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In vitro activity of methanol extracts of plants used as spices against *Mycobacterium tuberculosis* and other bacteria

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ABSTRACT

This study determined the phenolic composition of two thyme species (Lamiaceae), *Origanum minutiflorum* O. Schwarz and P.H. Davis and *Thymbra spicata* L. var. *spicata,* and assessed their antibacterial and antimycobacterial activities. "Kekik" is a collective term used in Turkey for plants that smell like thyme. *O. minutiflorum*, (locally "Sutculer kekigi", endemic) and *T. spicata* var. *spicata* (locally "Karakekik") are widely used in Turkey and are important export commodities.

The activity of the methanol extracts of these plants is given here for the first time. *T. spicata* var. *spicata* exhibited a high level of activity against *Mycobacterium tuberculosis* (minimum inhibitory concentration MIC 196 μg/ml), and moderate activity (MIC 640 μg/ml) against *Escherichia coli, Salmonella typhimurium, Enterobacter aerogenes*, and *Staphylococcus epidermidis*. Carvacrol, rosmarinic acid, hesperidin and naringenin were identified as the major phenolic compounds for *T. spicata* var. *spicata.* Carvacrol, rosmarinic acid, eriodictiol and luteolin were identified as the major phenolic compounds for *O. minutiflorum.* The effective constituents of methanol extracts of these plants are given here for the first time.

1. Introduction

One of the important genera yielding kekik for commerce is *Thymbra. Thymbra spicata* var. *spicata* is traded under the Turkish name of "Karakekik" (Black kekik). Baser (2002), citing the work of Tümen, Ermin, Özek, Kürkçüoglu, and Baser (1994) states that *Thymbra* (Lamiaceae) is represented in Turkey by four taxa belonging to two species (Unal, Topcuoglu, & Gokceoglu, 2005). *Thymbra* spp. is also used as condiment, herbal tea, and the production of essential oils for kekik water (Tümen et al., 1994). Although the antibacterial activities of their essential oils have long been recognized, further uses have recently been identified. Baser (2002) showed that carvacrol was one of the main constituents of the oil samples of *T. spicata.* Avci, Kupeli, Eryavuz, Yesilada, and Kucukkurt (2006) determined that *T. spicata* was associated with hypocholesterolaemic activity in mice fed a high-cholesterol diet. Moreover, *T. spicata* oil were found to be more effective against fungal growth (Soylu, Soylu, & Kurt, 2006).

The genus *Origanum* (Lamiaceae) is represented throughout the world by 50 species and in Turkey by 22 species or 32 taxa, 21 being endemic to Turkey. "Sütcüler kekiği", (*Origanum minutiflorum*) (endemic) is of commercial importance in Turkey (Baydar, Sagdic, Ozkan, & Karadogan, 2004). The main portion of kekik exports from Turkey include *O. minutiflorum* as the most widely traded. In commercial terms, *O. minutiflorum* represents the majority of Turkey's oregano exports. It is used to increase the flavour of food and also for herbal tea in the regions where it grows (Baser, 2002). Origanum mostly grow in the Mediterranean region and the Balkans (Baser, 2002). *O. minutiflorum*, which is rich in essential oils including thymol, carvacrol and pinene, shows antibacterial (Dadalioglu & Evrendilek, 2004), antifungal (Askun, Tumen, & Satil, 2008), anti-yeast (Souza, Stamford, Lima, & Trajano, 2007) and aqueous extracts show antioxidant properties (Dorman, Bachmayer, Kosar, & Hiltunen, 2004). In addition, they have been shown to be effective preservatives in food (Souza et al., 2007). The aim of the study was to investigate methanol extracts and to determine the major constituents responsible for the antimicrobial properties of these plants. Although their valuable compounds of essential oils have been known for a long time, the effective constituents of methanol extracts of these plants are given here for the first time.

Antimycobacterial drugs cause unpleasant side effects and trigger changes in the antibiotic target, thereby reducing the efficacy of drug therapies. *Mycobacteria* have recently increased their virulence and tuberculosis (TB) is the most lethal infection in the world. Between 1980 and 2005, 90 million cases of TB worldwide were reported to the WHO (World Health Organisation). The WHO stated "The global incidence of TB was estimated to be 136 cases per 100,000 population per year in 2005. In addition, the

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WHO region of the Americas and the WHO African region represent a total of 8.8 million new cases of TB and 1.6 million deaths from TB every year" (World Health Organisation, 2008a).

There were 9.5 million TB-related child deaths globally in 2006 (World Health Organisation 2008b). Today, one of the most important global health problems is changes in behavior of TB such as resistance to anti-TB drugs and the influence of the HIV epidemic (World Health Organisation, 2008a).

2. Material and methods

2.1. Plant materials

Aerial parts (Herba in flowering stage) of *T. spicata* var. *spicata* were collected in June 2005 and *O. minutiflorum* was collected in July 2006 from the Balikesir and Antalya regions of Turkey, respectively. The plants were identified by Associate Prof. Dr. F. Satil at Balıkesir University, Turkey. Reference specimens were deposited in the herbarium of Balikesir University, Department of Biology. The locality, altitude, collection time and herbarium number of the species are given in Table 1.

2.2. Preparation of extracts

Plants were air-dried at room temperature. Extracts of *O. minutiflorum* (115 g) and *T. spicata* var. *spicata* (115 g) were extracted with 1 l of methanol (98%) at room temperature over a period of 10 days, according to the Seshadri (1962) method. The methanol extracts were dried *in vacuo* at 40 °C. The total yield quantities were 1.25 g and 1.38 g, respectively. All stocks were stored in a deepfreeze at −20 °C.

2.3. HPLC conditions

HPLC was performed using a Shimadzu HPLC device according to preparation techniques for phenolic compounds (Caponio, Alloggio, & Gomes, 1999). The following equipment was used for reverse-phase chromatography: DAD detector (lmax = 278) and SIL-10AD vp auto sampler; SCL-10Avp system controller; LC-10ADvp pump and DGU-14A degasser. The column oven was CTO-10Avp and the column was Agilent Zorbax EclipseXDB-C18 (250 × 4.60 mm) 5 μm. The mobile phase consisted of A: 3% acetic acid and; B: methanol and the flow speed was 0.8 ml/min. The column temperature was 30 °C and the injection volume was 20 μl.

2.4. Microorganisms and inoculum

A total of eight Gram-positive and Gram-negative bacteria were used to study antibacterial activity. Gram-positive bacteria were: *Staphylococcus aureus* (6538-P), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212) and *Bacillus cereus* (CCM 99). Gram-negative bacteria were: *Escherichia coli* (ATCC 11230), *Salmonella typhimurium* (CCM 583) *Enterobacter aerogenes* (CIP 6069) and *Klebsiella pneumoniae* (CCM 2318).

Table 1
Herbarium data of plants.

Genus species authority	Locality	Altitude (m)	Collection time	Herbarium number
Thymbra spicata L. var. *spicata*	Balikesir, Gokceyazi, İvrindi	140 m	14/06/2005	FS1437
Origanum minutiflorum O. Schwarz and P.H. Davis (Endemic)	Antalya, Beydagi	1400 m	21/07/2005	FS1439

2.5. Antibacterial activity test

Stock solutions of all extracts were prepared in 10% dimethylsulphoxide (DMSO). Determination of minimal inhibitory concentration (MIC) by the microdilution method were performed according to the National Committee of Clinical Laboratory Standard guidelines (National Committee for Clinical Laboratory Standards (NCCLS, 2000) and Koo et al. (2000), Sterile 96-well microplates were used for the assay (0.2 ml volume, Fisher Scientific).

Samples were diluted to twice the desired initial test concentration with Trypton Soya Broth-Soybean Casein Digest Medium USP, (TSB), (Oxoid, Code: CM0129); samples that were difficult to dissolve were sonicated. All wells were filled with TSB (80 μl). The test sample (80 μl) was added to the first well and serial twofold dilutions were made down to the desired minimum concentration. Serial dilutions were performed so that extract concentrations in the range of 10 240 to 5 μg/ml were obtained. Day-old cultures of bacteria grown on Trypton Soya Agar (TSA) (Oxoid, Code: CM0131) plates were suspended in TSB until turbidity was equal to a 0.5 McFarland Standard (Koneman, Allen, Janda, Schreckenberger, & Winn, 1997). Gentamycin (Oxoid) were used for positive controls. Serial dilutions were performed so that Gentamycin concentrations in the range of 128 to 0.06 μg/ml were obtained. The plates were inoculated with the bacterial suspension (10 μl per well) and incubated at 37 °C overnight. All tests were made in triplicate in three different experiments. The lowest concentrations which did not show any growth of tested organism after macroscopic evaluation was determined as MIC.

2.6. Antimycobacterial activity test

MGIT *Mycobacteria* Growth Indicator Tubes, containing 4 ml of modified Middlebrook 7H9 Broth Base were used. Each test tube includes a fluorescent – quenching-based oxygen sensor embedded in silicone in the bottom of the tube. The fluorescent compound is sensitive to the presence of dissolved oxygen in the broth. The initial concentration of dissolved oxygen quenches the fluorescent emission from the compound. Actively respiring microorganisms consume the oxygen and allow the fluorescence to be observed using a 365 nm UV transilluminator (Chaudhuri et al., 1995; Palaci et al., 1996; Walters & Hanna, 1996).

The extracts were tested in duplicate against the reference strain, *Mycobacterium tuberculosis* H37Ra (ATCC 25177), for their inhibitory activity in duplicate. Inoculum was prepared both from solid media (Lowenstein–Jensen Medium) and from a positive BACTEC *Mycobacteria* Growth Indicator Tube (MGIT) according to the manufacturer's (Becton, Dickinson and Company) instructions.

To prepare inoculum from a culture of Lowenstein–Jensen Medium less than 15 days old, a suspension was prepared in Middlebrook 7H9 Broth. The turbidity of the suspension was adjusted to the McFarland standard 1.0. The suspension was vortexed for several minutes and allowed to precipitate larger particles then to sit for 20 min. The supernatant was transferred to an empty, sterile tube and allowed to sit for a further 15 min. After being transferred to a new sterile tube, the suspension was adjusted to a 0.5 McFarland turbidity standard by visual comparison. One ml of the adjusted suspension was diluted in 4 ml of sterile saline.

To prepare inoculum from a positive BACTEC MGIT tube, the positive tubes were used beginning from the day after it first became positive (day 1 positive) up to and including the fifth day (day 5 positive). The positive tubes older than five days were subcultured into fresh growth-medium. The tubes which were day-1 and day-2 positive proceeded to the inoculation procedure for the susceptibility test. The tubes between day 3 and day 5 positive were diluted using a 1 ml of the positive broth with 4 ml of sterile saline, the total is 5, then this diluted suspension were used for inocula-

Table 2
Amount of chemicals in the methanol extracts of *T. spicata* var. *spicata* and *O. minutiflorum*.

Chemicals (μg/ml)	*Thymbra spicata* var. *spicata*	*Origanum minutiflorum*
Gallic acid	[a]	[a]
Catechin	[a]	[a]
Caffeic acid	[a]	[a]
Epicatechin	[a]	[a]
Vitexin	[a]	21.1
Rutin	1175	209
Naringin	[a]	[a]
Hesperidin	9428	[a]
Apigenin glucoside	81.7	555
Rosmarinic Acid	26644	3764
Eriodictiol	1444	1273
Quercetin	945	122
Naringenin	1529	334
Luteolin	277	722
Apigenin	250	396
Carvacrol	81481	99065
Acecetin	[a]	[a]

[a] Not determined.

tion procedures. Each assay was performed according to the MGIT manual Fluorometric susceptibility test procedure recommended by the manufacturer, Becton, Dickinson and Company.

OADC supplement (0.5 ml) (a mixture of oleic acid, albumin, dextrose and catalase) was added to each tube. This extract (0.1 ml) was added to each MGIT tube. The final concentration of the extracts adopted to evaluate the antimycobacterial activity was within the range of 1.5–0.012 mg/ml. Inoculum (0.5 ml) was added to each tube except for the negative control. An uninoculated MGIT tube was used as a negative control. The positive control tube contained only organisms and OADC, but not the plant extract.

Any suspicious growth of other bacteria was checked using blood agar for each test. The vials were incubated at 37 °C and were tested daily, starting on the second day of incubation, using a MicroMGIT Flourescence reader with a long wave UV light. MIC was determined as the lowest dilution giving a negative result using a MicroMGIT Florescence reader within 2 days of when the controls turned positive.

3. Results

The methanol extracts prepared from aerial parts of *T. spicata* var. *spicata* and *O. minutiflorum*, the herbarium data of these species shown in Table 1, were analyzed by HPLC. The quantity of chemicals in the methanol extracts are given in Table 2. Chromatograms of phenols in the methanol extracts were compared to chromatograms of standards (Figs. 1–3). The results were evaluated according to the literature.

Extracts were tested against *S. aureus*, *S. epidermidis*, *E. faecalis*, *B. cereus*, *E. coli*, *S. typhimurium*, *E. aerogenes*, and *K. pneumoniae* for antibacterial activity (Table 3). *M. tuberculosis* was used for the antimycobacterial activity test (Table 3). Gram (−) bacteria and Gram (+) bacteria were tested for susceptibility to reference drug, Gentamicin. Standard drugs used against *M. tuberculosis* were rifampin, ethambutol and isoniazid. All bacteria were found to be sensitive to standard drugs given final concentrations (Table 3).

The major phenolic compounds for *T. spicata* var. *spicata* determined by HPLC analyses of the methanol extracts were carvacrol, rosmarinic acid, hesperidin, naringenin eriodictiol, rutin, and quercetin. A number of minor compounds were identified, includ-

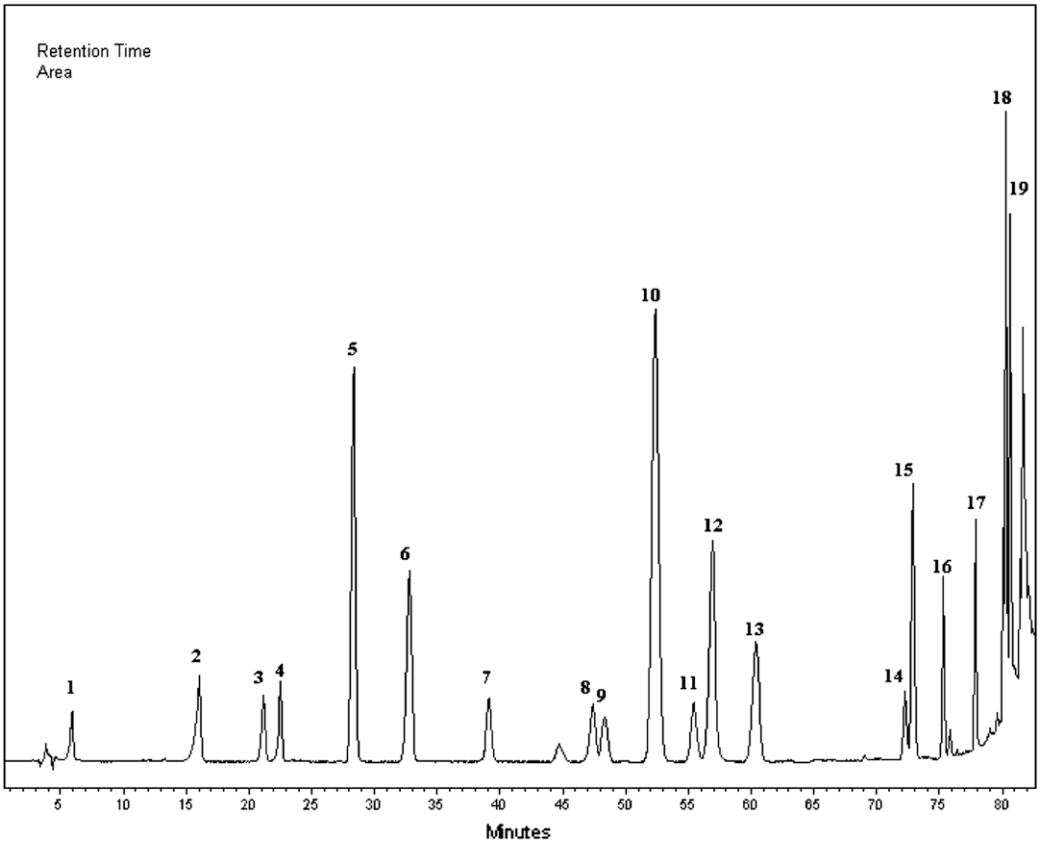

Fig. 1. Chromatogram of standards: (1) gallic acid, (2) catechin, (3) caffeic acid, (4) epicatechin, (5) vitexin, (6) rutin, (7) naringin, (8) hesperidin, (9) Apigenin glucoside, (10) rosmarinic acid, (11) eriodictiol, (12) quercetin, (13) naringenin, (14) luteolin, (15) apigenin, (16) carvacrol, (17) acetacin.

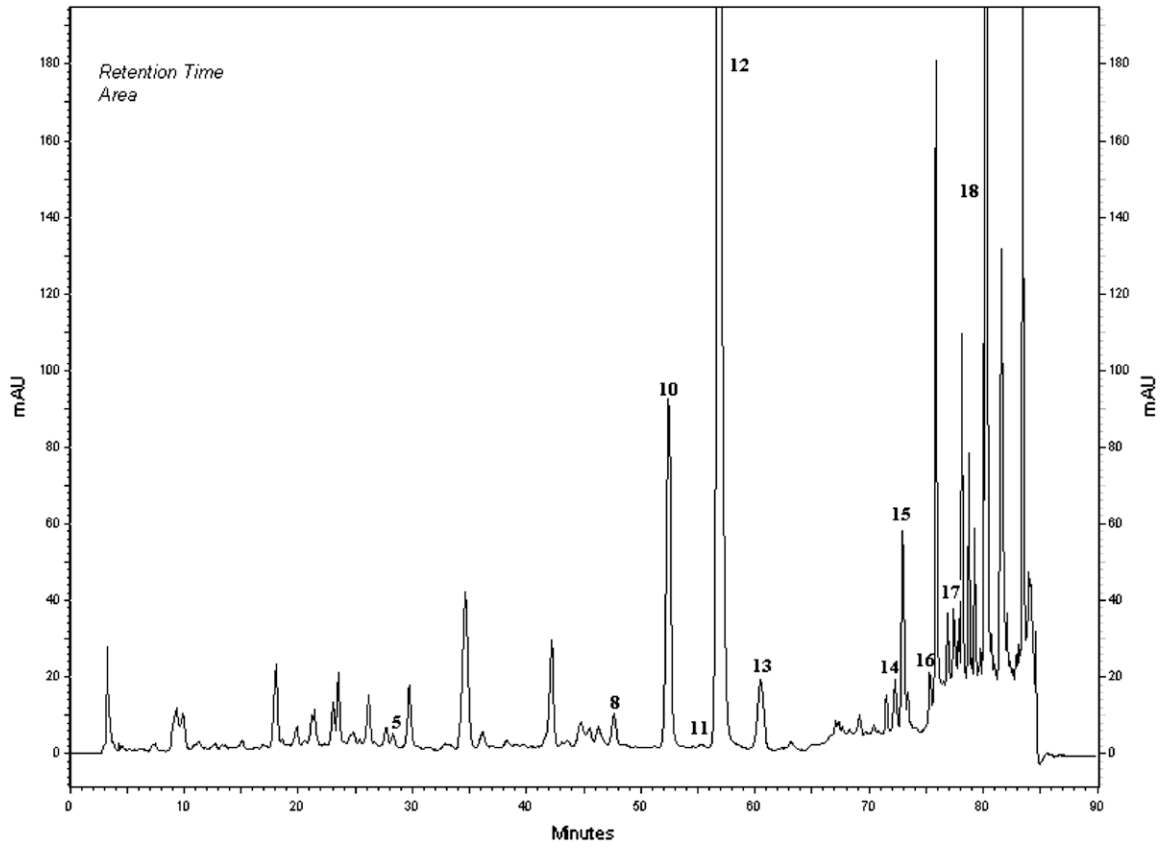

Fig. 2. HPLC chromatogram of methanol extracts of *Thymbra spicata* var. *spicata*.

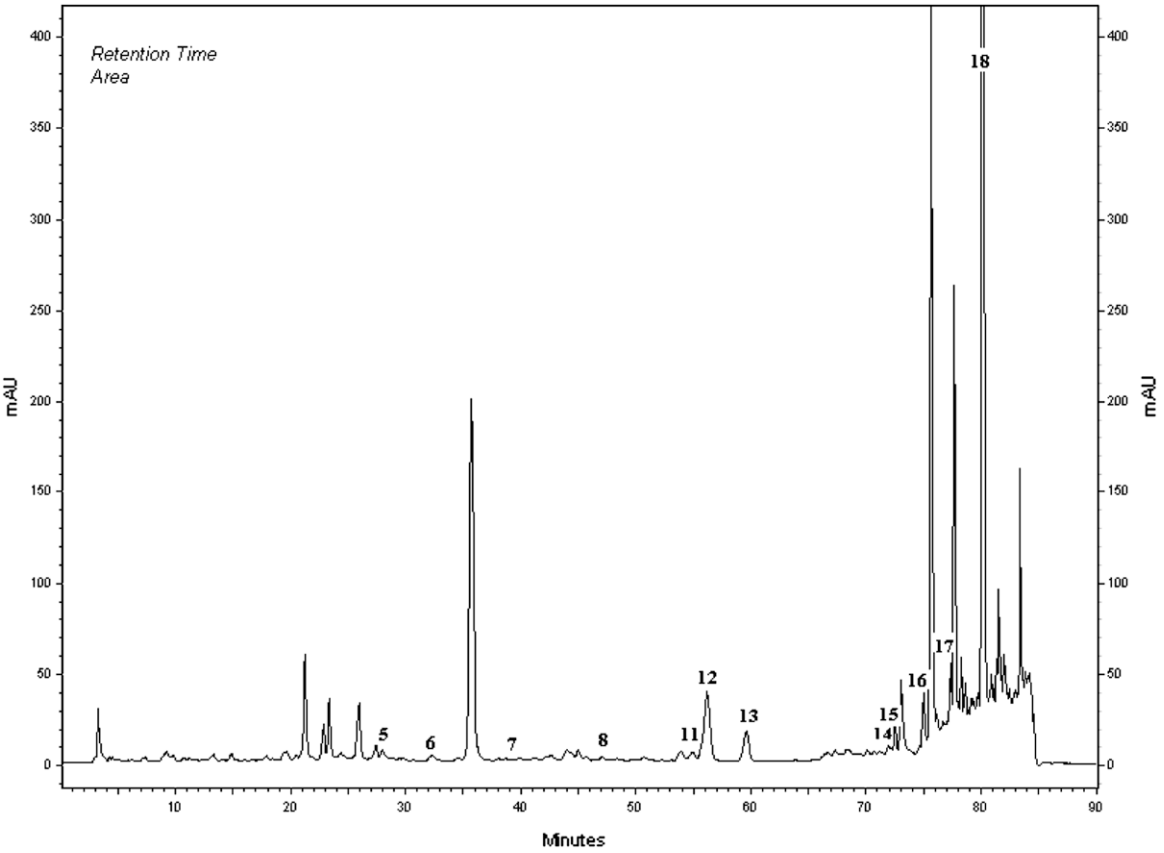

Fig. 3. HPLC chromatogram of methanol extracts of *Origanum minutiflorum*.

Table 3
Antibacterial activity of methanol extracts of the plants as MIC (μg/ml) and susceptibility test results against *M. tuberculosis* H37Ra (ATCC 25177) obtained by MGIT fluorometric manual method.

Bacteria	MIC (μg/ml)			Standard drugs
	T. spicata var. *spicata*	*Origanum minutiflorum*	Standard drug	
Ec	640	5120	2	Gentamycin
St	640	5120	2	
Ef	1280	5120	32	
Ea	640	1280	2	
Sa	1280	2560	4	
Se	640	1280	2	
Kp	5120	10240	8	
Bc	5120	10240	8	
Mt	196	392	0.8	Streptomycin
			1.0	Rifampin
			3.5	Ethambuthol
			0.1	Isoniasid

Sa: *Staphylococcus aureus* (6538-P); Se: *Staphylococcus epidermidis* (ATCC 12228); Ef: *Enterococcus faecalis* (ATCC 29212); Bc: *Bacillus cereus* (CCM 99); Ec: *Escherichia coli* (ATCC 11230), St: *Salmonella typhimurium* (*CCM 583*) Ea: *Enterobacter aerogenes* (CIP 6069); Kp: *Klebsiella pneumoniae* (CCM 2318), and MT: *Mycobacterium tuberculosis* H37Ra (ATCC 25177).

ing apigenin, luteolin, and apigenin glucoside. The major phenolic compounds for *O. minutiflorum* were carvacrol, rutin, rosmarinic acid, eriodictiol, luteolin, and apigenin glucoside. Additionally, quercetin, naringenin, vitexin, and apigenin were identified by HPLC analyses.

T. spicata var. *spicata* was most effective (MIC 640 μg/ml) against Gram (−), *E. coli, S. typhimurium* and *E. aerogenes* and Gram (+), *S. epidermidis.* Other bacteria (*K. pneumoni*, *B. cereus, E. faecalis, S. aureus*) showed activity between 1280 and 5120 μg/ml. *O. minutiflorum* was shown to be most effective against *S. epidermidis* and *E. aerogenes* (MIC 1280 μg/ml). MIC values for other bacteria varied between 5120 and 0240 μg/ml (3). Of the two plants studied, *T. spicata* var. *spicata* showed greater antimycobacterial efficacy (MIC 196 μg/ml) than *O. minutiflorum* (MIC 392 μg/ml) (Table 3).

4. Discussion

A review of the literature on the antimicrobial activity of different plant extracts shows that methanol extracts have a high level of activity. Parekh and Chanda (2007) reported that the crude methanol extract of *Woodfordia fruticosa* contains certain constituents such as tannins with significant antibacterial properties, which enables the extract to overcome the barrier in Gram-negative cell wall.

Parekh, Jadeja, and Chanda (2005) reported that methanol extracts were more active than aqueous extracts for all 12 plants studied. Methanol provided more consistent antimicrobial activity compared to those extracted in water. These activities might depend on the compounds being extracted by each solvent, the polarity of the solvents, and their intrinsic bioactivity.

In our previous research (Askun et al., 2008), on the effects of plant-derived methanol extracts (including *T. spicata* and *O. minutiflorum*) against fungi, formed a good basis for developing research on antimicrobial and antimycomicrobial activity.

Silme and Yegen (2006) reported that the practical significance of carvacrol was that the chitinase and the indole-3-acetic acid (IAA) is produced by biodegradation of carvacrol. These results suggest the possible application of bacterial biodegradation of carvacrol in plant protection science as a biological fungicide.

Plant methanol extracts contain many chemicals such as alkaloids, amino acids, flavonoids, glycosides, phytosterols, saponins, steroids, tannins and triterpenoids (Kumar et al., 2009). We therefore anticipate the possibility that they might yield a different spectrum of antibacterial components from those previously described. To date, there are relatively few published accounts of the effect of plant extracts on *M. tuberculosis* (Adeniy, Groves, & Gangadharam, 2004; Rojas et al., 2006).

Apart from their culinary usage, *Origanum* and *Thymbra* species are used to cure stomach-aches and respiratory colds (Kocabas & Karaman, 2001). The *in vitro* antibacterial and antimycobacterial activities may support the use of *Thymbra* and *Origanum* species in traditional medicine to treat microbial infections. They are also used to preserve plants. For example, figures, one of the most important export products of western Turkey, are soaked in boiled water with thyme, then left to dry (Tumen, 1989).

It has previously been shown that carvacrol (Botelho et al., 2007) and rosmarinic acid (Chakraborty et al., 2007) are capable of inhibiting Gram (−) bacteria. The antibacterial effects of luteolin against Gram (+) bacteria have been recently studied (Obied, Bedgood, Prenzler, & Robards, 2007.

Extracts of natural products are a common starting point in the search for new antimycobacterial agents. According to our results, *T. spicata* var. *spicata* and *O. minutiflorum* were especially active against *M. tuberculosis* and bacteria. The antimycobacterial activity of *T. spicata* var. *spicata* was better than that of *O. minutiflorum.* These results form a good basis for selection of candidate plant species for further phytochemical and pharmacological investigation.

Baydar et al. (2004) investigated the effect of *O. minutiflorum* and *T. spicata* oils on bacteria by identifying their eight major constituents. The oil constituents with the highest yields were cavracrol, *c*-terpinene and *p*-cymene. The carvacrol yield of *T. spicata* (75.5%) is lower than *O. minutiflorum* (84.6%). They found, using paper disc diffusion methods, that both oils showed similar activity against bacteria. *B. amyloliquefaciens* and *P. vulgaris* were the most susceptible, and were the only bacteria to be inhibited by the oils at 1/200 concentration. At 1/300 concentration, neither of the two oils had an inhibitory effect against any of the bacteria. When comparing carvacrol yields in our methanol extracts to oils, the carvacrol yield of *T. spicata* (81481.0 μg/ml) is lower than *O. minutiflorum* (99064.6 μg/ml). However, we determined that rosmarinic acid, one of the important flavonoids, is obtained using methanol as a polar solvent. Moreno, Scheyer, Romano, and Vojnov (2006) reported that rosmarinic acid was the most effective antimicrobial against Gram-positive bacteria, Gram-negative bacteria and yeast.

With regard to the antimycobacterial activity of *O. minutiflorum* (carvacrol yields 99064.6 μg/ml), it would be expected to be more active than *T. spicata var. spicata* (carvacrol yields 81481.0 μg/ml). However, the results indicate that the opposite is true: *T. spicata var. spicata* extract has a higher efficacy than that of *O. minutiflorum* (see Table 2). With a high quantity, rosmarinic acid might be responsible for this antimycobacterial activity. In addition to this, Mandalari et al. (2007) reported that, as pair wise combinations of eriodictyol, naringenin and hesperidin showed both synergistic and indifferent interactions that were dependent on the test indicator organism and their cell wall structure.

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