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Short Communication

Antibacterial Activity of *Otostegia limbata*

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ABSTRACT

The aim of this work was to examine the antimicrobial effect of *Otostegia limbata* extracts obtained with solvent of different polarities on six different bacteria. Powdered aerial parts of the *O. limbata* plant were extracted with ethanol and then dry later made solution in threev solutions, dimethylsulphoxide (DMSO) and methanol.. Antimicrobial activities of three extracts were tested on 6 bacterial strains using Disk Diffusion Method (DDM), Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The results values showed that *Staphylococcus aureus* was most sensitive against all three extracts. The highest zone of inhibition (ZOI) value of *S. aureus* against DMSO extract was 19±1.5. No antimicrobial activity was observed against the *Pseudomonas aeruginosa,* while only ethanolic extract showed some antimicrobial activity against *Escherichia coli* with ZOI value of 11.5±1.1 and the extracts inhibited the growth of the bacterial isolates in a concentration dependent manner with MICs ranging from 0.5-2 mg mL^{-1}. This is the first report of the antibacterial potency of *O. limbata.* The findings provide the evidence that *O. limbata* as a good medicinal plant for further investigations.

Key Words: Antibacterial; Zone of inhibition; Disk diffusion method; Minimum inhibitory concentration; Minimum bactericidal concentration

INTRODUCTION

The use of herbal medicines has always been part of human culture, as some plants possess important therapeutic properties, which can be used to cure human and other animal diseases. Rios and Receo (2005) reported that certain plants had healing potentials. Indeed this antimicrobial principle was well accepted long before mankind discovered the existence of microbes. The healing property of these medicinal plants is usually due to the presence of secondary metabolites, which differ from one plant to another. The use of plant, plant extract or chemical derived from plants to treat disease is therapeutic modality. To date over 75 pure compounds derived from higher plants are used in herbal medicine but most of those applied in modern medicine are now produced synthetically (Suck, 1989; Anwannil & Atta, 2005). A large number of phytochemicals belonging to several classes show inhibitory effect on all types of micro-organisms in vitro (Cowan, 1999) and some plant extracts have shown activity on both Gram negative and Gram positive organisms (Nascimento *et al.,* 2000).

Labiatae species are recognized for their medicinal uses. Because of their vital oils content, antimicrobial activity of different species of this family was demonstrated (Skaltsa *et al.*, 2003). It was reported that *Mentha* spp. exhibit antimicrobial activity against *Escherichia coli*, *Bacillus subtilis*, *Salmonella enteritidis* and *Staphylococcus aureus* (Sivropoulou *et al.,* 1995; Tassou *et al.,* 2000). The genus *Otostegia* Bioss. (Labiatae) comprises 20 species. Except *Otostegia persica,* no previous antimicrobial investigation of this species has been reported. The tested extracts of *O. persica* exhibited significant antibacterial activity, which supported the idea of using it as a candidate for further antimicrobial and phytochemical research (Asghari *et al.,* 2006).

There are reports on the isolation of two new tricyclic clerodane-type diterpenoids (limbatolide D & E) from the roots of *O. limbata.* It is also reported that juice of *O. limbata* plant is used to treat bleeding gums of children and opthalmia (Ahmad *et al.,* 2006). Their structures and the relative configuration were established on the basis of spectral methods, especially two-dimensional (2D) NMR techniques (Ahmad *et al.,* 2007). *O. limbata* grows in Northern-West of Pakistan in hilly areas near Abottabad.

The aim of this work was to examine the effect of the dimethyl sulfoxalate (DMSO), methanolic and ethanolic extracts of *O. limbata* on the growth of six bacterial strains using various approaches.

MATERIALS AND METHODS

Microorganisms. Cultures of *B. subtilis*, *Listeria monocytogenes*, *S. aureus*, *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella spp*. were obtained from Department of Biological Sciences, Forman Christian College Lahore, Pakistan. Purity plate of each of the

bacterial isolates was obtained by culturing the bacterial isolates on their selective media. Biochemical tests were replicated to re-identify and confirm the identity of the isolates.

Plant material. Aerial parts of the plant were collected from hills near Abottabad, Pakistan. Extraction was completed by macerating l00 g of powdered dry plant in 900 mL of 70% ethanol as solvent for 48 h at room temperature with the help of a percolator apparatus (2 L capacity). The extracts were filtered using Whatman filter paper (No. 4) and dried under reduced pressure at 40°C using rotary evaporator. The dried extracts were then extracted with 350 mL of DMSO, followed by 350 mL each of ethanol and methanol. Concentration of extracts was determined by difference in the weight of evaporated volume from the primary volume of the extracts.

Antimicrobial Activity

Disc diffusion method. This method was described by Bauer *et al.* (1966). The MHA plates were prepared by pouring 15 mL of molten media into sterile Petri plates and allowed to solidify for 5 min. Then 0.1% inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 min. Different concentrations of extracts (0.25, 0.50, 1, 2 & 4 mg $disc^{-1}$) were loaded on 6 mm sterile disc, which were placed on the surface of medium and the compound was allowed to diffuse for 5 min. After overnight incubation at 37°C, diameter of the zone of inhibition (ZOI) of bacterial growth around each disc was measured with transparent ruler. A 0.3 g mL^{-1} of Erythromycin treated tubes were used as control. These studies were replicated six times.

Minimum inhibitory concentration (MIC) test. This test utilized the lowest concentration of an antimicrobial extract to inhibit the visible growth of a microorganism after overnight incubation (Jennifer, 2001). The potential extract was determined according to the macro-broth dilution technique (Tilton & Howard, 1987; Baron & Finegold, 1990). Standardized suspensions of the test organisms were inoculated in a series of sterile tubes of nutrient broth containing twofold dilutions of leaf extracts and incubated at 37°C for 24 h. The cultures were prepared at 24 and 48 h broth cultures of tested microorganisms.

Minimum bactericidal concentration (MBC). The MBCs were determined by first selecting tubes that showed no growth during MIC determination. For this a loopful from each tube was sub-cultured onto extract free agar plates and incubated for further 24 h at 37°C. The lowest concentration at which no growth was observed was noted.

RESULTS AND DISCUSSION

Extract of *O. limbata* plants showed some degree of activity against one or more of the bacterial strains. DMSO, methanolic and ethanolic extracts even at the concentration of 8 mg mL^{-1}, extracts showed great antimicrobial effect especially against *L. monocytogens* and *S. aureus* strains; the latter being the most sensitive. The antimicrobial activity profile indicated that *O. limbata* extract was active more against Gram-positive than Gram-negative bacteria. The activity against both the types of bacteria indicated the presence of broad spectrum antibacterial compounds (Table I). Ethanolic extract at concentration 8 mg mL^{-1} was the most promising against *E. coli* and showed the zone of inhibition 11.5±1.1 mm, while DMSO and methanolic extracts had no effect at the *E. coli* strain (Table I). It is interesting to be noted that some Gram-positive bacteria were sensitive but not the Gram-negatives, as emphasized elsewhere (Cosentino *et al.*, 1999; Karaman *et al.*, 2003). The MIC for *O. limbata* was about 8 mg mL^{-1}. Determination of inhibition zone of *O. limbata* including 8 to 0.25 mg mL^{-1} sample solutions concentrate revealed that sample solutions could give partial inhibition with several bacterial colonies (Table II). This indicated that some observed strain of microbe had resistance against sample solutions. Deans and Ritchie (1987) and Deans *et al.* (1995) observed that the susceptibility of Gram-positive and Gram-negative bacteria to plant volatile oils had a little influence on growth inhibition. However, some oils appeared more active with respect to Gram reaction, exerting a greater inhibitory activity against Gram-positive bacteria. It was often reported that Gram-negative bacteria were more resistant to the essential oils present in plants (Smith-Palmer *et al.*, 1998). The cell wall structure of Gram-negative bacteria is constituted essentially with Lipopolysaccharides (LPS). This constituent avoids the accumulation of the oils

Table I. Zone of Inhibition at the concentration of 8 mg mL^{-1} against 6 bacterial strains

Bacterial strains	Growth inhibition diameter (mm)		
	DMSO	Ethanol extract	Methanol extract
Salmonella sp.	8.4±1.0	8.1±0.5	7.9±0.5
E. coli	6.0	11.5±1.1	6.0
P. aeruginosa	6.0	6.0	6.0
B. subtilis	7.5±0.5	7.1±0.5	6.0
L. monocytogens	13.8±1.5	14.5±1.4	11.0 ±1.1
S. aureus	19±1.5	18±1.0	16±1.0

The data are expressed as mean ± SD
The diameter of paper disc was 6 mm

Fig. 1. Zone of Inhibition at the concentration of 8 mg mL^{-1} against bacterial strains

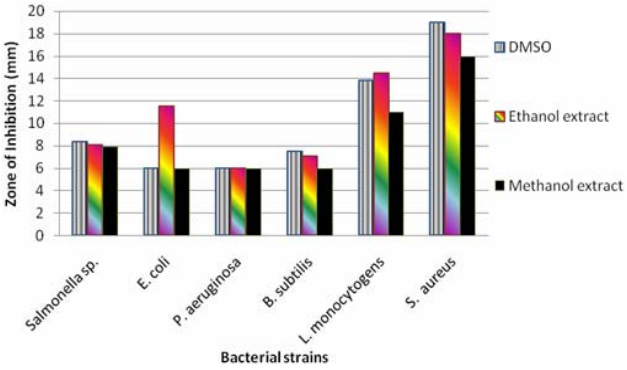

Table II. MIC of *Otostegia limbata* of all extracts against tested microorganisms

Microorganism concentration	Test	Extract	Concentration of extract (mg mL^{-1})						Negative control	Positive control
			8	4	2	1	0.5	0.25		
E. coli	MIC	DMSO	NR	NR	NR	NR	NR	NR	NR	NR
		Ethanolic	-	-	-	+	+	+	-	+
		Methanolic	NR	NR	NR	NR	NR	NR	NR	NR
B. subtilis	MIC	DMSO	-	-	-	-	+	+	-	+
		Ethanolic	-	-	-	-	+	+	-	+
		Methanolic	NR	NR	NR	NR	NR	NR	NR	NR
Salmonella sp.	MIC	DMSO	-	-	-	+	+	+	-	+
		Ethanolic	-	-	-	+	+	+	-	+
		Methanolic	-	-	-	+	+	+	-	+
L. monocytogenes	MIC	DMSO	-	-	-	-	+	+	-	+
		Ethanolic	-	-	-	-	+	+	-	+
		Methanolic	-	-	-	-	+	+	-	+
S. aureus	MIC	DMSO	-	-	-	-	-	+	-	+
		Ethanolic	-	-	-	-	-	+	-	+
		Methanolic	-	-	-	-	+	+	-	+

The bacterial suspensions were used as positive control
Extracts in broth were used as negative control
NR = No Result

Table III. MBC of *Otostegia limbata* of all extracts against tested microorganisms

Conc.(mg/ml) Microorganism	Test	Extract	Concentration of extract (mg mL^{-1})						Negative control	Positive control
			8	4	2	1	0.5	0.25		
E. coli	MBC	DMSO	NR	NR	NR	NR	NR	NR	NR	NR
		Ethanolic	-	-	-	-	+	+	-	+
		Methanolic	NR	NR	NR	NR	NR	NR	NR	NR
B. subtilis	MBC	DMSO	-	-	-	-	-	+	-	+
		Ethanolic	-	-	-	+	+	+	-	+
		Methanolic	NR	NR	NR	NR	NR	NR	NR	NR
Salmonella sp.	MBC	DMSO	-	-	-	+	+	+	-	+
		Ethanolic	-	-	+	+	+	+	-	+
		Methanolic	-	-	+	+	+	+	-	+
L. monocytogenes	MBC	DMSO	-	-	-	-	+	+	-	+
		Ethanolic	-	-	-	+	+	+	-	+
		Methanolic	-	-	+	+	+	+	-	+
S. aureus	MBC	DMSO	-	-	-	-	-	+	-	+
		Ethanolic	-	-	-	-	-	+	-	+
		Methanolic	-	-	-	-	-	+	-	+

The bacterial suspensions were used as positive control
Extracts in broth were used as negative control
NR = No Result

on the cell membrane (Bezić *et al*., 2003).

Absolute ethanol extract was highly effective against *E. coli* strain since 22.8±1.0 mm diameter of zone of inhibition was observed although it was not the part of the study. The response of methanolic extract, compared to the other two extracts, was not much promising, although methanol extracts also showed antimicrobial effect and positive results against *Salmonella* spp., *L. monocytogens* and *S. aureus* strains. However, against Gram negative bacterial the extracts showed no activity. (Table I & II) *S. aureus* was the most sensitive, which was inhibited by all three extract (Fig. 1) and on the other hand, no inhibition was observed in the *P. aeruginosa*. As far as antimicrobial activity is concerned, *P. aeruginosa* was the only bacterium that was not susceptible to the oil (Imelouane *et al*., 2009a). It is known to have high level of intrinsic resistance to virtually all known antimicrobials and antibiotics due to a combination of a very restrictive outer membrane barrier, highly resistant even to synthetic drugs (Skočibušić *et al*., 2006). This bacterium is resistant to antimicrobial agents and diterpenes present in Salvia species (Darias *et al*., 1990). Some organisms exhibited only slight susceptibility (Table II & III). Phenolic extract of sage and thyme showed antibacterial activity against *S.. aureus* and *Enterococcus sp.*, respectively. On the other hand, *E. coli* was more affected by the ethanolic extract of parsley. The phenolic extract did not show pronounce effect on the tested Gram-positive organisms (El Astal *et al.*, 2005). Similarly, Gram-negative bacteria were more sensitive to the essential oil of thyme and did not show any selectivity towards Gram positive-bacteria (Imelouane *et al.*, 2009b).

CONCLUSION

Ethanol and DMSO extracts of *O. limbata* showed the best antimicrobial activity, which supports the taxonomic

relationship of this plant to Labiatae family. This can be considered as a component of broad spectrum antimicrobial agents. Among the microorganisms, *B. subtilis* was the most resistant to ethanolic extracts while *S. aureus* was the most sensitive at a concentration of 8 mg mL^{-1}.

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