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RESEARCH ARTICLE

Fatty acids and other lipid composition of five *Trifolium* species with antioxidant activity

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Abstract

The contents of fatty acids and other lipids of *Trifolium balansae* Boiss, *Trifolium stellatum* Lin., *Trifolium nigrescens* Viv. subsp. *petrisavi* (Clem) Holmboe, *Trifolium constantinopolitanum* Ser., and *Trifolium resupinatum* L. var. *resupinatum* L. (Leguminosae) were determined by gas chromatography-mass spectrometry (GC-MS). The whole plant hexane extracts of five *Trifolium* species contained eight fatty acids consisting of linolenic (31.1%) and palmitic (18.9%) acids as the most abundant unsaturated and saturated fatty acids, respectively. The total unsaturation for the oils of five *Trifolium* species was 30.6–42.2%. In addition to fatty acids, some alkanes and phytol were also obtained. The antioxidant activity and contents of fatty acids and lipids of five *Trifolium* species *T. balansae*, *T. stellatum*, *T. nigrescens* subsp. *petrisavi*, *T. constantinopolitanum,* and *T. resupinatum* var. *resupinatum* are presented for the first time in this study.

Keywords: *Antioxidant activity; fatty acid; GC-MS;* Leguminosae*; lipid;* Trifolium *species*

Introduction

Trifolium (*Leguminosae*) genus is represented with 103 species in Turkey (Zohary & Davis, 1970). Trakya (European Turkey), with 67 *Trifolium* taxa, would seem to be a centre of diversity (Zohary & Heler, 1984). In Turkish folk medicine, some *Trifolium* species such as *Trifolium repens* Lin., *Trifolium arvense* Lin., *Trifolium pratense* Lin. are used as expectorant, analgesic, antiseptic and tonic (Baytop, 1984). Also, some of them are important feeding material for sheep and cattle in the Mediterranean (De Rijke et al., 2001; Oleszek & Stochmal, 2002). In previous studies, megastigmane glycosides (Mohamed et al., 1999), chalcanol glucosides (Mohamed et al., 2000), triterpene saponins (Mohamed et al., 1995), flavonoids (Simonet et al., 1999; Oleszek & Stochmal, 2002), steroids, phytylesters and lipids (Sabudak et al., 2006; Isik, 2005) have beeen isolated from *Trifolium* species. However, no chemical analysis has been carried out on the oil *Trifolium balansae* B., *Trifolium stellatum* L., *Trifolium nigrescens* V. subsp. *petrisavi* (Clem) Holmboe, *Trifolium constantinopolitanum* S., and *Trifolium resupinatum* L. var. r*esupinatum* L. Oils constitute one of the three major classes of food product, the others being proteins and carbohydrates. Fatty acid is the main component of oils.

The aim of the present paper is to analyze fatty acid and lipid composition of *Trifolium balansae*, *Trifolium stellatum*, *Trifolium nigrescens* subsp. *petrisavi*, *Trifolium constantinopolitanum,* and *Trifolium resupinatum* var. *resupinatum* and to determine their antioxidant activities. This is the first investigation of fatty acid and other lipid composition of the five *Trifolium* species with their antioxidant activity. The antioxidant potential was investigated by β-carotene bleaching method, (2,2-diphenyl-2-picrylhydrazyl) DPPH free radical scavenging activity, metal chelating activity and total phenolic content of the hexane extracts of the whole plants.

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Materials and methods

Plant material

T. balansae, *T. stellatum*, *T. nigrescens* subsp. *petrisavi*, *T. constantinopolitanum,* and *T. resupinatum* var. *resupinatum* were collected from Corlu-Tekirdag, Turkey in May 2006. The plant was identified by N. Guler of Trakya University, Turkey, a voucher specimen was deposited in the Herbarium of Department of Biology, Faculty of Arts and Science, Trakya University (EDTU 9500, 9501, 9502, 9503 and 9504, respectively).

Oil extraction

The oil was extracted from dried powdered whole plant of each *Trifolium* species at 70°C for 12 h in a Soxhlet extractor using *n*-hexane as a solvent (yield of extract: 1.12–1.30%). The solvent was evaporated by rotary evaporator. The obtained oil was esterified to determine fatty acid composition by GC-MS.

Derivatization of fatty acids

The hexane extracts (100 mg) were dissolved in toluene (1 mL) in a round-bottomed flask fitted with a condenser, and H_2SO_4 in methanol (2 mL, 1%) was added. The mixtures were left overnight in a round-bottomed flask at 50°C, then a NaCl solution (5 mL, 5%) was added and the required esters were extracted with hexane (2.5 mL), thus, the organic layer was separated. The hexane layer was washed with a potassium bicarbonate solution (4 mL, 2%) and dried over anhydrous Na_2SO_4 and filtered. The organic solvent was removed under reduced pressure on a rotary evaporator to give fatty acid methyl esters and other lipids (Yayli et al., 2001; Kilic et al., 2005; Topcu et al., 2007).

GC-MS conditions

The fatty acid methyl esters were analyzed using to Thermo Trace 2000 GC series gas chromatography and Thermo DSQ mass spectrometer. SGE BP x 70 column (25 m x 0.25 mm, 0.25 µm film thickness) was used. The carrier gas was helium at a rate of 1 mL/min. GC oven temperature was kept at 100°C for 5 min and programmed to 240°C at a rate of 4°C/min and kept constant for 5 min. The injection temperature and source temperature were 250° and 220°C, respectively. MS interface temperature was 240°C. The injection volume was 0.5 µL with a split ratio of 1:30. EI-MS were taken at 70eV ionization energy. Mass range was from *m/z* 50 to 650 amu. Scan time 0.5 sec with 0.1 interscan delays. The library search was carried out using NIST and Wiley (Gas Chromatography-Mass Spectrometry) GC-MS libraries and (TUBITAK National Metrology Institute (UME)) library. Supelco™ 37 components of (Fatty acid Methyl ester) FAME mixture (Catalog no: 47885-U) were used for the comparison of the GC chromatograms. The relative percentages of separated compounds were calculated from total ion chromatography by the computerized integrator.

Antioxidant activity tests

Four methods were used to determine antioxidant potential of the oils, the first one was carried out to measure total phenolic concentration in the extracts.

Determination of total phenolic concentration

The concentrations of phenolic content in all extracts were expressed as micrograms of pyrocatechol equivalents (PEs), determined with FCR according to the method of Slinkard and Singleton (1977) (Table 2). The solution (1 mL) of the extracts in methanol was added to 46 mL of distilled water and 1 mL of (Folin-Ciocalteu Reactant) FCR, and mixed thoroughly. After 3 min, 3 mL sodium carbonate (2%) was added to the mixture and shaken intermittently for 2 h at room temperature. The absorbance was read at 760 nm. The concentration of phenolic compounds was calculated according to the following equation that was obtained from standard pyrocatechol graph:

Absorbance = 0.0517 pyrocatechol (mg) + 0.0297 (R^2: 0.9928)

Determination of the antioxidant activity with the β-carotene bleaching method

The antioxidant activity of hexane extracts of five *Trifolium* species was evaluated using β-carotene-linoleic acid model system (Miller, 1971). β-Carotene (0.5 mg) in 1 mL of chloroform was added to 25 µL of linoleic acid, and 200 mg of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL distilled water saturated with oxygen, was added by vigorous shaking. This mixture (4000 µL) was transferred into different test tubes containing different concentrations of the sample. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The emulsion system was incubated for 2 h at 50°C. A blank, devoid of β-carotene, was prepared for background subtraction. BHT and α-tocopherol were used as standards.

Free radical scavenging activity

The free radical scavenging activity of hexane extracts of five *Trifolium* species was determined by the DPPH

assay described by Blois (1958). In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 4 mL of this solution was added to 1 mL of sample solutions in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = x 100

Metal chelating activity

The chelating activity five *Trifolium* extracts on Fe^{2+} was measured as reported by Decker and Welch (1990). The extracts were added to a solution of 2 mM $FeCl_2$ (0.1 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture reached equilibrium, the absorbance was determined at 562 nm. The percentage chelating activity of samples on Fe^{2+} was calculated as follows:

Metal chelating activity (%) = [1-(absorbance of sample)/(absorbance of control] x 100

Reference compounds used are quercetin and EDTA.

Statistical analysis

Experimental results concerning this study were mean ± SD of three parallel measurements. Significant differences between means were determined by Student's t-test, p values <0.05 were regarded as significant, p values <0.01 were regarded as very significant.

Results and discussion

Composition of fatty acid and lipid

The experimental results are summarized in Table 1 which shows the percentage content of individual fatty acids and lipids. There were eight fatty acids identified by comparison with the fatty acid methyl ester standards. The oil of five *Trifolium* species showed a similar fatty acid profile. The major fatty acids were linolenic acid (16.6%–31.1%), palmitic acid (11.1%–18.9%) and linoleic acid (5%-11.3%). In all of these species, the dominant fatty acid was linolenic acid. In the previous studies, linolenic acid was also found to be the dominant fatty acid in *T. repens* and *T. pratense* (Thomson & Knight, 1978; Body, 1974). Lauric, myristic, stearic, oleic and arachidic acids were detected in smaller amounts. The total saturated fatty acid percentage was in the range of 16.7% and 23.3% while the total unsaturation for the oil was 27.7%–42.2%. Palmitic acid (11.1%– 18.9%) and linolenic acid (16.1%–31.1%) are the most abundant saturated and unsaturated fatty acids, respectively.

The oil contained several essential fatty acids and, therefore, has potential nutritional value. The presence of essential fatty acids, namely, linoleic acid and linolenic acid confers on the oil considerable nutritional value (Eromosele & Eromosele, 2002). Especially, linoleic acid is important for its metabolic role in the synthesis of prostoglandins (Al-Jassir et al., 1995). Linolenic acid was found to be at a maximum concentration in *T. constantinopolitanum*. This indicates that *T. constantinopolitanum* oil is a rich source of linolenic acid and, therefore, the unsaturated nature of this oil qualifies it to be promising edible oil.

In this study, the level of palmitic acid (11.1%-18.9%) was higher than the value for *Sesamum indicum* L. (6.8%-8.7%) (Mohamed & Awatif, 1998). Palmitic acid

Table 1. The composition (%) of fatty acid and lipids of the oil from five *Trifolium* species.

	Percentage [%]				
Compound	*Trifolium balansae*	*Trifolium stellatum*	*T. nigrescens* subs. *petrisavi*	*T. constantino politanum*	*T. resupinatum* var. *resupinatum*
Tetradecane, $C_{14}H_{30}$	1.9	1	0.9	1.0	0.8
Pentadecane, $C_{15}H_{32}$	1.5	1.3	0.8	0.8	0.6
Eicosane, $C_{20}H_{42}$	6.3	8.9	17.3	4.5	6.5
Phytol, $C_{20}H_{40}O$	8.9	8.5	5.1	7.2	11.5
Lauric acid, (C12:0)	1.5	1.2	1.3	1.0	1.4
Myristic acid, (C14:0)	1.1	1.1	1.7	0.9	1.2
Palmitic acid, (C16:0)	11.1	16.1	18.9	14.4	18.3
Stearic acid, (C18:0)	1.3	0.7	0.8	1.1	0.7
Oleic acid, (C18:1)	3.3	2.0	4.5	1.7	2.3
Linoleic acid, (C18:2)	5.0	9.6	11.3	9.4	6.9
Linolenic acid, (C18:3)	22.3	16.1	22.3	31.1	26.2
Arachidic acid, C20:0)	1.7	0.7	0.6	0.4	0.8
Total saturation	16.7	19.8	23.3	17.8	22.4
Total unsaturation	30.6	27.7	38.1	42.2	35.4

Table 2. Total phenolic contents of hexane extracts of five *Trifolium* species*.

Sample	Yield of extract[%]	Phenolic content [μg PEs/mg extract]§
T. balansae	1.30	73.90 ± 1.47
T. stellatum	1.21	69.38 ± 1.43
T. nigrescens	1.12	75.08 ± 0.66
T.constantinopolitanum	1.27	82.71 ± 1.28
T. resupinatum	1.20	66.10 ± 1.49

*Values expressed are means ± SD of three parallel measurements ($P < 0.05$).
§ PEs, pyrocatechol equivalents. $R^2 = 0.9928$ $y = 0.0517x - 0.0297$.

Table 3. Antioxidant activity (% Inhibition) of hexane extracts of five *Trifolium* species*, quercetin, BHT and α-tocopherol in β-carotene-linoleic acid system*.

Sample	10 μg	25 μg	50 μg
T. balansae	10.87 ± 1.12	32.51 ± 1.30	68.58 ± 0.90
T. stellatum	39.72 ± 0.77	46.05 ± 0.61	56.59 ± 1.00
T. nigrescens	50.64 ± 1.76	59.85 ± 1.40	75.19 ± 0.96
T.constantinopolitanum	45.22 ± 0.82	59.74 ± 0.09	83.94 ± 5.71
T. resupinatum	47.14 ± 0.24	52.43 ± 0.71	61.24 ± 1.68
α-Tocopherol§	77.96 ± 0.50	80.19 ± 0.19	82.88 ± 0.72
BHT§	85.00 ± 1.55	88.18 ± 0.87	90.01 ± 0.34

*Values expressed are means ± SD of three parallel measurements ($P < 0.05$).
§Reference compounds.

Table 4. DPPH radical scavenging activity (% Inhibition) of hexane extracts of five *Trifolium* species*, BHT and α-tocopherol*.

Sample	10 μg	25 μg	50 μg	100 μg
T. balansae	2.78 ± 0.15	3.85 ± 0.09	5.63 ± 0.01	9.19 ± 0.20
T. stellatum	4.14 ± 0.04	6.96 ± 0.07	11.66 ± 0.13	21.05 ± 0.25
T. nigrescens	2.39 ± 0.17	4.37 ± 0.39	7.67 ± 0.75	14.27 ± 1.47
T.constantinopolitanum	5.47 ± 0.18	9.09 ± 0.00	15.14 ± 0.28	27.22 ± 0.86
T. resupinatum	3.12 ± 0.03	4.79 ± 0.20	7.56 ± 0.47	13.12 ± 1.02
α-Tocopherol§	62.00 ± 0.00	91.44 ± 0.45	94.45 ± 0.44	97.45 ± 0.91
BHT§	15.55 ± 0.40	30.13 ± 0.06	47.15 ± 0.12	64.85 ± 0.23

*Values expressed are means ± SD of three parallel measurements ($P < 0.05$).
§Reference compounds.

is used an indicator of adulteration of cotton seed oil obtained by palm olein. Cotton seed oil has a palmitic acid content of 21%-26% whereas palm olein contains around 40% palmitic acid (Aparico & Aparico Ruiz, 2000).

The epicuticular waxes cover the surfaces of the aerial organs of plants are usually composed of a mixture of long chain aliphatic compounds such as *n*-alkanes, esters, aldehydes, ketones, alcohols and acids. Waxes are important for repelling water and controlling the gas balance between a plant and its environment; other possible functions may be related to the protection of epidermal cells against mechanical damage and inhibition of fungal and insect attacks (Taxa et al., 1996).

The *n*-alkanes are thought to be endogenous to a plant; they are formed as a result of the decarboxylation of long-chain fatty acids (Iyer et al., 1998), in the range C_8-C_{35}, those most abundant being between C_{21} and C_{35} (Bastic et al., 1978; Lanzon et al., 1994; McGill et al., 1993). The hydrocarbons of five *Trifolium* species were tetradecane (0.8–1.9%), pentadecane (0.6–1.5%) and eicosane (4.5–17.3%). Eicosane is the most abundant hydrocarbon in *T. nigrescens* subsp. *petrisavi*.

Another natural product found in higher plants is phytol, which commonly occurs as part of the chlorophyll molecule. sPhytol (11.5%) was found to be highest concentration in *T. resupinatum* var. *resupinatum* oil. Phytol is also known as a cancer preventive agent (Alberto et al., 2002).

Table 5. The metal chelating effect (% Inhibition) of hexane extracts of five *Trifolium* species and quercetin on ferrous ions*.

Sample	50 μg	100 μg	200 μg
T. balansae	23.25 ± 0.03	25.32 ± 0.87	29.45 ± 0.55
T. stellatum	20.66 ± 0.13	21.16 ± 0.14	22.16 ± 0.00
T. nigrescens	22.79 ± 0.49	25.24 ± 0.42	30.14 ± 0.07
T. constantinopolitanum	19.31 ± 0.65	19.99 ± 0.22	21.35 ± 0.07
T. resupinatum	21.80 ± 0.40	22.59 ± 0.59	24.19 ± 0.02
Quercetin§	23.50 ± 0.19	27.51 ± 0.11	35.56 ± 0.12
EDTA§	62.42 ± 0.22	90.34 ± 0.55	96.01 ± 0.24

*Values expressed are means ± SD of three parallel measurements ($P < 0.05$).
§Reference compounds.

Antioxidant potential

All the five *Trifolium* extracts showed more or less antioxidant activity in β-carotene-linoleic acid system (Table 3), however, the highest activity was observed for *T. nigrescens* and *T. constantinopolitanum*. DPPH radical scavenging activity tests were carried out at four different concentrations, however, inhibition percentages were very low. As seen in Table 4, relatively better inhibitions were obtained for *T. constantinopolitanum* and *T. stellatum* oils, but fairly weak radical scavenging

effect was observed, in general. Metal chelating effects of all the tested oils (Table 5) were found to be close to that of quercetin which can be explained as a weak and very slight increasing activity depending on the concentration.

It can be concluded that these five oils were found to be only effective in the β-carotene-linoleic acid system which indicates their antioxidant potential. This finding was parallel with the unsaturated fatty acid content in the oils.

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