ANTIBACTERIAL ACTIVITY OF *THYMUS DAENENSIS* METHANOLIC EXTRACT

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ABSTRACT

Medicinal plants are potential of antimicrobial compounds. The present study deals with the antibacterial activity of methanolic extract of *Thymus daenensis*. Aerial parts of the plant were collected from Alvand mountainside (Hamadan, Iran) in May 2005, air-dried and extracted by methanol. The dried extract was redissolved in methanol to make a 100mg/ml solution and then filtered. Antibacterial activity of the extract was evaluated against various Gram-positive and Gram-negatives bacteria using disk diffusion technique. Blank paper disks were loaded with 40µl of the methanol solution and then dried up. The impregnated disks were placed on Mueller-Hinton agar inoculated with bacterial suspension equal to 0.5 McFarland.

The extract inhibited the growth Gram-positive bacteria, i.e., *Staphylococcus aureus*, *Micrococcus luteus*, *Entrococcus faecalis*, *Streptococcus pyogenes*, but it showed no activity against Gram-negative bacteria. The most significant effect was seen against S.aureus including *MRSA*, which are important nosocomial pathogens. MIC90 of the extract was determined against Gram-positive bacteria (3.12 mg/ml) and 11 *MRSA* strain (1.56 mg/ml).

Keywords: Antibacterial activity, *Thymus daenensis*, disk diffusion.

INTRODUCTION

Infectious diseases are the second leading cause of death worldwide (Fazly-Bazzaz *et al.,* 2005). Treatment of infections continues to be a problem in modern time because of severs side effects of some drugs and growing resistance to antimicrobial agents. Hence, search for newer, safer and more potent antimicrobials is a pressing need. Herbal medicines have received much attention as a source of new antibacterial drugs since they are considered as time-tested and comparatively safe both for human use and for environment (Fazly-Bazzaz *et al.,* 2005).

The landscape of Iran includes great ecological diversity and has a rich flora which has not been studied for its phytochemistry and bioactivity. Lamiaceae (formerly Labiatae) is of the most important plant families in which *Thymus* with about 215 species is a significant genus (Zaidi *et al.,* 2005).

Thymus species are commonly used as tonic, carminative, digestive, antitussive, expectorant and for the treatment of cold in Iranian traditional medicine. Recent studies imply that these species have strong antibacterial activities (Vila, 2002). Extracts of *Thymus vulgaris* of Greek origin were examined as potential sources of phenolic compounds with antimicrobial activity against selected microbes (Proestos *et al.,* 2005). In other research, aqueous and ethanolic extracts (10-200 mg/ml) of *Thymus capitatus* inhibited the growth of several bacteria and fungi (Kandil *et al.,* 1994). It is believed that flavonoids are responsible of these activities (Özçelik *et al*. 2006; Maheswara *et al*., 2006; Friedman, 2007; Cowan, 1999). They are known to be synthesized by plants in response to microbial infection (Dixon *et al.,* 1983). Abundantly distributed in edible plants, flavonoids appear to be of most potent next generation drugs for infection treatment as a number of them possess unique and sufficient antibacterial activity. In the present study, thus, *Thymus daenensis* methanolic extract (rich of flavonoids (Markham, 1982) was evaluated for antibacterial activities against standard and clinical isolates of both Gram-positive and Gram-negative bacteria (Mothana and Lindequist, 2005).

The essential oil obtained from the aerial parts of *T. daenensis* was analyzed, the main components were thymol, *p*-cymene, β-caryophyllene and methyl carvacrol (Nickavar *et al.,* 2005).

MATERIALS AND METHODS

Plant material

Aerial parts of *T. daenensis* were collected from Alvand mountainside (Hamadan, Iran) and identified by Dr. Gh. Amin at Faculty of Pharmacy, Tehran University Medical Sciences.

Extraction of plant material

The air-dried plant materials were ground into fine powder and Soxhelet extracted using methanol (Merck).

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Table 1: Diameter of inhibition zones (mm) produced by methanolic extract against bacteria tested.

Bacterial strains	Positive control			Negative control	Methanolic extract concentration (mg/ml) and zone of inhibition (mm)							MC (mg/ml)
	V	O	CIP		100	50	25	12.5	6.25	3.12	1.56	
S. aureus ATCC 25923	17*	15	23	6	*22*	*21*	*12*	*8*	*6*	*6*	*6*	12.5
S. aureus ATCC 29737	18	20	24	6	*19*	*18*	*13*	*9*	*6*	*6*	*6*	12.5
S. aureus ATCC 6538	17	17	24	6	*20*	*19*	*15*	*10*	*9*	*6*	*6*	6.25
MRSA ATCC 33591	17	7	22	6	*26*	*22*	*16*	*12*	*11*	*6*	*6*	6.25
E. faecalis ATCC 29212	19	NT	24	6	*8*	*6*	*6*	*6*	*6*	*6*	*6*	100
VRE	7	NT	22	6	*13*	*10*	*9*	*8*	*6*	*6*	*6*	12.5
M. luteus ATCC 9341	NT	NT	NT	6	*18*	*17*	*16*	*10*	*6*	*6*	*6*	12.5
S. pyogenes ATCC 8668	NT	NT	22	6	*25*	*20*	*18*	*16*	*15*	*14*	*6*	3.12
K. pneumoniae ATCC 700603	NT	NT	21	6	-	-	-	-	-	-	-	-
P. aeroginosa ATCC 27853	NT	NT	23	6	-	-	-	-	-	-	-	-
E. coli ATCC 8739	NT	NT	22	6	-	-	-	-	-	-	-	-
E. coli ATCC 35218	NT	NT	21	6	-	-	-	-	-	-	-	-
E. coli ATCC 25922	NT	NT	23	6	-	-	-	-	-	-	-	-
Salmonella spp.	NT	NT	22	6	-	-	-	-	-	-	-	-

*Diameter of paper discs used was 6 mm.
NT: not tested; V: Vancomycin (dicks 30 µg); O: Oxacillin (disks 1 µg); CIP: Ciprofloxacin (disks 5 µg)
Italic numbers indicate diameters of inhibition zones in mm.
MRSA: methicillin-resistant *Staphylococcus aureus*
VRE: vancomycin-resistant *Enterococcus faecium*
MC : Minimum concentration of methanolic extract producing a clear zone.

Solvent was removed from filtered extract under reduced pressure to produce a thick dark green extract. The resulting extract was kept in a dark and cold place in sterile vials for further tests.

Test organisms

Various Gram-positive and Gram-negative bacteria from standard cultures (received from collections) and clinical isolates present in our laboratory were used as test strains. Standard strains were obtained from MAST (MAST Ltd., UK) or Persian Type Culture Collection, Iranian Research Organization for Science and Technology (PTCC, Iran). Clinical strains of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) were isolated from clinical samples at Milad Hospital (Tehran, Iran). The organisms were reconstituted from frozen stocks, maintained on soybean-casein digest agar (SCDA) slanting media and subcultured regularly on fresh slants.

Antibacterial assay

Antibacterial activity of the crude extract was investigated against 14 bacterial strains by the paper disk diffusion technique (Bauer *et al*., 1966). The extract was redissolved in methanol to make a 100 mg/ml solution and then filtered. From this solution, 40 µl aliquots were (concentrations 100, 50, 12.5, 6.25, 3.12 and 1.56 mg/ml of extract) transferred onto blank paper disks (6 mm diameter) (Vila, 2002). Dried disks were placed onto Mueller-Hinton agar medium (Merck) previously inoculated with a bacterial suspension (0.5 McFarland ca. 10^8 CFU/ml) and incubated at 35 ± 1ºC for 24 h. Plates were then examined for the presence of zones of inhibition, and the diameters were measured, if any.

Table 2: Diameter of inhibition zones (mm) produced by methanolic extract against MRSA isolates tested

Bacterial strains	Positive control		Negative control	Methanolic extract concentration (mg/ml)							MC (mg/ml)
				100	50	25	12.5	6.25	3.12	1.56	
	V	O		Zone inhibition (mm)							
MRSA1	+	_	_	*25*	*24*	*19*	*15*	*10*	_	_	6.25
MRSA2	+	_	_	*19*	*18*	*15*	*11*	_	_	_	12.5
MRSA3	+	_	_	*18*	*17*	*14*	*12*	_	_	_	12.5
MRSA4	+	_	_	*26*	*25*	*19*	*13*	*12*	*9*	_	3.12
MRSA5	+	_	_	*27*	*26*	*20*	*16.5*	*11*	_	_	6.25
MRSA6	+	_	_	*29*	*28*	*26*	*17.5*	*12*	_	_	6.25
MRSA7	+	_	_	*21*	*20*	*16*	*12*	*9*	_	_	6.25
MRSA8	+	_	_	*21*	*20*	*17*	*12*	*10*	_	_	6.25
MRSA 9	+	_	_	*28*	*27*	*22*	*19*	*11*	_	_	6.25
MRSA10	+	_	_	*24*	*23*	*16*	*11*	*9*	_	_	6.25
MRSA ATCC 33591	17	_	_	*22*	*21*	*17*	*11*	*9*	_	_	6.25

NT: not tested
V: Vancomycin (dicks 30 µg); O: Oxacillin (disks 1 µg); CIP: Ciprofloxacin (disks 5 µg)
Italic numbers indicate diameters of inhibition zones in mm
MRSA: methicillin-resistant *Staphylococcus aureus*
MC: Minimum concentration of methanolic extract producing a clear zone.

Oxacillin disks (1 µg) and vancomycin dicks (30 µg) (Padtan Teb, Iran) as well as ciprofloxacin disks (5 µg) were used as positive controls, where found appropriate. Methanol containing disks were also used as negative controls. Experiments were carried out duplicately, mean of four readings has been indicated in tables 1 and 2 (Zaidi *et al.*, 2005).

Minimum concentration (MC)

MC of the extract was determined against Gram-positive bacteria and 11 strains of MRSA at 7 concentrations (100-1.56 mg/ml) by paper disk diffusion technique just as previous method mentioned before (tables 1 and 2). The reconstituted extract was diluted to give 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.12 mg/ml and 1.56 mg/ml. The MC was regarded as the lowest concentration of the extract which didn't permit the growth of the susceptible bacteria (Vila, 2002).

RESULTS AND DISCUSSION

The inhibitory effects of methanolic extract of *T. daenensis* against different test organisms are shown in tables (1 and 2). The results indicate significant antibacterial activity against standard strains of Gram-positive bacteria including *S. aureus*, MRSA, *S. pyogenes*, *E. faecalis* and VRE but it is shown no activity against Gram-negative bacteria. Zone of inhibition produced by the extract in paper disk diffusion assay (Zaidi *et al.*, 2005) against the susceptible bacteria ranged from 8 to 29 mm while the vancomycin, ciprofloxacin and oxacillin as positive controls yielded inhibitory zones measuring 12-18 mm. Regarding *E. faecalis* ATCC 29212, methanolic extract could inhibit the growth only at 100 mg/ml, however the extract could effectively inhibit other bacterial tested at concentration as low as 6.25-12.5 mg/ml. It was interesting to note that the MRSA strains indicated more sensitivity to the investigated extract and MC90 against MRSA strains was 6.25 mg/ml. The extract contains many phytochemicals substances including terpenoids, tannins and polyphenolic compounds as well as flavonoids. The different components diffusing at different rates may have been responsible for the varying zones of inhibition obtained in our assays against the susceptible microorganisms. This plant is assumed to have compounds which have a potential antimicrobial activity its important compounds are flavonoids. Flavonoid's activity is probably due to their ability to complex with extra cellular and soluble proteins and to complex with bacterial cell walls and lipophilic flavonoids may also disrupt bacterial membranes. If the components could be separated and tested further we might find use as individual antibacterial agent against Gram-positive infections (Vila, 2002).

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