ORIGINAL ARTICLE

Antimicrobial activity and chemical composition of the essential oils of *Thymbra spicata* var. *intricata*

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In this study, *T. spicata* var. *intricata*, endemic to Turkey, were collected from various localities of Mugla, Turkey. The essential oils were obtained using the hydrodistillation method. The antimicrobial activities of the essential oils on micro-organisms, including multiple antibiotic resistant bacteria, were evaluated using the disc diffusion method. The chemical composition of the essential oil was determined by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The essential oils were effective against Gram-positive and Gram-negative bacteria, which included multiple antibiotic resistant strains. However, *Pseudomonas aeruginosa* ATCC 27853 and *Pseudomonas fluorescens* MU 87 were resistant to these oils. The essential oils were very effective against *Candida albicans*. The antimicrobial activity of the essential oils showed some variations depending on the localities from which they were collected. A total 24 components were identified in the essential oil. The main components were characterized as carvacrol (75.74%), γ-terpinene (9.28%), *p*-cymene (7.17%), myrcene (1.39%), β-caryophyllene (1.13%) and thymol (0.15%), respectively.

Key words: Antimicrobial activity, chemical composition, *Thymbra spicata* var. *intricata*

INTRODUCTION

The leafy parts of plants such as *Origanum*, *Thymbra* and *Satureja* species are used in traditional medicine in the treatment of various diseases. They are known as thyme or ''kekik,'' which is the name given to those species with a thymol/carvacrol type odour, in Turkey.[1] *Thymbra* is represented by two species and four taxa in Turkey.[2] *T. spicata* (black thyme) growing wild in some Eastern Mediterranean countries[3] and the dried leaves are used as spice and herbal tea.[3,4] The essential oils of this plant have wide industrial applications, from the flavouring of foods, liqueur production, perfumery and antiseptic to being used as antimicrobial agents.[5] The essential oil of *T. spicata* is characterized by high content of carvacrol, γ-terpinene and *p*-cymene, respectively. Moreover, its essential oil contains a low percentage of myrcene, α- terpinene, bornylacetate, borneol and thymol.[1]

The two varieties of *T. spicata* are known by different local names and have traditional uses in various regions of Turkey.[6] In South Anatolia *T. spicata* var. *spicata* is known as "Sater" or "Zater".[4] In Southwest Anatolia, *T. spicata* var. *intricata* is called "Karabaş kekiği" or "Karakekik".[6]

The *T. spicata* var. *intricata*, which is endemic in Turkey, comprises 10-40 cm shrubs that grow at altitudes of 150 to 1520 m, in dry stony places, rocks and limestone cliffs.[2] In folk medicinal traditions, infusion of this plant is used to soothe a sore throat, treath mouth ulcer, stomachache, headache and toothache.[6]

The antimicrobial properties[1,7-10] and essential oil composition[3,10-12] of *T. spicata* var. *spicata* have been investigated extensively. The chemical composition of *T. spicata* var. *intricata* has also been studied before.[6] However, the antimicrobial properties of the essential oils of *T. spicata* var. *intricata* have been relatively unexplored until recently.

The antimicrobial activities of essential oils against tested bacteria differed, depending on location and seasonal variations.[13] For this reason, the materials of this plant were collected from different areas of Mugla province, Turkey, in order to determine the effects of the locations on the antimicrobial activity of essential oils of *T. spicata* var. *intricata* against micro-organisms, especially multiple antibiotic resistant bacteria and chemical composition of *T. spicata* var. *intricata*.

MATERIALS AND METHODS

Plant Materials

Four types of plant materials were collected at the flowering stage (May-September) from different

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localities of Mugla region, Turkey. Voucher specimens of the plants were taxonomically identified and deposited at the Herbarium of the Department of Biology at the Mugla University in Turkey.

Isolation of Essential Oils

The essential oils of dried aerial parts of *T. spicata* var. *intricata* were obtained via hydrodistillation by using a Clevenger type apparatus for 4 hours. The oils were dried over anhydrous sodium sulphate and stored under nitrogen in a sealed vial until required.[14]

Micro-organisms and Condition for Cultivation

In this study; *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* CNCTC 8/77, *Micrococcus luteus* NRRL B-4375 and *C. albicans* ATCC 10239 were used. Also *P. fluorescens* MU 87, *Pseudomonas stutzeri* MU 70, *Stenotrophomonas maltophilia* MU 64, *S. maltophilia* MU 99, *Cryseomonas luteola* MU 65, *S. aureus* MU 38, *S. aureus* MU 44 and S*taphylococcus epidermidis* MU 30, which are multiple antibiotic resistant bacteria, were used.

S. aureus, *S. epidermidis*, *E. coli*, *M. luteus* and *B. subtilis* were cultured in Nutrient Broth (NB) (Difco) at 37 ± 0.1°C; *S. mutans* were cultured in Brain Heart Infusion Broth (BHIB) (Difco) at 37 ± 0.1°C; *P. aeruginosa*, *P. fluorescens*, *P. stutzeri*, *S. maltophilia* and *C. luteola* were cultured in Nutrient Broth (NB) (Difco) at 30 ± 0.1°C; and *C. albicans* were cultured in Sabouraund Dextrose Broth (SDB) (Difco) at 30 ± 0.1°C.

Antimicrobial Assay

The antimicrobial activity of the essential oils of plants were assayed by the disc diffusion method.[15,16] The inoculum size of each group of bacteria and yeast were prepared by using a No. 0.5 McFarland tube, to give a concentration of 1×10^8 bacteria and 1×10^6 yeast per milliliter. Mueller Hinton Agar (MHA) (Difco), Brain Heart Infusion Agar (BHIA) (Difco) and Sabouraund Dextrose Agar (SDA) (Difco), sterilized in a flask and cooled to 45-50°C, were distributed to sterilized Petri dishes with a diameter of 9 cm (15 ml) after injecting cultures (0.5 ml) of bacteria and yeast and distributing the medium in petri dishes homogeneously. The plates were held for 15-20 minutes at room temperature. Each essential oil (20 μl) was applied, under suction, to the sterile 6 mm discs (Schleicher and Schuell). Plates injected with the above-mentioned materials were located on the solid agar medium by pressing slightly. Plates injected with yeast were incubated at 30°C for 48 hours, those injected with *S. aureus*, *S. epidermidis*, *S. mutans*, *E. coli*, *M. luteus* and *B. subtilis* were incubated at 37°C for 24 hours and those injected with *P. aeruginosa*, *P. fluorescens*, *P. stutzeri*, *S. maltophilia* and *C. luteola* were incubated 30°C for 24 hours. At the end of the incubation periods, the diameters of the inhibition zones formed on the MHA, BHIA and SDA were evaluated in millimetres. Studies were performed in triplicate.

Analysis of Chemical Compositions

Gas chromatography analyses of the essential oil was performed using Shimadzu GC-17 AAF series gas chromatography, equipped with a flame ionization detector (FID) and a DB1 fused silica column (30 m × 0.25 id., film thickness 0.25 μm). The oven temperature was held at 60°C for 5 minutes, then programmed to 220°C at 3°C/minute and held at isothermal for 15 minutes; injector temperature and detector temperatures were 250°C and 270°C, respectively; carrier gas was He. GC-MS analyses were carried out on a Varian Saturn 2100, equipped with a DB1 MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). For GC-MS detection, an electron ionisation system with ionization energy of 70 eV was used. The oven temperature was held at 60ºC for 5 minutes, then increased to 220°C with 3°C/minute increments and held at this temperature for 15 minutes. Injector temperature was 280°C, and the carrier gas was He (20 psi).

Identification of Essential Oil Components

The components of the essential oil were identified by comparison of their mass spectra with those of a computer library (NIST 2002 and WILEY) or with authentic compounds, and confirmed by comparison of their retention indices (RI), either with those of authentic compounds, or with data published in the literature.[17]

RESULTS AND DISCUSSION

The *in vitro* antimicrobial activity of the essential oils of four materials of *T. spicata* var. *intricata* on Gram-positive and Gram-negative bacteria and *C. albicans*, collected from various localities of Mugla, Turkey, were studied. These results are shown in Tables 1 and 2. The maximum activity was on *B. subtilis* ATCC 6633 (21-23 mm) and the minimum activity was on *S. aureus* ATCC 25923 (13-14 mm), among the Gram-positive bacteria. The inhibition zones, especially on methicillin-resistant *S. aureus* MU 38 and oxacillin resistant *S. aureus* MU 44 were 13-15 mm and 18-20 mm, respectively. Furthermore, the inhibition zones on oxacillin-resistant *S. epidermidis* MU 30 were 17-18 mm.

On the other hand, the maximum activity was observed on *S. maltophilia* MU 64 (28 mm), MU 99 (27-29 mm) and *C. luteola* MU 65 (26-27 mm), among the Gram-negative bacteria. The antibacterial activity of this plant on multiple antibiotic resistant strains was especially notable. The antibiotic resistance patterns of the multiple antibiotic resistance strains are shown in Table 3.

These plant materials showed strong antibacterial activity on *C. luteola* and *S. maltophilia*. It is important that the essential

Table 1: Antimicrobial activity of *T. spicata* var. *intricata* on Gram-positive bacteria

Gram-positive bacteria	Essential oils of the plant samples			
	***T. spicata* var. *intricata* from Dugerek**	***T. spicata* var. *intricata* from Kizildag**	***T. spicata* var. *intricata* from Yatagan-Turgut**	***T. spicata* var. *intricata* from around the Ula Pool**
	Inhibition zone diameter (mm)			
B. subtilis ATCC 6633	23	21	21	22
M. luteus NRRL B- 4375	17	18	17	18
S. aureus ATCC 25923	13	13	14	14
S. aureus MU 38	14	15	13	15
S. aureus MU 44	19	18	20	18
S. epidermidis MU 30	18	17	17	17
S. mutans CNCTC 8/77	21	18	20	18

Table 2: Antimicrobial activity of *T. spicata* var. *intricata* on Gram-negative bacteria

Gram-negative bacteria and yeast	Essential oils of the plant samples			
	***T. spicata* var. *intricata* from Dugerek**	***T. spicata* var. *intricata* from Kizildag**	***T. spicata* var. *intricata* from Yatagan-Turgut**	***T. spicata* var. *intricata* from around the Ula Pool**
	Inhibition zone diameter (mm)			
E. coli ATCC 25922	16	14	14	16
C. luteola MU 65	27	27	26	27
P. aeruginosa ATCC 27853	7	7	7	7
P. fluorescens MU 87	8	8	8	8
P. stutzeri MU 70	19	19	19	19
S. maltophilia MU 64	28	28	28	28
S. maltophilia MU 99	29	27	29	29
C. albicans ATCC 10239	24	21	23	30

Table 3: Antibiotic resistance patterns of *S. maltophilia* strains

Strains	Resistance patterns
S. aureus MU 38	P, AK, DA, CN, ME, TEC, TE, OX
S. aureus MU 44	AMC, P, MEZ, AK, AX, CFP, DA, CN, OX
S. epidermidis MU 30	P, AK, DA, CN, OX, TEC, TE
C. luteola MU 65	MEZ, PRL, TIM, CAZ, FEP, CRO, CTX, KF, ATM, P, CIP, NOR, C, AM
P. fluorescens MU 87	MEZ, PRL, TIM, CRO, CTX, KF, ATM, P, C, SXT, AM
P. stutzeri MU 70	MEZ, CAZ, FEP, CRO, CTX, KF, ATM, P, C, SXT, AM
S. maltophilia MU 64	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, CIP, NOR, C, SXT, TVA, AM, PRL, ATM, SAM, AMC
S. maltophilia MU 99	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, NOR, C, SXT, TVA, AM, PRL, ATM, SAM, AMC

MEZ: Mezlocillin (75 µg); TIM: Ticarcillin + clavulanic acid (75 + 10 µg); CAZ: Ceftazidime (30 µg); FEP: Cephepim (30 µg); CRO: Ceftriaxone (30 µg); CTX: Cefotaxime (30 µg); KF: Cephalothin (30 µg); IPM: Imipenem (10 µg); P: Penicillin (10 U); AK: Amikacin (30 µg); TOB: Tobramycin (10 µg), NET: Netilmicin (30 µg); CN: Gentamicin (10 µg); TE: Tetracycline (30 µg); CIP: Ciprofloxacin (5 µg); NOR: Norfloxacin (10 µg); C: Chloramphenicol (30 µg); SXT: Trimetoprim + sulfamethoxazole (1.25 + 23.75 µg);TVA: Trovafloksasin (10 µg); AM: Ampicillin (10 µg); PRL: Piperacillin (100 µg); ATM: Aztreonam (30 µg); SAM: Sulbactam + Ampicillin (10 + 10 µg); AMC: Amoxicillin + Clavulanic acid (20 + 10 µg); DA: Clindamycin (2 µg); E: Erythromycin (15 µg); ME: Methicillin (5 µg); OX: Oxacillin (1 µg); TEC: Teicoplanin (30 µg)

oils of *T. spicata* var. *intricata* have antibacterial activity on *S. maltophilia* MU 64 and *S. maltophilia* MU 99, which are multiple antibiotic-resistant bacteria, because, *S. maltophilia* is a biogenic amine producer in food.[18] Also *S. maltophilia* has become an important agent of nosocomial infections.[19] Treatment of *S. maltophilia* infections is often difficult because *S. maltophilia* is inherently resistant to most of the available antimicrobial agents, including β-lactams, quinolones and aminoglycosides.[20,21]

The essential oils also inhibited the growth of multiple antibiotic resistant *Staphylococcus* strains, tested. The effects of its essential oils on *S. aureus* MU 44 and *S. epidermidis* MU 30 were very high. *S. aureus* is one of the most common causes of both hospital-and community-acquired infection worldwide.[22] *S. aureus* is a major cause of cutaneous infections, furunculosis, impetigo and abscesses, organ infections, osteomyelitis, endocarditis and arthritis, and toxinoses, such as, food poisoning, septic shock, scalded skin syndrome and toxic shock syndrome.[23] The presence of antibiotic-resistant staphylococci is of concern due to the possible spread of resistance determinants among the *Staphylococcus* species. This could lead to the survival, growth and spread of enterotoxigenic staphylococci and staphylococci of clinical significance.[24]

The inhibition zones of the essential oils of these materials collected from different locations, on bacteria, were similar

[Tables 1 and 2]. The effect of their essential oils on *P. aeruginosa* ATCC 27853, *P. fluorescens* MU 87, *P. stutzeri* MU 70 and *S. maltophilia* MU 64 were the same. Although the antibacterial activities of the essential oils were very similar, the anticandidal activity showed variations depending on the locations. Also, the anticandidal activities were very high. The effect of essential oils was 21-30 mm on *C. albicans*. The maximum activity on *C. albicans* was shown by the essential oils that were collected from around the Ula Pool. Their activity on *C. albicans* was higher than the activity of nystatine (19 mm) (Data not showed).

This study demonstrates that *T. spicata* var. *intricata* had antimicrobial activity on Gram-positive and Gram-negative bacteria and *C. albicans*. Furthermore, the essential oil of the plant had an inhibiting effect on antibiotic resistant strains, such as, *S. maltophilia*, *C. luteola*, *S. aureus* and *S. epidermidis*.

The yield of essential oils obtained from air-dried plant materials was 3.17-3.5% (v/w). Twenty-four components were detected in the essential oils obtained from *T. spicata* var. *intricata* and all of them were detected by using GC, GC-MS analytical methods and literature knowledge. The main components were characterized as carvacrol (75.74%), γ-terpinene (9.28%), *p*-cymene (7.17%), myrcene (1.39%), β-caryophyllene (1.13%) and thymol (0.15%), respectively [Table 4]. Although the percentage of oxygen-containing monoterpenes is very high in essential oils (78.21%), they have poor content with regard to oxygen-containing sesquiterpenes (0.62 %) [Table 5]. *T. spicata* var. *intricata* essential oil is rich in phenolic compounds. Phenolic compounds such as carvacrol are widely reported to possess high levels of antimicrobial activity.[25-29] Carvacrol-rich essential oils are of special commercial interest as ingredients in animal feed and for the preservation of food, because of their high potency as antibacterial and antifungal agents.[1,30] Essential oils, which have antimicrobial activity, are used as integral ingredients in prepared foods or added as flavouring agents to foods.

Most of these reports indicate that carvacrol, γ-terpinene, *p*-cymene and thymol are the main and characteristic constituents of *T. spicata* essential oils. Only one study exists of essential oil composition of *T. spicata* var. *intricata*. In the study, in eight samples of *T. spicata* var. *intricata* collected from various locations of Turkey, with the exception of one sample, the main components of the essential oils of *T. spicata* var. *intricata* were detected to be carvacrol (9.21%-70.98%), γ-terpinene (8.33%-22.33%) and *p*-cymene (6.44%-23.54%), respectively.[6] Components of *T. spicata var. intricata* essential oils in our study are in compliance with the previous study of *T. spicata* var. *intricata* [Table 4]. However, the carvacrol content of the essential oil in our study was higher than that in the previous study.

Table 4: The essential oil constituents of *T. spicata* var. *intricata*

Compound	RI[a]	(%)	Identification methods
α-Thujene	901	tr	a, b, c
α-Pinene	914	0.87	a, b, c
Camphene	925	0.08	a, b, c
Sabinene	954	tr	a, b, c
β-Pinene	960	0.38	a, b, c
Myrcene	977	1.39	a, b, c
α-Phellandrene	989	0.12	a, b, c
Limonene	1002	0.52	a, b, c
α-Terpinene	1007	0.23	a, b, c
p-Cymene	1015	7.17	a, b, c
1,8-Cineole	1023	0.13	a, b, c
γ-Terpinene	1047	9.28	a, b, c
Linalool	1082	0.27	a, b, c
Camphor	1106	tr	a, b, c
Menthone	1114	0.1	b, c
Borneol	1132	0.62	a, b, c
Menthol	1138	0.14	a, b, c
Terpinen-4-ol	1142	0.95	a, b, c
Pulegone	1174	0.11	a, b, c
Thymol	1211	0.15	a, b, c
Carvacrol	1216	75.74	a, b, c
β-Caryophyllene	1277	1.13	a, b, c
Spathulenol	1326	0.17	b, c
Caryophyllene oxide	1328	0.45	b, c

RI: Retention index on DB1 column; a: co-injection with authentic compounds; b: MS; c: literature comparison; t: traces(< 0.08%); [a]In DB-1 fused silica capillary column

Table 5: Class composition of *T. spicata* var. *intricata* essential oil

Class of compounds	Percentage
Monoterpene hydrocarbons	19.81
Oxygen containing monoterpenes	78.21
Sesquiterpene hydrocarbons	1.13
Oxygen containing sesquiterpenes	0.62
Others	0.23
Total	100.00

The result of this study supports the folkloric use of these plants. Therefore, this result may suggest that the essential oils of this variety possess compounds with antimicrobial properties, which can be used as antimicrobial agents in new drugs for therapy of infectious diseases in human and as natural food preservatives.

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