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Bacterial Susceptibility to and Chemical Composition of Essential Oils from *Thymus kotschyanus* and *Thymus persicus*

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Susceptibility of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumonia*, and *Pseudomonas aeroginosa* to the essential oils extracted from two varieties of Thyme, i.e., *Thymus kotschyanus* Boiss. and Hohen. and *Thymus persicus* L. at preflowering and flowering stages were studied. The disk diffusion method was used to evaluate the zone of microbial growth inhibition at various concentrations of the oils. Minimal inhibitory concentration and minimal bactericidal concentration of the oils were determined and compared with each other. The oils from the above plants were found to be strongly bactericidal with that of *T. kotschyanus* being more effective. *T. kotschyanus* and *T. persicus* oils analyzed by gas chromatography (GC) and GC/mass spectrometry (MS) lead to identification of 33 and 26 components, respectively. The profile of the oil components from *T. persicus* was similar to that of *T. kotschyanus* in almost all of the compounds but at different concentrations. The major components of *T. kotschyanus* oil before and at the flowering stages were carvacrol (35.06, 22.75%), thymol (26.60, 16.52%), γ-terpinene (7.81, 0.34%), γ-terpinene (4.34, 0%), borneol (2.29, 4.52%), myrcene (0.26, 12.65%), thymolquinone (0, 11.39%), nerol (0, 6.10%), and β-caryophyllene (0, 5.54%), respectively, and those of *T. persicus* at the same stages were carvacrol (38.96, 27.07%), thymol (6.48, 11.86%), P-cymene (7.51, 10.16%), γ-terpineol (0, 9.51%), nerol (15.66, 9.41%), γ-terpinene (6.11, 6.51%), and thymol acetate (5.29, 5.30%), respectively. The contribution of oil components to its antibacterial property is discussed. High aromatic compound content of the phenol-rich oils seems to account for strong antibacterial activity.

KEYWORDS: ***Thymus kotschyanus*; *Thymus persicus*; essential oil; antimicrobial**

INTRODUCTION

Essential oils are rich sources of biologically active compounds (*1*). Recently, there has been a profound interest in the antimicrobial properties of extracts from aromatic plants, particularly essential oils (*2*). Many oils and extracts from different plants have been investigated for their antimicrobial properties against a series of bacteria and yeasts (*3*−*7*). Essential oils have been found to be antibacterial (*8*), antifungal (*9*), and therapeutic in cancer treatment (*10*). Some oils have been shown to have applications in food preservation (*11*). Some oils have pharmacological properties (*12*), and some are used in aromatherapy (*13*). Thus, the use of natural antimicrobial compounds seems to be important not only in the preservation of food but also in the control of human and plant diseases of microbial origin. The genus *Thymus* has been credited with a long list of pharmacological properties. Dietary supplementation of thyme oils retained more favorable antioxidant capacity during life span of experimental animals (*14*). Thyme oils present high antimicrobial effects on various microorganisms (*15*−*17*). The infraspecific variability of oils in the *Thymus* genus has been the subject of several studies reviewed by Stahl-Biskup (*18*). Bacterial and fungal infections pose a greater threat to health, most notably in immunosupressed subjects, hence the need to find inexpensive and effective antimicrobial agents. The quantitative composition and the relative proportions of the oil components are widely influenced by the genotype, the ontogenic development, and the environmental and growing conditions (*19*, *20*). In light of the above facts, it seems necessary to evaluate the chemical composition and antimicrobial activities of the oils of Iranian *Thymus persicus* L. and *Thymus kotschyanus* Boiss. and Hohen., which have not been reported to date. The present study was planned to evaluate their antibacterial activity taking into account the chemical composition of the oils.

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Table 1. Diameter of Microbial Inhibition Zones (mm) Determined by Disk Diffusion Assay at Various Dilutions of Essential Oils from *T. kotschyanus* and *T. persicus*[a]

		corresponding effects of oils on microorganisms									
		E. coli		*S. aureus*		*B. subtilis*		*K. pneumonia*		*P. aeroginosa*	
plant name	oil dilution	P	F	P	F	P	F	P	F	P	F
T. kotschyanus	1	50	40	75	75	75	75	75	57	12	12
	1/2	42	31	64	53	70	62	60	44	8	7
	1/4	28	21	34	30	46	44	44	32	R	R
	1/8	22	12	25	21	40	32	18	15	R	R
	1/16	18	R	15	14	27	21	12	12	R	R
T. persicus	1	51	62	57	75	73	75	48	52	9	R
	1/2	36	46	44	64	54	58	34	38	R	R
	1/4	24	24	34	40	34	40	21	26	R	R
	1/8	18	22	27	30	26	32	8	11	R	R
	1/16	10	14	15	16	14	20	R	R	R	R

[a] P, preflowering stage; F, flowering stage; and R, resistant.

Table 2. Minimal Inhibitory (MIC) and Bactericidal Concentrations (MBC) of the Essential Oils from *T. kotschyanus* and *T. persicus* against 10^7 CFU/mL of Microbial Suspensions[a]

			corresponding MIC/MBC effects of oils on microorganisms									
			E. coli		*S. aureus*		*B. subtilis*		*K. pneumonia*		*P. aeroginosa*	
plant name	oil dilutions	oil in broth (ppm)	P	F	P	F	P	F	P	F	P	F
T. kotschyanus	1	10	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–
	1/2	5	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–
	1/4	2.5	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–
	1/8	1.25	±	–/–	+/+	±	+/+	+/+	±	±	–/–	–/–
	1/16	0.625	–/–	–/–	±	±	+/+	±	–/–	–/–	–/–	–/–
T. persicus	1	10	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–
	1/2	5	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–
	1/4	2.5	+/+	+/+	+/+	+/+	+/+	+/+	±	+/+	–/–	–/–
	1/8	1.25	±	±	±	±	±	+/+	–/–	–/–	–/–	–/–
	1/16	0.625	–/–	–/–	±	–/–	–/–	±	–/–	–/–	–/–	–/–

[a] P, preflowering stage; F, flowering stage; +, effective; and –, not effective.

MATERIALS AND METHODS

Cultures and Media. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 9372), *Klebsiella pneumonia* (ATCC 13183), and *Pseudomonas aeroginosa* (ATCC 27853) were cultured in nutrient broth (Merck) at 37°C for 24 h. Broth cultures were diluted in sterile nutrient broth to a concentration of approximately 10^8 CFU/mL. Subsequent dilutions were made from the above suspension. Both spectrophotometer absorbance reading at 580 nm and plate counts using nutrient broth dilution blank and nutrient agar were used to confirm the viable cell concentration. In subsequent trials, only spectrophotometer absorbance readings were used to estimate cell concentration. Diluted cultures were then used in the tests.

Oil Isolation. *T. kotschyanus* Boiss. and Hohen. and *T. persicus* L. were collected from the Damavand area of Iran during March−June 2000. The plant leaves were collected in March and April before the flowering stage. The leaves were also collected in May and June, where the plants set to flowers. The fresh aerial parts, i.e., the leaves, were hydrodistilled for 90 min in a full glass apparatus. The oils were isolated using a Clevenger type apparatus. The extraction was carried out for 2 h after a 4 h maceration in 500 mL of water. The samples from *T. kotschyanus* and *T. persicus* before the flowering stage yielded 0.55 and 0.57% w/w and at the flowering stage yielded 1.65 and 0.90% w/w oil, respectively. The oils were stored in dark glass bottles in a freezer until they were used.

Oil Analysis. The essential oils were analyzed by gas chromatography (GC) (9-A-Shimadzu) and GC/mass spectrometry (MS) (Varian-3400) column (DB-1, 60 m × 0.25 mm fused silica capillary column; film thickness, 0.25 μm) using a temperature program of 40−220 °C at a rate of 4 °C/min; injector temperature, 260 °C; carrier gas, helium. The constituents were identified by comparison of their mass spectra with those in the computer library and with authentic compounds. The identifications were confirmed by comparison of their retention indices with those of authentic compounds or with literature data.

Selection of Dilution Solvent. Various solvents such as ethanol, methanol, acetone, butanol, and diethyl ether were tested for their antibacterial activities using the disk diffusion method. The solvent showing no antimicrobial activity, i.e., methanol, was selected as a diluting medium for the oils. This solvent also served as the control. Dilutions (1/2, 1/4, 1/8, and 1/16) of oils were made with methanol. These dilutions were used in antibacterial analysis. Undiluted oil was taken as dilution 1.

Antibacterial Analysis. The fresh oils were tested for their antibacterial activities. The disk diffusion method (*21*, *22*) was used for antibacterial screening as follows. Sterile Mueller−Hinton agar medium (Merck) was prepared and distributed into Petri plates of 90 mm diameter. This medium was used for antibiogram assays. The disk size used was 6 mm (Whatman No. 1) paper. Different dilutions of the oils were made with methanol. The microbial suspension was streaked over the surface of the Mueller−Hinton agar using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. Under aseptic conditions, the disks were placed on the agar plates and then 10 μL from each of the oil dilutions was put on the disks. A 10 μL dilution solvent (methanol) was added to the disks on the control plates. The plates were then incubated at 37 °C for 24−48 h in order to get reliable microbial growth. Diameters of microbial inhibition zones were measured using vernier calipers.

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were assessed according to the modified procedure of Kivanc and Akgul (*8*). MIC was determined by a broth dilution method in test tubes as follows: 50 μL from each of various dilutions of the oils was added to 5 mL of nutrient broth tubes containing 10^7 CFU/mL of live bacterial cells. The tubes were then incubated on an incubator shaker as to evenly disperse the oil throughout the broth in

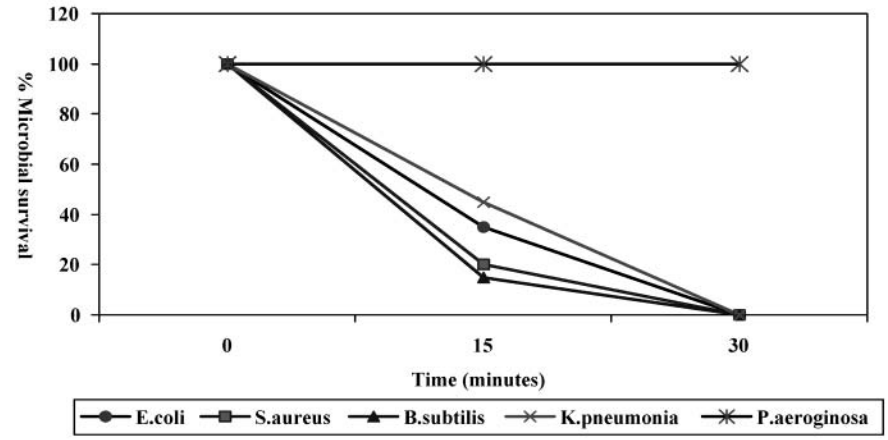

Figure 1. Kinetics of microbial destruction at MBC levels of the fresh oil from *T. kotschyanus* before the flowering stage.

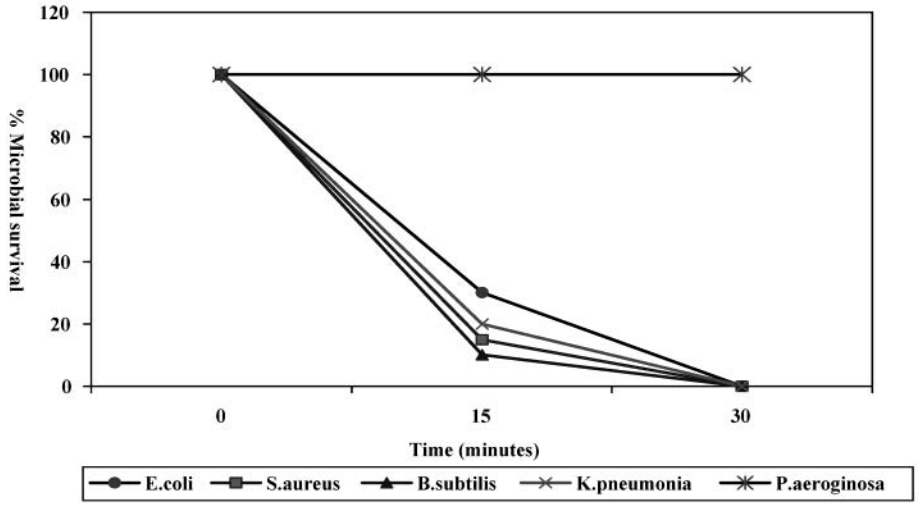

Figure 2. Kinetics of microbial destruction at MBC levels of the fresh oil from *T. kotschyanus* at the flowering stage.

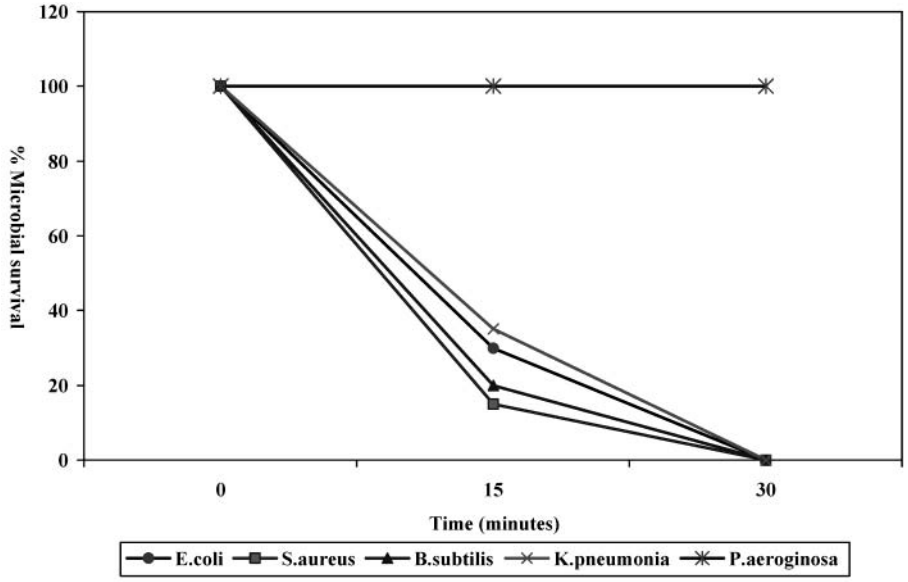

Figure 3. Kinetics of microbial destruction at MBC levels of the fresh oil from *T. persicus* before the flowering stage.

the tubes. The highest dilution (lowest concentration), showing no visible growth, was regarded as MIC. Cells from the tubes showing no growth were subcultured on nutrient agar plates to determine if the inhibition was reversible or permanent. MBC was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates.

Bactericidal Kinetics of the Oils. A 50 μL amount of each oil at the dilution determined by MBC was added to 5 mL of each microbial suspensions containing 10^7 cells/mL and was then incubated at 37 °C for 15, 30, 45, and 60 min in an incubator shaker. Samples were taken after the time intervals and were cultured on nutrient agar for 24 h at 37 °C. A 50 μL amount of dilution solvent was added to the control

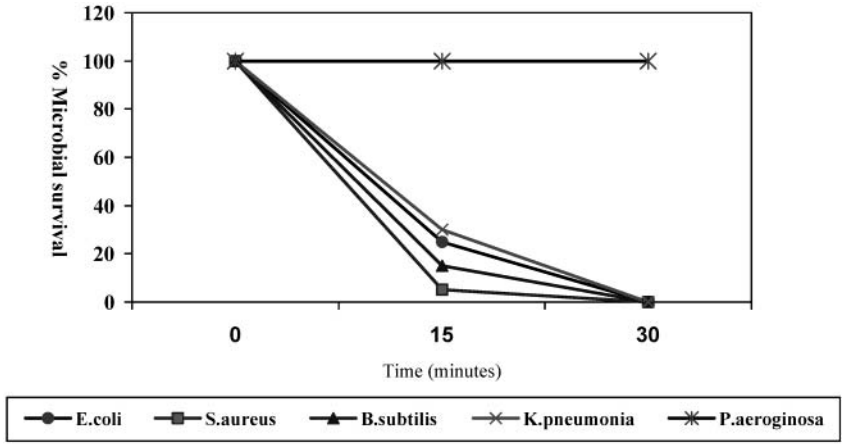

Figure 4. Kinetics of microbial destruction at MBC levels of the fresh oil from *T. persicus* at the flowering stage.

Table 3. Percentage Composition of *T. kotschyanus* and *T. persicus* Oils[a]

no.	*T. kotschyanus* compds	RI	% before flowering	% at flowering	*T. persicus* compds	RI	% before flowering	% at flowering
1	α-thujene	923	1.37	0.21	α-thujene	923	0.93	trace
2	α-pinene	931	1.77	0.44	camphene	943	0.51	0.28
3	camphene	943	0.47	1.04	β-pinene	968	0.25	0.39
4	sabinene	971	0.33		myrcene	979	1.18	0.91
5	β-pinene	983	1.78	0.30	α-terpinene	1007	0.89	0.49
6	myrcene	998	0.26	12.65	P-cymene	1012	7.51	10.16
7	α-phellandrene	1006	1.49		1,8-cineole + limonene	1019	2.17	2.67
8	α-terpinene	1018	4.34		γ-terpinene	1048	6.11	6.51
9	P-cymene	1023	2.17		*trans*-sabinene hydrate	1052	0.72	1.31
10	1,8-cineole + limonene	1027		2.77	linalool	1087	1.26	0.91
11	γ-terpinene	1055	7.81	0.34	camphor	1121	- - -	0.25
12	*trans*-sabinene hydrate	1058	1.71	1.61	borneol	1150	1.57	2.90
13	linalool	1087		0.45	terpinene-4-ol	1163	0.25	0.39
14	camphor	1123		0.64	α-terpineol	1175	- - -	9.51
15	verbenol	1123		0.23	methyl thymol	1217	0.62	1.02
16	borneol	1154	2.29	4.52	methyl carvacrol	1228	0.24	0.13
17	terpinene-4-ol	1164	0.31	0.20	nerol	1247	15.66	9.41
18	α-terpinenol	1175		0.36	geranial	1251	0.35	0.33
19	methyl thymol	1217	0.34	1.05	thymol	1278	6.48	11.86
20	thymolquinone	1226		11.39	carvacrol	1282	38.96	27.07
21	methyl carvacrol	1228	1.22	0.58	thymol acetate	1364	5.29	5.30
22	nerol	1247		6.10	β-caryophyllene	1423	2.04	3.00
23	geranial	1251	0.61	1.69	germacrane B	1492	0.31	trace
24	thymol	1278	26.60	16.52	δ-cadinene	1514	2.80	3.19
25	carvacrol	1282	35.06	22.75	spathulenol	1568	0.20	trace
26	geranyl acetate	1364	2.56	1.47	caryophyllen oxide	1574	0.47	0.67
27	β-bourbonene	1406		0.23				
28	β-caryophyllene	1423		5.54				
29	α-humulene	1452		0.19				
30	germacrane D	1479	0.21	0.13				
31	β-bisabolene	1492	0.87	1.48				
32	γ-cadinene	1514	1.48	0.93				
33	caryophyllene oxide	1574		1.68				

[a] RI, retention indices; and trace, less than 0.1%.

tubes instead of essential oil. Microbial colonies were counted after the incubation period, and the total number of viable cells per milliliter was calculated. The calculation was converted to percent dead cells using routine mathematical formulas.

RESULTS

Variable zones of microbial growth inhibition were noted in both of the plants essential oils (**Table 1**). The oils from *T. kotschyanus* at 1, 1/2, and 1/4 dilutions and from *T. persicus* at 1 and 1/2 dilutions were strongly bactericidal. *P. aeroginosa* showed a static reaction to the oils from *T. kotschyanus* and resistance to the oils from *T. persicus* (**Table 1**). The fresh oils were tested for their bacteriostatic and bactericidal effects employing MIC and MBC techniques (**Table 2**). *T. kotschyanus* oils from both stages at 1/4 dilution were bactericidal against all of the microbial species with the exception of *P. aeroginosa.* The bactericidal effect of *T. kotschyanus* oil at the preflowering stage was noted against *S. aureus* and *B. subtilis* at 1/8 and 1/16 dilutions, respectively (**Table 2**). The bactericidal effect of *T. persicus* oil at the preflowering stage was seen against *E. coli*, *S. aureus*, and *B. subtilis* at 1/4 dilution. At the same stage, *K. pneumonia* required a higher oil concentration (1/2 dilution) to be killed. The Gram negative *E. coli*, Gram positive *S. aureus*, and encapsulated *K. pneumonia* were killed at 1/4 dilution of oil extracted at the flowering stage of *T. persicus* while the spore-forming bacillus (*B. subtilis*) was easily killed at a higher

dilution of 1/16. *P. aeroginosa* was not affected (**Table 2**). More than 50% of any bacterial population, with the exception of the resistant genus *P. aeroginosa*, was rendered nonviable in 15 min. One hundred percent lethal effects were observed within 30 min of the exposure to the oils (**Figures 1−4**). Chemical analysis of the components of the oil from *T. kotschyanus* and *T. persicus* resulted in the identification of 33 and 26 components, respectively (**Table 3**). The major components of *T. kotschyanus* oil before and at the flowering stages were carvacrol (35.06, 22.75%), thymol (26.60, 16.52%), γ-terpinene (7.81, 0.34%), γ-terpinene (4.34, 0%), borneol (2.29, 4.52%), myrcene (0.26, 12.65%), thymolquinone (0, 11.39%), nerol (0, 6.10%), and β-caryophyllene (0, 5.54%), respectively. Those of *T. persicus* at the same stages were carvacrol (38.96, 27.07%), thymol (6.48, 11.86%), P-cymene (7.51, 10.16%), α-terpineol (0, 9.51%), nerol (15.66, 9.41%), γ-terpinene (6.11, 6.51%), and thymol acetate (5.29, 5.30%), respectively.

DISCUSSION

Average values of three trials constitute the results. Both the Gram positive and the Gram negative bacteria were very sensitive even to higher essential oil dilutions especially of *T. kotschyanus* Boiss. and Hohen. (**Table 1**). Lawrence (*23*) and Shu Chi-Kuen and Lawrence (*24*) have established that the composition of essential oils will depend on the plant species, the chemotypes, and the climatic conditions; therefore, their antimicrobial activities could vary. This suggestion has been supported in the present study. The differences observed in antimicrobial activities of essential oils suggest susceptibility of microorganisms to various chemical components of the oils. This becomes more evident with the different reactions of the same microbial strains to the oil of the same plant species extracted at two life stages (**Tables 1** and **2**). On the basis of diameter of microbial inhibition zones, oils from *T. kotschyanus* and *T. persicus* at the MBC dilutions exhibited a 21−44 and 24−40 mm range with different bacteria, respectively (**Table 1**). Although the oils exhibited significant antibacterial activity, the level of inhibition could be lower in diffusion plate tests (*9*). MBCs of the oils were put against microbial suspensions in the nutrient broth to measure the time required for maximum efficacy of the oils to render the microbial population nonviable (**Figures 1−4**). Keeping in mind that microorganisms could show lower sensitivity when exposed to the action of the oils in the presence of nutrients (*3*), it can be concluded that the oils under study are strongly bactericidal. Bagci and Digrak (*4*) classified oils into three groups according to the strength of their antimicrobial activity. Such a difference in antimicrobial activity of the essential oils can be seen in this study. The profile of *T. persicus* oil composition is similar in almost all of the components to that of *T. kotschyanus* but at different quantities (**Table 3**). The variation in the quantity or quality of the oils supports Piccaglia et al. (*20*) who stated that the quantitative composition and the relative proportions of the oil components are widely influenced by the genotype, the ontogenic development, and the environmental and growing conditions. This further supports the suggestion of Lawrence (*23*) and Shu and Lawrence (*24*) on dependence of oil composition on the plant species, the chemotypes, and the climatic conditions. It also implies the possibility of different medicinal uses of the same plant species grown in different regions (*25*). The high carvacrol content of the oils accounts for their strong antimicrobial activity as already pointed out by numerous authors regarding other phenol-rich oils (*16*, *26*). In both plants, the carvacrol content decreases at the flowering stage, which could be a probable reason for the decline in antimicrobial activity of *T. kotschyanus* oil as compared to preflowering stage. The thymol and carvacrol contents in our plants were less than the thyme species studied by Tzakou (*15*), Juliano (*16*), and Karaman (*17*). The results indicate that stronger antibacterial effects were achieved using *T. kotschyanus* and *T. persicus* oils as compared to those studied by other investigators (*15*−*17*). Although the compounds present in the greatest proportions are not necessarily responsible for the greatest share of the total activity (*27*), the pattern of oil activity on microorganisms may however be correlated to its components. Essential oils with high monoterpenes hydrocarbons have been reported to be very active against bacteria (*28*). In this study, aromatic compounds such as carvacrol, thymol (phenols), and P-cymene contributed to more than 75% of the chemical composition of thyme oils with strong antibacterial properties. These results indicate the potential antibacterial properties of essential oils and hence a hope for the near future to find emergence of antibacterial compounds from natural sources.

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