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ORIGINAL ARTICLE

Chemical profile and biological activities of *Veronica thymoides* subsp. *pseudocinerea*

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Abstract

Context: In Turkey, *Veronica* species (Plantaginaceae) have been used as a diuretic and for wound healing in traditional medicine.

Objective: To examine the fatty acid and essential oil profiles, the antioxidant, anticholinesterase, antimicrobial, and DNA damage effects of *Veronica thymoides* P.H. Davis subsp. *pseudocinerea* M.A. Fischer as a potential source of natural active compounds.

Materials and methods: GC/MS was used to analyze essential oil and fatty acid obtained from whole plant. The antioxidant activity was evaluated by the β-carotene-linoleic acid test system, DPPH-free and ABTS cation radicals scavenging, and cupric reducing antioxidant capacity assays. The anticholinesterase and antimicrobial activities were determined by Ellman and broth macrodillution methods, respectively. The effect of the methanol extract on DNA cleavage was investigated.

Results: Hexatriacontene (21.0%) was found to be the main constituent in essential oil, and linoleic acid (25.2%) and palmitic acid (20.6%) in fatty acid. Methanol extract demonstrated the best IC_{50} values in lipid peroxidation (49.81 ± 0.31 μg/ml) and DPPH-free radical scavenging activity (15.32 ± 0.17 μg/ml). Methanol and water extracts possessed strong ABTS cation radical scavenging activity with IC_{50} values 9.15 ± 0.28 and 8.90 ± 0.14 μg/ml, respectively. The acetone extract exhibited moderate butyrylcholinesterase inhibitory activity. The highest antimicrobial activity was determined in methanol extract against *Escherichia coli* with 31.25 μg/ml MIC value. Inhibition of methanol extract on plasmid DNA cleavage by OH radicals was found to be 93.32% at 500 μg/ml.

Conclusion: The methanol extract having strong antioxidant and DNA damage effects could be investigated phytochemically to find natural active compounds.

Keywords

Anticholinesterase, antimicrobial, antioxidant, DNA damage, fatty acid, essential oil

History

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Introduction

In Turkey, the genus *Veronica* L. (Plantaginaceae) is represented by 79 species, 26 of them are endemic (Davis, 1978). *Veronica* species, known in Turkish as ''At teresi, Yavsan otu, Ciban otu'', have been used as diuretic and for wound healing in Turkish folk medicine (Baytop, 1984). They have also been used as restoratives, tonics, and in the treatment of respiratory diseases in Chinese and Native American traditional medicines (Stojkovic et al., 2013).

Phytochemical studies have revealed that iridoid glucosides are the chemotaxonomic markers for *Veronica* species; the species are also rich in phenylethanoid and flavonoid glycosides (Saracoglu et al., 2004). A large variety of flavone aglycones such as luteolin, apigenin, chrysoeriol, scutellarein, and isoscutellarein were also reported from *Veronica* species (Grayer-Barkmeijer, 1978; Saracoglu et al., 2004). Phenylethanoid and flavonoid glycosides were obtained from *V. thymoides* subsp. *pseudocinerea* such as 3′-hydroxyscutellarein 7-*O*-(6″-*O*-protocatechuoyl)-β-glucopyranoside, 3′-hydroxyscutellarein 7-*O*-(6″-*O*-trans-feruloyl)-β-glucopyranoside, luteolin 7-*O*-β-glucopyranoside, and (+)-syringaresinol 4′-*O*-β-glucopyranoside (Saracoglu et al., 2004). Extensive research showed that *Veronica* species had various biological activities. Harput et al. (2011) investigated radical scavenging effects of 14 *Veronica* species, and they reported that *V. officinalis* L. exhibited the strongest DPPH and NO radical scavenging activity. *Veronica cuneifolia* subsp. *cuneifolia* D. Don possessed stronger radical scavenging and cytotoxic activities

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than *V. cymbalaria* (Saracoglu et al., 2011). The different extracts of three *Veronica* species were found to protect liver cells from CCl_4-induced liver damage by their antioxidative potential on hepatocytes (Zivkovic et al., 2012).

Veronica thymoides P.H. Davis subsp. *pseudocinerea* M.A. Fischer which is an endemic *Veronica* species has been grown in Kahramanmaras, Kastomonu, Gumushane, and Ankara (Turkey). Saracoglu et al. (2004, 2008) reported that acylated flavonoids, phenol glycosides, and iridoid glucosides were obtained from this plant, and three of the isolated iridoid glucosides, veronicoside, amphicoside, and verminoside, showed significant cytotoxic effect against human and murine cancer cell lines (Saracoglu & Harput, 2012). The purpose of the current study was to examine the fatty acid and essential oil compositions of *V. thymoides* subsp. *pseudocinerea*, the antioxidant, anticholinesterase, antimicrobial, and DNA damage effects of the extracts with their total phenolic and flavonoid contents for the first time.

Materials and methods

Chemicals

A Thermo pH-meter, Gel documentation system, an Elma S15 ultrasonic bath, Horizontal electrophoresis (Biorad, Hercules, CA), Horizontal electrophoresis power supply (Wealtec, Sparks, NV), Shimadzu UV spectrophotometer, automatic pipettes (Eppendorf), a BioTek Power Wave XS and a vortex (LMS Co. LTD, Vietnam, VI) were used for the activity assays. Ethanol, hexane, diethyl ether, chloroform, toluene, dichloromethane, methanol, potassium acetate, BHT (butylated hydroxytoluene), sulfuric acid, aluminum nitrate nonahydrate, aluminum chloride, ABTS, potassium persulfate, sodium acetate, nutrient broth, boric acid, nutrient agar, and sodium bicarbonate were purchased from Merck (Darmstadt, Germany), DPPH, β-carotene, H_2O_2, linoleic acid, quercetin, pyrocathecol, Tween 40, acetic acid, sodium methoxide, gel loading dye, DTNB [5,5-dithiobis-(2-nitro benzoic acid)], copper (II) chloride dihydrate ($CuCl_2.2H_2O$), neocuproine (2,9-dimethyl-1,10-phenanthroline), EDTA, acetylcholinesterase, butyrylcholinesterase, trisma base from Sigma (Steinheim, Germany), α-tocopherol, acetylthiocholine iodide from Aldrich (Germany), galanthamine hydrobromide from Sigma-Aldrich (Steinheim, Germany), butyrylthiocholine iodide from Fluka (Germany), steril blank disc, and antibiotic disc from Oxoid (Hampshire, United Kingdom), acetone, petroleum ether, sodium dihydrogen phosphate, sodium hydrogen phosphate, ammonium acetate, and sodium carbonate from Reidel de Haen (Seelze, Germany).

Plant material

Veronica thymoides subsp. P.H. Davis *pseudocinerea* M. A. Fischer (whole plant) was collected and identified by S. Demirci from Kahramanmaras (Southeastern Turkey) in June (2012). This specimen has been stored in the Herbarium of Istanbul University (ISTE 97136).

Preparation of essential oil

The dried and crumbling into small pieces plant material (100 g) was subjected to hydrodistillation with water for 3 h using a Clevenger-type apparatus. The obtained essential oil which was dried over anhydrous Na_2SO_4 and stored at +4 °C was diluted with CH_2Cl_2 (1:3 v/v) for the analysis.

GC/MS conditions of essential oil

MS analyses were performed on Thermo Electron Trace 2000 GC model gas chromatography and Thermo Electron DSQ quadrupole mass spectrometry. A non-polar Phenomenex DB5 fused silica column (30 m × 0.32 mm, 0.25 μm film thickness) was used with helium at 1 ml/min (20 psi) as a carrier gas. The GC oven temperature was kept at 60 °C for 10 min and programmed to 280 °C at a rate of 4 °C/min and then kept constant at 280 °C for 10 min. The split ratio was adjusted to 1:50, the injection volume was 0.1 μl and EI/MS was recorded at 70 eV ionization energy. The mass range was *m/z* 35–500 amu. Identification of the compounds was based on the comparison of their retention times and mass spectra with those obtained from authentic samples and/or the NIST and Wiley spectra as well as the literature data.

GC/MS conditions and esterification of fatty acid

Esterification of the petroleum ether extract and GC/MS procedure described by Sabudak et al. (2009) were applied. Thermo Scientific Polaris Q GC-MS/MS was used.

Preparation of the petroleum ether, acetone, methanol, and water extracts of the plant

The dried and powdered (100 g) plant material was sequentially macerated with petroleum ether, acetone, methanol, and water for 24 h at 25 °C (three times). After filtration and evaporation of the solvents, the crude extracts were obtained with following yields: 1.4% petroleum ether extract of *V. thymoides* subsp. *pseudocinerea* (VTP), 1.1% acetone extract of *V. thymoides* subsp. *pseudocinerea* (VTA), 5.2% methanol extract of *V. thymoides* subsp. *pseudocinerea* (VTM), and 1.9% water extract of *V. thymoides* subsp. *pseudocinerea* (VTW) (w/w).

Determination of total phenolic and flavonoid contents

Phenolic and flavonoid contents which were expressed as pyrocatechol and quercetin equivalents, respectively, were determined as reported in the literature (Moreno et al., 2000; Slinkard & Singleton, 1977). The following equations were used to calculate total phenolic and flavonoid contents of the extracts:

$$\text{Absorbance} = 0.0123 \text{ pyrocatechol } (\mu g) + 0.0349 \ (R^2 = 0.9910)$$

$$\text{Absorbance} = 0.1701 \text{ quercetin } (\mu g) - 0.7078 \ (R^2 = 0.9939)$$

Antioxidant activity assays

β-Carotene–linoleic acid test system, DPPH free radical scavenging activity, ABTS cation radical decolorization,

and cupric reducing antioxidant capacity (CUPRAC) assays were carried out to determine the antioxidant activity (Apak et al., 2004; Blois, 1958; Miller, 1971; Re et al., 1999).

Anticholinesterase activity

A spectrophotometric method developed by Ellman et al. (1961) was established to indicate the acetyl- and butyryl-cholinesterase inhibitory effects.

Antimicrobial activity

Five different microorganisms including Gram-positive bacteria (*Streptococcus pyogenes* ATCC19615 and *Staphylococcus aureus* ATCC 25923), Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922), and yeast (*Candida albicans* ATCC10231), which were purchased from Refik Saydam Sanitation Center (Ankara, Turkey), were used for detecting the antimicrobial activity of the samples.

Broth macrodillution method

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) which is the lowest concentration of extracts to result in no growth of the inoculum, by using the broth macrodillution method (Islam et al., 2013; NCCLS, 2009). Ampicillin and fluconazole were used as positive controls for bacteria and yeast, respectively.

DNA damage protective activity

Measurement of the DNA damage protective activity of the methanol extract was checked on pBluescript M13 (+) plasmid DNA. Plasmid DNA was oxidized with OH radicals which generated from UV photolysis of H_2O_2 in the presence of the extract and checked on 1% agarose according to Kizil et al. (2009) (Paul et al., 2013). Percent inhibition of the DNA cleavage was calculated using the method described by Fukuhara and Miyata (1998).

Statistical analysis

The results of the activity assays were mean ± S.D. of three parallel measurements. The statistical significance was estimated using Student's *t*-test, p values <0.05 were regarded as significant.

Results

Reactive oxygen species and free radicals cause breaks in DNA strands and damage the cell membrane and protein (Choi et al., 2007). It is well known that oxidative stress is one of the primary factors of degenerative diseases. Epidemiological studies have suggested that it is very important to add antioxidant rich foods such as fruits, vegetables, and cereals to a diet for the prevention of chronic diseases (some types of cancer, neourodegenerative, and cardiovascular diseases) (Ajila et al., 2008). Therefore, screening of medicinal and edible plants to find new antioxidants has become important in recent years.

Phytochemical identification by GC/MS analysis

The present work is the first report on *V. thymoides* subsp. *pseudocinerea* fatty acid composition. As shown in Table 1, 14 components constituting 99.3% of the fatty acid were identified, and linoleic acid (25.2%) and palmitic acid (20.6%) were found to be the major constituents.

A literature survey revealed that there was an essential oil study on *Veronica* species. Cyclohexene, 4-methylene, 1-[1-methylethyl], β-pinene, 1S-α-pinene, β-phellandrene, β-myreene, and germacrene-D were the main components of *V. linariifolia* Pall. ex Link. essential oil (Li, 2002). The essential oil composition of *V. thymoides* subsp. *pseudocinerea* is given in Table 2. In the GC/MS analysis,

Table 1. Fatty acid composition of the petroleum ether extract.

Rt (min)[a]	Constituents[b]	Composition %
12.00	Lauric acid	0.4
14.39	10-Undecenoic acid	0.5
18.60	Myristic acid	1.1
25.27	Palmitic acid	20.6
28.86	11,13-Dimethyl-12-tetradecen-1-ol acetate	0.5
29.75	Phytol	1.2
30.64	Linoleic acid	25.2
30.77	Oleic acid	7.7
30.86	Linolenic acid	12.4
31.54	Stearic acid	4.1
36.23	Nonacosanol	2.0
37.38	Arachidic acid	14.2
39.36	Docosane	1.1
43.82	Behenic acid	8.3
	Total	99.3

[a]Retention time (as minutes).
[b]Compounds listed in the order of elution from a HP-5 MS column. A nonpolar Phenomenex DB-5 fused silica column.

Table 2. Chemical composition of the essential oil obtained from *V. thymoides* subsp. *pseudocinerea*.

RI[a]	Rt (min)[b]	Constituents[c]	Composition %
1484	30.42	Valencene	2.2
1498	30.86	α-Selinene	7.5
1505	31.02	β-Himachalene	3.8
1746	35.52	2-Methyl heptadecane	2.0
1800	36.45	Octadecane	2.8
1890	36.74	2-Methyl-1-hexadecanol	2.3
2185	38.34	*Z*-8-Octadecen-1-ol acetate	3.5
2171	38.98	Butyl phthalate	5.3
2109	40.01	Heneicosane	2.2
2259	40.13	2,5-di-*tert* Octyl-*p*-benzoquinone	5.1
2366	40.59	Arachidic acid	8.0
1986	40.66	Hexadecanoic acid	5.4
2413	41.13	3-Ethyl-5-(2-ethylbutyl)octadecane	1.6
2700	43.30	Heptacosane	1.9
2852	43.64	1-Hexacosanol	2.2
2900	44.10	Nonacosane	3.3
3094	44.41	Ethyl *iso*-allocholate	4.2
3508	45.11	17-Pentatriacontene	8.6
3600	46.50	Hexatriacontene	21.0
4400	47.12	Tetratetracontene	6.0
		Total	98.9

[a]Retention indices (DB-5 column).
[b]Retention time (as minutes).
[c]Compounds listed in the order of elution from a HP-5 MS column. A nonpolar Phenomenex DB-5 fused silica column.

hexatriacontene (21.0%) was found to be the main constituent in 20 components constituting 98.9% of the essential oil.

Antioxidant activity assays

The antioxidant activity of the petroleum ether, acetone, methanol, and water extracts prepared from the whole plant was established by using β-carotene bleaching, DPPH free radical scavenging, ABTS cation radical decolorization, and cupric reducing antioxidant capacity assays with their total phenolic and flavonoid contents. As shown in Table 3, total phenolic contents of all extracts were found to be higher than their flavonoid contents. The water extract had the richest phenolic and flavonoid contents. Among the tested extracts, the methanol extract exhibited the highest lipid peroxidation and DPPH free radical scavenging activity at all concentrations (10, 25, 50, and 100 μg/ml) (Table 4). The methanol (IC_{50} 9.15 ± 0.28 μg/ml) and water extracts (IC_{50} 8.90 ± 0.14 μg/ml) possessed strong ABTS cation radical scavenging activity at 100 μg/ml (Table 4). The methanol and water extracts indicated almost the same reducing effect in CUPRAC assay at 100 μg/ml (Figure 1). These results showed that there was a relationship between phenolic contents and antioxidant potential of the extracts. The antioxidant activity of the extracts increased with arising their phenolic contents. In the present work, the methanol and water extracts which were rich in phenolic compounds indicated high antioxidant activity. Phenylethanoid glycosides, polar phenolic compounds commonly found in *Veronica* species, may be responsible for this activity. Harput et al. (2011) investigated the antioxidant activity of 14 *Veronica* species, and they also reported that high phenolic contents and antioxidant activity of *Veronica* species were related to each other.

Anticholinesterase activity

To our knowledge, the current study is the first anticholinesterase activity study on *Veronica* species. While none of the extracts possessed an anticholinesterase effect, only the acetone extract indicated moderate butyrylcholinesterase inhibitory activity (62.42% inhibition) at 200 μg/ml.

Antimicrobial activity and minimum inhibition concentration

Antimicrobial activity

Stojkovic et al. (2013) reported that the MICs and MBCs values of *V. montana* L. water extract were higher than those of streptomycin. Dulger and Ugurlu (2005) indicated that the methanol extract of *V. lycica* E. Lehm. possessed strong antimicrobial activity against Gram-positive bacteria and

Table 3. Total phenolic and flavonoid contents of the extracts[a].

Samples	Phenolic content (μg/mg PEs extract)[b]	Flavonoid content (μg/mg QEs extract)[c]
Petroleum ether extract of *V. thymoides* subsp. *pseudocinerea*	93.90 ± 1.20	15.94 ± 0.24
Acetone extract of *V. thymoides* subsp. *pseudocinerea*	193.50 ± 2.36	51.70 ± 0.21
Methanol extract of *V. thymoides* subsp. *pseudocinerea*	248.37 ± 3.68	47.02 ± 0.21
Water extract of *V. thymoides* subsp. *pseudocinerea*	360.16 ± 6.54	52.59 ± 0.07

[a]Values expressed are means ± S.D. of three parallel measurements ($p < 0.05$).
[b]PEs, pyrocatechol equivalents ($y = 0.0123x + 0.0349$, $R^2 = 0.9910$).
[c]QEs, quercetin equivalents ($y = 0.1701x - 0.7078$, $R^2 = 0.9939$).

Table 4. Antioxidant activity of the extracts and standards.

	IC_{50} (μg/ml)		
Samples	Lipid peroxidation	DPPH free radical	ABTS cation radical
Petroleum ether extract of *V. thymoides* subsp. *pseudocinerea*	105.26 ± 0.18	>200	>200
Acetone extract of *V. thymoides* subsp. *pseudocinerea*	166.49 ± 0.79	38.17 ± 0.33	20.55 ± 0.45
Methanol extract of *V. thymoides* subsp. *pseudocinerea*	49.81 ± 0.31	15.32 ± 0.17	9.15 ± 0.28
Water extract of *V. thymoides* subsp. *pseudocinerea*	185.54 ± 0.81	88.34 ± 0.55	8.90 ± 0.14
α-TOC	15.54 ± 0.12	18.76 ± 0.41	9.88 ± 0.23
BHT	10.35 ± 0.67	48.86 ± 0.50	10.67 ± 0.11

Values are means ± S.D., $n = 3$, $p < 0.05$, significantly different with Student's *t*-test.

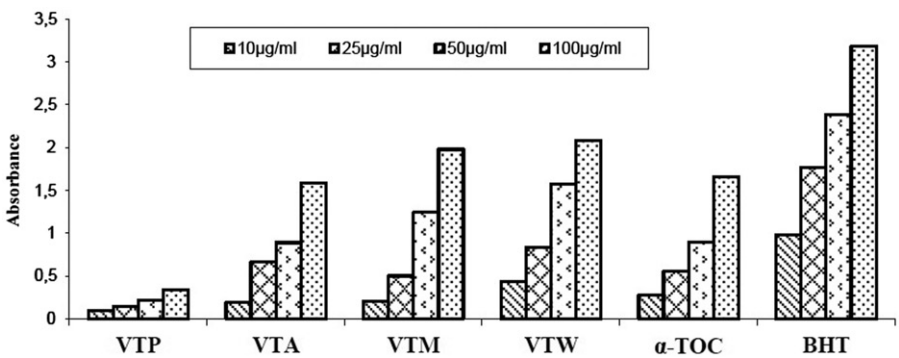

Figure 1. Cupric reducing antioxidant capacity of the extracts, α-tocopherol and BHT. Values are means ± S.D., $n = 3$, $p < 0.05$, significantly different with Student's *t*-test.

yeast cultures. In the current study, the petroleum ether and water extracts of *V. thymoides* subsp. *pseudocinerea* showed no antimicrobial activity against tested microorganisms even with 30 mg/ml concentration (data were not given). The acetone and methanol extracts exhibited almost the same weak antimicrobial activity against *S. pyogenes* and *S. aureus* (Table 5). When we compare the MIC values of the extracts with those of positive controls, the antimicrobial activity of the extracts were found to be quite low. The highest activity was indicated in the methanol extract against *E. coli* with 31.25 µg/ml MIC value. The acetone extract has no antimicrobial activity against *E. coli* and *C. albicans*.

Table 5. Antimicrobial activity of *V. thymoides* subsp. *pseudocinerea* extracts compared to positive controls.

	MIC (µg/ml)		
Microorganisms	Acetone extract	Methanol extract	Positive controls[a]
Gram-positive			
S. aureus	50 ± 0.2	52 ± 0.4	1.95 ± 0.3
S. pyogenes	45 ± 0.4	62.5 ± 0.5	7.815 ± 0.1
Gram-negative			
E. coli	–	31.25 ± 0.3	7.815 ± 0.4
P. aeruginosa	> 250	55 ± 0.4	–
Yeast			
C. albicans	–	> 250	3.125 ± 0.2

–, not active.
[a]Ampicilline for bacteria and fluconazole for yeast.

DNA damage protective activity

Since the methanol extract showed the best antioxidant and antimicrobial effects among the tested extracts, its protection ability (100, 250, 350, and 500 µg/ml) against oxidative DNA damage was measured using pBluescript M13 (+) plasmid DNA. The band intensity and electrophoretic pattern of plasmid DNA are shown in Figure 2(A) and (B), respectively. The addition of the extract (Figure 2B, lanes 6–9) to the reaction mixture suppressed the formation of circular relaxed DNA and induced a partial recovery of supercoiled DNA. Figure 2(B), lane 2, represents the effects of OH radical generated from UV photolysis of H_2O_2. The methanol extract (200 µg) alone had no significant effect on DNA cleavage (Figure 2B, lane 5). DNA damage inhibition activity of the methanol extract at 100, 250, 350, and 500 µg/ml concentrations were found to be 77.95, 79.89, 87.21, and 93.32%, respectively (Figure 2B, lanes 6–9). The results showed that the methanol extract of *V. thymoides* subsp. *pseudocinerea* had potent activity to protect DNA from oxidation.

Conclusion

The present study is the first fatty acid, essential oil, antioxidant, anticholinesterase, antimicrobial, and DNA damage protective activity report on *V. thymoides* subsp. *pseudocinerea*. The water and methanol extracts which were found to be rich in phenolic contents exhibited stronger ABTS cation radical scavenging activity than standard compounds, α-TOC and BHT. The methanol extract also showed the significant inhibition of lipid peroxidation and DPPH free

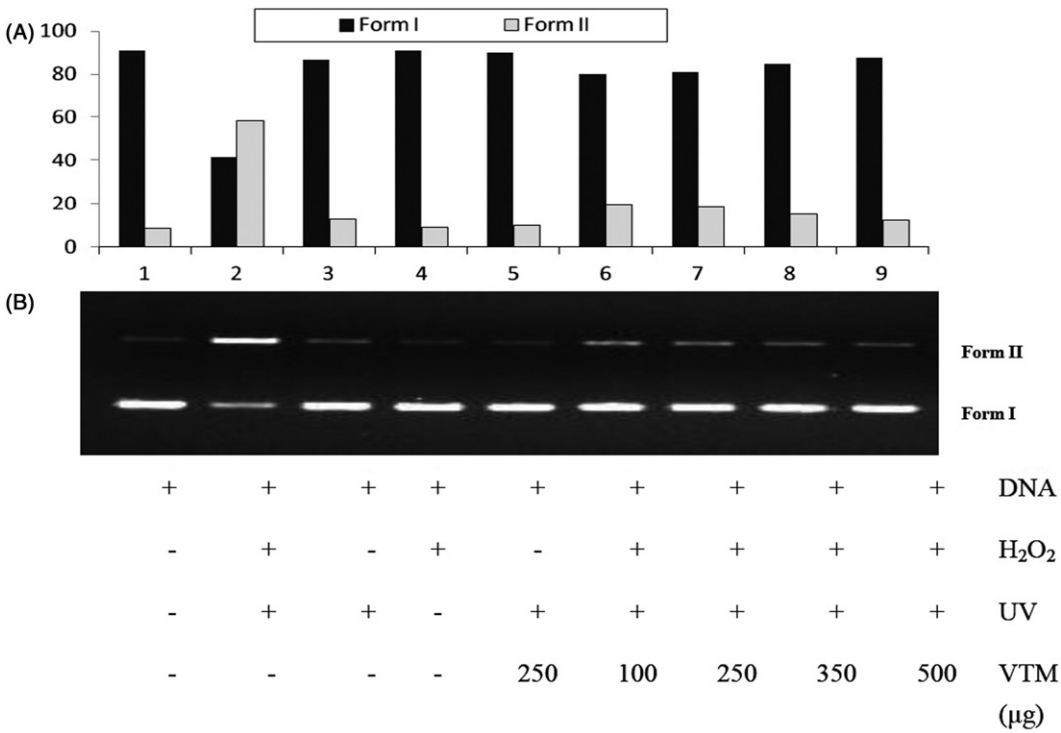

Figure 2. The quantified band intensity for the scDNA (Form I), ocDNA (Form II) with Quantity One 4.5.2. version software (A). Electrophoretic pattern of pBluescript M13+ DNA after UV-photolysis of H_2O_2 in the presence or absence of methanol extract of *V. thymoides* subsp. *pseudocinerea*. Reaction vials contained 200 ng of supercoiled DNA (31.53 nM) in distilled water, pH 7). Electrophoresis was performed using 1% agarose at 40 V for 3 h in the presence of ethidium bromide (10 mg/ml) (B). Electrophoresis running buffer. TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.2). Gel was scanned on Gel documentation system (Gel-Doc-XR, BioRad, Hercules, CA). Bands on the gels were quantified using discovery series Quantity One programme (version 4.5.2. BioRad Co.).

radical scavenging activities. The antioxidant and DNA damage effects of the methanol extract may mostly be related to the phenolic polar compounds. The polar extracts of *V. thymoides* subsp. *pseudocinerea* can be investigated in terms of both phytochemical and biological aspects to find active natural compounds.

Declaration of interest

There is no conflict of interest.

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