Research Article

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Phytochemical screening, anticancer and antioxidant activities of *Origanum vulgare* L. ssp. *viride* (Boiss.) Hayek, a plant of traditional usage

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Abstract

BACKGROUND: A detailed phytochemical analysis of *Origanum vulgare* L. ssp. *viride* (Boiss.) Hayek was carried out and the antioxidant activities of five different crude extracts were determined. The antiproliferative activities of the extracts were determined using the xCELLigence system (Real Time Cell Analyzer).

RESULTS: Differences between the essential oil and volatile organic compound profiles of the plant were shown. The main component of the essential oil was caryophyllene oxide, while the main volatile organic compounds were sabinene and eucalyptol as determined by HS-GC/MS. Phenolic contents of the extracts were determined qualitatively and quantitatively by HPLC/TOF-MS. Ten phenolic compounds were found in the extracts from *O. vulgare* and *Origanum acutidens*: rosmarinic acid (in highest abundance), chicoric acid, caffeic acid, *p*-coumaric acid, gallic acid, quercetin, apigenin-7-glucoside, kaempferol, naringenin and 4-hydroxybenzaldehyde.

CONCLUSION: This study provides first results on the antiproliferative and antioxidant properties and detailed phytochemical screening of *O. vulgare* ssp. *viride* (Boiss.) Hayek.

Keywords: *Origanum vulgare* ssp. *viride*; *Origanum acutidens*; phenolics; antioxidant; antiproliferative

INTRODUCTION

Origanum species have been used for thousands of years as spices and in ethnomedicine. *Origanum vulgare* (L.) is a medicinal perennial plant and *Origanum acutidens* (Hand.–Mazz.) is an endemic, herbaceous perennial plant growing mainly in the Central Anatolia region of Turkey. Many herbs are commonly used in home-type cure therapies, complementary medicine and modern medicine because of their perceived antioxidant, antimicrobial, anticancer, etc. properties. *Origanum* species are counted among these herbs, since they show high activities according to their assessment for the above biological properties.[1,2] In addition to these properties, they are consumed worldwide as spices. As they possess such biological activities, their potential must be revealed by scientific studies and explained to the public, since these herbs are available in almost all public markets.

It is well known that reactive oxygen species (ROS) have the potential to cause serious health problems, including cancer.[3,4] Since antioxidants can pair with these ROS more quickly than body chemicals can, one way to diminish the effect of ROS is to take antioxidants into the body. Medicinal plants are potential sources of natural compounds with biological activities and therefore attract the attention of researchers worldwide. Information on the antioxidant activity and phenolic compounds of medicinal plants associated with anticancer properties is scarce. Free radicals are active chemical species involved in biological processes whose high existence can give rise to several diseases (cancer, etc.).[5] Many people suffer from such diseases, especially from middle age onwards. Consumption of plants with high contents of antioxidant compounds such as polyphenols and flavonoids should feature in everyone's diet, since such species have high potential to diminish the negative effects of free radicals as a result of their electronic and molecular structures. *Origanum vulgare* species enjoy wide industrial, pharmaceutical and traditional usage around the world because of their proven biological (antimicrobial, fungicidal, antioxidant, etc.) properties.[6,7] Our recent study revealed that the anticancer activity of flavone glycosides was higher than that of 5-fluorouracil.[8]

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There is much discussion in the literature about the mechanisms of the antioxidative action of phenolic compounds.[9–11] Polyphenolic compounds are able to act as antioxidants by a free radical-scavenging mechanism, with the formation of less reactive phenoxyl radicals. The high potential of flavonoid compounds to scavenge free radicals may be explained by their ability to donate a hydrogen atom from their hydroxyl group. The reaction produces radicals, and a stable molecule then undergoes a change to a resonance structure by redistributing the unpaired electron on the aromatic core. Thus flavonoid phenoxyl radicals exhibit a much lower reactivity compared with other phenols that would react further to form unreactive compounds, probably by radical termination. The increase in activity has been observed to depend mainly on the position and/or pattern of hydroxylation rather than on the number of hydroxyl groups.

The results of some studies revealed that *O. vulgare* species – either their essential oils or organic solvent extracts – have high antioxidant capacity, which may be attributed to their high phenolic content.[12,13] Other studies indicated that there is diversity in the characterization of the phenolic compounds of samples obtained from different countries and extracted using different procedures. Phenolic acid content did not vary among different samples and was lower that of flavonoids.[14] Rosmarinic acid was the most abundant phenolic acid in all studies. *Origanum* species were found to have high antioxidant, antimicrobial and antifungal activities, which could be attributed to their high content of polyphenolic compounds.[15] Although there are many studies on *O. vulgare* species, few can be found on *O. vulgare* L. ssp. *viride* (Boiss.) Hayek (OVV), a subspecies commonly used in traditional medicine in northern Turkey.

The aim of this work was to perform a detailed study of OVV. The essential oil of OVV was obtained by hydrodistillation and analyzed by gas chromatography/mass spectrometry (GC/MS). Extracts of OVV in different solvents were prepared. The anticancer and antioxidant activities of these extracts were assessed and their phenolic contents were determined by high-performance liquid chromatography/time-of-flight mass spectrometry (HPLC/TOF-MS) based on their high-resolution *m/z* values and comparison of their *m/z* values and retention times with those of authentic samples in our laboratory. The quantitation of phytochemical composition was compared with that of *O. acutidens* studied at slightly modified extraction conditions.

MATERIALS AND METHODS

Chemicals and solvents

Trichloroacetic acid (TCA), Folin–Ciocalteu reagent (FCR) and potassium ferricyanide ($K_3[Fe(CN)_6]$) were purchased from E. Merck (Darmstadt, Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), formic acid, fetal bovine serum (FBS), penicillin/streptomycin and Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) were purchased from Sigma-Aldrich (Darmstadt, Germany). All solvents used in HPLC analysis were of HPLC grade and purchased from E. Merck. All other solvents used in the extraction steps were distilled before use.

All phenolic standards used in quantitation of phenolic compounds of the extracts were of analytical grade and supplied by Prof. Dr Isa Telci (Gaziosmanpasa University, Tokat, Turkey).

Plant material

OVV was harvested in Giresun, a city in the Black Sea region of Turkey. A voucher specimen (OMUB 5991) was deposited at the Herbarium of the Biology Department, Ondokuz Mayis University, Samsun, Turkey The aerial parts of the plant were air dried and then stored at room temperature in the dark until study. *Origanum acutidens* (OA) was collected at flowering stage from its natural habitat in Ortaköy village, Bingöl, Turkey during July 2013.

Preparation and analyses of essential oil of OVV

To obtain the essential oil of OVV, 40 g of the plant was boiled in 750 mL of distilled water in a round bottom flask for 3 h using a Clevenger apparatus. The resulting essential oil was filtered through a 0.45 μm filter cartridge and diluted with acetone. An Agilent 7890A GC system coupled with an Agilent 5975C inert triple-axis MS detector (Agilent Technologies, USA; SEM Ltd. Istanbul, Turkey) was used for identification of the essential oil components. A GC instrument with a flame ionization detector, together with an Agilent HP-5MS column (30 m × 0.25 mm, 0.25 μm), was used to quantitatively analyze the components. The carrier gas was helium at a flow rate of 1.25 mL min^{-1}. The oven temperature program was as follows: from 60 to 140 °C at 5 °C min^{-1}, 5 min hold; from 140 to 220 °C at 4 °C min^{-1}; from 220 to 280 °C at 20 °C min^{-1}, 1 min hold. The injection volume was 1 μL. Components were identified by matching their mass spectra with those in the NIST and Wiley libraries and by comparing their retention indices with literature values.[16–18] Analyses were performed in triplicate.

Analyses of volatile organic compounds

Volatile organic compound (VOC) analyses of OVV aerial parts were performed using an Agilent G1888 network headspace sampler coupled with an Agilent 7890A GC system. Analyses were based on different headspace (HS) oven temperatures (80, 100 and 120 °C) and different equilibration times (10, 20 and 30 min) during which plant samples were held in an HS oven. Results were evaluated as % area of VOCs of OVV. The oven temperature program, using an Agilent DB-WAXETR column (60 m × 0.32 mm, 0.25 μm), was as follows: from 60 to 150 °C at 5 °C min^{-1}, 2 min hold; from 150 to 220 °C at 3 °C min^{-1}, 2 min hold. The injection volume was 1 μL and the carrier gas was helium at a flow rate of 1.5 mL min^{-1}. To perform the analyses, 0.2 g aerial part samples of OVV were placed in 20 mL glass vials and sealed with polytetrafluoroethylene septa and aluminum seals.

Preparation of plant extracts and determination of phenolic compounds

Sampling of OVV was randomized. The dried aerial parts of plants were powdered in a mill. For water (W) extraction, a 10 g sample was mixed with 250 mL of boiling water and filtered through Whatman No. 1 filter paper. The filtrate was lyophilized in a Christ Alpha 1-2 LD Plus lyophilizer (CHRIST Model; Martin-Christ, Osterode, Germany). For ethanol (ET), methanol (ME), ethyl acetate (EA) and hexane (HE) extraction, 10 g samples were powdered in a mill and extracted in a Soxhlet apparatus with solvent until the extracting solution became colorless. The extracts were filtered through Whatman No. 1 filter paper. The filtrates were frozen and lyophilized in a Christ Alpha 1-2 LD Plus lyophilizer. All extracts were placed in plastic bottles and stored at −20 °C until use.

The dried aerial parts of AO were powdered in a mill. For water (W) extraction, a 50 g sample was boiled with 1 L of distilled water for 2 h and filtered through Whatman No. 1 filter paper. The aqueous extract was subsequently extracted with ethyl acetate (EA) and *n*-butanol (*n*-BuOH). The plant residue was finally extracted with

1:1 (v/v) methanol/chloroform (MeCh). The organic layers of EA, *n*-BuOH and MeCh were concentrated under reduced pressure.

Phenolic compounds of the plant extracts were quantitated in an Agilent 1260 Infinity HPLC system coupled with an Agilent 6210 TOF-MS detector and an Agilent Zorbax SB-C18 column (100 mm × 4.6 mm 3.5 µm). Mobile phases A and B were water/1 mL L^{-1} formic acid and acetonitrile respectively. The flow rate was 0.8 mL min^{-1}, the column temperature was 35 °C and the injection volume was 5 µL. The elution program was as follows: 0–1 min, 10% B; 1–12 min, 40% B; 12–14 min, 90% B; 14–17 min, 90% B; 17–18 min, 10% B; 18–25 min, 10% B. Analyses were conducted in triplicate.

Determination of fatty acids

A solution of 20 mg of OVV extract in 3 mL of hexane was mixed with 3 mL of 1 mol L^{-1} KOH/MeOH solution and rigorously vortexed. The hexane phase was filtered (0.45 µm filter) and analyzed in an Agilent 7890A GC system coupled with an Agilent 5975C inert triple-axis MS detector and an Agilent HP-5MS column (30 m × 0.25 mm, 0.25 µm). The carrier gas was helium at a flow rate of 1 mL min^{-1}. The temperature program was as follows: 120 °C for 4 min; from 120 to 200 °C at 3 °C min^{-1}, 10 min hold; from 200 to 280 °C at 15 °C min^{-1}, 15 min hold. The injection volume was 1 µL. Analyses were done in triplicate.

Antioxidant assays

All antioxidant tests and analyses were performed in triplicate and results were averaged.

Determination of total phenolic components

Total phenolic components of the OVV extracts were estimated by a colorimetric assay based on procedures described by Singleton and Rossi,[19] with some modifications. Briefly, 0.1 mL of extract (containing 0.1 mg of extract) was mixed with 46 mL of water, then 1 mL of FCR was added followed by 3 mL of 20 g L^{-1} Na_2CO_3. After incubation at 25 °C for 2 h, the absorbance of the reaction mixture at 760 nm was measured. A standard curve was prepared using 0–100 µg mL^{-1} solutions of gallic acid in ethanol ($y = 0.681x + 0.0096$, $R^2 = 0.9968$). The total phenolic content of each extract was determined from the standard curve and expressed as g gallic acid equivalent kg^{-1} dried plant material.

Total flavonoids in the extracts were determined by a colorimetric method described by Chang *et al.*,[20] with some modifications. Briefly, 0.1 g of extract was dissolved in 1 mL of methanol. A 0.1 mL aliquot of this solution was mixed with *x* mL of 100 g L^{-1} $AlCl_3 \cdot 6H_2O$ and 0.1 mL of 1 mol L^{-1} potassium acetate and kept at 25 °C for 30 min. The absorbance of the reaction mixture at 415 nm was measured. A standard curve was prepared using 0–100 µg mL^{-1} solutions of quercitin in ethanol ($y = 0.7727x - 0.002$, $R^2 = 0.9992$). The total flavonoid content of each extract was determined from the standard curve and expressed as g quercetin equivalent kg^{-1} dried plant material.

Determination of total antioxidant activity

Total antioxidant activity of the OVV extracts was evaluated spectrophotometrically according to Prieto *et al.*[21] using a phosphomolybdenum method based on the reduction of Mo(VI) to Mo(V) by antioxidants and the subsequent formation of specific green phosphate/Mo(V) compounds. A 0.3 mL aliquot of 100 µg mL^{-1} sample solution was combined with 2.7 mL of reagent solution (0.6 mol L^{-1} H_2SO_4, 28 mmol L^{-1} sodium phosphate, 4 mmol L^{-1} ammonium molybdate) in a test tube. The tube was capped and incubated in a boiling water bath at 95 °C for 90 min. After cooling to room temperature, the absorbance of the reaction mixture at 695 nm was measured. For the blank, 0.3 mL of ethanol was mixed with 2.7 mL of reagent solution. A typical blank solution containing 2.7 mL of reagent solution and the appropriate volume of methanol used to dissolve the sample was incubated under the same conditions as the rest of the samples. Stock solutions of α-tocopherol in methanol were prepared. The antioxidant activity of each extract was determined as equivalents of α-tocopherol using an extinction coefficient of 4×10^3 L mol^{-1} cm^{-1} and expressed as µmol α-tocopherol equivalent g^{-1} dried plant material.

Inhibition of linoleic acid peroxidation

The tests were performed according to a modified method of Choi *et al.*[22] Extracts at a concentration[23,24] of 100 µg mL^{-1} were mixed separately with linoleic acid solution (0.28 mg linoleic acid, 0.28 mg Tween-20) in 500 mL of 100 µmol L^{-1} phosphate buffer (pH 7.4) and 150 µL of 10 µmol L^{-1} ascorbic acid solution. Linoleic acid peroxidation was initiated by the addition of 0.1 mL of 10 µmol L^{-1} $FeSO_4$ and incubation at 37 °C for 60 min. The mixtures were cooled and 1.5 mL of 100 mL L^{-1} TCA in 5 mL L^{-1} HCl was added. Then 3 mL of 10 mL L^{-1} (thiobarbituric acid) in 50 mmol L^{-1} NaOH was added and the mixtures were kept in a water bath at 90 °C for 60 min. After cooling, 2 mL aliquots were taken from each sample, vortexed with 2 mL of butanol and centrifuged at 1000 × *g* for 30 min. The upper layers were separated for spectrophotometric measurements. The absorbance of each solution at 532 nm (A_{532}) was measured and linoleic acid peroxidation inhibition was calculated as

$$\text{linoleic acid peroxidation inhibition } (\%) = \left(1 - A_{532(\text{sample})}\right) / A_{532(\text{control})}$$

Reducing power

Reducing power of the extracts was determined according to the method of Oyaizu.[25] Extracts and standard antioxidants at 50–500 µg mL^{-1} in 1 mL of distilled water were mixed separately with 2.5 mL of 0.2 mol L^{-1} phosphate buffer (pH 6.6) and 2.5 mL of 10 g L^{-1} $K_3[Fe(CN)_6]$ and incubated at 50 °C for 20 min. Then 2.5 mL of 100 mL L^{-1} TCA was added to each mixture and the mixtures were centrifuged at 5000 × *g* for 20 min. Finally, 2.5 mL aliquots of the supernatants were mixed with 2.5 mL of distilled water and 0.5 mL of 1 g L^{-1} $FeCl_3$. The absorbance of each solution at 700 nm was measured. Higher absorbance indicates better reducing power under the reaction conditions.

Metal-chelating activity

Chelation of ferrous ions by the extracts was determined by the method of Dinis *et al.*[26] Extracts and standard antioxidants at 100 µg mL^{-1} were added separately to 0.05 mL of 2 mmol L^{-1} $FeCl_2$ solution. The reaction was initiated by the addition of 0.2 mL of 5 mmol L^{-1} ferrozine and each mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the resulting solution at 562 nm (A_{562}) was then measured. Metal-chelating activity was calculated as[27]

$$\text{metal-chelating activity } (\%) = \left[\left(A_{562(\text{control})} - A_{562(\text{sample})}\right) / A_{562(\text{control})}\right] \times 100$$

Superoxide anion-scavenging activity

Superoxide anion-scavenging activity of the extracts was measured according to a slightly modified method of Nishikimi *et al.*[28] Superoxide radicals were generated in a phenazine methosulfate (PMS)/nicotinamide adenine dinucleotide (NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). Briefly, 1 mL aliquots of extracts and standard antioxidants at 100 μg mL^{-1} were mixed separately with 1 mL of 156 μmol L^{-1} NBT solution in 100 mmol L^{-1} phosphate buffer (pH 7.4) and 1 mL of 468 μmol L^{-1} NADH solution in 100 mmol L^{-1} phosphate buffer (pH 7.4). The reaction was started by adding 100 μL of 60 μmol L^{-1} PMS solution in 100 mmol L^{-1} phosphate buffer (pH 7.4). Each mixture was incubated at 25 °C for 5 min and the absorbance at 532 nm (A_{532}) was measured against a blank sample. A decrease in absorbance of the mixture indicates an increase in superoxide anion-scavenging activity. The inhibition of superoxide anion generation was calculated as[27]

$$\text{inhibition of superoxide anion generation } (\%) = \left[\left(A_{532(\text{control})} - A_{532(\text{sample})}\right)/A_{532(\text{control})}\right] \times 100$$

Free radical-scavenging activity

The effect of all extracts on DPPH radical (DPPH•) scavenging was estimated according to the method of Blois,[29] wherein the bleaching rate of a stable free radical(DPPH•) is monitored at a characteristic wavelength in the presence of samples. Briefly, 0.5 mL of 0.1 mmol L^{-1} DPPH• solution in ethanol was added to 3 mL of each extract and standard antioxidant solution at 100 μg mL^{-1} in water separately. The mixtures were shaken vigorously and left at room temperature for 30 min, after which the absorbance of each at 517 nm (A_{517}) was measured. A decrease in absorbance of the DPPH• solution indicates an increase in DPPH• radical-scavenging activity. This activity was calculated as[30,31]

$$\text{DPPH}^{\bullet}\text{ radical-scavenging activity } (\%) = \left[\left(A_{517(\text{control})} - A_{517(\text{sample})}\right)/A_{517(\text{control})}\right] \times 100$$

Hydrogen peroxide-scavenging activity

Hydrogen peroxide-scavenging activity was measured according to a slightly modified method of Zhao *et al.*[32] Briefly, 1 mL of 0.1 mmol L^{-1} hydrogen peroxide and 1 mL of each extract and standard antioxidant at 100 μg mL^{-1} separately were mixed with 10 mL of 30 g L^{-1} ammonium molybdate, 10 mL of 2 mol L^{-1} H_2SO_4 and 7 mL of 1.8 mol L^{-1} KI. The mixtures were titrated with 5 mmol L^{-1} sodium thiosulfate ($Na_2S_2O_3$) until the disappearance of yellow color. Hydrogen peroxide-scavenging activity was calculated as

$$\text{hydrogen peroxide} - \text{scavenging activity } (\%) = \left[\left(V_0 - V_1\right)/V_0\right] \times 100$$

where V_0 is the volume of $Na_2S_2O_3$ solution without extract and V_1 is the volume of $Na_2S_2O_3$ solution mixed with extract or standard antioxidant.

Cell culture and xCELLigence

The xCELLigence Real Time Cell Analyzer-Single Plate (RTCA-SP) instrument (Roche Applied Science, Basel, Switzerland) was used to visualize the antiproliferative effects of the OVV extracts on human cervical cancer (HeLa) cells. This instrument is a combination of four parts: an E-Plate 96, a Single Plate (SP) station that is kept in an incubator and holds the E-Plate 96, an analyzer and a computer with RTCA software. The wells of the E-Plate 96 have an inner volume of 243 ± 5 μL and their bottoms are coated with golden electrodes.[33] The system measures impedance differences in order to derive cell index values at time points whose intervals can be set by the operator. These impedance differences and thus the cell index values depend on the cell activity at the bottom of the wells. The higher the cell population growing at the bottom and the greater the spreading of the cells, the higher is the cell index value. This system allows the user to analyze cell behavior in a label-free environment and produces a real-time profile of the cells.[34]

The HeLa cell line was cultured in DMEM-HG supplemented with 100 mL L^{-1} heat-inactivated FBS and 20 mL L^{-1} penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

To determine the antiproliferative effects of the OVV extracts, HeLa cells were first detached from the tissue culture flask by treatment with trypsin/ethylenediaminetetraacetic acid solution. After detachment, the same volume of culture medium was added to this cell suspension and gently mixed. Then the suspension was partitioned into Falcon tubes and centrifuged. Meanwhile, 50 μL of culture medium was added to each well of the E-Plate 96 and left in the hood for 15 min and in the incubator for 15 min to allow the electrodes to equilibrate with the culture medium. After this period, the E-Plate 96 was inserted into the RTCA-SP station and a background measurement was performed. Immediately afterwards, the E-Plate 96 was ejected from the station and 100 μL of cell suspension adjusted to a concentration of 2.5×10^4 HeLa cells per 100 μL^{-1} was added to each well. Three wells were left blank to check if there would be an increase due to the culture medium. The plate was left in the hood for another 30 min for the cells to adhere to the bottom, after which the plate was inserted into the RTCA-SP station and a measurement lasting 80 min was started. After this period, the plate was ejected from the station and plant extracts (in dimethyl sulfoxide (DMSO)/culture medium; the final concentration of DMSO in the wells was less than 10 mL L^{-1}) were added to the wells at four different concentrations (250, 100, 50 and 25 μg mL^{-1}), and the final volume of each well was adjusted to 200 μL with culture medium. Then the plate was connected to the station and a measurement lasting 48 h was started. The measurement was made in triplicate.

Statistical analysis

The experiments were performed in triplicate. Antioxidant results are given as mean ± standard deviation (SD) of three parallel measurements. One-way analysis of variance (ANOVA) was performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Results of proliferation tests were evaluated as mean ± SD of three measurements of cell index values by ANOVA at 1% significance level and compared by Tukey's one-way comparison test for differences using Minitab 14 (Minitab Inc., State College, PA, USA).

RESULTS AND DISCUSSION

A number of assays have been developed to detect antioxidant activity of natural products, food additives and synthetic compounds. These assays have been widely used to measure total antioxidant capacity, but there are difficulties in measuring this parameter owing to limitations associated with methodological

Table 1. Extraction yields and quantitation results for total phenolics and flavonoids and individual phenolic and flavonoid compounds of *Origanum vulgare* and *Origanum acutidens*

Origanum vulgare	W	EA	ME	ET
Extraction yield[a]	1.55	0.96	2.37	1.28
Total phenolics[b]	39.56 ± 0.42	9.73 ± 0.07	56.83 ± 1.65	22.12 ± 0.30
Total flavonoids[c]	33.57 ± 0.34	7.62 ± 1.18	35.25 ± 0.56	15.96 ± 2.15
Phenolics[d]				
Gallic acid	0.016 ± 0.001	0.012 ± 0.001	0.027 ± 0.001	0.014 ± 0.000
Caffeic acid	0.172 ± 0.010	0.258 ± 0.019	0.367 ± 0.008	0.180 ± 0.008
4-Hydroxybenzaldehyde	ND	0.009 ± 0.001	ND	0.002 ± 0.000
p-Coumaric acid	0.109 ± 0.002	0.054 ± 0.002	0.365 ± 1.050	0.065 ± 0.003
Rosmarinic acid	7.599 ± 0.115	4.303 ± 0.113	19.269 ± 1.035	6.958 ± 0.071
Chicoric acid	0.323 ± 0.830	0.160 ± 0.004	0.910 ± 0.040	0.355 ± 0.007
Flavonoids[d]				
Apigenin-7-glucoside	0.018 ± 0.002	0.012 ± 0.001	0.077 ± 0.005	0.036 ± 0.003
Quercetin	0.022 ± 0.001	0.020 ± 0.001	0.039 ± 0.002	0.027 ± 0.002
Naringenin	ND	0.061 ± 0.002	0.060 ± 0.003	0.043 ± 0.001
Kaempferol	0.011 ± 0.003	0.069 ± 0.003	0.057 ± 0.003	0.044 ± 0.002
Origanum acutidens	W	EA	*n*-BuOH	MeCh
Phenolics[d]				
Gallic acid	0.015 ± 0.001	0.019 ± 0.003	0.036 ± 0.031	0.009 ± 0.002
Caffeic acid	0.024 ± 0.002	0.478 ± 0.015	0.092 ± 0.003	0.096 ± 0.012
4-Hydroxybenzaldehyde	0.348 ± 0.009	1.104 ± 0.120	1.381 ± 0.023	0.468 ± 0.011
p-Coumaric acid	0.068 ± 0.003	0.002 ± 0.001	0.267 ± 0.011	ND
Rosmarinic acid	0.392 ± 0.012	4.858 ± 0.435	6.951 ± 0.539	0.525 ± 0.004
Chicoric acid	ND	0.003 ± 0.001	ND	ND
Flavonoids[d]				
Apigenin-7-glucoside	ND	0.005 ± 0.001	ND	ND
Quercetin	ND	0.007 ± 0.002	ND	0.005 ± 0.002
Naringenin	ND	0.007 ± 0.001	ND	0.012 ± 0.001
Kaempferol	ND	ND	ND	0.002 ± 0.002

Units: [a] g kg^{-1}; [b] g gallic acid equivalent kg^{-1}; [c] g quercetin equivalent kg^{-1}; [d] g kg^{-1}. ND, not detected.

issues and free radical sources.[35] In this study the antioxidant activity of different extracts of *O. vulgare* L. ssp. *viride* (Boiss.) Hayek was compared with that of several well-known antioxidants, namely *tert*-butylhydroquinone (TBHQ), Trolox (TRLX), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and α-tocopherol (TOC). The antioxidant activity of samples was evaluated in a series of *in vitro* tests, including lipid peroxidation, reducing power, total antioxidant activity using a phosphomolybdenum method, DPPH free radical scavenging, hydrogen peroxide scavenging, superoxide anion scavenging and metal chelation. Also, antiproliferative effects of the extracts were tested against HeLa cells. In addition, phytochemical properties of *O. vulgare* L. ssp. *viride* and *O. acutidens* were compared (Table 1).

Extraction yields were 1.55, 0.96, 2.37, 1.28 and 0.33 g kg^{-1} for W, EA, ME, ET and HE extractions respectively (Table 1) and 0.246 g of essential oil was obtained. The highest extraction yield was obtained from ME and the lowest from HE. The yield of essential oil is lower than that reported for *O. vulgare* ssp. *vulgare*.[36]

Essential oil analysis

The composition of the essential oil of OVV was determined and the relative abundances of individual components are given in Table 2. It is clear that the compound with the highest abundance was caryophyllene oxide (25.01%), followed by linalool (8.32%), 1,8-cineol (7.98%), caryophyllene (6.40%), spathulenol (6.31%), p-cymene (4.11%) and caryophyllenol II (4.03%). The total components identified accounted for 91.49% of the essential oil.

Analyses of OVV essential oils reveal that their components can vary depending on growth habitat. In this respect, the results of a study on *O. vulgare* ssp. *viride* (syn. *Origanum heracleoticum*) by Afsharypour *et al.*[37] were compared with the findings of our study. Those authors classified the content of the essential oil into two main groups, i.e. monoterpenes (52.5%) and sesquiterpenes (44.0%). The main components of the monoterpene fraction were linalyl acetate (20.1%), which was also the main component of the whole essential oil, sabinene (13.4%) and γ-terpinene (5.6%), while those of the sesquiterpene fraction were β-caryophyllene (14.5%), caryophyllene oxide (4.7%) and germacrene D (4.6%). In contrast, the main component of the essential oil of OVV studied in our laboratory was caryophyllene oxide (25.01%). Such differences were also clear for other components.

Volatile organic compound analyses

Table 3 lists the VOCs of OVV that were analyzed qualitatively and quantitatively by HS-GC/MS. The results revealed that sabinene, eucalyptol, linalool and *o*-cymene were the main VOC components of OVV, with different abundances under different conditions. The numbers of detected VOCs were 14, 23, 24, 29 and 30 for conditions A (10 min at 80 °C), B (20 min at 80 °C), C (30 min at 80 °C), D (20 min at 100 °C) and E (20 min at 120 °C) respectively

Table 2. Components of OVV essential oil

Compound	Relative abundance (%)	RI	RI^{Ref}
α-Thujene	0.40 ± 0.04	927	932
α-Pinene	0.73 ± 0.03	935	939
Camphene	0.98 ± 0.04	949	954
Sabinene	3.03 ± 0.03	971	977
β-Pinene	0.98 ± 0.02	975	979
β-Myrcene	0.32 ± 0.03	985	993
p-Cymene	4.11 ± 0.17	1022	1026
Limonene	1.13 ± 0.04	1027	1030
1,8-Cineol	7.98 ± 0.24	1031	1031
β-Ocimene	0.23 ± 0.02	1059	1054
Terpinolene	0.27 ± 0.03	1092	1088
Linalool	8.32 ± 0.46	1102	1100
Borneol/isoborneol	1.81 ± 0.08	1182	1165
4-Terpineol	3.30 ± 0.01	1195	1178
α-Terpineol	2.86 ± 0.01	1209	1191
Bornylacetate/isobornylacetate	0.72 ± 0.08	1292	1287
β-Bourbonene	1.19 ± 0.01	1395	1386
Caryophyllene	6.40 ± 0.18	1426	1423
Humulene	0.94 ± 0.02	1460	1456
Alloaromadendrene	0.48 ± 0.00	1468	1458
Germacrene D	1.60 ± 0.04	1489	1485
β-Bisabolene	2.34 ± 0.07	1515	1511
δ-Cadinene	0.52 ± 0.02	1531	1528
Elemol	1.53 ± 0.01	1557	1542
(−)-Spathulenol	6.31 ± 0.06	1585	1582
Caryophyllene oxide	25.01 ± 0.26	1592	1589
Humulene epoxide	2.37 ± 0.01	1615	1606
γ-Eudesmol	0.84 ± 0.01	1633	1635
τ-Cadinol	0.75 ± 0.02	1650	1660
Caryophyllenol II	4.03 ± 0.09	1666	1675
Total	91.49		

Values are mean ± SD of triplicate determinations. Refs 16–18.

Table 3. Volatile organic compounds of OVV determined by HS-GC/MS

Compound	Relative abundance (%)				
	A	B	C	D	E
α-Pinene	4.80	4.11	4.06	3.13	2.67
Camphene	0.99	1.57	2.25	1.83	1.61
β-Pinene	3.26	2.88	2.86	2.41	1.91
Sabinene	26.40	18.98	19.82	12.68	10.07
Myrcene	2.45	3.35	1.40	0.79	0.57
α-Terpinene	ND	ND	ND	0.15	0.26
Limonene	2.47	3.28	3.19	1.97	1.64
Eucalyptol	11.76	17.92	22.44	23.93	19.04
Ocimene	1.81	1.07	0.705	0.29	ND
3-Carene	1.80	3.20	1.96	1.31	1.19
3-Octanone	3.15	3.24	2.98	1.75	1.37
o-Cymene	4.63	7.67	7.91	4.80	4.29
6-Methyl-5-heptene-2-one	ND	0.30	0.31	0.20	0.19
3-Octanol	ND	0.42	0.39	0.32	0.27
1-Octene-3-ol	1.53	1.77	1.53	1.23	1.08
cis-Linalool oxide	ND	1.32	1.35	2.73	2.15
trans-Sabinene hydrate	ND	1.96	1.89	3.00	2.29
trans-Linalool oxide	ND	1.01	1.19	2.17	2.18
Linalool	4.82	10.15	9.44	13.02	12.23
cis-Sabinene hydrate	ND	ND	ND	1.48	1.32
cis-β-Terpineol	ND	0.94	0.73	ND	ND
β-Caryophyllene	ND	1.43	1.47	3.07	3.37
Myrtenal	ND	ND	ND	ND	0.12
Sabina ketone	ND	ND	ND	0.32	0.42
Aromadendrene	ND	ND	ND	0.39	0.54
α-Caryophyllene	ND	ND	ND	0.54	0.68
α-Terpineol	1.02	ND	1.15	2.03	2.21
p-Menth-1-en-8-ol	ND	1.10	ND	ND	ND
Borneol	ND	ND	0.44	1.04	1.32
Germacrene D	ND	0.41	0.41	0.85	0.92
β-Bisabolene	ND	ND	ND	0.57	0.87
Caryophyllene oxide	ND	0.63	0.86	3.14	4.89
Spathulenol	ND	ND	ND	ND	0.57

Conditions: A, 10 min at 80 °C; B, 20 min at 80 °C; C, 30 min at 80 °C; D, 20 min. at 100 °C; E, 20 min at 120 °C. ND, not detected.

Although it is necessary to use a solvent during both extraction and analysis of essential oils, HS-GC allows analyses to be carried out without a solvent. In this part of the study, different VOC profiles of OVV were determined under different conditions. First, the oven temperature was kept constant at 80 °C while the holding time of vials in the oven was varied. The effect of holding time can be clearly seen in Table 3. There is an obvious difference in the relative abundances of components, particularly the main ones: sabinene showed a tendency to decrease with increasing holding time, while several other VOCs showed an increase. With increasing holding time, the number of determined compounds was also increased.

Alteration of the oven temperature also had interesting effects on the VOC profile of OVV. The main components were still the same, with varying abundances. While the amounts of sabinene and *o*-ocimene decreased, those of eucalyptol and linalool increased. There is also another point to observe: although caryophyllene oxide, which was the main component of the essential oil of OVV, was not detected under condition A, the amount of this component was notably higher under condition E; there was an increase from 0.63 under condition B to 4.89 under condition E. This is clear evidence of a high abundance of caryophyllene oxide in OVV. However, owing to its high boiling point, this compound cannot vaporize at lower temperatures in shorter times. In addition, caryophyllenol II, 4-terpineol, elemol and γ-eudesmol were major components of the essential oil of OVV that could not be determined by HS analyses.

There were also clear differences in composition between the essential oil and VOCs of OVV. While the essential oil was rich in components with high melting/boiling points, i.e. caryophyllene oxide (m.p. 62–63 °C), spathulenol (b.p. 269–270 °C), linalool (b.p. 198–199 °C), caryophyllene (b.p. 262–264 °C) and caryophyllenol II (b.p. 309.8 °C), the VOC profiles were rich in components with lower boiling points, i.e. sabinene (163–164 °C) and eucalyptol (176–177 °C). This can be explained by the diffusion and extraction effect of solvent, in this case boiling water, into the plant surfaces. In the HS sampler oven the sole effect causing the plant to release its components is heating, whereas in hydrodistillation the solvent effect is combined with the heating effect.

Table 4. Antioxidant test results

Sample	50 μg mL^{-1}	100 μg mL^{-1}	250 μg mL^{-1}
Antioxidant activity (μmol α-tocopherol equivalent g^{-1})			
W	157590 ± 5770	162435 ± 133792	501840 ± 19474
EA	32725 ± 11420	55462.5 ± 35161	88187.5 ± 4508
ME	156740 ± 5289	183940 ± 126459	386240 ± 4808
ET	106760 ± 1923	220660 ± 166849	292400 ± 16348
HE	28220 ± 17791	28900 ± 2404	48280 ± 1923
TOC	108800 ± 1923	138380 ± 100013	247520 ± 11059
BHA	158100 ± 12502	252620 ± 188006	301580 ± 35582
BHT	73610 ± 22840	80240 ± 14906	89930 ± 5049
TRLX	81260 ± 8174	90950 ± 63229	116620 ± 18752
TBHQ	166600 ± 10097	232900 ± 168291	358190 ± 8415
Reducing power (A_{700})			
W	0.321 ± 0.016	0.382 ± 0.021	1.039 ± 0.085
EA	0.167 ± 0.004	0.151 ± 0.059	0.353 ± 0.011
ME	0.324 ± 0.012	0.418 ± 0.036	0.948 ± 0.003
ET	0.203 ± 0.066	0.370 ± 0.007	0.711 ± 0.008
HE	0.207 ± 0.001	0.215 ± 0.008	0.214 ± 0.031
TOC	0.143 ± 0.012	0.179 ± 0.011	0.420 ± 0.013
BHA	0.392 ± 0.000	0.636 ± 0.000	1.347 ± 0.001
BHT	0.645 ± 0.004	0.872 ± 0.012	1.040 ± 0.074
TRLX	0.315 ± 0.002	0.414 ± 0.006	0.885 ± 0.008
TBHQ	0.272 ± 0.024	0.539 ± 0.004	1.023 ± 0.044
Free radical-scavenging activity (%)			
W	37.69 ± 0.05	36.54 ± 17.95	58.85 ± 2.72
EA	66.92 ± 33.72	87.69 ± 1.09	52.69 ± 7.07
ME	73.85 ± 3.26	83.08 ± 0.07	88.08 ± 0.54
ET	62.31 ± 0.18	76.92 ± 5.44	83.85 ± 3.26
HE	44.23 ± 26.65	0.77 ± 0.09	48.08 ± 10.33
TOC	56.15 ± 15.23	76.92 ± 6.53	82.69 ± 0.54
BHA	90.00 ± 4.35	91.92 ± 4.90	94.23 ± 1.63
BHT	92.31 ± 0.00	94.23 ± 1.63	97.31 ± 1.63
TRLX	90.77 ± 1.09	94.23 ± 1.63	95.00 ± 1.63
TBHQ	17.31 ± 0.54	81.15 ± 1.63	87.69 ± 1.09

Table 5. (A) Superoxide-scavenging activity (%), (B) metal-chelating activity (%), (C) hydrogen peroxide-scavenging activity (%) and (D) lipid peroxidation (%) at 100 μg mL^{-1} concentration

Sample	A	B	C	D
W	85.81 ± 1.20	51.06 ± 0.52	40.00 ± 9.43	95.50 ± 0.71
EA	44.32 ± 1.40	88.78 ± 1.20	43.33 ± 1.57	90.00 ± 3.54
ME	68.31 ± 1.87	46.40 ± 2.92	44.44 ± 9.43	84.75 ± 8.84
ET	59.08 ± 8.63	51.43 ± 0.90	51.11 ± 9.24	83.50 ± 5.66
HE	37.89 ± 4.35	46.88 ± 7.03	52.11 ± 3.14	84.50 ± 9.90
TOC	29.23 ± 3.08	49.21 ± 1.20	50.00 ± 7.86	88.00 ± 0.09
BHA	33.11 ± 7.22	83.60 ± 0.00	38.89 ± 6.86	80.00 ± 2.83
BHT	33.21 ± 7.22	55.56 ± 0.90	67.78 ± 7.26	78.75 ± 0.35
TRLX	41.11 ± 1.81	80.11 ± 2.54	45.56 ± 4.71	82.00 ± 2.12
TBHQ	40.30 ± 0.80	56.77 ± 2.02	36.67 ± 1.57	42.25 ± 3.89

Antioxidant assays

Antioxidant capacity is widely used as a parameter for medicinal bioactive and functional components in food. In this study, antioxidant capacities of OVV extracts were determined by the formation of a green phosphomolybdenum complex and compared with those of TOC, BHA, BHT, TRLX and TBHQ as positive controls. The formation of the complex at 95 °C was measured by the intensity of absorbance of the reaction mixture at extract concentrations of 50, 100 and 250 μg mL^{-1}, as shown in Table 4. The phosphomolybdenum assay is based on the reduction of Mo(VI) to Mo(V) by antioxidant compounds and the formation of a green phosphate/Mo(V) complex that absorbs at 695 nm. The experimental data indicated that all OVV extracts were likely to have the potential for phosphomolybdenum reduction, but with statistically significant differences ($P < 0.01$). The antioxidant activities of W and ME extracts were higher than those of other extracts and standards in terms of phosphomolybdenum reduction potential.

In the present work, antioxidant activities, reducing powers and free radical-scavenging activities were determined at 50, 100 and 250 μg mL^{-1} concentrations of W, EA, ME, ET and HE extracts of OVV and compared against positive controls (TOC, BHA, BHT, TRLX and TBHQ) at the same concentrations (Table 4). Because of the phenolic and flavonoid contents of the extracts (Table 1), free hydroxyl groups were a source of hydrogen atoms in the neutralization of radical species of flavonoid contents, altering the stability of a flavonoid radical formed by the abstraction of a hydrogen atom from another hydroxyl group. The di- and polyhydroxy groups in the phenolic rings of flavonoids can donate single electrons, resulting in the production of a resonance-stabilized structure and the formation of less reactive flavonoid phenoxyl radicals. This is the basis of their ability to prevent lipid peroxidation chain reactions. Lipid peroxyl radicals are converted to hydroperoxides by reaction with α-tocopherol. The high potential of phenolics to scavenge free radicals may be explained by their ability to donate hydrogen atoms from their hydroxyl groups. Plant phenolics have been studied in relation to the prevention of cancer,[8] which may be connected with antioxidant potential. In addition, it was reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation.[31] According to these results, the various solvent extracts of OVV should be acknowledged as promising sources of non-toxic natural antioxidants that could be used for cultivation and breeding programs.

Lipid peroxidation

Lipid peroxidation involves a series of free radical-mediated chain reaction processes and is measured by the amount of peroxide, the primary product of lipid oxidation, produced during the initial stages of oxidation. In this assay, hydroperoxide produced by linoleic acid added to the reaction mixture, oxidized by air during the experimental period, was measured indirectly. Ferrous chloride and thiocyanate react with each other to produce ferrous thiocyanate (red color) by means of hydroperoxide. Total antioxidant activities of W, EA, ME, ET and HE extracts and positive controls (TOC, BHA, BHT, TRLX and TBHQ) were determined by the ferric thiocyanate method in the linoleic acid system. The effects of solvent extracts and standards on lipid peroxidation in linoleic acid emulsion are shown in Table 5. Over the incubation period, the activities of ME, HE and ET extracts at 100 μg mL^{-1} concentration (84.75, 84.50 and 83.50 μg mL^{-1} respectively) were higher than those of all positive controls except TOC (88.00 μg mL^{-1}) at the same concentration ($P < 0.05$). W and EA extracts had the highest activity among all standards and solvent extracts tested. These results clearly indicated that phenolic compounds and flavonoids had effective and powerful antioxidant activities (Tables 1 and 5).

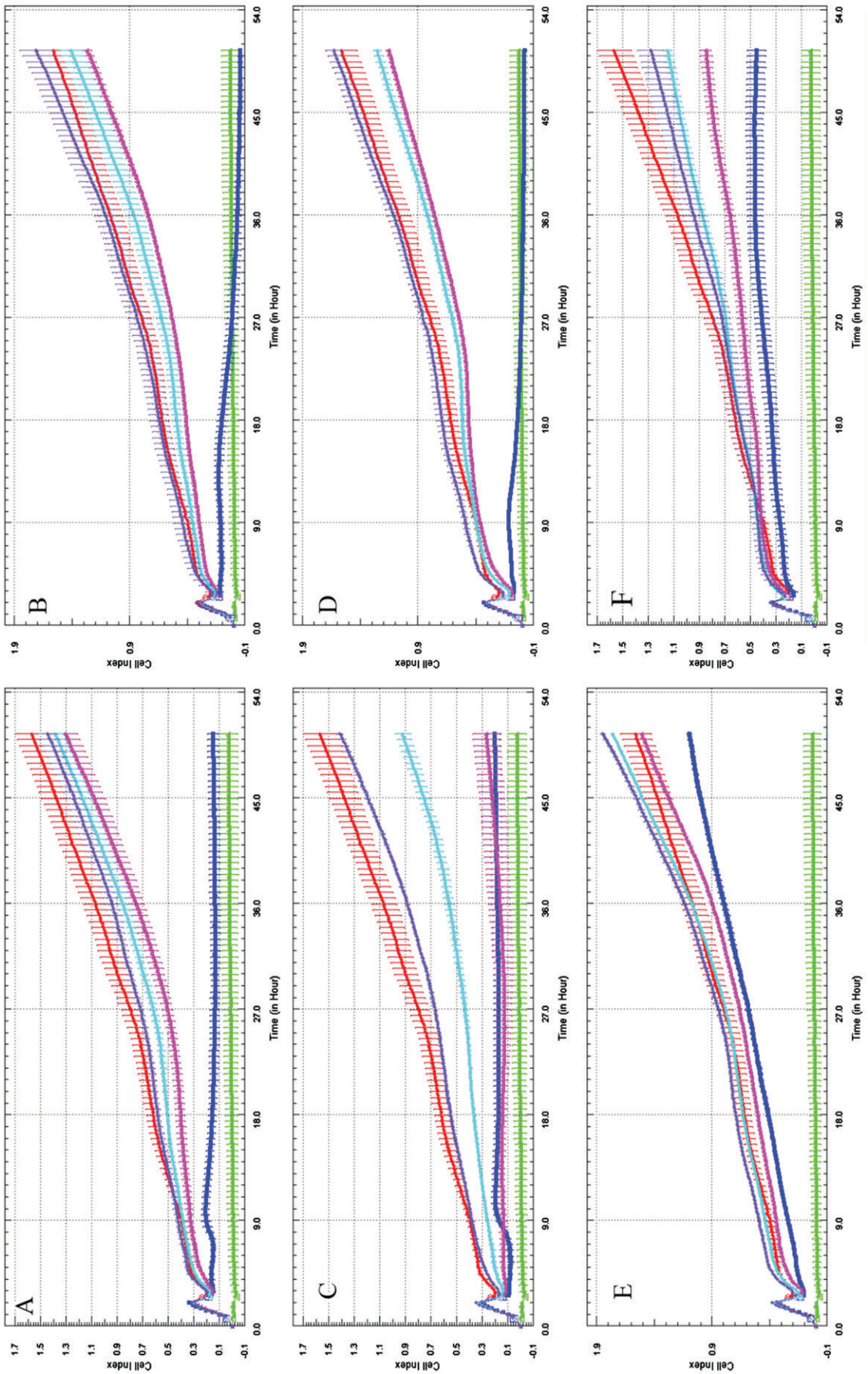

Figure 1. Proliferation assays of (A) ME, (B) ET, (C) EA, (D) W and (E) HE extracts of *Origanum vulgare* ssp. *viride* and (F) cis-platin as positive standard at various concentrations (μg mL^{-1}): ——, 250; ——, 100; ——, 50; ——, 25; ——, control; ——, medium.

Table 6. Cell index values from antiproliferative tests of extracts on HeLa cells

	Cell index			
Extract	250 μg mL^{-1}	100 μg mL^{-1}	50 μg mL^{-1}	25 μg mL^{-1}
12 h after treatment				
ME	0.177 ± 0.033c	0.383 ± 0.039c	0.468 ± 0.019ab	0.526 ± 0.038a
ET	0.128 ± 0.041c	0.378 ± 0.020c	0.442 ± 0.030b	0.578 ± 0.079a
EA	0.186 ± 0.036c	0.152 ± 0.030d	0.326 ± 0.010c	0.484 ± 0.010a
W	0.067 ± 0.007c	0.443 ± 0.017bc	0.486 ± 0.028ab	0.632 ± 0.038a
HE	0.348 ± 0.014b	0.447 ± 0.002bc	0.554 ± 0.010a	0.628 ± 0.011a
Cis-Pt	0.318 ± 0.050b	0.444 ± 0.038bc	0.507 ± 0.034ab	0.514 ± 0.066a
Control	0.545 ± 0.051a	0.545 ± 0.051ab	0.545 ± 0.051a	0.545 ± 0.051a
24 h after treatment				
ME	0.137 ± 0.039de	0.500 ± 0.046c	0.603 ± 0.035b	0.707 ± 0.027b
ET	0.025 ± 0.043de	0.527 ± 0.013bc	0.611 ± 0.054b	0.824 ± 0.086ab
EA	0.175 ± 0.037d	0.131 ± 0.063d	0.428 ± 0.021c	0.660 ± 0.016b
W	0.008 ± 0.009e	0.540 ± 0.011bc	0.593 ± 0.021b	0.842 ± 0.035ab
HE	0.575 ± 0.027b	0.652 ± 0.006ab	0.767 ± 0.010a	0.831 ± 0.022ab
Cis-Pt	0.408 ± 0.057c	0.565 ± 0.042bc	0.687 ± 0.040ab	0.716 ± 0.070b
Control	0.779 ± 0.079a	0.779 ± 0.079a	0.779 ± 0.079a	0.779 ± 0.079ab
36 h after treatment				
ME	0.138 ± 0.043de	0.842 ± 0.075bc	0.926 ± 0.053b	1.017 ± 0.055abc
ET	0.000 ± 0.000e	0.821 ± 0.025bc	0.948 ± 0.081b	1.209 ± 0.106ab
EA	0.188 ± 0.043d	0.180 ± 0.093c	0.597 ± 0.040c	0.959 ± 0.015c
W	0.000 ± 0.000e	0.826 ± 0.020bc	0.883 ± 0.031b	1.204 ± 0.061ab
HE	0.856 ± 0.030b	0.976 ± 0.021ab	1.184 ± 0.0150a	1.250 ± 0.073a
Cis-Pt	0.467 ± 0.067c	0.705 ± 0.042c	0.926 ± 0.035b	1.002 ± 0.082bc
Control	1.155 ± 0.103a	1.155 ± 0.103a	1.155 ± 0.103a	1.155 ± 0.103abc
48 h after treatment				
ME	0.151 ± 0.047d	1.308 ± 0.104abc	1.380 ± 0.062bcd	1.443 ± 0.081bc
ET	0.000 ± 0.000d	1.276 ± 0.045bc	1.409 ± 0.105b	1.718 ± 0.141ab
EA	0.204 ± 0.049d	0.273 ± 0.106e	0.920 ± 0.061e	1.411 ± 0.029bc
W	0.000 ± 0.000d	1.156 ± 0.025c	1.251 ± 0.040 cd	1.631 ± 0.075ab
HE	1.100 ± 0.023b	1.502 ± 0.026ab	1.761 ± 0.014a	1.851 ± 0.026a
Cis-Pt	0.450 ± 0.075c	0.849 ± 0.048d	1.145 ± 0.036de	1.279 ± 0.105c
Control	1.563 ± 0.136a	1.563 ± 0.136a	1.563 ± 0.136ab	1.563 ± 0.136abc

For each time, values with different letters in a column are significantly different (ANOVA $P < 0.01$, Tukey test).

Free radical-scavenging activity

The effect of antioxidants on DPPH• scavenging may be explained in terms of their hydrogen-donating ability. DPPH• is a stable free radical and accepts an electron or hydrogen radical to form a stable diamagnetic molecule. The DPPH•-scavenging model is a widely used method of evaluating antioxidant activities in a relatively short time compared with other methods.[38] The free radical-scavenging activity of OVV extracts and standards at 50, 100 and 250 μg mL^{-1} concentrations was evaluated as % inhibition in the DPPH radical model system. Among the extracts, the highest inhibition of 88.08% was found for ME extract at 250 μg mL^{-1}, which was higher than that of TOC and TBHQ (Table 4). The DPPH•-scavenging capacity of samples at 250 μg mL^{-1} decreased in the following order: BHT > TRLX > BHA > ME > TBHQ > ET > TOC > W > EA > HE. The results were statistically significant ($P < 0.05$).

Reducing power

OVV extracts showed effective reducing power as determined by the potassium ferricyanide reduction method, comparable to that of the standards. The yellow color of the test solution changes to various shades of green and blue depending on the reducing power of the sample. The reducing power of OVV extracts may serve as a significant indicator of their potential antioxidant activity.[39] The reductive ability was investigated via the Fe^{3+}–Fe^{2+} transformation in the presence of OVV extracts using the method of Oyaizu.[25] The reducing power capacities of OVV extracts are compared with those of BHA, BHT, TRLX, TBHQ and TOC in Table 4.

The reducing power of antioxidant samples increased with increasing sample concentration. At different concentrations, OVV exhibited more effective reducing power than the control, and differences were statistically significant ($P < 0.05$). The reducing capacities of some OVV extracts were greater than those of the standards. The reducing power of samples at 250 μg mL^{-1} decreased in the following order: BHA > BHT > W > TBHQ > ME > TRLX > ET > TOC > EA > HE. Thus the W extract of OVV exhibited higher activity than the other extracts.

Superoxide-scavenging activity

Superoxide anions are precursors of active free radicals that have the potential to react with biological macromolecules and thereby

Table 7. Fatty acid methyl esters and non-volatile components of hexane extract of OVV

Compound	Relative abundance (%)
Caryophyllene oxide	0.97 ± 0.04
Tetradecanoic acid methyl ester	0.15 ± 0.00
Hexadecanoic acid methyl ester	3.80 ± 0.11
9,12-Octadecadienoic acid methyl ester	1.26 ± 0.03
9,12,15-Octadecatrienoic acid methyl ester	4.18 ± 0.10
trans-Phytol	0.68 ± 0.02
Octadecanoic acid methyl ester	1.19 ± 0.03
Heneicosane	0.31 ± 0.01
cis-Phytol	0.19 ± 0.01
Docosane	0.53 ± 0.03
Eicosanoic acid methyl ester	1.59 ± 0.74
Tricosane	0.82 ± 0.03
Tetracosane	1.43 ± 0.13
Docosanoic acid methyl ester	2.03 ± 0.07
3-Ethyl-5-(2-ethylbutyl)octadecane	1.42 ± 0.22
Pentacosane	1.67 ± 0.37
Hexacosane	4.09 ± 0.17
Tetracosanoic acid methyl ester	2.42 ± 0.23
13-Dodecyl-hexacosane	1.52 ± 0.22
Heptacosane	1.99 ± 0.03
14-beta-H-Pregna	2.86 ± 0.24
Octacosane	0.93 ± 0.14
1-Eicosanol	38.02 ± 1.33
Nonacosane	1.05 ± 0.06
Cyclotetracosane	7.85 ± 0.29
Hentriacontane	1.64 ± 0.06
Total	84.59

Values are mean ± SD of triplicate determinations.

induce tissue damage. They have also been implicated in several pathophysiological processes owing to their transformation into more reactive species such as hydroxyl radicals that initiate lipid peroxidation. Superoxide has also been observed to directly initiate lipid peroxidation.[40] As shown in Table 5, extracts of OVV at 100 μg mL^{-1} significantly inhibited superoxide anion formation to different extents ($P < 0.05$). The superoxide-scavenging activity of samples decreased in the following order: W > ME > ET > EA > TRLX > TBHQ > HE > BHT > BHA > TOC. OVV extracts are superoxide scavengers and their capacity to scavenge superoxide may contribute to their total antioxidant activity. The distinct effects of some plant extracts and synthesized chemicals have been previously noted in two different assay systems. Therefore the observation of different effects of some plants on lipid peroxidation and free radical and superoxide anion radical formation is not surprising, since the ways of production of oxidative stress in these methods are different.[41]

Metal-chelating activity

The chelation of ferrous ions by OVV extracts was estimated by the method of Dinis *et al.*[26] In the presence of other chelating agents, complex formation is disrupted, as a result of which the red color of the complex gradually disappears. Measurement of the rate of color reduction allows estimation of the chelating activity of the coexisting chelator.[42] In this assay, extracts of OVV and standard antioxidants interfered with the formation of ferrozine–Fe^{2+} complex, suggesting they have chelating activity and capture ferrous ions before ferrozine. As shown in Table 5, the formation of ferrozine–Fe^{2+} complex is disrupted in the presence of extracts, indicating that OVV extracts chelate iron. The high metal-chelating activity of EA extract of OVV at 100 μg mL^{-1} showed marked hydrogen peroxide-scavenging activity. The metal-chelating activity of samples decreased in the following order: EA > BHA > TRLX > TBHQ > BHT > ET > W > TOC > HE > ME ($P < 0.05$). It was noted that chelating agents that form σ-bonds with a metal are secondary antioxidants owing to reduction of the redox potential. Iron can stimulate lipid peroxidation by the Fenton reaction and can also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation.[40]

Hydrogen peroxide-scavenging activity

Hydrogen peroxide can be formed *in vivo* by oxidizing enzymes such as superoxide dismutase and catalase. It can cross membranes and slowly oxidize a number of compounds.[40] The ability of *Hypericum* species to scavenge hydrogen peroxide was reported by Zhao *et al.*[32] The effects of OVV extracts on hydrogen peroxide scavenging are shown in Table 5. The extracts had markedly higher hydrogen peroxide-scavenging activity than some standard antioxidants ($P < 0.05$). The hydrogen peroxide-scavenging activity of samples decreased in the following order: BHT > HE > ET > TOC > TRLX > ME > EA > W > BHA > TBHQ.

Hydroxyl radicals abstract hydrogen atoms from membrane lipids and bring about lipid peroxidation.[40] Clearly, the ability of OVV extracts to scavenge hydroxyl radicals results directly in the prevention of propagation of the process of lipid peroxidation. Hydrogen peroxide is very important owing to its ability to penetrate biological membranes. Although hydrogen peroxide is not reactive and effective by itself, it can sometimes be toxic to cells, because it may give rise to an increase in the amount of hydroxyl radicals. Addition of hydrogen peroxide to cells in culture can lead to transition metal ion-dependent hydroxyl radical-mediated oxidative DNA damage.[43] Thus removal of hydrogen peroxide is very important for the protection of the living system.

Determination of total phenolics and flavonoids

A study performed to investigate extraction yields of three members of the Lamiaceae family, rosemary, marjoram and oregano, revealed that the extracts had a high content of rosmarinic acid.[44] Our results showed that rosmarinic acid was the main component of OVV extracts, followed by caffeic acid, chicoric acid and *p*-coumaric acid (Table 1). 4-Hydroxybenzaldehyde was the compound found in lowest amount in OVV extracts (not detected at all in W and ME extracts). In contrast, 4-hydroxybenzaldehyde was the second most abundant component of OA extracts. The W extract of OA was partitioned with EA and *n*-BuOH successively. The plant residue was also extracted with MeCh. Rosmarinic acid was mostly extracted by boiling water and taken into EA (4.858 g kg^{-1}) and *n*-BuOH (6.951 g kg^{-1}) with repeated extractions from the aqueous solvent. The final extraction of the plant residue with MeCh gave only 0.525 g kg^{-1} rosmarinic acid, and the final quantity of rosmarinic acid left in water was 0.392 g kg^{-1}. Moreover, HPLC/TOF-MS analysis together with principal component analysis showed that the two species (OVV and OA) differed significantly in their phenolic and flavonoid contents (Table 1).

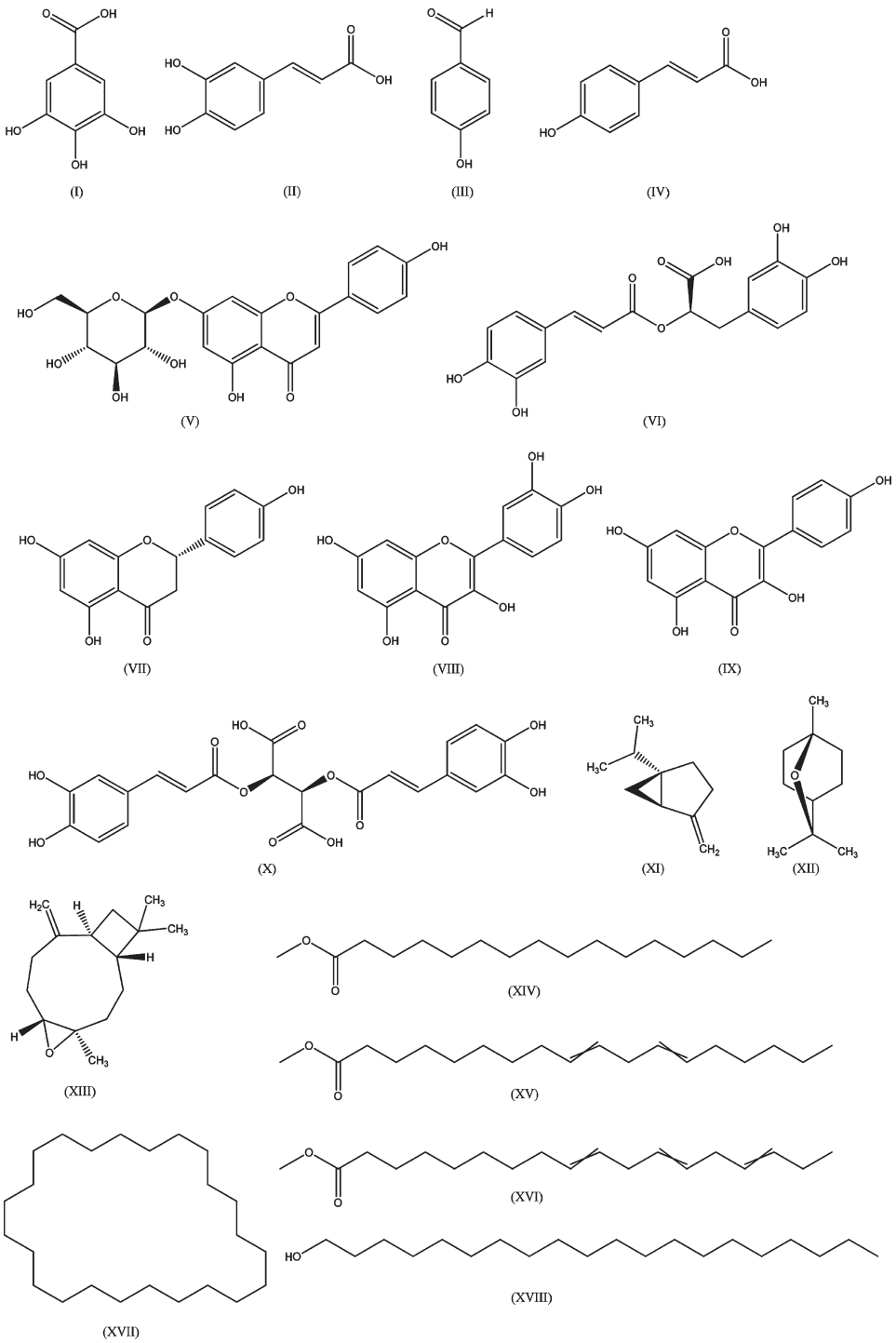

Figure 2. Phenolic components determined by HPLC/TOF-MS, i.e. gallic acid (I), caffeic acid (II), 4-hydroxybenzaldehyde (III), *p*-coumaric acid (IV), apigenin-7-glucoside (V), rosmarinic acid (VI), naringenin (VII), quercetin (VIII), kaempferol (IX) and chicoric acid (X), main essential oil components, i.e. sabinene (XI), eucalyptol (XII) and caryophyllene oxide (XIII), and main fatty acids and hydrocarbons, i.e. hexadecanoic acid methyl ester (XIV), 9,12-octadecadienoic acid methyl ester (XV), 9,12,15-octadecatrienoic acid methyl ester (XVI), cyclotetracosane (XVII) and 1-eicosanol (XVIII).

Total phenolic and flavonoid contents of the extracts were calculated as gallic acid and quercetin equivalents (eq.) respectively (Table 1). The highest-yield ME extract (2.37 g kg^{-1}) also had the highest levels of phenolics (56.83 g gallic acid eq. kg^{-1}) and flavonoids (35.25 g quercetin eq. kg^{-1}). However, only 36.84 and 0.65% respectively of these contents could be identified by authentic compounds available in our laboratory.

Role of rosmarinic acid

Plant extracts that are rich in rosmarinic acid (RA) have been used as preserving agents for many years. However, the benefits of RA are not limited to this use. It also has many biological activities (antiviral, antioxidant, antibacterial, antihepatitis, antitumor, etc.).[45,46] Although RA can be found in varying amounts in lower and higher plant families, members of the Lamiaceae and

Nepetoideae families produce RA much more in their metabolic pathways.[47] Yoshida *et al.*[48] isolated ten compounds, including RA, from *Centella asiatica* by activity-guided fractionation and tested the main fractions and isolated compounds against human adenocarcinoma (MK-1), HeLa and murine melanoma (B16F10) cells. Their results showed that one of the main fractions that contained RA and the RA that was isolated from this fraction had antiproliferative activity against HeLa and B16F10 cells. In addition, they suggested that the activity of this fraction originated from its high RA content. Tepe *et al.*[46] performed a series of tests *in vitro* to reveal the antioxidant potential of methanol extracts of two *Salvia* species and compared their results with values for pure RA tested by the same methods. They found that these extracts had good antioxidant properties and that there was a direct relation between RA content of the extracts and antioxidant potential.

Antiproliferative activities

Figure 1 shows the results of xCELLigence real-time monitoring of the proliferation of HeLa cells treated with different solvent extracts obtained from OVV. Differences in antiproliferative activity between solvent extracts are dependent on their phytochemical composition. The cell index measurements provide a clear indication that the antiproliferative activities of all solvent extracts are similar (Figs 1A–1D and Table 6) except for HE extract (Fig. 1E and Table 6), which is inactive. A recent study on the antiproliferative activity of fatty acids demonstrated that the viability of cancer cells declined below 25% after the incubation of fatty acids and hydrocarbons with cancer cells, including the HeLa cell line.[49] However, the major compound of HE extract is 1-eicosanol (38.02%; Table 7 and Fig. 2) in the present work as compared with oleic, palmitic and linoleic acids (28.30, 26.74 and 20.61% respectively) in the literature.[49] This could explain the lower activity of HE extract in the present work and also the lower proliferative activity at lower concentrations of 50 and 25 $\mu g\,mL^{-1}$ (Fig. 1E).

Figure 1C shows that HeLa cells were inactivated by EA extract at concentrations of 250 and 100 $\mu g\,mL^{-1}$ (pink and light blue lines), while lower activities were obtained at lower concentrations of 50 and 25 $\mu g\,mL^{-1}$ (dark blue line) in a dose-dependent manner. Although the antiproliferative activity of EA extract increased at concentrations of 250 and 100 $\mu g\,mL^{-1}$, higher activities were obtained only at 250 $\mu g\,mL^{-1}$ for ME (Fig. 1A), ET (Fig. 1B) and W (Fig. 1D) extracts. HE extract showed lower activity even at higher concentrations (Fig. 1E).

It was reported that methanol extracts of *Sideritis libanotica* ssp. *linearis*, containing flavonoids, showed significant anticancer activity against three cell lines, namely Vero cells (African green monkey kidney), C6 cells (rat brain tumor) and HeLa cells (human uterus carcinoma).[50] It was observed that methanol extracts inhibited the proliferation of these cell lines in a dose-dependent manner (from 25 to 250 $\mu g\,mL^{-1}$).[51] Similar findings were obtained in the present study: ME extract (Fig. 1A) as well as ET extract (Fig. 1B) showed higher activity at higher concentrations and contained several phenolic compounds as determined by HPLC/TOF-MS (Table 1). Rosmarinic acid was the most abundant phenolic compound determined in W, EA, ME and ET extracts (Table 1 and Fig. 2).

The correlation between activity and phenolic compounds seems to depend on the chemical structure of natural products and the type of cancer cells. Many polyphenols and flavonoids have been reported to inhibit proliferation and angiogenesis of tumor cells *in vitro*.[52] The anticarcinogenic activity of OVV is due to major bioactive compounds such as rosmarinic acid, as reported in a similar study on *Rosmarinus officinalis*.[51]

CONCLUSION

In recent years, there has been a growing demand for the of use natural remedies, as synthetic drugs can cause severe side effects. OVV would seem to be useful as a natural therapeutic remedy, since its different solvent extracts have potential antioxidant and antiproliferative activities. The results of this study can be seen as scientific support for the traditional and folkloric usage of OVV in northern Turkey. The antioxidant tests showed that there was a similarity between the activities of the extracts. The W extract of OVV had the highest activities in most of the tests. However, there was no correlation between the anticancer activity and antioxidant activities of the extracts. While EA extract had the highest anticancer activity, it showed the best antioxidant activity only in metal chelation. The preparation procedure of W extract of OVV is the same as that people follow when they prepare herbal tea at home. It might be concluded that this method is the best way to take antioxidants into the body and benefit from them. The findings suggest that the phenolic and flavonoid compounds exhibit antioxidant activity at lower concentrations and anticancer activity at higher concentrations in cells. The quantity of rosmarinic acid was found to be higher than that of other phenolic compounds in OVV and OA.

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