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Original Article

Antibacterial Activity of *Thymus pubescens* Methanolic Extract

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Abstract

During the last two decades, various medicinal plants have been studied for their possible antimicrobial activity to discover new antimicrobial agents capable of resolving problems such as the development of drug resistance in pathogenic microorganisms as well as the side effects of some present antibiotics. In this study, the antibacterial activity of methanolic extract of *T. pubescens* (rich in flavonoids) was investigated.

The aerial parts of the plant were collected from Alvand mountainside (Hamadan, Iran) in May 2005 and identified by Hamadan Natural Sources Organization. The air-dried plant materials were ground to fine powder and then extracted by soxhelet apparatus using methanol. The extract was tested at a concentration of 100 mg/ml against a panel of Gram-positive and Gram-negative bacteria using the disk diffusion technique. This methanolic extract demonstrated antibacterial activity against Gram-positive bacteria including *Staphylococcus aureus,* Methicillin-resistant *S. aureus* (MRSA)*, Streptococcus pyogenes*, *Enterococcus faecalis,* Vancomycin-resistant *E. faecalis* (VRE) and *Micrococcus luteus*, and produced inhibition zones with 8-16 mm diameters. However, it showed no activity against Gram-negative bacteria, such as *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella* spp. Minimum concentrations (MC) of the extract forming a clear zone were determined against susceptible bacteria. MC_{90} of the extract against 10 MRSA strains tested was 1.56 mg/ml, indicating its good activity against this important nosocomial pathogen. *T. pubescens* seems to be a good candidate for further phythochemical studies in an attempt to find new chemical entities combating resistant bacteria.

Keywords: Antibacterial activity; *Thymus pubescens*; Disk diffusion; MRSA.

Introduction

Infectious diseases are the second leading cause of death worldwide. Treatment of infections continues to be problematic in modern time because of the severe side effects of some drugs and the 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 the environment (1).

Iran has a great deal of ecological diversity and has a rich herbal flora which is still much unstudied regarding phytochemistry and bioactivity. Lamiaceae (formerly Labiatae) is one of the most important plant families in which *Thymus* with about 215 species, is a significant

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genus (2).

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 (3). Extracts of *Thymus vulgaris* of Greek origin were examined as potential sources of phenolic compounds and showed antimicrobial activity against selected microbes (4). In another research, aqueous and ethanolic extracts (10-200 mg/ml) of *Thymus capitatus* inhibited the growth of several bacteria and fungi (5). It is believed that flavonoids are responsible for these activities (6-9). They are known to be synthesized by plants in response to microbial infection (10). Analysis of the essential oil obtained from the aerial parts of *T. pubescens* revealed the main components to be carvacrol, thymol, α-terpineol and *p*-cymene (11). Abundantly distributed among the edible plants, flavonoids seem to include some of the most potent next generation drugs for the treatment of infections, since some of them possess unique antibacterial potency. Therefore, in the present study, *T. pubescens* methanolic extract, being rich of flavonoids (12), was evaluated for its antibacterial activities against the standard and clinical isolates of various Gram-positive and Gram-negative bacteria.

Experimental

Plant material

The aerial parts of *T. pubescens* Boiss. & Kotschy ex Celak (syn. *T. xylorrhizus* Boiss. & Kotschy) (Lamiaceae) were collected from the Alvand mountainside (Hamadan, Iran) in May 2005 and identified by Hamadan Natural Sources Organization. The plant parts were air-dried and finally ground to a fine powder.

Extraction of plant material

The dried ground material was extracted by Soxhelet apparatus using methanol (Merck). The extract was filtered and its solvent removed under reduced pressure to produce a thick dark green paste. The resulting extract was kept in a sterile vial in a dark and cold place for further tests.

Test organisms

Various Gram-positive and Gram-negative bacteria including both standard and clinical isolates 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 methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) were isolated from patients in Milad Hospital (Tehran, Iran). The organisms were maintained on soybean casein digest agar (SCDA) and transferred onto fresh slants on a regular basis.

Antibacterial assay

Antibacterial activity of the crude extract was investigated against 14 bacterial strains by the paper disk diffusion technique (13). The extract was redissolved in methanol to make a 100 mg/ml solution and then filtered. From this solution, 40-µl aliquots were transferred onto blank paper disks with a diameter of 6 mm (3). Dried disks were placed onto Mueller Hinton agar medium (Merck) previously inoculated with a bacterial suspension (*ca.* 10^8 CFU/ml) and incubated at 35±1 °C for 24 h. Plates were then examined for the presence of growth inhibition zones, and diameters were measured, if any. Oxacillin disks (1 µg) and vancomycin disks (30 µg) (Padtan Teb, Iran) as well as ciprofloxacin disks (5 µg) were used as positive controls, where appropriate. A disk loaded by 40 µl methanol instead of the extract solution served as the negative control. The experiments were carried out four times and the results were presented as mean±SD.

Minimum concentration producing growth inhibition zone (MC)

MC of the extract was determined by testing 7 concentrations of the extract against Gram-positive bacteria, including 11 strains of MRSA, by the paper disk diffusion technique. The reconstituted extract was diluted to give concentrations of 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.12 mg/ml and 1.56 mg/ml. The lowest concentration of the extract that could inhibit the bacterial growth was considered as MC (3).

Table 1. Antibacterial effects of *Thymus pubescens* methanolic extract against various bacteria, as obtained by the disk diffusion technique (n=4).

Bacterial strain	Diameter of inhibition zone (mm)				MIC (mg/mL)
	Positive control			Methanolic extract	
	V	O	C		
S. aureus ATCC 25923	NT	15.0±0.5	23.8±1.2	13.8±0.5*	3.12±0.00
S. aureus ATCC 29737	NT	20.2±0.4	24.4±0.9	13.2±0.7	12.50±0.00
S. aureus ATCC 6538	NT	17.2±1.3	26.6±0.7	12.1±1.0	6.25±0.00
MRSA ATCC 33591	NT	7.4±0.7	22.2±0.5	13.8±1.1	1.56±0.00
E. faecalis ATCC 29212	19.2±1.5	NT	24.3±1.1	11.8±0.5	100.00±0.00
VRE	6.8±0.9	NT	22.1±0.5	9.1±1.0	100.00±0.00
M. luteus ATCC 9341	NT	NT	20.9±0.5	16.3±0.6	3.12±0.00
S. pyogenes ATCC 8668	NT	NT	22.1±1.3	11.2±0.9	6.25±0.00
K. pneumoniae ATCC 700603	NT	NT	21.2±0.6	6.0±0.0	>100
P. aeruginosa ATCC 27853	NT	NT	23.7±0.6	6.0±0.0	>100
E. coli ATCC 8739	NT	NT	22.3±0.7	6.0±0.0	>100
E. coli ATCC 33218	NT	NT	21.1±0.2	6.0±0.0	>100
E. coli ATCC 25922	NT	NT	22.7±0.7	6.0±0.0	>100
Salmonella spp.	NT	NT	22.3±0.4	6.0±0.0	>100

*Diameter of paper disks used was 6 mm. Data are presented as Mean±SD.
NT: not tested, V: Vancomycin disk (30 µg), O: Oxacillin disk (1 µg), C: Ciprofloxacin disk (5 µg), MRSA: methicillin-resistant *Staphylococcus aureus*, VRE: vancomycin-resistant *Enterococcus faecium*, MIC: Minimum concentration of the methanolic extracts producing a clear zone.

Results and Discussion

The inhibitory effects of methanolic extract of *T. pubescens* against different test organisms are shown in Tables 1 and 2. The extract indicated significant antibacterial activity (growth inhibition zone diameters ranging from 8 to 16 mm) against Gram-positive bacteria including *S. aureus,* MRSA*, S. pyogenes, E. faecalis* and VRE. Similar results have been reported for essential oils from *T. pubescens* (14) and aqueous-methanolic extracts of *T. vulgaris* (15). This strong antibacterial effect could be due to flavonoids, which have been shown to be active against MRSA (16, 17). On the other hand, the extract had no activity against Gram-negative bacteria. This could be related to the outer membrane acting as a permeability barrier in these bacteria (18). Inversely, essential oil of *T. pubescens* has been shown to have strong

Table 2. Antibacterial effects of *Thymus pubescens* methanolic extract against MRSA isolates obtained by the disk diffusion technique (n=4).

Bacterial strains	Diameter of inhibition zone (mm)			MIC (mg/ml)
	Cefoxitin (1 µg)	Methanol control	Methanolic extract (100 mg/ml)	
$MRSA_1$	6.0±0.0*	6.0±0.0	11.5±1.0	1.56±0.00
$MRSA_2$	6.0±0.0	6.0±0.0	10.3±1.3	4.69±1.63
$MRSA_3$	6.0±0.0	6.0±0.0	14.0±1.5	1.56±0.00
$MRSA_4$	6.0±0.0	6.0±0.0	11.1±0.6	1.56±0.00
$MRSA_5$	6.0±0.0	6.0±0.0	12.3±1.7	1.56±0.00
$MRSA_6$	6.0±0.0	6.0±0.0	13.2±1.4	1.56±0.00
$MRSA_7$	6.0±0.0	6.0±0.0	12.0±0.0	1.56±0.00
$MRSA_8$	6.0±0.0	6.0±0.0	11.3±1.0	1.56±0.00
$MRSA_9$	6.0±0.0	6.0±0.0	14.4±1.7	1.56±0.00
$MRSA_{10}$	6.0±0.0	6.0±0.0	13.0±1.1	1.56±0.00
MRSA ATCC 33591	6.0±0.0	6.0±0.0	14.0±1.5	1.56±0.00

*Diameter of paper disks used was 6 mm. Data are presented as Mean±SD.
MRSA: methicillin resistant *Staphylococcus aureus*, MIC: Minimum concentration of the methanolic extracts producing a clear zone.

inhibitory effects on Gram-negative bacteria even in diluted forms (14).

Using the disk diffusion technique, minimum concentration of the extract producing a clear zone was also determined using the disk diffusion technique, which was 100 mg/ml. For *E. faecalis* ATCC 29212 and VRE, methanolic extract could inhibit the growth only at 100 mg/ml; however, the extract could effectively inhibit other test bacteria even at concentrations as low as 1.56-12.5 mg/ml. It was interesting to note that the MRSA strains were the most sensitive species to the investigated extract, with the MC_{90} against these isolates being 1.56 mg/ml. *T. pubescens* contains various compounds including terpenoids, tannins and polyphenolic compounds, as well as flavonoids. The latter being more likely to possess antimicrobial activity. Flavonoids' activity is probably due to their ability to form complex with extra-cellular and soluble proteins, as well as bacterial cell wall. lipophilic flavonoids may also disrupt bacterial membranes (19). Negative control did not show any growth zone. It is concluded that, separation of components of this extract and evaluation of their antimicrobial activity may lead into new agents against important nosocomial pathogens, such as MRSA.

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