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ANTIMICROBIAL ACTIVITY OF LEAF EXTRACTS OF THREE MEDICINAL PLANTS

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ABSTRACT

Crude extracts from leaves of three medicinal plants namely: *Chenopodium album, Moringa oleifera* and *Terminalia arjuna* in four buffers: phosphate buffer saline (PBS), potassium phosphate buffer (PPB), sodium acetate buffer (SAB) and sodium phosphate buffer (SPB), were screened for their antimicrobial activity. The quantity of proteins in the extracts was estimated and antimicrobial activity of the extracts determined on the basis of inhibition zone using Agar well Diffusion Assay and Minimal Inhibition Concentration (MIC). The maximum protein (22.96 mg protein/100 ml) was found in the leaf extract of *M.oleifera* extracted in PBS buffer. Minimum protein was 7.29 mg/100 ml in *T. arjuna in* the sodium phosphate buffer.All the plants showed significant activity against eight pathogens tested. The PBS buffer extract of *M.oleifera* showed the maximum inhibition against *E.coli* (31±0.23mm) and *A. niger* (30±0.05 mm) while the minimum inhibition was seen against *P. vulagris* but none for *Fusarium spp.* MIC values of all extracts tested against five bacterial species ranged from 11 to 188 µg/ml and against the three fungi between 24 to 387µg/ml, respectively. Extracts in phosphate buffer saline and sodium acetate buffer, had higher activity as compared to that in potassium phosphate and sodium phosphate buffers.

KEYWORDS: Chenopodium album, Moringa oleifera and Terminalia arjuna, antimicrobial activity, Buffers, Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa , Proteus vulgaris, Apergillus niger, Tricophyton rubrum and Fusarium spp.

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INTRODUCTION

Plants have been the best source for natural products in terms of drugs and active compounds for maintaining human health and control of infections because of microbes that pose serious health problems[1, 2]. Over the past two decades, there has been a lot of interest in the investigations on natural materials as sources of new antibacterial and antifungal agents[3]. Therefore, plants as antimicrobials are being investigated to better understand their properties, safety and efficacy[4].The aim of the present study was to study the antimicrobial activity of leaf extracts of plants namely *Chenopodium album, Moringa oleifera* and *Terminalia arjuna* against bacteria and fungi. *Chenopodium album, Moringa oleifera* and *Terminalia arjuna* are the plants widely distributed in Asia. *Chenopodium album* is commonly known as *Bathua* used as a leafy vegetable and belongs to the family Chenopodiaceae, is distributed throughout the world. This is also known to be medicinal plant in scientific and folk literature, having antipruritic, antinociceptic[5], and sperm immobilizing activity[6]. Medicinally, it has been used to treat various symptoms attributable to nutritional deficiencies and curing anorexia, cough, dysentery, diarrhoea, piles and kills small worms. It is also having sedative and refrigerant properties. Aqueous and methanolic extracts of *C. album* have been shown to be antibacterial against human pathogenic bacteria, viz. *E. coli, S. typhimurium, S. aureus, P. vulgaris* and *P. aueruginosa*. The aqueous leaf extract revealed strong antibacterial activity on *S. aureus* while methanolic leaf extract proved to be having strong antibacterial activity against *P. aeruginosa*[7,8]. *Moringa oleifera* (Lam.) is a highly nutritious plant used for vegetable and pickling purposes and also medicinal values[9].Different parts of the plant such as the leaves, roots, seed, bark, fruit, flowers and immature pods act as cardiac and circulatory stimulants, and for treatment of fever, bronchitis, eye and ear infections, diarrhoea. It also possesses antitumour, antipyretic, antiepileptic, anti-inflammatory, antispasmodic and diuretic activity[10], antiulcerative[11], cholesterol lowering[12], antihypertensive, antioxidant, antidiabetic, and hepatoprotective[13], antibacterial and antifungal activities[14]. *Terminalia arjuna*, commonly known as *Arjuna*belongs to the family Combretaceace and found throughout the greater part of northern India. It is one of the most versatile medicinal plants having a wide spectrum of biological activity. It contains flavonoids as arjunone, arjunolone along with biacalein, luteolin, quercetin-7-O-rhamnoside, triterpenoid saponins as arjunic acid, arjunolic acid, glycoside, steroids, tannins etc. The bark of *T. arjuna* is anti-dysentric, antipyretic, astringent, cardiotonic, lithotriptic and health tonic while the powder of the bark acts as a diuretic in cirrhosis of the liver and gives relief in symptomatic hypertension[15]. *Arjuna* leaves have also been shown to possess analgesic and anti-inflammatory properties[16].

MATERIALS AND METHODS

(i)Bacterial and fungal strains

Authentic cultures of bacterial and fungi were obtained from IMTECH, Chandigarh. Bacterial strains were *S. aureus* (MTCC 7443), *E. coli* (MTCC730),*B. subtilis* (MTCC441), *P. aeruginosa* (MTCC7925), *P. vulgaris* (MTCC1771) and fungal strains were *A. niger* (MTCC 281), *Fusarium spp*. (MTCC 3871) and *T. rubrum* (MTCC 3272).

(ii)Medicinal plants

The medicinal plants under study were selected on the basis of review and ethno pharmacologic reports. Leaves of the following plants viz. *Chenopodium album, Moringa oleifera* and *Terminalia arjuna* were screened for the study. The leaves of selected medicinal plants were collected from herbal garden of Amity University, Gurgaon. The leaves of selected plants were collected and washed with sterile distilled water and then dried using laminar air flow and stored in air tight containers till further analysis.

(iii) Extraction of crude extracts

Crude leaf extracts were prepared using10g of leaf samples by grinding in 150 ml of any one of four different buffers, namely i) Phosphate buffer saline (PBS, pH 7.2), ii) 10mM Potassium phosphate buffer (PB, pH 7.0), iii) 10mM of sodium phosphate buffer (SP, pH 7.0) iv) 3M of sodium acetate buffer (SA, pH 5.2) respectively, with the help of ice cold mortar & pestle. The extracts thus obtained were centrifuged at 12,000 rpm for 30 min at 4°C. Residue was discarded and supernatant collected. The resultant supernatant (crude extract) was subjected to Bradford assay for total protein quantification. The extracts were then saturated to 80% ammonium sulfate. The supernatant and pellet (resuspended in 5 ml of respective buffer) thus obtained were re-subjected to Bradford assay before testing their antimicrobial activity[17].

(iv)Culture media and preparation of inocula

Stock cultures were maintained at 4ºC on slants of Nutrient agar (NA, Hi-Media) for bacteria and potato dextrose agar (PDA) for fungus, and then they were used to check antimicrobial activity of the resuspended pellet and supernatant of selected plants against *S. aureus, E. coli, B. subtilis, P. aeruginosa, P. vulgaris, A. niger*, *T. rubrum and Fusarium spp*. The microbial strains from the stock culture were inoculated on the agar plates using sterilized platform of Laminar Air Flow and incubated at 37°C for bacterial growth for 24 hr and fungal growth for 2–3 days. Nutrient broth (1.3 g/100 ml) and Potato dextrose broth (2.4 g/100 ml) was mixed in distilled water and autoclaved at 121°C for 15 min. A loop full from stock culture of the bacteria and fungi was mixed in the 10 ml of broth mediums and incubated overnight at 37°C and then used for streaking onto the agar plates to check antimicrobial activity.

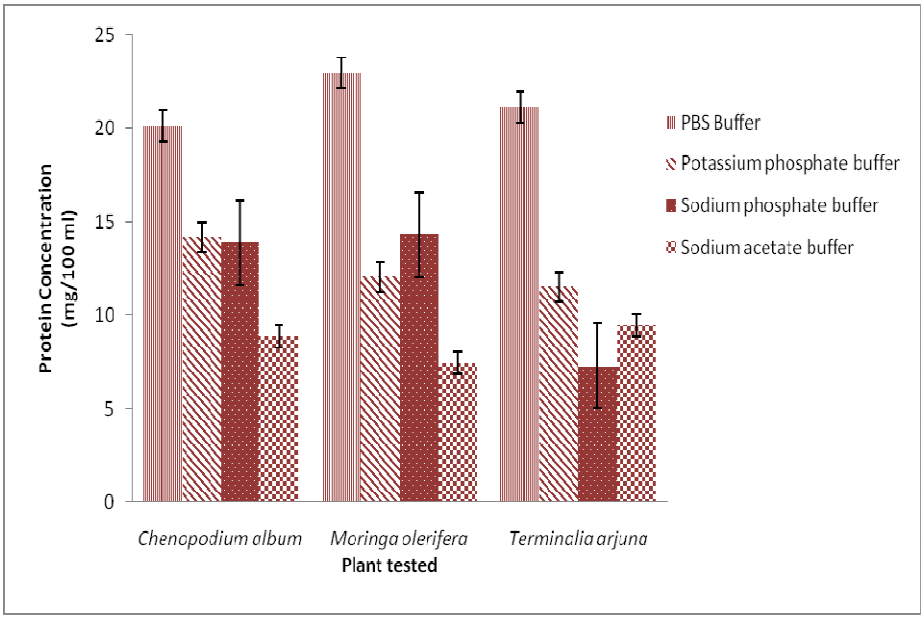
(v) Assay of Antimicrobial activity

The antimicrobial activity of the pellet and supernatant was determined by Agar well diffusion assay. Nutrient agar plates (2.34 gm NA in 100 ml of distilled water) were prepared for bacteria and potato dextrose agar plates (3.9 gm PDA in 100 ml of distilled water) was prepared for fungus. Petri plates containing 25ml of agar medium were inoculated with 100µl of bacterial and fungal cultures by the spread plate technique and were allowed to dry in a sterile chamber.6mm wells were created aseptically using sterile agar borer. A 50 µl of the leaf extracts were loaded into the wells and allowed to get absorbed into the agar medium. The plates were then incubated at 37°C for 24 h for bacteria and 2-3 days for fungi. Rifampicin and Chloramphenicol were used as positive control and water as a negative control. The plates then examined for the presence of growth inhibition zones, and diameters were measured in millimeters. The experiment had three replicates for each treatment and was repeated twice[18].

RESULTS AND DISCUSSION

For the extraction of proteins from plants, many factors are important such as pH (acidic and basic), ionic composition of the system, temperature, solvent volume, types of polymers (protein/peptide), concentration of the targeted protein/peptide and many other parameters may influence the extraction and stability of protein[19]. Hence no single buffer is universal and appropriate for the extraction of all the targeted proteins[20]. The concentration of protein in crude extracts from three plants varied depending on the extraction buffer (Figure 1). The maximum proteins in leaf extracts were in the *M. oleifera* i.e. 22.96 mg protein/100 ml in PBS buffer. Minimum extraction of protein 7.29 mg/100 ml in *T. arjuna* was observed in the sodium phosphate buffer (Figure 1).Overall comparison of these buffer systems showed that phosphate buffer saline allowed maximum extraction of protein with highest protein concentration in *M. oleifera* while it was lowest in sodium phosphate buffer. Crude extract of these plants were subjected to ammonium sulphate precipitation to 80% saturation level. The pellet (resuspended) and supernatants thus obtained were subjected to test for antimicrobial activity against eight microbes, namely *S. aureus, E. coli, B. subtilis, P. aeruginosa, P. vulgaris, A. niger*, *T. rubrum and Fusarium spp* (Table 1). The supernatants did not show any antifungal or antibacterial activity. Zero or lower activities registered by these plant extracts indicate that either the plant had no antimicrobial activity or poor activity because of very low protein concentration. Antimicrobial activities were observed for the resuspended pellet against the eight microbes tested. Among the four buffers, maximum antimicrobial activity was registered upon extraction in PBS from all the three plants tested. This may be due to the fact that buffers with the recommended pH show insignificant penetration through biological membranes, and have maximum buffering capacity at the pH tried where the protein exhibits optimalstability[21].Strong antifungal and antimicrobial activities were observed for the resuspended pellet extract, suggesting that the activity was most likely either due to some protein or peptide. Earlier studies have reported that there are plants where the isolated proteins were not effective and the activity was actually due to the presence of certain secondary metabolites or other organic compounds[22]. Therefore, treatment of the pellet extract with proteinase K (10 µl of 20 mg/mL) was tried. It abolished the activity confirming that antimicrobial activities were only due to proteins or peptides present in the extract (data not given) and not due to other organic compounds[23].

Figure 1
Estimation of protein (mg/100ml) from crude leaf extracts of the medicinal plants in different buffers



The leaf extracts of *Chenopodium album* in all four buffers exhibited significant antimicrobial activity, varied greatly in the zone of inhibition from 7 to 21 mm. But there was no antibacterial activity of *C. album* leaf extracts against *P. aeruginosa* and also antifungal activity against *Fusarium spp.* irrespective of any of the four buffers tested (Table 1).

- The leaf extract of *C. album* in PBS showed strongest antimicrobial activity against *E.coli* (21±0.01mm) followed by *S. aureus* (19±0.04 mm) and *A. niger* (15±0.13mm) respectively and minimum activity was found against *P. vulgaris* (9±0.14mm) and *T. rubrum* (7±0.09 mm) respectively.
- In PPB extracts highest activity was registered against *S. aureus* (13±0.03 mm) followed by *E.coli* (12±0.23 mm), *A. niger* (12±0.18mm), *T. rubrum* (11±0.13 mm) while having moderate activity against *B. subtilis* (10±0.06 mm) but no activity against *P. vulgaris*.
- Leaf extracts in SPB showed highest antimicrobial activity against *A. niger* (14±0.15 mm), *S. aureus* (14±0.00 mm), *E.coli* (12±0.03 mm) and *T. rubrum* (10±0.23 mm) respectively but none against *B. subtilis and P. vulgaris*.
- The SAB extracts had maximum activity against *S. aureus* (18±0.12 mm) followed by *E.coli* (15±0.16 mm), *A. niger* (15±0.24 mm), *B. subtilis* (12±0.03 mm) but nil against *P. vulgaris*.

M. oleifera leaf extracts in all the buffers registered significant antimicrobial activity against all the bacteria and fungi tested (Table 1).The extracts of *M. oleifera* in PBS showed the highest activity followed by that in SAB.

- The leaf extracts in PBS had maximum antibacterial activity against *E.coli* (31±0.23 mm) followed by B. subtilis (17±0.14 mm), *P. vulgaris* (17±0.11 mm) and antifungal activity against *A. niger* (30±0.05 mm) followed by *T. rubrum* (27±0.03 mm).
- In PPB, highest antimicrobial activity was seen against *S. aureus* (18±0.15 mm) followed by *E.coli* (17±0.01 mm), *A. niger* (17±0.12 mm), *B. subtilis* (16±0.09 mm) and moderate activity against *P. vulgaris* (15±0.03 mm), *T. rubrum* (14±0.01 mm) but minimum activity was found against *P. aeruginosa* (11±0.02 mm) and *Fusarium spp* (9±0.00 mm).
- Leaf extracts of *M. oleifera* in SPB showed antimicrobial activity against all the microbes, the highest against *E.coli* (18±0.08 mm) and lowest against *P. aeruginosa* (11±0.03 mm) whereas highest antifungal activity was against *A. niger* (16±0.11mm) and lowest against *Fusarium spp* (10±0.01mm) respectively.
- In case of SAB maximum activity was against *E.coli* (28±0.00 mm) followed by *S. aureus* (25±0.03 mm), *A. niger* (25±0.00 mm), and least against *Fusarium spp* (10±0.14 mm). Similarly, in case of *T. arjuna,* PBS extract exhibited maximum activity against bacteria and fungi with zones of inhibition in the range of 25mm-10mm (Table 1) followed by that in SAB with zone of inhibition in the range of 17mm-8mm.
- In PPB, the highest antimicrobial activity was registered against *E.coli* (19±0.14) followed by *B. subtilis* (16±0.08), *A. niger* (16±0.23), and minimum activity against *P. vulgaris* (11±0.16), *T.rubrum* (11±0.06) and *S. aureus* (8±0.17).But the activity was nil against *P. aeruginosa* and *Fusarium spp*.
- Similarly, SPB extract showed moderate activity against *B. subtilis* (13±0.13), *A. niger* (15±0.15) and poor against *E.coli* (10±0.01), and *T. rubrum* (11±0.01) but none against *S. aureus*, *P. vulgaris, P. aeruginosa and Fusarium spp.*

In literature, these plants are reported to be rich in primary and secondary metabolites[24-26]. Various reports indicate the antimicrobial activity exhibited in extracts of these plants i.e. our results corroborate the previous findings. Aqueous and methanol leaf extracts of *C. album* were evaluated against five human pathogenic bacteria viz. *E. coli*, *S. typhimurium*, *S. aureus*, *P. vulgaris* and *P. aeruginosa*[7]. Nayak *et al.*[26] evaluated the antimicrobial activity and anthelmintic activity of solvent extracts of *Chenopodium album* against different pathogens. Antimicrobial activity[28], anti-inflammatory activity[29], hypotensive activity[30] and antihepatotoxic properties[15]of *Moringa oleifera* have been reported. Antibacterial and antifungal activity of *M. oleifera* leaves in aqueous, acetone and ethanolic extract have already been reported against other pathogens[31, 32]. Similarly, aqueous extracts of *T. arjuna* bark showed activity against *P. vulgaris, K. aerogenes, E. coli* and *P. aerogenes*[33], *S. epidermidis*[34], ear pathogens namely *S. aureus, P. aeruginosa, P. mirabilis, Acitenobacter sp.*[35]. Similarly root extracts of *arjuna* showed activity against *Aspergillus niger* and *Candida albican*[36], methanolic extract showed antifungal and antihelicobactor activity[37]. In other study, the organic solvent extracts of five *Terminalia* species (*T. arjuna, T. chebula, T. bellerica, T. catappa* and *T. alata*) were tested and found to inhibit the plant pathogenic fungi i.e. *A. flavus, A. alternata, A. niger, A. brassicicola,* and *H. tetramera*[38].

Table 1
Antimicrobial activity of the pellet (resuspended) from crude extracts of the medicinal plants against pathogenic microbes

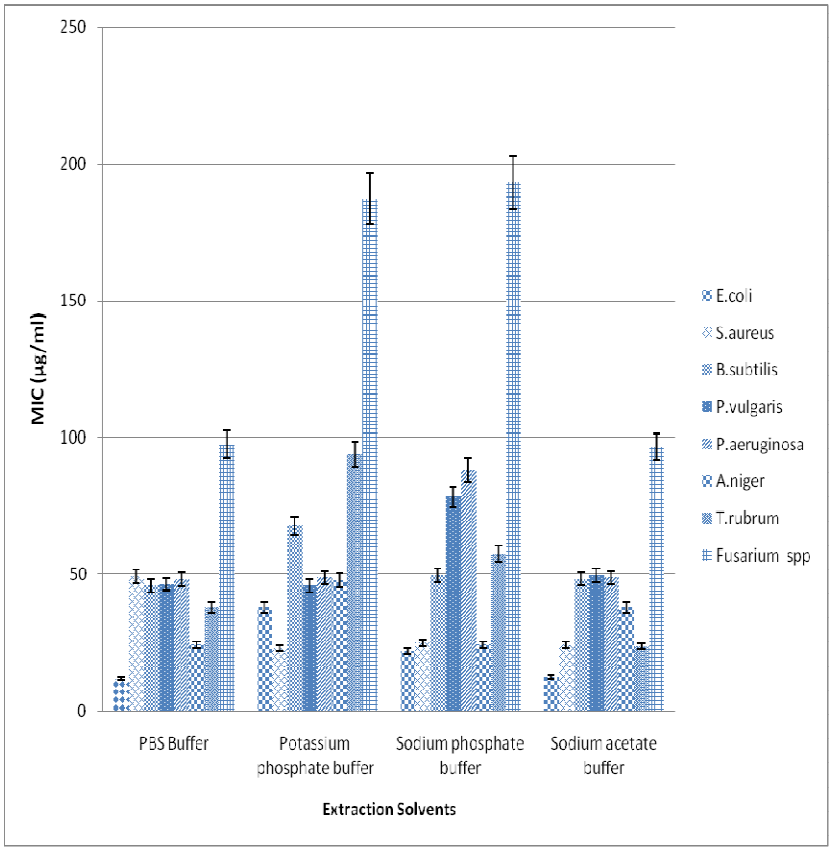
Buffer Extractant	Medicinal Plants	Zone of inhibition (mm)							
		Bacteria					Fungi		
		E.coli	*S.aureus*	*B.subtilis*	*P.vulgaris*	*P.aeruginosa*	*A.niger*	*T.rubrum*	*Fusarium spp*
PBS Buffer (pH 7.2)	*Chenopodium album*	21±0.01	19±0.04	12±0.01	9±0.14	0	15±0.13	10±0.00	0
	Moringaoleifera	31±0.23	12±0.16	17±0.14	17±0.11	15±0.15	30±0.05	27±0.03	19±0.00
	Terminalia arjuna	25±0.12	18±0.02	24±0.11	15±0.03	10±0.01	25±0.15	18±0.12	11±0.03
Potassium phosphate buffer (pH 7.0)	*Chenopodium album*	12±0.23	13±0.03	10±0.06	0	0	12±0.18	11±0.13	0
	Moringaoleifera	17±0.01	18±0.15	16±0.09	15±0.03	11±0.02	17±0.12	14±0.01	9±0.00
	Terminalia arjuna	19±0.14	8±0.17	16±0.08	11±0.16	0	16±0.23	11±0.06	0
Sodium phosphate buffer (pH 7.0)	*Chenopodium album*	12±0.03	14±0.00	0	0	0	14±0.15	10±0.23	0
	Moringaoleifera	18±0.08	16±0.25	14±0.01	12±0.12	11±0.03	16±0.11	13±0.05	10±0.01
	Terminalia arjuna	10±0.01	0	13±0.13	0	0	15±0.15	11±0.01	0
Sodium acetate Buffer (pH 5.2)	*Chenopodium album*	15±0.16	18±0.12	12±0.03	0	0	15±0.24	7±0.09	0
	Moringaoleifera	28±0.00	25±0.03	18±0.11	15±0.03	15±0.02	25±0.00	19±0.18	10±0.14
	Terminalia arjuna	16±0.09	11±0.15	14±0.08	10±0.21	8±0.02	17±0.19	11±0.01	0
Rifampicin		36	29	29	26	24	35	30	29
Chloramphenicol		41	36	34	29	28	40	29	28
Water		0	0	0	0	0	0	0	0

Minimum inhibitory concentration (MIC) evaluation of leaf extracts was determined against the bacterial and fungal strains, necessary for their effective use in further study (Table 2). It was observed that the extract of *M. oleifera* had highest activity. Out of the four extracts of *M. oleifera* (Figure 2), the extract in PBS buffer had highest MIC value (11.95±0.01µg/ml) against *E.coli.* The minimum inhibitory concentrations of *M. oleifera* reported in earlier studies reveal its high antibacterial potential against *E. coli* and *S. aureus*[39]. The present study corroborates their findings. Webster *et al.*[40] also reported that crude plant extracts are generally a mixture of active and non-active compounds, and MICs of less than 100 µg /ml suggest good antimicrobial activity. In this study, MICs values were found to be potent or less than 100 µg/ml in most of the extracts, proving high antimicrobial activity in plants under study. Present evaluation suggests antimicrobial activity in these plants to be due to the proteins while previous study[27,31] reported that antimicrobial activity was due to phytochemicals like saponin, triterpenoids, steroids, glycosides, anthraquinone, and flavonoids. This also suggests that the selected medicinal plants have the antimicrobial potential against various pathogens not only due to any single but cumulative and/or complex (synergistic) effects of different components.

Table 2
Minimum inhibitory concentration (MIC, µg/ml±SD) of the leaf extracts of the medicinal plants against the bacterial and fungal strains

Buffer Extractant	Medicinal Plants	MIC (µg/ml)							
		Bacteria					Fungi		
		E.coli	*S.aureus*	*B.subtilis*	*P.vulgaris*	*P.aeruginosa*	*A.niger*	*T.rubrum*	*Fusarium spp*
PBS Buffer (pH 7.2)	*C.album*	45.13±0.04	46.40±0.04	88.12±0.08	97.50±0.09	-	78.75±0.07	98.43±0.09	386.25±0.38
	M.oleifera	11.95±0.01	49.21±0.04	45.93±0.04	46.40±0.04	48.25±0.04	24.35±0.02	37.96±0.37	97.50±0.09
	T.arjuna	47.810±0.04	91.80±0.09	48.75±0.04	90.00±0.09	180.0±0.18	45.00±0.04	44.06±0.04	-
PP buffer (pH 7.0)	*C.album*	94.68±0.09	185.62±0.18	187.5±0.18	-	185.6±0.18	96.56±0.09	94.68±0.09	-
	M.oleifera	37.96±0.37	22.96±0.02	67.81±0.04	45.93±0.04	48.75±0.04	47.810±0.04	93.75±0.09	187.5±0.18
	T.arjuna	25.03±0.25	393.75±0.39	-	180.0±0.18	-	48.75±0.04	95.62±0.09	-
SP buffer (pH 7.0)	*C.album*	92.81±0.09	47.810±0.04	-	183.75±0.1	-	45.75±0.04	180.0±0.18	-
	M.oleifera	22.03±0.02	25.030±0.25	49.68±0.04	78.28±0.04	88.12±0.08	24.370±0.02	57.50±0.09	193.1±0.19
	T.arjuna	-	187.5±0.18	93.75±0.09	-	-	90.00±0.09	95.62±0.09	-
SA Buffer (pH 5.2)	*C.album*	58.75±0.04	25.03±0.25	104.68±0.09	-	-	57.18±0.02	-	-
	M.oleifera	12.51±0.10	24.35±0.02	48.28±0.04	49.68±0.04	48.75±0.04	37.96±0.37	23.67±0.02	96.56±0.09
	T.arjuna	37.96±0.37	94.68±0.09	82.81±0.09	172.8±0.18	187.50±0.18	47.34±0.04	151.8±0.18	-

Figure 2
Minimum inhibitory concentration (MIC,µg/ml) of Moringaoleifera in four different buffer extracts



CONCLUSION

The results (Tables 1 and 2) of the present study showed that plant extracts screened against eight microbes exhibited significant antimicrobial effects. In particular, phosphate saline buffer extracts of these plants offer effective separation of bioactive components for the inhibition. These plant species were having high antimicrobial activity nearly comparable to that of the commercially available antibiotics.The present study focused on preliminary screening of extracts having antimicrobial activity through their proteins for the treatment of various diseases. Further studies are, however, required to confirm the most potential cause of effectiveness as antimicrobial agents. The present results may form the basis for selection of plant species for investigations in the potential discovery of new bioactive components. Further studies therefore have been initiated for the isolation and structure elucidation of the antimicrobial bioactive components from these plants.

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