLES OF *B.* 'SHUBRA' (LANE 7-12) AND *B.* 'TEXAS KING' (LANE 1 TO 5) FLOWER EXTRACT. LANE 5- 25% PROTEIN PELLET , LANE 4- 50% PROTEIN PELLET, LANE 3- 75% PROTEIN PELLET, LANE 2- 90% PROTEIN PELLET AND LANE 1- 95% PROTEIN PELLET OF *B.* 'SHUBRA'. LANE 6-LADDER AND LANE 12- 25% PROTEIN PELLET, LANE 11- 50% PROTEIN PELLET, LANE 10- 75% PROTEIN PELLET, LANE 9- 90% PROTEIN PELLET, LANE 8- 95% PROTEIN PELLET OF *B.* 'TEXAS KING'. A TO J- REPRESENT DIFFERENT PROTEINS BANDS OF *B.* 'TEXAS KING' AND LANE K TO R- REPRESENT DIFFERENT PROTEINS BAND OF *B.* 'SHUBRA'

TABLE 9: APPROXIMATE MOLECULAR WEIGHT OF B. 'SHUBRA' CULTIVAR PROTEIN SAMPLE

S. no.	Sample	Leaf extract			Flower extract		
		Relative mobility	Number of bands	Molecular weight (kDa)	Relative mobility	Number of bands	Molecular weight (kDa)
1	25% protein pellet	0.181	1	72.44	0.702	2	6.80
					0.843		5.11
2	50% protein pellet	0.236	2	58.88	0.121	3	22.13
		0.363		36.30	0.632		7.85
					0.864		4.89
3	75% protein pellet	0.223	3	61.65	0.121	2	22.13
		0.363		36.30	0.891		4.64
		0.618		14.12			
4	90% protein pellet	0.200	4	66.06	0.181	1	19.58
		0.363		36.30			
		0.400		31.62			
		0.581		26.30			
5	95% protein pellet	0.200	3	66.06	---	---	---
		0.327		41.49			
		0.363		36.30			
6	Supernatant	0.200	1	66.06	---	---	---

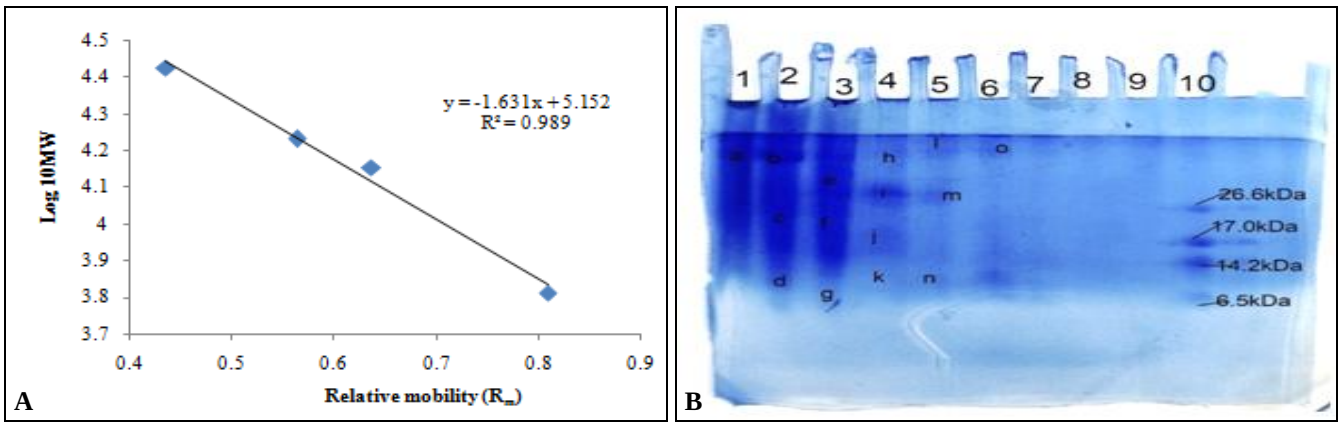

FIG. 7: (A) STANDARD GRAPH FOR ESTIMATION OF MOLECULAR WEIGHT OF ANTIMICROBIAL PROTEIN/PEPTIDES IN LEAF EXTRACT OF *B.* 'SHUBRA' CULTIVAR. (B) TRICINE SDS-PAGE ANALYSIS OF DIFFERENT PROTEIN SAMPLES OF *B.* 'SHUBRA'LEAF EXTRACT (LANE 1 TO 6). LANE 1- 25% PROTEIN PELLET, LANE 2-50% PROTEIN PELLET, LANE 3- 75% PROTEIN PELLET, LANE 4- 90% PROTEIN PELLET, LANE 5- 95% PROTEIN PELLET AND LANE 6- SUPERNATANT, LANE 10- LADDER. AT OO REPRESENTS DIFFERENT PROTEIN BANDS IN 25%, 50%, 75%, 90%, 95% AND IN SUPERNATANT OF LEAF EXTRACT

The molecular weight of antimicrobial proteins / peptides from the leaves of *Bougainvillea* 'Texa King' and *Bougainvillea* 'Shubra' cultivars ranged between 14.1 to 72.4kDa. Whereas, antimicrobial proteins/ peptides from flower extract of both the cultivars ranged between 4.6 to 23.4 kDa. From the result obtained this can be interpreted that different parts of the same plant species have different types of antimicrobial proteins/peptides in relation to number and molecular weight. The flower extract showed lower molecular weight proteins; therefore, the possibility that these are the peptides of interest with antimicrobial activity increases.

Narwal *et al*., (2001) isolated and purified antiviral proteins from the leaves of *Bougainvillea xbuttiana*, which imparted resistance against tobacco mosaic virus (TMV) and sun hemp rosette virus (SRV) in their respective test hosts *viz. N. glutinosa*, *N. tabacum* var Samsun-NN and C*yamopsis tetragonoloba* respectively [12]. Two polypeptides of MW 28,000 and 24,000 D were observed on SDS-PAGE. These two polypeptides were found to be highly basic, rich in lysine with pI around 10.0 and 10.5 respectively. Similarly, antimicrobial proteins/ peptides of less than 26.6 kDa were found in leaf extract of *Datura inoxia*, which showed activity against *S. aureus* and *B. subtilis* whereas no activity was showed against *C. albicans* and *E. coli* [25]. In the present study, also no activity was recorded against gram-negative bacteria. From the earlier and present study, it could be inferred that PAMPs are less potent against gram-negative bacteria.

CONCLUSION: The present study is an attempt to isolate antimicrobial protein/peptides from leaves and flowers of *B.* 'Texas King' and *B.* 'Shubra' cultivars. Antibacterial activity was recorded in leaf and flower extract of *B.* 'Texas King' cultivar against *B. licheniformis* and *S. aureus* respectively, whereas *B.* 'Shubra' cultivar leaf extract showed activity against *S. aureus*. The antifungal activity was found in *B.* 'Shubra' (leaf and flower) extract and *B.* 'Texas King' leaf extract whereas no activity was recorded with flower extract of *B.* 'Texas King'. These protein extracts have been found to be active against selective microbes; however, none of them is active against gram-negative bacteria (*E. coli*). Extracts not showing activity against certain gram-positive bacteria selected in the present study might be attributed to low concentration or variation in minimum inhibitory concentration (MIC) value.

The study also reveals that different proteins/ peptides of interest can be extracted from different parts of the same plant species, thereby harvesting only that particular part of the plant rather than sacrificing the whole plant, which will help in the sustainable conservation of biodiversity. In our studies, flowers may be the suitable source for isolation of antimicrobial peptides, rather than leaves, which requires confirmation by further purification and characterization. Therefore, these proteins are the putative AMPs showing antimicrobial activity which can be used for medical purpose in the development of new antibiotics.

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CONFLICTS OF INTEREST: The authors declare that they have no conflict of interest.

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