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Diversity and ecotypic variation in the antioxidant and antigenotoxic effects of *Thymus kotschyanus* Boiss & Hohen

Vahid Afshari,[a†] Fatemeh Elahian,[b†] Yasaman Ayari,[a] Alireza Yazdinezhad[a] and Seyed Abbas Mirzaei[c]*

Abstract: *Thymus* species are amongst the most popular medicinal plants because of their biological effects and pharmacological properties, and they are widely used in folk medicine for many disorders. Fourteen ecotypic *Thymus kotschyanus* essential oils were isolated, and the biological functionalities of ecotypic oils were characterized in terms of free radical scavenging and antigenotoxic properties. The most abundant ecotypic oil constituents were thymol and carvacrol (27.2–75.6%). The oils produced scavenging capacity, bleaching inhibitory capacity, and COMET-inhibitory capacity values in a dose-dependent manner ranging from 0.15 to 4.79 mg/ml, 0.04 to 3.1 mg/ml and 0.03 to 5.00 mg/ml, respectively. The benefits of *T. kotschyanus* essential oils from different regions vary, and they represent an inexpensive source of natural substances that have the potential to be used as antioxidative and antigenotoxic agents. Although the impacts of the microenvironment, climate difference and agronomical condition on the medical benefits of the plant were complex and highly context dependent, some correlations among the biological effects, main essential constituents, and the geographical environment were predicted. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: antigenotoxicity; antioxidant; essential oil; geographical variation; *Thymus kotschyanus*

Introduction

The *Lamiaceae* (*Labiatae*), or mint family, is the 7th largest family of flowering plants, with a diverse distribution that contains approximately 236 genera and up to 7200 species. *Thymus* is the most useful genus of this family and the most famous fragrant plant that is mainly cultivated in Mediterranean countries, southern Greece and the northern part of Africa.[1,2]

The thyme genus is complex from the taxonomical and systematic points of view, with a significant polymorphism in morphological features and also in essential oil compositions. The aerial parts of these plants have been widely used in folk medicine for digestive and upper respiratory infections.[3,4] Thyme oils have been extensively proved for their wide spectrum of biological activities, such as their acaricidal, bactericidal, insecticidal, fungicidal, virucidal and antioxidant activities.[3,5–7] Their therapeutic potentials, such as their anti-inflammatory, anti-arthralgia, anti-cough, antidepressant and immunostimulating properties, have also been fully reviewed.[8–11] *Thymus kotschyanus* is one important member of 200 species of the *Thyme* genus to which the same actions in folk medicine are attributed,[12] but many important biological properties such as antigenotoxicity and antioxidant activities have not been surveyed and remained unclear yet.

There is much evidence that the physical and biological behaviours of many organisms, including amphibians, insects, fishes, mammals, plants and reptiles, have been influenced by climate change.[13,14] Plants' responses to the ecosystem changes are complex and highly context-dependent. The climate variations have introduced significant and detectable impacts and alterations in plant immunology, genetics, morphology, phenology, phenotype, physiology, population divergence and evolution over millions of years. Chemical ecology is the scientific study of chemicals that mediate the interactions and relationships between living organisms and their environment. Those species that had the capacity for chemical adaptation in the face of habitat changes survived and others became extinct.[15,16] Variation in the biological activities of the ecotypic genus will reflect the ecological conditions and environmental factors affecting the chemical composition of essential oils and may exhibit variations in the medicinal properties. Here, we highlight and emphasize the potential impacts of these expected climate variations on the chemical composition of the ecotypic essential oils of *T. kotschyanus* and demonstrate biological diversity in their antioxidative and antigenotoxic behaviours. We also describe quantitative methods concerning antioxidation and antigenotoxicity for the first time. We hope the present study will promote research in this field.

* Correspondence to: S. A. Mirzaei, Clinical Biochemistry Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran. E-mail: mirzaei.a@skums.ac.ir, dr_amirzaei@yahoo.com

† These authors contributed equally to this work.

[a] Department of Pharmaceutical Biotechnology, School of Pharmacy, Zanjan University of Medical Sciences, Iran

[b] Cellular and Molecular Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

[c] Clinical Biochemistry Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

Experimental

Chemicals and media

Agarose, carvacrol, thymol, standard alkane solution (C_8-C_{20}), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,6-di-tert-butyl-4-methylphenol (BHT), linoleic acid and β-carotene were purchased from Sigma-Aldrich (Sigma-Aldrich, Deisenhofen, Germany). Dextrose monohydrate and peptone were obtained from Merck (Darmstadt, Germany). Mueller-Hinton media, TopVision LM GQ Agarose and Ficoll-Paque™ PLUS were purchased from Scharlau (Barcelona, Spain), Fermentas (Vilnius, Lithuania), and GE Healthcare (Uppsala, Sweden), respectively. Other chemicals and solvents were of analytical grade (>99% purity) and were purchased from commercial sources in Iran.

Plant material

Fresh aerial parts of *T. kotschyanus* Boiss. & Hohen (*Avishan kuhi* in Persian) were collected during the flowering stage (June 2012) from 14 different ecological locations in Iran according to WHO and Flora Iranica protocols.[17] Briefly, 15 mature plants were randomly selected on a dry day, imaged, harvested and mixed to obtain a randomly selected mixture set. At least three sets have been collected from each identical location and were individually analysed for all experiments in parallel. The species were identified by Professor M. Musavi (Agriculture and Natural Resources Research Center, Zanjan) and the herbarium voucher specimens were deposited at the School of Pharmacy of Zanjan University of Medical Sciences. The essential oils were obtained by 3-h hydrodistillation of 100 g of dried and ground aerial tissues (flowers, leaves and stems) in 500 ml of deionized water using a modified Clevenger-type apparatus comprising a water-cooled oil receiver to minimize formation of overheating artifacts.[18] The essential oils were dried over anhydrous sodium sulphate and stored in air-tight glass containers at 4 °C in the dark for a maximum of 3 months before further analyses.

Yield and refractive index

The essential oil yield was calculated as the volume (millilitres) of oils per weight (gram) of air-dried aerial tissues for three replications. The oil density was measured using a liquid micropycnometer. A benchtop RX-7000a refractometer (Atago, Tokyo, Japan) was used for determination of the refractive indices of the oils according to the manufacturer's instructions. All assays were performed in at least three independent experiments.

UV absorbance and determination of the extinction coefficient

An absorption spectrum of the oils diluted in methanol in the wavelength range of 230–1000 nm was obtained using a microplate reader (Infinite®-M200; Tecan, Grödig, Austria). According to the Beer–Lambert equation, the slope of the line obtained by plotting the mean absorbance at any individual wavelengths versus serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 mg/ml) of the test material represents the extinction coefficient (molar absorptivity). All experiments were performed three independent times.[19]

Gas chromatography (GC) analyses

Oils obtained from aerial parts of *T. kotschyanus* were analysed using an Agilent HP-6890 gas chromatograph (Agilent Technologies, USA) with an HP-5 (5% phenyl- 95% methylpolysiloxane) capillary column (30 m × 0.32 mm, film thickness 0.25 μm) equipped with a flame ionization detector. N_2 (1 ml/min) was the carrier gas, and 1.0 μl of each sample was injected with a split ratio of 1:10. The oven temperature was initially kept at 60 °C for 3 min and then raised to 250 °C at the rate of 3 °C/min. Injector and detector temperatures were set at 250 and 290 °C, respectively. All quantifications were carried out by a data-handling program supplied by the manufacturer of the instrument. The composition was reported as a relative percentage of the total peak area of each component in relation to the total area of the chromatogram in the GC/FID. Quantitative data obtained from thymol and carvacrol were used for standardization and quantification. All experiments were carried out three independent times, and average values were reported.[20]

Gas chromatography-mass spectrometry (GC-MS)

The essential oils were analysed on an Agilent gas chromatograph (GC-7890A; USA) equipped with a mass detector (EIMS-5975C; electron ionization energy of 70 eV) and an HP-5 MS (5% Phenyl- 95% methylpolysiloxane) capillary column (30 m×0.25 mm; film thickness 0.25 μm). The column oven was programmed at an initial 40 °C for 5 min, followed by 40 to 230 °C at a linear increasing rate of 10 °C/min, and finally from 230 to 280 °C at a ramping of 30 °C/min. The injector and detector temperature were set at 240 and 250 °C, respectively. Helium was the carrier gas (1 ml/min flow rate), and a sample of 1.0 μl was injected using a split ratio mode (1:10). Retention indices (RI) of the compounds were calculated using a homologous series of n-alkanes (C_8-C_{20}) injected in the conditions equal to the samples. Identification of the essential oil constituents was based on computer matching with the NIST library and Wiley7n.L library, as well as by comparison of the retention indices and fragmentation pattern of the mass spectra with those published in the literature. The spectra of authentic compounds (thymol and carvacrol) were further confirmation of their identification. All experiments were carried out three independent times, and average values were reported.[20–23]

DPPH scavenging assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) was commercialized as a stable free radical. It exhibits a strong purple colour (maximum absorption at 517 nm) when dissolved in methanol. The purple colour will disappear in the presence of antioxidants. A 2-ml sample of a fresh methanolic stock solution of DPPH (80 μg/ml) was added to 2 ml of doubling dilutions of *T. kotschyanus* essential oil with a final concentration range of 0.01 to 1.28 mg/ml in methanol and left in the dark for 30 min. Neutralization of DPPH was measured against the negative control (NC) at 517 nm by a Tecan Infinite M200 spectrophotometer according to the following equation:

$$\%SC = \frac{(A_{NC} - A_{\text{Sample}})}{A_{NC}} \times 100$$

Negative controls consisted of all the reagents except the antioxidant components. BHT (0.5 to 128 μg/ml) and L-ascorbic acid (0.5 to 128 μg/ml) were used as standard antioxidant agents. The SC_{50} (scavenging capacity-50) is the oil concentration causing 50% neutralization effects. The SC_{50} was determined from the equation of the best-fitting linear or non-linear regression curve plotted from the scavenging percent versus oil concentrations. All experiments were recorded three independent times and the mean values ± SE were reported.[24]

β-Carotene bleaching assay

The antioxidant activity of *T. kotschyanus* essential oil was studied according to modified procedures from Demirci *et al.* using bleaching of a β-carotene/linoleic acid emulsion system.[25] Briefly, 1 mg of β-carotene was dissolved in 2 ml of chloroform and mixed with 40 mg of linoleic acid and 200 mg of Tween-20. After the evaporation of chloroform using a rotary evaporator at 40 °C for 30 min, 100 ml of oxygenated deionized water was added with vigorous shaking. Then, 350 μl of oil within the final double dilation range of 0.01 to 1.28 mg/ml in ethanol was transferred into 2500 μl of the β-carotene mixture. All tubes were subjected to thermal autoxidation at 50 °C in light for 120 min, and the absorbance level at different time intervals at 470 nm was monitored using a Tecan spectrophotometer. The antioxidant power was calculated using the following equation:

$$\%BIC = \left[1 - \frac{(A_{\text{Sample}}0\text{min} - A_{\text{Sample}}120\text{min})}{(A_{NC}0\text{min} - A_{NC}120\text{min})}\right] \times 100$$

The negative control (NC) consisted of all reagents except the test samples. Appropriate blanks without β-carotene were also included for the background subtraction. The BIC_{50} (bleaching inhibitory capacity) was calculated as the oil concentration causing 50% bleaching inhibition. BIC_{50} was calculated from the best linear or non-linear regression plot of the percentage antioxidant activity versus oil concentrations. All experiments were conducted three times, and the mean values ± SE were reported. BHT (0.5 to 128 μg/ml) and L-ascorbic acid (0.5 to 128 μg/ml) were used as standard antioxidant agents.

Antigenotoxicity activity (COMET-assay)

The antigenotoxic effects of the essential oils were assessed under an alkaline condition using a COMET-assay. Briefly, fresh blood samples were obtained from healthy volunteers, and the lymphocytes were isolated using Ficoll-Paque™ PLUS according to the manufacturer's protocol. The cell count was adjusted to 2×10^5 cells/ml with phosphate-buffered saline (PBS). Serial dilutions of the oils (final concentrations: 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0 mg/ml) containing up to 2.5% DMSO and 100 μM H_2O_2 solution (the H_2O_2 concentration optimized in the lab to induce more than 75% tail DNA in the lymphocytes in the essential oil–free tubes) were provided and stored for 5 min at room temperature. Then, 50 μl of lymphocyte suspension containing 1×10^4 cells was transferred to each dilution, and the combination was stored for 30 min at 4 °C. Afterwards, the cell mixture was centrifuged at 800 × *g* (Eppendorf, Hamburg, Germany) for 5 min at 4 °C, and the supernatant was discarded. The cells were resuspended in 50 μl of PBS, mixed with 50 μl of preheated 1.5% w/v low melting agarose at 37 °C, spread on dried microscopic slides pre-coated with 1.5% w/v normal melting agarose, and stored on ice for 30 min for solidification. The cells were sandwiched with 100 μl of 0.75% w/v low melting agarose. After the solidification, the slides were immersed in the ice-cold lysing solution (2.25 M NaCl, 90 mM EDTA, 9 mM Tris base, 0.7% w/v NaOH, 10% v/v DMSO, 1% v/v Triton X-100; pH = 10) for at least 4 h. The slides were then washed and electrophoresed at 25 V and 300 mA for 45 min using the alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA; pH>13). The alkaline conditions were then neutralized using a neutralizing buffer (0.4 M Tris; pH = 7.5) and washed three times with ice-cold water. Finally, the slides were stained with 20 μl ethidium bromide (2 μg/ml). Eventually, the cells were imaged at 20× magnification by the green dichroic mirror of an Olympus fluorescent microscope (BX51; Tokyo, Japan) equipped with a 12.8 megapixel Olympus CCD camera (DP72; Philippine). Treated cells with PBS or essential oils alone without hydrogen peroxide confirmed no spontaneously COMET production. L-ascorbic acid (0.5 to 128 μg/ml) or BHT (0.5 to 128 μg/ml) co-treated with 100 μM H_2O_2 were the positive standards, demonstrating standard antigenotoxic activities. At least 150 individual cells were pictured for the statistical analysis. The DNA damage was analysed using Tritek COMET-Score™ software version 1.5.2.6, and the tail DNA% ([Tail DNA÷(Head DNA+Tail DNA)]×100) was recorded. Higher DNA percentages in the tail represent greater DNA damage. The CIC_{50} (COMET-inhibitory capacity-50) is the essential oil concentration that reduced the tail DNA percent to 50% of the cells treated with 100 μM hydrogen peroxide alone. CIC_{50} was calculated from the best-fit equation of a linear or non-linear regression curve of the tail DNA per cent versus the oil concentration. This unique quantification method has not been reported yet. All experiments were performed three independent times, and the results were expressed as the mean values ± SE.[26–28]

Statistical analysis

All experiments were carried out at least three independent times, and average values ± standard errors were reported. These results were analysed using one-way analysis of variance (ANOVA) running Tukey's post-hoc tests. *P*-values less than 0.05 were considered statistically significant.

Results

Morphological and physicochemical properties of the ecotypes

Thymus kotschyanus is a perennial flowering plant that grows to 20–30 cm tall with a compact mounded feature. Short hairs are located on tiny ovate leaves (4–7 mm). The flowers occur in dense terminal heads that are usually bisexual, verticillastrate and coloured white, yellow, or purple with a tubular-campanulate calyx (Supplementary Information Fig.1). The hydrodistillation of the aerial parts produced strong yellow oil with a distinct sharp odour with a yield of 0.31 to 0.82% (v/w). The highest yields were found in warm climates (TK4 and TK12), and the yields decreased significantly ($P < 0.001$) in cold regions (TK7 and TK11). The data also indicated that plants grown in loamy clay soil (TK3, TK4, TK12 and TK13) showed a positive increase in yield compared with those grown in sandy soil (TK7, TK9 and TK11). Most of the mean values obtained from the optical rotation analyser (data not shown) and the refractometer (1.482 to 1.532 nD) were not significantly different ($P > 0.05$) and were grouped together (Table 1). The maximum UV absorbance of the oils was observed in UVB at 280 nm (Supplementary Information Fig. 2). According to the Beer–Lambert equation, the molar extinction coefficient of the oils was calculated to be 2.25 to 19.75 L/(cm × g) at 280 nm, as determined from the slope of the plot of A280 versus serial oil dilutions (Supplementary Information Fig. 3). The results revealed a lack of homogeneity of variances among the oils ($P < 0.001$) that indicated the molar absorptivity was ecotype specific (Table 1).

Chemical composition of essential oils

The chemical compositions of *T. kotschyanus* essential oils differed considerably with regard to the ecotypes, and they are presented in Table 2. A total of 60 compounds representing more than 96% of the oils were identified. Carvacrol was the major constituent of the essential oils (5.3–54.2%), followed by thymol (8.1–28.4%), geraniol (up to 26.0%), alpha-terpineol (up to 24.8%), β-citronellol (up to 12.2%) and α-terpinyl acetate (up to 11.6%). Trace components (<0.1%) such as 2-β-pinene, linalool oxide, isoborneol, β-bourbonene, jasmone, cis-thujopsene, β-gurjunene, cis-nerolidol and α-bisabolol were also detected. Table 2 indicates that oxygenated monoterpenes, including thymol, carvacrol, alpha-terpineol and linalool were the dominant class of constituents (64.6 to 90.6%), sesquiterpene hydrocarbons including β-caryophyllene and β-bisabolene comprised moderate levels of the oil (3.0 to 14.7%), and monoterpene hydrocarbons and oxygenated sesquiterpenes were present in low amounts (0 to 15.8%). The hydrocarbon compounds accounted for ≈13.9% of the oils, whereas oxygenated compounds were ≈82.4%. The quantitative differences across the ecotypic populations are as a result of different ecological and geographical origin factors, different genetic and nutritional statuses, and can be considered to have a chemotaxonomic significance.

Antioxidant activity

The antioxidant properties of all 14 essential oils were evaluated by two different methods. A DPPH assay was employed to measure the free radical scavenging capacities. The electron or hydrogen donor oils that quench and stabilize DPPH to DPPH-H are the best at scavenging. Table 3 indicates that TK5 and TK12 showed a dose-

Table 1. Physical and geographical properties of the ecotypic *Thymus kotschyanus* essential oils

Ecotype	Geographical location	City, Province	Altitude (m)	Annual Temperature (°C)		Annual Humidity %	Density (g/ml) at 25°C	ε_{280nm} [l/(cm × g)]	Refractive index at 25°C	Yield (v/w) %
				Min	Max					
TK1	36 43 91N 49 47 81E	Rodbar, Gilan	1840	-2	37	80.2±11.48	1.027±0.06 ^{a}	2.25±0.67	1.482±0.017 ^{a}	0.61±0.25 ^{c}
TK2	36 34 00N 49 55 00E	Ghazvin, Ghazvin	2000	-11	39	45.0±10.31	0.924±0.02 ^{c}	13.10±1.53	1.501±0.092 ^{a}	0.50±0.11
TK3	36 26 00N 50 07 00E	Ghazvin, Ghazvin	1500	-5	40	51.5±15.73	0.834±0.00 ^{f}	14.70±1.44	1.532±0.095 ^{b}	0.75±0.01
TK4	32 01 42N 53 32 42E	Nodoushan, Yazd	1418	-4	44	28.2±19.34	0.879±0.02 ^{d}	11.94±0.49	1.511±0.010 ^{b}	0.81±0.15 ^{a}
TK5	38 09 57N 44 30 41E	Salmas, West Azarbaijan	2323	-11	36	58.6±16.32	0.845±0.00 ^{e}	12.31±1.61	1.501±0.082 ^{b}	0.35±.07
TK6	37 27 00N 48 06 00E	Khalkhal, Ardabil	1417	-15	29	67.4±17.28	0.887±0.06 ^{d}	8.97±0.72	1.498±0.058 ^{a}	0.63±0.05 ^{c}
TK7	37 20 00N 48 40 00E	Ardabil, Ardabil	2500	-20	33	65.0±18.52	0.836±0.08 ^{e}	10.38±0.64	1.503±0.030 ^{b}	0.31±0.05 ^{d}
TK8	36 16 00N 49 23 00E	Yengejeh, Ghazvin	1400	-4	35	55.0±18.73	1.002±0.00 ^{b}	1.13±0.97	1.499±0.085 ^{a}	0.68±.07 ^{b}
TK9	36 34 00N 49 20 00E	Charbin, Ghazvin	1800	-2	36	53.1±20.35	0.848±0.04 ^{e}	3.36±0.35	1.487±0.037 ^{a}	0.41±0.05
TK10	36 04 56N 46 30 09E	Divandareh, Kordestan	2017	-22	35	58.8±19.94	0.830±0.08 f	11.41±0.29	1.503±0.013 ^{b}	0.60±0.04 ^{c}
TK11	35 57 11N 46 48 53E	Saqqez, Kordestan	1569	-20	30	47.9±17.64	0.827±0.02 ^{f}	2.29±0.32	1.482±0.022 ^{a}	0.31±0.02 ^{d}
TK12	34 27 03N 50 26 15E	Qom, Qom	1515	-5	44	31.5±21.17	0.820±0.00 f	16.23±0.84	1.514±0.039 ^{b}	0.82±0.07 ^{a}
TK13	34 12 38N 50 51 05E	Jafarieh, Qom	2560	1	37	37.5±19.83	0.770±0.00 ^{g}	19.75±1.60	1.512±0.009 ^{b}	0.67±0.05 ^{b}
TK14	34 08 57N 50 55 24E	Dastjerd, Qom	3070	-5	35	31.1±15.94	0.780±0.08 ^{g}	4.99±0.30	1.487±0.032 ^{a}	0.57±0.08

Values in the same column with common letters reveal the homogeneity of variances among the oils ($P>0.05$) using ANOVA analysis.

Table 2. The essential oil composition (%) of *T. kotschyanus* collected from various geographical locations

Compounds	RI	TK1 36 43 91N 49 47 81E	TK 2 36 34 00N 49 55 00E	TK 3 36 26 00N 50 07 00E	TK 4 32 01 42N 53 32 42E	TK 5 38 09 57N 44 30 41E	TK 6 37 27 00N 48 06 00E	TK 7 37 20 00N 48 40 00E
Alpha-pinene	938	0.3	1.3	0.4	0.1	0.1	0.2	
Camphene	953			0.3				
2-Beta-pinene	978	0.1			0.1			0.2
Beta-myrcene	990	0.1	0.1	0.4		0.1	0.1	0.1
p-Cymene	1025		1.7	7.6	1.2	7.7	0.2	0.4
Z-β-Ocimene	1037				1.9			0.4
Limonene	1028	2.9						
1,8-Cineole	1030		0.7	0.6			0.1	0.1
E-β-Ocimene	1051	0.1						0.1
Gamma-terpinene	1059		0.3	5.4	1.2	3.2	0.5	0.3
Linalool oxide	1074		0.1				0.0	
Alpha-terpinolene	1087	0.3		0.4	0.1	1.9	0.1	
Linalool	1098	0.9	0.7	1.4	0.3	3.0	0.5	1.4
Cis-rosoxide	1109							
Cis-beta-terpineol	1144		0.9	0.8	0.4	0.1	1.0	0.2
Camphor	1148	0.2		0.3			0.3	
Isopulegol	1150							
Citronellal	1154	0.1						
Isoborneol	1161			0.1				
Borneol	1169	0.1	2.9	4.9	2.1		0.8	1.6
4-Terpineol	1175			2.0	0.5		0.8	0.3
Alpha-terpineol	1189	25.6	3.4	0.2	0.7		0.9	0.9
Cis-dihydrocarvone	1195			0.3				
Citronellol	1227	12.6						
Neral (Citral)	1238	0.8					1.5	
Geraniol	1253	7.8	0.9			9.1	26.1	14.0
Geranial	1265	0.9						
Thymol	1303	22.6	10.1	28.0	22.0	12.1	18.7	29.5
Carvacrol	1308	5.5	51.0	35.8	55.9	42.7	32.0	42.1
Alpha- terpinyl acetate	1350	12.0	2.5		0.1		0.2	0.3
Thymol acetate	1354			0.1				
Eugenol	1358		0.6	0.1		0.1	0.1	0.1
Geranyl acetate	1381	0.2	0.2	0.5	0.1	1.0	2.7	0.3
Beta-cubebene	1386	0.1					0.4	
Beta-bourbonene	1389	0.1	0.1	0.0	0.1	0.2	0.1	0.1
Jasmone (cis)	1393		0.3	0.1		0.2		0.1
Caryophyllene	1420	1.9	4.3	1.5	3.9	8.1	2.8	1.1
Cis-thujopsene	1432					0.1		
Beta.-gurjunene	1435		0.1		0.3		0.1	0.1
Alpha-himachalene	1451	0.3	0.1					0.2
Alpha-humulene	1456	0.1	2.1	0.6	1.4	0.3	0.8	0.1
Geranyl propanaote	1479					0.4	0.3	
Gama-muurolene	1481	0.1	0.6	0.1	0.4	0.1		0.1
Beta(cis)-guaiene	1494		1.3		1.2			
Alpha-muurolene	1501					0.2		
Beta-bisabolene	1507	0.5	4.2	0.6	1.3		0.8	0.8
Cis-alpha-bisabolene	1508			0.4				
Gamma-cadinene	1515	0.1	0.5	0.5	0.2	0.8	0.5	0.2
Delta-cadinene	1524		1.3			0.8		0.5
Trans-calamenene	1530	0.3		0.3			0.2	
Cis-nerolidol	1533	0.3				0.1		
Geranyl botanoate	1565					0.3		
Caryophyllenyl alcohol	1573					0.2		
Spathulenol	1579	0.1	1.1	0.4	0.2	0.2	0.4	0.3
Caryophyllene oxide	1584	0.4	2.4	0.9	0.6	2.2	2.0	0.6
Globulol	1586	0.1	0.3			0.2		
Cubenol	1648		0.4	0.3	0.1	0.1	0.3	0.1
Alpha-cadinol	1655	0.1	0.2	1.3		0.2	0.1	0.1
Alpha-bisabolol	1687				0.2	0.3	2.5	0.4
(Z,E)-Farnesol	1702					0.2		0.1
Others		2.6	3.5	3.6	4.0	4.1	2.0	2.8

Values are the mean of two different experiments. SD values were ignored to reduce the complexity of the table.

Table 2. (Continued)

Compounds	TK 8 36 16 00N 49 23 00E	TK 9 36 34 00N 49 20 00E	TK 10 36 04 56N 46 30 09E	TK 11 35 57 11N 46 48 53E	TK 12 34 27 03N 50 26 15E	TK 13 34 12 38N 50 51 05E	TK 14 34 08 57N 50 55 24E
Alpha-pinene		0.3	0.1	1.4	0.2	1.1	
Camphene		0.3	0.1	0.2			
2-Beta-pinene		0.1					
Beta-myrcene		0.4	0.2	1.8		0.4	
p-Cymene					7.8		
Z-β-Ocimene						0.2	
Limonene		0.1		4.9			
1,8-Cineole	0.6	0.3	0.2		2.2	0.2	1.1
E-β-Ocimene		0.5		0.5			
Gamma-terpinene	0.2	0.5	0.9		0.2	4.4	
Linalool oxide							0.1
Alpha-terpinolene			0.1	1.1			
Linalool		1.1	0.3	4.3		3.6	3.4
Cis-rosoxide		0.1					
Cis-beta-terpineol	1.9	0.2	0.2		0.3	1.5	0.2
Camphor		0.2	0.1				1.0
Isopulegol		0.2					
Citronellal			0.1				0.8
Isoborneol				0.2			
Borneol	1.7	4.4	1.5	1.3	1.1	3.6	4.6
4-Terpineol	1.3	0.4	0.4	0.4		1.5	4.9
Alpha-terpineol	7.6	1.1	0.2	8.0			0.1
Cis-dihydrocarvone							
Citronellol							
Neral (Citral)		2.9	0.9				2.5
Geraniol	4.8	18.1	20.6	6.5			8.0
Geranial	0.1						
Thymol	21.3	22.1	10.7	14.2	16.4	9.0	15.7
Carvacrol	46.8	22.2	49.7	32.4	54.7	55.5	34.7
Alpha- terpinyl acetate	0.1						
Thymol acetate						0.3	
Eugenol	0.1		0.1			0.2	0.1
Geranyl acetate		0.9	0.8	0.3	0.6	0.3	1.0
Beta-cubebene		1.7		1.0			
Beta-bourbonene		1.2	0.2	0.3		0.1	
Jasmone (cis)		0.1	0.1			0.1	
Caryophyllene	2.0	1.7	2.2	5.1	5.3	5.2	3.8
Cis-thujopsene							
Beta.-gurjunene	0.1		0.7	0.3	0.1		0.2
Alpha-himachalene		0.1			2.8		2.6
Alpha-humulene	0.5	1.2	0.1	2.1		0.8	2.6
Geranyl propanaote						0.5	
Gama-muurolene	0.5	0.5	0.5	0.3		0.3	0.7
Beta(cis)-guaiene						0.4	0.4
Alpha-muurolene					0.2		0.3
Beta-bisabolene	0.9	5.4	0.6	2.4		1.1	1.2
Cis-alpha-bisabolene			0.4	0.3			
Gamma-cadinene		0.8	0.4			0.9	
Delta-cadinene	1.1	0.4	0.7	1.1	1.7	0.8	
Trans-calamenene							
Cis-nerolidol		0.3					
Geranyl botanoate		1.2					
Caryophyllenyl alcohol							
Spathulenol	0.6	0.3	0.8	4.0	1.2	0.2	1.0
Caryophyllene oxide	4.9	3.1	1.3	0.6		2.0	1.5
Globulol							
Cubenol	0.1	0.4	0.1	0.2	0.6	0.2	0.4
Alpha-cadinol		0.2	0.1		0.3	0.1	1.4
Alpha-bisabolol		2.1	1.0	0.8	2.2	1.2	1.6
(Z,E)-Farnesol							
Others	3.0	3.2	3.9	4.2	2.2	4.4	4.1

Table 3. Antioxidant and antigenotoxic activity values for *T. kotschyanus* essential oils from different ecotypes

Sample	BHT	VIT C	TK1	TK2	TK3	TK4	TK5	TK6
DPPH radical scavenging activity [SC_{50}, mg/ml]	0.02±0.000[a]	0.03±0.000[a]	0.25±0.023[b]	0.43±0.049[c]	0.22±0.020[b]	0.22±0.020[b]	0.15±0.000[a]	0.45±0.006[c]
Bleaching inhibitory capacity [BIC_{50}, mg/ml]	0.03±0.000[a]	0.01±0.000[a]	>1.28±0.000[f]	0.09±0.006[b]	0.05±0.000[a]	0.12±0.001[b]	0.09±0.005[a]	0.28±0.046[c]
COMET-inhibitory concentration [CIC_{50} (mg/ml)]	0.018±0.000[a]	0.008±0.000[a]	0.35±0.017[b]	3.60±0.133[d]	0.05±0.000[a]	0.35±0.003[b]	0.02±0.000[a]	0.16±0.023[a]
Sample	TK7	TK8	TK9	TK10	TK11	TK12	TK13	TK14
DPPH radical scavenging activity [SC_{50}, mg/ml]	0.25±0.028[b]	>1.28±0.000[f]	>1.28±0.000[f]	0.41±0.052[c]	0.49±0.035[c]	0.15±0.013[a]	0.35±0.017[b]	0.75±0.040[c]
Bleaching inhibitory capacity [BIC_{50}, mg/ml]	0.11±0.004[b]	0.36±0.046[d]	0.45±0.011[d]	0.07±0.006[a]	0.10±0.000[b]	0.24±0.012[c]	0.04±0.000[a]	0.09±0.017[a]
COMET-inhibitory concentration [CIC_{50} (mg/ml)]	>5.00±0.000[e]	4.16±0.005[e]	2.13±0.225[c]	0.03±0.005[a]	0.08±0.025[a]	0.75±0.003[b]	0.05±0.005[a]	0.21±0.052[a]

[a]The SC_{50} representing scavenging concentration-50 and BIC_{50} representing bleaching inhibitory concentration-50 are the oil concentrations scavenging 50% of DPPH or peroxyl free radicals, respectively.
[b]Values in the same column with common letters reveal the homogeneity of variances among the oils ($P > 0.05$) using an ANOVA analysis.
[c]The extrapolated SC_{50} values for TK8 and TK9 are 4.79 and 2.38 mg/ml, respectively. The extrapolated BIC_{50} value for TK1 is 3.1 mg/ml.
[d]The CIC_{50} is the essential oil concentration that protects 50% of the DNA from damage.
[e]The extrapolated CIC_{50} value for TK7 is 10.23 mg/ml.

dependent scavenging potency similar to BHT and ascorbic acid ($P > 0.05$), and TK8 and TK9 demonstrated the lowest scavenging activities ($P < 0.001$). In contrast, β-carotene bleaching assays determined the lipid peroxidation inhibition capacities in the phase of initiation and the phase of propagation. The oils significantly inhibited the bleaching of β-carotene in a concentration-dependent manner in comparison with the negative control. TK3, TK10, TK13 and TK5 were the most potent antioxidants, with activities comparable to those of BHT and ascorbic acid ($P > 0.05$). TK1, TK9 ($P < 0.001$) and TK8 ($P < 0.05$) exhibited the lowest activities (Supplementary Information Fig. 4).

Antigenotoxicity activity

Table 3 and Figure 1 indicate that lymphocytes pretreated with essential oils show a significant decrease in the COMET-tail caused by a genotoxic standard (hydrogen peroxide). A possible mechanism of essential oil antigenotoxicity involves either activation of DNA repair mechanisms or the quenching of hydroxyl free radicals and the prevention of phosphodiester bond breakage. The DNA protection was dose-dependent, and ascorbic acid, BHT, TK5, TK11, TK13, TK3 and TK10 were grouped together ($P < 0.05$) in the potent class (Supplementary Information Fig. 5). TK7 and TK8 exhibited the lowest biological activities ($P < 0.001$).

Discussion and conclusion

Probably the most important source of reactive oxygen species (ROS) is the immune system, this capacity first playing an important bactericidal and fungicidal role.[29] An internal antioxidant defense network in humans is interlocking and complex. Human cells and tissues have been adapted to synthesize potent endogenous antioxidants to quench a great majority of free radical production cascades. Unfortunately, the persistence of these reactions and failures in the repair systems would ultimately trigger variety oxidative stresses such as cumulative DNA damage, cancerous malignancies, inflammation, autoimmune diseases, brain dysfunctions and also heart failures. There are many doubts that internal redox-active agents are always adequate *in vivo*.[29]

Essential oils in nature play a variety of important roles, attracting individual insects and bees, promoting the dispersion of pollen and seeds, or acting as antibacterial, antiviral, antifungal, insecticidal or herbicidal agents. The antioxidant and antigenotoxic activities of the volatile oils of thymus species are biological properties of significant interest that may play important roles in normal cellular functions.[30,31] Essential oils are highly complex mixtures of volatile compounds, and the biological activities are expected to be related to the constituent composition and structural configuration of the components.[4,8,16,32]

The production of secondary metabolites is believed to be stimulated by stressful environments. In particular, dry weather and water shortages reduce the leaf area and consequently result in a greater oil accumulation, which is in complete agreement with our data. For example, TK4, TK3 and TK12, which are collected from hot and dry environments, produced the maximum amount of oil, whereas TK11 and TK7 produced the minimum amount of oil. TK13 oil, with the highest extinction coefficient at 280 nm, could serve as a potent UVB sunscreen. It seems that growth at higher altitudes in mountainous regions led to this phenomenon.

Our data also demonstrated that TK3, TK4, TK5 and TK12 were the best free radical scavengers using the DPPH method and that TK3, TK5, TK10, TK13 and TK14 were the strongest peroxidation

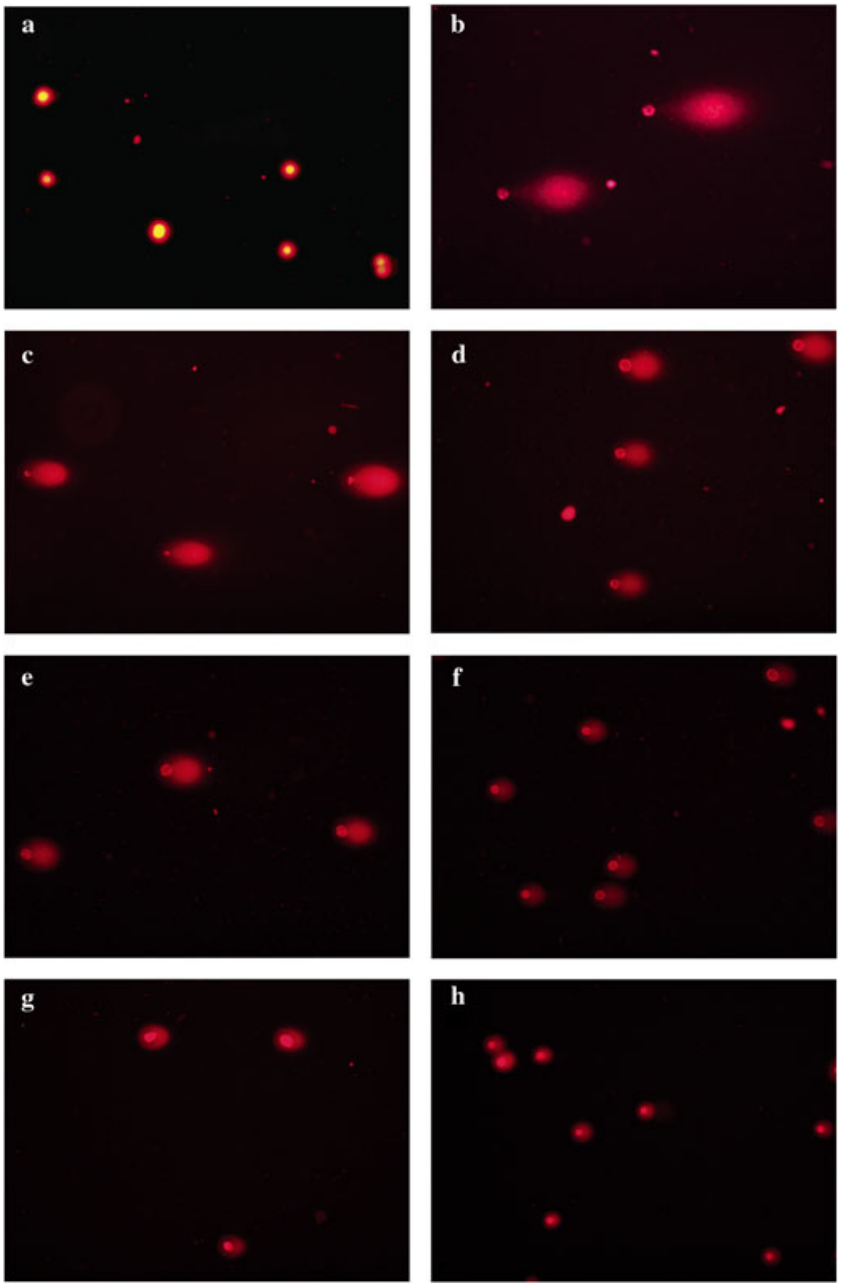

Figure 1. Representative COMET-images from (a) untreated lymphocytes (negative COMET), (b) H_2O_2-treated lymphocytes (positive COMET) and (c–H) simultaneous treatment with TK12 oil at 0.05, 0.1, 0.5, 1, 2.5 or 5.0 mg/ml and 100 μM H_2O_2. DNA was stained with ethidium bromide and imaged with the green filter of the Olympus fluorescent microscope at 20× magnification.

inhibitors that could be interpreted by the major constituents. These phenomena are directly related to phenolic compounds and most probably to p-cymene and linalool, which are strong radical scavengers that are capable of donating hydrogen atoms to convert DPPH into the stable DPPH-H or of transferring electrons that may possess peroxyl radical scavenging properties for the lipid peroxidation test.[25,33] Furthermore, the essential oils showed relatively more potency on β-carotene antioxidant activity than the DPPH method ($P > 0.001$). It seems the phenolic structures are lipophilic and have a relatively low capacity to dissolve in an aqueous solution of DPPH. In contrast, the β-carotene technique employs an emulsified system consisting of two polar and apolar phases. Polar antioxidant residues remain in the aqueous phase, and apolar antioxidants (mainly carvacrol, thymol, and eugenol) concentrate in the lipid cores and exhibit cumulative lipid peroxidation inhibition properties. In addition to the phenolic constituents, a literature review showed that the distribution of polyunsaturated structures (such as bisabolol, bisabolene, borneol, cadinene, citronellol, cubenol, farnesol, geranial, geraniol, guaiene, humulene, linalool, muurolene, myrcene, neral, ocimene and terpinene) in the polar or apolar phases behave in a manner similar to ascorbic acid (in the polar phase) or β-carotene (in the apolar phase) and may be responsible for the high antioxidant effects of TK5 and TK10 in the DPPH method or TK13 and TK10 in the β-

carotene method.[34–36] In contrast, it seems that high levels of caryophyllene oxide (an oxidant constituent) in TK8 and TK9 caused antagonistic activities in both antioxidation methods.

Hydroxyl-free radicals are produced from hydrogen peroxide at high levels in the aqueous phase, and water soluble antioxidants or chemopreventive agents would protect cells from oxidative damage and quench or minimize the DNA damage.[37] TK3, TK5, TK10 and TK4 oils showed high dose-dependent antigenotoxic effects that were more common to the DPPH method. TK7 and TK8 possessed the lowest antigenotoxic activity because of the low antioxidant level, which was expected. Unfortunately, the microenvironment and ecological effects on antioxidants and antigenotoxicity are too complex and are not reviewed in the literature.

For some oils, no significant correlation between the biological activities and the major components has been found. This suggested that the constituents present in significant proportions were not necessarily responsible for a significant share of the total activity and that minor components exhibited potent synergistic or antagonistic effects that led to complex activity.[38,39] More comprehensive investigations of the biological correlations among taxa are needed to determine the trajectory of the monophyletic groups' evolution. In the near future, this knowledge could help to predict the adaptation response and the extinction risk of plant species as the result of the contemporary unprecedented environmental changes.[40–42]

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Conflict of interest

We certify that there is no conflict of interest with any financial organization.

Authors' contributions

S.A. Mirzaei coordinated the study, designed the antioxidant research and revised the final manuscript. F. Elahian designed the genotoxicity and statistical analysis of the data, wrote the corresponding portions of the manuscript and participated in an intellectual discussion of the data and manuscript writing. A. Yazdinezhad performed the GC and GC-MS spectrometry and wrote the corresponding portions of the manuscript. V. Afshari and Y. Ayari were students who collected the samples and performed most of the experiments for their PharmD theses.

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