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Chemical Composition, and Antibacterial and Free-Radical-Scavenging Activities of the Essential Oils of a Citronellol Producing New Chemotype of *Thymus pubescens* Boiss. & Kotschy ex Celak

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Abstract: The composition of the essential oils obtained from the flowering aerial parts of two populations of *Thymus pubescens,* collected from Mishov-Dagh, was determined by the GC-MS analyses. A total of 18 compounds, representing about 95% of the total oils, were identified in both samples of the essential oils. The essential oils of these two populations showed the presence of high amounts of citronellol (42.0% and 42.6%), geranyl acetate (14.0 and 14.0%), geraniol (13.0 and 13.1%), citronellyl acetate (3.9 and 3.8%), L-linalool (7.8% and 7.9%), *cis*-nerodiol (5.9% and 5.5%) and citronellyl acetate (3.9% and 3.8%). However, in the published literature, carvacrol, thymol and *p*-cymene were reported to be the major compounds in *T. pubescens.* This significant difference in the composition of the essential oils was a clear evidence of chemical polymorphism with in the *T. pubescens* taxon, suggesting that these two populations of *T. pubescens* were in deed a new chemotype of this species, and the name *Thymus pubescens* Boiss. & Kotschy ex Celak chemotype Citronellol for this new chemotype has been proposed. The antibacterial and free-radical-scavenging properties of the essential oils of *T. pubescens* have also been evaluated.

Keywords: *Thymus pubescens*; Lamiaceae; chemotype; essential oil; citronellol; DPPH; antibacterial activity.

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1. Introduction

The genus *Thymus* L. (Lamiaceae) comprises about 350 species of perennial shrubs that grow usually on rocky ground and limestones in the world. There are 14 *Thymus* species available in the Iranian flora, and 4 of these species are endemic. *Thymus pubescens* Boiss. et Kotschy ex Celak (common name: 'Avishan-e-korkaloud') grows wildly and abundantly in the North and North-West of Iran [1-3]. *Thymus pubescens* has been used by the local people as a food additive, and as herbal remedy for gastro-intestinal disorders. There are several reports on the chemical compositions of *T. pubescens* from different parts of Iran available to date [4-9]. All those previous reports indicated thymol, carvacrol, *p*-cymen and γ–terpinene as the major components in the oils. There are evidences that within the genus *Thymus*, chemical polymorphism of the essential oils is a widespread phenomenon, and more than 20 essential oil chemotypes exist in different species of the genus *Thymus* [10-14]. We now report on the essential oils composition of two populations of a citronellol-producing new chemotype of *T. pubescens* that grows exclusively in the area of Misho-Dagh, Payam village, Iran, and on the evaluation of the free-radical-scavenging and the antibacterial activities of the essential oils.

2. Materials and Methods

2.1. Plant Material

The aerial parts of *Thymus pubescens* Boiss. & Kotschy ex Celak were collected at the flowering stage from two wild populations from Misho-Dagh Mountain, Payam village, East Azerbaijan-Iran, in May 2004 (sample 1: 37°,30',0.2", at an altitude of 1750 m and sample 2: 46°,17',49.2" at an altitude of 2100 m). Voucher specimens (Tbz-FPh-152 & 153) were deposited at the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences.

2.2 Distillation of plant materials

The aerial parts of the flowering plants of two populations of *T. pubescens* were subjected to hydro-distillation for 3h using a Clevenger-type apparatus. The resulting oils were subsequently dried over anhydrous sodium sulphate and dissolved in *n*-hexane (1 mL) for analysis.

2.3 GC-MS and GC-FID analyses

The essential oils were analyzed using a Shimadzu GCMS-QP5050A gas chromatograph-mass spectrometer (GC-MS) fitted with a fused methyl silicon DB-5 column (60 m x 0.25 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas at a flow rate of 0.9 mL/min. The oven temperature was kept at 50°C for 2 min, and programmed to rise to 230°C at a rate of 2°C/min and then kept constant for 8 min. The injector temperature was 250°C and split ratio was adjusted at 1:51. The mass spectral (MS) data were obtained at the following conditions: ionization potential 70 eV; ion source temperature 200°C; quadrupole temperature 100°C; solvent delay 3 min; EM voltage 3000 volts. Identification of compounds was based on direct comparison of the Kovats indices and MS data with those for standard compounds, and computer matching with the NIST NBS54K Library, as well as by comparison [15, 16].

For quantitation (area %), the GC analyses were also performed on an Agilent 6890 series apparatus fitted with a FID detector. The FID detector temperature was 300 °C. To obtain the same elution order as with GC-MS, simultaneous auto-injection was performed on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

2.4 The DPPH assay

The free-radical-scavenging property of the essential oils was assessed using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay as described in the literature [17, 18]. DPPH was purchased from Fluka Chemie AG, Bucks. The essential oil was dissolved in $CHCl_3$ to obtain the stock concentration of 1 mg/mLl. Dilutions were made to obtain concentrations of 5×10^{-1}, 2.5×10^{-1}, 1.25×10^{-1}, 6.25×10^{-2}, 3.13×10^{-2} and 1.56×10^{-2} mg/mL. Diluted solutions (5 mL each) were mixed with DPPH (5 mL; 0.08 mg/mL) and allowed to stand for 30 min for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive control, quercetin.

2.5 Antibacterial assay

Bacterial cultures of four strains of Gram-negative bacteria, *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella typhimurium* (ATCC 4420) and *Serratia marcescens* (ATCC 33077), and six strains of Gram-positive bacteria, *Bacillus anthracis* (ATCC 9372), *Micrococcus luteus* (ATCC 10240), *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus saprophyticus* (ATCC 15305) and *Streptococcus pneumonia* (ATCC 12401) were used to evaluate the antibacterial properties of the essential oils. Bacterial cells used in this project were purchased in lyophilized form from The Institute of Pasture, Iran. These were cultured in LB agar medium after dissolving in sterile distilled water. The plates were incubated 24h at 37°C. Single colony from the plate was transferred into 4 mL fluid LB medium and incubated over night at 37°C and 200 rpm in shaking incubator. The cells were harvested by centrifugation at 3000 rpm for 15 min and 4˚C. Subsequently, they were washed twice and re-suspended in Ringer solution to provide bacterial concentrations between 10^6–10^7 cfu/mL [19].

The antimicrobial activity of the essential oils was monitored by 2 different methods. Disc-diffusion method was performed by standard NCCLS methodology using Mueller-Hinton (MH) plates (with some modifications), inoculated with a 0.5 McFarland standard of the selected bacterial strain [20]. Filter paper discs (Whatman No. 3; 6 mm diameter) were sterilized by autoclaving. A 10 µL of the 1, 1/5 and 1/10 dilutions of the essential oils in *n*-hexane was applied to each paper disc and allowed to air-dry. The dry discs were placed on the seeded MH agar plates; the essential oil dilutions were tested in triplicate, with 4 discs of each dilution on one plate. Amikacin (30 µg, Padtan Teb Inc.) and *n*-hexane were used, respectively, as a positive and a negative controls. The plates were incubated at 37°C overnight, after which the zones of inhibition around each disc were measured. The ratio between the diameter of the inhibition zones (mm) produced by each dilutions and the inhibition zone around the amikacin disc (mm) was used to express antibacterial activity. The antibacterial assays were repeated in Soy been Casein Digest Broth containing Tween 20 to reduce or eliminate influence of improper diffusion of the oil [20-22].

The macro tube dilution method recommended by the NCCLS was used to determine the MIC value of essential oils [23]. Suspensions with a turbidity equivalent to that of a 0.5 McFarland standard were prepared by suspending growth from LB agar plates in 2 mL sterile saline, and further diluted 1:10 to obtain a final inoculum of 5 x 10^5 cfu/mL. A 100 µL of the inoculum was applied to the test tube contained 5 mL Mueller-Hinton broth as well as 5 mL of the essential oils dilutions (1/2, 1/4, 1/8, 1/16, 1/32 in *n*-hexane). The MIC of each sample was determined according to the first dilution that did not show any microbial growth. All assays were performed in triplicate.

3. Results and Discussion

The ground aerial parts of the flowering plants of two populations of *T. pubescens* were subjected to hydro-distillation for 3h using a Clevenger-type apparatus to afford odorous pale yellow oils with a yield of 1.51 and 1.73 % (v/w). The chemical compositions of these essential oils, as determined by the GC-MS analyses and identified based on direct comparison of the retention times and MS data with those for standard compounds, and computer matching with the NIST NBS54K Library, as well as by comparison [15, 16], are listed in Table 1.

Table 1. GC-MS and GC-FID data of the components of the essential oils of two populations of *Thymus pubescens* Boiss.et kotschy ex Celak chemotype Citronellol, collected from Mishov-Dagh Mountain in Iran

Compounds	K.I.	Real % Area		MS fragment ions (intensity)	Mol. mass	Mol. formula
		Sample 1	Sample 2			
Myrcene	978	0.3	0.2	41(100), 53(12.09), 69(40.89), 77(10.22), 93(54.53), 107(1.40), 121(3.09), 136(1.14)	136	$C_{10}H_{16}$
ortho-Cymene	1012	0.4	0.5	41(9.07), 65(7.19), 77(5.16), 91(20.86), 105(3.55), 119(100), 134(19.27)	134	$C_{10}H_{14}$
1,8-Cineole	1016	1.6	1.5	43(47.27), 55(9.52), 71(10.56), 81(13.29), 93(8.02), 108(12.03), 121(1.33), 139(4.85), 154(3.43)	154	$C_10H_{18}O$
Linalool	1089	7.8	7.9	41(71.19), 43(83.12), 55(63.79, 71(100), 80(21.63), 93(67.16), 107(6.76), 121(17.98), 136(4.71), 154(0.08)	154	$C_{10}H_{18}O$
Citronellal	1136	1.2	1.0	41(100), 55(32.34), 69(51.97), 83(7.66), 95(29.57), 111(10.06), 121(14.42), 136(3.46), 154(1.85)	154	$C_{10}H_{18}O$
Isoborneol	1146	0.2	0.2	43(33.52), 59(17.58), 67(11.18), 81(16.92), 95(100), 110(23.30), 121(14.33), 136(7.81), 150(0.02)	154	$C_{10}H_{18}O$
Citronellol	1225	4	42.6	41(100), 55(39.78), 69(65.91), 81(23.97), 95(21.34), 109(9.48), 123(10.50), 138(2.89), 156(1.36)	156	$C_{10}H_{20}O$
Nerol	1229	1.0	1.0	41(100), 53(9.61), 69(55.60), 93(14.13), 95(8.53), 109(4.76), 134(2.69), 154(.033)	154	$C_{10}H_{18}O$
Geraniol	1236	13.0	13.1	41(100), 53(11.63), 69(64.92), 84(5.15), 93(16.87), 111(4.68), 123(7.07), 136(1.66), 154(0.14)	154	$C_{10}H_{18}O$
Geranial	1252	0.2	0.2	41(100), 55(5.11), 69(57.49), 81(2.83), 95(5.82), 109(12.26), 123(8.73), 139(3.25), 152(1.95)	152	$C_{10}H_{16}O$
Citronellyl acetate	1334	3.9	3.8	43(100), 55(30.66), 69(35.50), 81(35.05), 95(36.04), 109(14.25), 123(23.12), 138(16.38), 149(0.009),168(0.05), 198(0.007)	198	$C_{12}H_{22}O_2$

Table 1 Continued..

Neryl acetate	1350	0.6	0.4	41(100), 53(17.43), 69(51.69), 93(59.01), 95(6.33), 121(17.09), 136(9.79), 137(0.68), 170(0.93), 184(0.80), 197(0.06)	196	$C_{12}H_{20}O_2$
Geranyl acetate	1369	14.0	14.0	41(100), 53(15.02), 69(77.06), 80(10.76), 93(40.84), 107(5.84), 121(13.89), 136(7.82), 154(1.35), 178(0.02), 196(0.03)	196	$C_{12}H_{20}O_2$
β-Bourbonene	1373	0.4	0.4	41(42.32), 53(15.92), 80(79.80), 81(100), 105(18.47), 119(12.20), 123(76.81), 145(0.91), 161(18.11), 189(0.82), 204(1.54)	204	$C_{15}H_{24}$
β-Caryophyllene	1403	1.4	1.4	41(100), 55(29.45), 69(38.25), 93(45.65), 105(36.05), 119(19.67), 133(30.72), 148(7.55), 161(14.79), 176(1.65), 189(5.01), 204(3.75)	204	$C_{15}H_{24}$
β-Curcumene	1502	0.4	0.3	41(37.06), 55(29.45), 69(38.25), 93(45.65), 105(35.05), 119(100), 133(30.72), 148(7.55), 161(4.79), 176(0.14), 189(0.48), 204(1.52),	204	$C_{15}H_{24}$
(Z)-Nerolidol	1521	5.9	5.5	41(100), 55(32.52), 69(62.66), 80(1.34), 93(49.27), 107(28.62), 119(12.00), 121(16.22), 136(6.71), 149(1.69), 161(14.77), 177(1.04), 189(1.56), 204(1.16), 222(.04)	222	$C_{15}H_{26}O$
Geranyl butanoate	1545	0.6	0.5	41(100), 57(41.53), 69(66.86), 79(16.68), 93(72.46), 107(8.13), 121(16.70), 136(10.86), 146(0.70), 167(0.75), 177(0.63), 187(0.85), 224(0.05)	224	$C_{14}H_{24}O_2$
Monoterpenes		0.7	0.6			
Oxygenated monoterpenes		67.0	67.6			
Monoterpenic esters		19.4	18.1			
Sesquiterpenes		2.2	2.1			
Oxygenated sesquiterpenes		5.9	6.0			
Total identified		95.2	93.8			

A total of 18 compounds, representing about 95% of the total oils, were identified in both samples. The main component of the essential oils was citronellol (42% and 42.6%). The other major compounds were geranyl acetate (14.0 and 14.0%), geraniol (13.0 and 13.1%), citronellyl acetate (3.9 and 3.8%), linalool (7.8% and 7.9%), *cis*-nerodiol (5.9% and 5.5%) and citronellyl acetate (3.9% and 3.8%) (Table 1). Oxygenated monoterpenes and monoterpene esters (>85%) were the major groups of terpenoids in these oils, but lesser amounts of sesquiterpenes (~8%) were also present.

The essential oil composition of several samples of *T. pubescens* collected from different parts of Iran was previously reported [4-9] (Table 2). It is interesting to note that there is a considerable difference between the chemical profiles of the essential oils of *T. pubescens* growing in Mishov-dagh and other locations in Iran. While carvacrol and thymol were found to be the main compounds of the oils of *T. pubescens* collected from most of the locations in Iran [4-9], the oils obtained from plants growing in Mishov-Dagh Mountain contained citronellol, geranyl and geraniol as major constituents. On the basis of this striking difference, the populations of *T. pubescens* growing in Mishov-Dagh Mountain could be classified as a new chemotype, Citronellol. Citronellol, geranyl acetate and

geraniol, the major monoterpenes of the Citronellol chemotype, were not detected in any other chemotypes described in the literature to date. On the other hand, neither carvacrol nor thymol, the phenolic compounds that are commonly found in *T. pubescens*, could be detected in the chemotype found in Mishov-Dagh Mountain.

Table 3 lists the major constituents of the essential oils of various species of the genus *Thymus* from different parts of Iran [24-39]. Although carvacrol and thymol were detected as the main compounds of several species of *Thymus*, they were present in small amounts or absent in the some other species, e.g. *T. carmanicus* [24], *T. citriodorus* [25], and *T. fedschenkoi* [26]. Similar profiles were also reported for some other species, e.g. *T. bracteosus* [27], *T. cilicicus* [28], *T. lotocephallus* [29] and *T. mastichina* [30], which do not grow in Iran. 1,8-Cineole, *p*-cymen, borneol and β-caryophyllene were found to be present in all species of *Thymus* and 1,8-cineole is the single common compound among all investigated chemotypes of *T. pubescens*. The taxonomic status of *T. pubescens* is not currently quite clear. Although the morphological characteristics of *T. fallax* [31] and *T. serpyllum* [32] are similar to those of *T. pubescens*, and *T. fallax* var. *pubescens* and *T. serpyllum* var. *kotschyanus* are considered synonymous to *T. pubescens* [3], the chemical profiles of their essential oils are significantly different. However, a high degree of similarities could be found in the chemical compositions of the essential oils of *T. pubescens*, *T. carnosus* [33], *T. kotschyanus* [34, 35] and *T. persicus* [36-39].

Table 2. Major components of the essential oils of *T. pubescens* collected at flowering stage from different regions of Iran [4-9].

Compound	Regions in Iran								
	Mishov dagh	Firouz koh	Damavand	Fashem	Sirchal	Darre-e –lar	Abali	Sadde lar	Amarlou
Citronellol	42.0	-	-	-	-	-	-	-	-
Geraniol	13.05	-	-	-	-	-	-	-	-
Geranyl acetate	13.96	-	-	-	-	-	-	-	-
Carvacrol	-	48.8	64.8	31.6	23.1	54.7	64.73	69.2	14.9
Thymol	-	13.9	11.9	17.1	7.8	1.1	1.6	0.98	37.9
p-cymene	-	12.7	2.9	9.4	6.8	9.7	6.6	8.9	13.1
Borneol	-	3.8	0.7	4.6	3.8	1.7	2.6	5.0	3.1
1,8-Cineole	1.57	2.4	0.7	2.5	2.7	3.2	1.6	1.7	0.5
α-Pinene	-	2.2	0.4	2.0	3.2	2.4	2.0	1.0	-
Thymoquinone	-	2.7		-	-	1.33	-	-	8.7
α-Terpinene	-	0.5	0.9	1.3	2.2	5.8	-	0.6	0.4
Linalool	7.8	1.0	-	0.6	0.8	0.78	-	0.5	4.4
Myrecene	0.29	-	1.0	1.4	3.0	2.5	-	-	0.5

The antibacterial effect of the essential oils of *T. pubescens* against four strains of Gram-negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Serratia marcescens*, and six strains of Gram-positive bacteria, *Bacillus anthracis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus* and *Streptococcus pneumonia* were screened by the filter paper disc diffusion method [19] (Table 4). The results were shown as inhibition zone diameter related to essential oil and standard antibiotic ± SD as well as % of activity in comparison with standard antibiotic. The essential oils showed weak to moderate inhibitory effects on the test microorganisms. The most marked effect was observed against *Staphylococcus epidermidis*. The essential oils were more effective against Gram-negative than Gram-positive strains. n order to determine the Minimum Inhibitory Concentrations (MIC) against sensitive strains, e.g. *Staphylococcus epidermidis, Staphylococcus aureus* and *Staphylococcus saprophyticus,* the macro tube dilution method as well as agar dilution method was conducted [20-22]. The MIC values of the essential oils were found to be 0.25, 0.5 and 0.5 v/v (diluted in *n*-hexane) when measured against *Staph. epidermidis, Staph. aureus* and *Staph. saprophyticus,* respectively.

Table 3. Major constituents of the essential oils of *Thymus* species growing in Iran [25-39].

Species	Main and common constituents
T. carmanicus	Pulegon (25.37%), borneol (6.37), 1,8-cineole (9.47), verbenone (8.o4%), *p*-cymene (6.13), β-pinene (5.99%)
T. carnosus	Thymol (36.1%), *p*-cymene (12.7%), γ-terpinene (19.1%), carvacrol (+), 1,8-cineole (+), borneol(+), β-caryophyllene (+)
T. citriodorus	Geraniol (54.4%), geranial (13.9%), neral (10.1%), nerol (5.2%), 3-octanone (3.3%) and borneol (3.2%)
T. fallax	Thymol (65%), γ-terpinene (10.8%), 1,8-cineole (+), *p*-cymene (+)
T. fedschenkoi	α-Terpineyl acetate (66%), *trans*-ocimene (5%), β-caryophyllene (4.4%), thymol (3%), borneol (+),1,8-cineole (+), *p*-cymen (+)
T. eriocalyx	Thymol(43.1%),linalool(12%),γ-terpinene(6.3%),borneol(4.9%),1,8-cineole(3.3%),β-caryophyllen(+)
T. eriocalyx	Linalool (1.8-60.4%), geraniol (trace-50.5%) and thymol (1.6-58.4%)
T. kotschyanus	Carvacrol (41.4%), thymol (19.5%), γ-terpinen (10.3), *p*-cymene (10.3), borneol (+), 1,8-cineole (+), β-caryophyllene (+)
T. kotschyanus	Pulegone (18.7%), isomenthone (17.8%), thymol (14.9%), 1,8- cineole (9.0%), piperitenone (6.3%) and carvacrol (5.5%).
T. persicus	Carvacrol (27.1%), thymol (11.9%), *p*-cymene (10.2%), γ-terpinene (6.5%), borneol (+), 1,8-cineole (+), β-caryophyllene (+)
T. serpyllum	Sabinene hydrate (22.7%), *p*-cymene (20.7%), thymol (18.7%), germacrene D (5.1%), borneol (3.1%),1,8-cineole (+), β-caryophyllene (+)

(+) = Present in trace amounts; not quantifiable

Both populations of *T. pubescens* lacked in any significant free-radical-scavenging property as determined by the DPPH assay [17, 18], which might be due to the absence of any phenolic compounds, e.g. carvacrol and thymol, in the essential oils.

Table 4. Mean inhibition zone diameter in disc diffusion assay ± SD of *Thymus pubescens* essential oil against four Gram-negative and six Gram-positive strains

Bacterial strains	Zone of inhibition in mm			
	Essential oil dilutions			Amikacin
	1	1/5	1/10	
Gram-negative				
Escherichia coli	-	-	-	23 ±0.8
Pseudomonas aeruginosa	6 ±2 (28%)	-	-	21 ±1
Salmonella typhimurium	8 ±1.7 (36%)	-	-	22 ±1.2
Serratia marcescens	-	-	-	18 ± 0.9
Gram-positive				
Bacillus anthracis	-	-	-	12 ± 0.9
Micrococcus luteus	6 ±2 (28%)	-	-	24 ±1
Staphylococcus aureus	9 ±1.6 (40%)		-	22 ±0.8
Staphylococcus epidermidis	13 ±1.3 (56%)	9 ±1.8 (39%)	-	23 ±1.2
Staphylococcus saprophyticus	6 ±1.2 (30%)	-	-	20 ± 1.1
Streptococcus pneumonia	-	-	-	18 ± 0.6

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