Investigating antimicrobial activity of hydroalcoholic extract and essential oil of *Tymbra spicata* against some pathogenic bacteria

Salar Bakhtiyari[1], Somaieh Sabzali[2], Arman Rostamzad[2], Gholam Basati[3*]

1. Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran
2. Department of Biology, Faculty of Basic Sciences, Ilam University, Ilam, Iran
3. Department of Clinical Biochemistry, Faculty of Allied Medical Sciences, Ilam University of Medical Sciences, Ilam, Iran

[*] **Corresponding author:** Tel: +98 841 2227147; fax: +98 841 2227147
Address: Department of Clinical Biochemistry, Faculty of Allied Medical Sciences, Ilam University of Medical Sciences, Bangananjab Avenue, Ilam, Iran
E-mail: basati-gh @medilam.ac.ir
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Abstract

Introduction: *Thymbra spicata* (*T. spicata*) is a conventional medicinal herb which is commonly used in the traditional medicine system of Iran. The aim of this study was to assess the antibacterial activity of the essential oil and hudroalcholic extract of the plant *T. spicata* on some important bacteria.
Materials and methods: The essential oil and extract of T. *spicata* were obtained by hydrodistillation and hydroalcoholic methods, respectively. Different concentrations of essential oil and hydroalcholic extract of the medicinal herb, *T. spicata,* were evaluated for their antibacterial activities against ten pathogenic bacteria (4 Gram-positive and 6 Gram-negative) by agar disc diffusion and macro Broth dilution method.
Results: All bacteria showed a profound susceptibility to the essential oil and hydroalcholic extracts of the *T. spicata*. The essential oil was shown to be more effective against the examined bacteria. The zone of inhibition of the essential oil showed to be maximum against *E. faecalis* (28 mm) and *M. morganii* (30 mm). The hydroalcholic extract of *T. spicata,* showed the highest antibacterial activity against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) with the zone of inhibitions, 19 mm and 15 mm, respectively. The minimum inhibitory concentration (MIC) values for the essential oil and hydroalcholic extracts ranged from 7-60μg/ mL and 5-19 mg/ml, respectively against the tested bacteria.
Conclusion: This study revealed that the potential using of *T. spicata* as a source of antibacterial agents could be effectively used for medicinal and pharmaceutical purposes.

Keywords: *Tymbra spicata*, essential oil, hydroalcholic extract

Introduction

Herbal remedies used in many types of alternative medicine provide an interesting and still principally unexplored source for the creation and development of potentially new drugs, which might help to overcome the growing problem of the microbial resistance and also the toxicity of the currently available commercial drugs (1). Nowadays, drug resistance to pathogenic microorganisms has become a very serious problem all over the world and hence, there has been an increased interest in investigating natural materials as sources of new antibacterial agents. Antimicrobial activities of various species

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of plants and their derivatives have been consequently reported (2, 3).

Thymbra spicata (*T. spicata* from *Lamiaceae* family) is a traditional medicinal plant which is commonly used in the traditional medicine system of Iran. This plant is a perennial herb that tends to grow on dry sunny hillsides and high dry meadows (4). The essential oil found in different parts of *T. spicata* makes it an important antibacterial and antioxidant natural source. It is used in food industry as spice and also utilized for the treatment of asthma and bronchitis in traditional medicine (5). This plant is known as a rich source of flavonoids, terpenoids, and isoprenoids such as thymol and carvacrol (6). Thymol, a naturally-occurring group of biocides, shows strong antimicrobial activity when used alone or accompanied with other biocides such as carvacrol. Furthermore, thymol can reduce bacterial resistance to commonly used antibiotics such as penicillin (**7**). The antimicrobial effects of thymol, varying from induction of antibiotic susceptibility in drug-resistant pathogens to powerful antioxidant properties, have been shown by several studies (8). It has also been demonstrated that thymol and carvacrol reduce bacterial resistance to antibiotics through a synergistic effect (**7**).

So far, no report has been published on antimicrobial properties of *T. spicata* extract. Therefore, we aimed to examine and compare the antimicrobial effects of hydroalcoholic extract and essential oil of *T. spicata* on some bacteria by determining the diameter of growth inhibited zone surrounding the discs.

Materials and methods

Plant collection and identification: The aerial parts of *T. spicata*, used in this study, were collected from hills around Ilam, Iran in May 2011. The taxonomic identity of this plant was authenticated by the voucher specimens deposited at the Department of Horticulture, Faculty of Agriculture, Ilam University.

Hydroalcoholic extract preparation: The aerial parts of *T. spicata* were cleaned with distilled water, shade dried at room temperature for 10 days and then ground to a fine powder. 200 gram of the powder was macerated in 1 liter of 50% (v/v) ethanol in distilled water and incubated for 10 days at 25˚C. The solution was filtered and its volume was adjusted to 350 mL with the 50% ethanol to give a clear dark brown filtrate (pH 6). The filtrate was afterward dried on the oven at 45˚C for two days.

Essential oil extraction: The essential oil of T. spicata was prepared based on the method recommended in British pharmacopoeia (9). Briefly, the aerial parts of *T. spicata* were dried in shadow, ground, and powdered in a domestic mixer. The powder was then hydrodistilled using a Clevenger apparatus for 3.5 hours. The resulted yellowish oil was dried over anhydrous sodium sulfate and kept at 4°C in a dark place.

Bacterial strains: The essential oil and hydroacholic extract of T. spicata were screened against six Gram-negative bacteria (*Escherichia coli*, *Peudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumonia*, *Enterobacter aerogenes*, and *morganella morganii*) and four Gram-positive bacteria (*Enterococcus faecalis* ATCC 2321, *Bacillus cereus*, *Staphylococcus aureus* ATCC 1885, and *Staphylococcus epidermidis* ATCC 2405).

Preparation of inoculums: All bacteria were grown in Brain Heart Infusion liquid medium at 37°C for 8 hours and used as inoculums. The turbidity of the suspensions was adjusted to the McFarland 0.5 turbidity standard.

Antibacterial assay for extract and essential oil: Antibacterial assay was performed using the disc diffusion method as previously described (10). Briefly, the total bacterial number for each inoculum was adjusted to 1.5×10^8 CFU ml^{-1} with reference to the McFarland turbidimetry,

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inoculated on the surface of sterile Muller Hinton Agar (MHA), and dispersed by a sterile cotton swab. The plates were then dried for 2-4 min. Seven concentrations (2, 1, 0.5, 0.250, 0.125, 0.062, and 0.031mg/mL) of hydroalcoholic extract and seven concentrations (500, 250, 125, 62, 31, 15, and 7 µg/mL) of essential oil of *T. spicata* were prepared. Subsequently, the sterile filter paper discs (6 mm diameter) (11,12) were saturated by adding 50 µL of each concentrations of the essential oil and extract, and placed on the surface of inoculated plates. The plates were incubated at 37°C for approximately 24 hours, and the zones of inhibition around each disc (mean ± SD of three replicates) were then measured by millimeter scale. Sterile paper discs impregnated only with ethanol served as negative control. In addition, discs containing standard concentration of seven antibiotics, including clindamycin, streptomycin, kanamycin, gentamicin, methicillin, ciprofloxacin, and vancomycin were used as positive controls to compare their antibacterial activity with the extract and essential oil.

Determination of MIC and MBC: Minimum inhibitory concentration (MIC) for the hydroalcoholic extract and essential oil of *T. spicata* was determined against the above mentioned bacterial strains using macro broth dilution assay method (13). In this assay, the 16-hours cultures were diluted with sterile physiologic saline solution to achieve suspensions containing nearly 1.5×10^8 CFU mL^{-1} with reference to the 0.5 McFarland Standard (14). Moreover, serial dilutions were prepared between 15 and 1 mg mL^{-1} for the extract and between 500 and 7 µg mL^{-1} for the essential oil. Standard bacterial suspensions were then added to the tubes containing 1 ml of Muller Hinton Broth (MHB) and different concentrations of the extract and essential oil. The tubes were incubated at 37°C for 24 hours. The growth of bacteria was determined via measuring optical density at 600 nm. Gentamicin and ethanol solutions were tested as positive and negative controls, respectively. Bacterial growth inhibition calculated by the following equation (15):

Inhibition % = $[(OD_c - OD_t) / OD_c] \times 100$

where ODc is the OD_{600} for the negative control (ethanol solution) and OD_t is the OD_{600} for the sample treated with the extract or essential oil of *T. spicata*.

Minimum bactericidal concentration (MBC) was determined by culturing one standard loop of the tubes showing no apparent growth on MHA and subsequent incubation at 37°C for 24 hours. The lowest concentration that inhibited colony formation on the agar was considered as the MBC.

Results

The antibacterial activity of the hydroalcoholic extract and essential oil of *T. spicata* was quantitatively assessed by measuring the inhibition zone diameter around each disc, and then compared with the standard antibiotic discs. The obtained results are given in Tables 1 and 2.

Our results showed that the extracts and essential oils of *T. spicata* could effectively inhibit the growth of both Gram-positive and Gram-negative. Among the various concentrations of *T. spicata* essential oils and hydroalcoholic extract, the concentrations 500 µg/ml and 76 mg/ml showed the best antibacterial activity, respectively (Tables 1 and 2). The obtained results exhibited that the extracts were more effective on Gram-positive bacteria than Gram-negative ones.

The MIC and MBC of extract and essential oil of *T. spicata* varied partly in relation to the tested bacteria (Tables 3 and 4). The lowest MIC values for the extract and essential oil (5 mg/ml and 7µg/ml, respectively) was observed to be against E. faecalis and S. epidermidis, respectively (Table 3).

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Table 1. Antibacterial activity of various concentrations of essential oils and hydroalcholic extracts of *Thymbra spicata* plant against four Gram-positive bacteria as compared with seven traditional antibiotics.

Substance	Concentration (µg/ml)	Diameter of inhibition zone (mm)			
		S. aureus	*E. faecalis*	*B. cereus*	*S. epidermidis*
Vancomycin	30	6	18	6	6
Ciprofloxacin	5	27.7	18.7	24	27.3
Clindamycin	2	9.3	8.3	6	8.7
Streptomycin	10	12.3	17.3	14.3	6
Methicillin	5	6	6	6	6
Gentamicin	10	23	22	24.3	19.3
Kanamycin	10	23	6	14	6
Essential Oil	500	25	28	26	24
	250	23	27	25	22
	125	21	25	23	20
	60	12	23	20	18
	30	11	22	14	15
	15	7	22	10	12
	7	7	14	8	10
Hydroalcholic Extract*	76	19	16	16	18
	38	16	13.5	13	14
	19	13	11	11	11
	9.5	11	10	9	12
	5	9	8	6	10
	2.5	6	7	6	8
	1.5	6	6	6	6

*Concentrations are in mg/ml.

Table 2. Antibacterial activity of various concentrations of essential oils and hydroalcholic extracts of *Thymbra spicata* plant against six Gram-negative bacteria as compared with seven traditional antibiotics.

Substance	Concentration (µg/ml)	Diameter of inhibition zone (mm)					
		P. aeruginosa	*E. coli*	*M. morganii*	*S. typhi*	*K. pneumonia*	*E. aerogenes*
Vancomycin	30	6	6	6	6	6	11
Ciprofloxacin	5	21	26.3	24.7	27.7	29	25
Clindamycin	2	6	6	6	6	6	6
Streptomycin	10	14.3	19.3	17.33	22.7	19.7	20.7
Methicillin	5	6	12.7	12.7	9.7	14.7	15
Gentamicin	10	6	6	6	6	6	6
Kanamycin	10	21	11	6	12	24	20
Essential Oil	500	27	25	30	25	26	28
	250	25	23	26	22	24	26
	125	22	20	23	20	22	24
	60	20	15	20	13	20	20
	30	10	11	18	12	18	18
	15	8	9	18	12	12	16
	7	6	7	12	9	7	13
Hydroalcholic Extract*	76	13	15	13	11	15	1
	38	11	11	10	9	12.5	9
	19	10	9	8	8	10.5	8
	9.5	9	7	6	6	7.4	7.5
	5	7	6	6	6	6	7
	2.5	6	6	6	6	6	6
	1.5	6	6	6	6	6	6

*Concentrations are in mg/ml.

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Accordingly, the highest MIC values for the extract and essential oil (19 mg/ml and 60µl/ml, respectively) was also observed to be against *E. coli*, *S. typhi*, *E. aerogenes* and *S.aureus* (Tables 3 and 4). The extract of *T. spicata* was recognized to be significantly more effective against Gram-positive bacteria (MICs ranging from 2.5 to 9.5 mg/ml) than against Gram-negative ones (MICs> 9.5 mg/ml).

Table 3. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of essential oils and hydroalcholic extracts of *Thymbra spicata* plant against four Gram-positive bacteria.

		Bacterium Species			
		S. aureus	*E. faecalis*	*B. cereus*	*S. epidermidis*
Essential Oil concentration (µg/ml)	MIC	60	7	15	30
	MBC	60	15	30	30
Hydroalcholic Extract concentration (mg/ml)	MIC	2.5	5	9.5	5
	MBC	5	9.5	19	5

MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration.

Table 4. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of essential oils and hydroalcholic extracts of *Thymbra spicata* plant against six Gram-negative bacteria.

		Bacterium Species					
		P. aeruginosa	*E. coli*	*M. morganii*	*S. typhi*	*K. pneumonia*	*E. aerogenes*
Essential Oil concentration (µg/ml)	MIC	30	30	15	60	15	15
	MBC	60	60	30	60	30	30
Hydroalcholic Extract concentration (mg/ml)	MIC	9.5	19	9.5	19	9.5	19
	MBC	19	38	19	38	19	38

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration

Discussion

The present study revealed the antibacterial activity of essential oil and hydroalcoholic extract of *T. spicata* plant against some Gram-positive and negative bacteria. Disc diffusion assay demonstrated that the essential oil of *T. spicata* was more effective than the hydroalcoholic extract of the plant. The most important components of the essential oil of *T. spicata* are carvacrol, p-cymene, β-myrcene, γ-terpinene, α-terpinene and trans-caryophyllene (6) that among which, carvacrol was shown to have a strong antibacterial effect (16,17, 18). Therefore, the antibacterial activity of the essential oil and hydroalcoholic extract of *T. spicata* may be partly attributed to carvacrol in the current study. Recently, the antibacterial activity of carvacrol and trans-caryophyllene, were examined against *E. coli*, *S. epidermidis*, *S. aureus*, *S. typhimurium*, *K. pneumoniae*, *P. Aeruginosa* and *E. faecalis* (18) and the effectiveness of carvacrol on the bacteria under study, except *P. aeruginosa*, has been demonstrated. In the present study,

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the essential oil of *T. spicata* was effective against the all mentioned bacteria including *P. aeruginosa.* The reason of the latter difference is not clear but it may be due to the presence of other compounds in the essential oil of *T. spicata*. In supporting the opinion, Tamara et al. reported that the antimicrobial activity of the essential oil of *T. spicata* comes from the combined effect of its constituted compounds (19). However, further studies are required to discern the issue. In another study by M. Akin and his colleague, the antibacterial activity of the essential oil of *T. spicata* was to be effective only against *B. cereus* and *E. coli* (20, 21). However, MIC was not determined in that study. In the current study, we tested higher concentrations of the essential oil of *T. spicata compared* with M. Akin and his colleague. This may account for the wider range of antibacterial activity that we found in the present study. In the present study, the results for essential oil and hydroalcholic extracts were partly parallel with each other, although the extract concentrations were three fold higher to be effective against the bacteria under study. The finding may be explained as the effective compounds of carvacrol which is more present in the oil phase, that is, the essential oil of the plant. Therefore, it is reasonable that the essential oil would be more effective against the examined bacteria than the hydroalcholic extract.

In conclusion, our results demonstrated that essential oil of *T. spicata* have potent antibacterial activity against a relatively typical candidate of Gram-negative and Gram-positive bacteria. Furthermore, the essential of the plant is more active against the bacteria under study than the hydroacholic extract.

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