

In vivo anti-inflammatory and *in vitro* antioxidant activities of Mediterranean dietary plants

Filomena Conforti^{a,*}, Silvio Sosa^b, Mariangela Marrelli^a, Federica Menichini^a,
Giancarlo A. Statti^a, Dimitar Uzunov^c, Aurelia Tubaro^b,
Francesco Menichini^a, Roberto Della Loggia^b

^a Department of Pharmaceutical Sciences, University of Calabria, Italy

^b Department of Materials and Natural Resources, University of Trieste, Italy

^c Natural History Museum of Calabria and Botanic Garden, University of Calabria, Italy

Received 23 August 2007; received in revised form 23 October 2007; accepted 14 November 2007

Available online 19 November 2007

Abstract

Five hydroalcoholic extracts of edible plants from Calabria region (Italy) used in local traditional medicine for the treatment of inflammatory diseases were evaluated for their *in vivo* topical anti-inflammatory activity (inhibition of croton oil-induced ear oedema in mice) and *in vitro* antioxidant and antiradical properties (inhibition of linoleic acid oxidation and bovine brain liposomes peroxidation, DPPH radical scavenging). All the extracts showed an anti-inflammatory effect: 300 $\mu\text{g}/\text{cm}^2$ provoked oedema reductions ranging from 21 to 27%. All the extracts exerted also radical scavenging and/or antioxidant properties, the most active plant being *Mentha aquatica* L. (Lamiaceae) which contained the highest amount of phenolics (337 mg/g) and of flavonoids (15.75 mg/g). Moreover, the content and the composition of sterols were assessed by GC–MS in the examined plants *Borago officinalis* L. (Boraginaceae) contained the highest number of sterols.

© 2007 Elsevier Ireland Ltd. All rights reserved.

Keywords: Anti-inflammatory activity; Antioxidant activity; Flavonoid content; Phenolic content; Radical scavenging activity; Sterols composition

1. Introduction

It is commonly accepted that in a situation of oxidative stress, reactive oxygen species (ROS) such as superoxide ($\text{O}_2^{\bullet-}$, OOH^\bullet), hydroxyl (OH^\bullet) and peroxy (ROO^\bullet) radicals are generated. The ROS play an important role in the pathogenesis of various serious diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation (Aruoma, 1998; Kris-Etherton et al., 2004).

The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several

anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or radical scavenging mechanism as part of their activity (Perry et al., 1999; Lin and Huang, 2002; Repetto and Llesuy, 2002). The mechanism of inflammation injury is attributed, in part, to release of reactive oxygen species from activated neutrophils and macrophages. This over production leads to tissue injury by damaging macromolecules and lipid peroxidation of membranes (Winrow et al., 1993; Gutteridge, 1995). In addition, ROS propagate inflammation by stimulating release of cytokines such as interleukin-1, tumor necrosis factor- α , and interferon- γ , which stimulate recruitment of additional neutrophils and macrophages. Thus free radicals are important mediators that provoke or sustain inflammatory processes and, consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation (Delaporte et al., 2002; Geronikaki and Gavalas, 2006).

Most clinically important medicines belong to steroidal or non-steroidal anti-inflammatory chemical therapeutics for treatment of inflammation-related diseases. Though these have

Abbreviations: ROS, reactive oxygen species; LDL, low-density lipoproteins; TLC, thin-layer chromatography plates; TBA, thiobarbituric acid; PBS, phosphate-buffered saline; BHT, butylated hydroxytoluene; DPPH, propyl gallate, 2,2-diphenyl-1-picrylhydrazyl; GC–MS, gas chromatography–mass spectrometry; MDA, malonaldehyde.

* Corresponding author. Tel.: +39 0984 493063; fax: +39 0984 493298.

E-mail address: filomena.conforti@unical.it (F. Conforti).

potent activity, long-term administration is required for treatment of chronic disease. Furthermore, these drugs have various and severe adverse effects. Therefore, naturally originated agents with very little side effects are desirable to substitute chemical therapeutics.

Epidemiological and experimental studies reveal a negative correlation between the consumption of diets rich in fruit and vegetables and the risks for chronic diseases, such as cardiovascular diseases, arthritis, chronic inflammation and cancers (Saleem et al., 2002; Prior, 2003; Chen et al., 2005; Zhang et al., 2005). These physiological functions of fruits and vegetables may be partly attributed by their abundance of phenolics.

There has been a growing interest in phenolic components of fruits and vegetables, which may promote human health or lower the risk for disease. Recent studies have focused on health functions of phenolics, including flavonoids from fruit and vegetables (Saleem et al., 2002; You-dim et al., 2002; Qian et al., 2004; Chen et al., 2006).

In the search for sources of natural antioxidants, in the last years some medicinal plants have been extensively studied for their antioxidant activity and radical scavenging activity (De las Heras et al., 1998; Desmarchelier et al., 2000; Schinella et al., 2002; VanderJagt et al., 2002).

The present paper deals with a preliminary screening of the following Italian plants: *Borago officinalis* L., *Capparis sicula* Veill. subsp. *sicula*, *Malva sylvestris* L., *Mentha aquatica* L. and *Raphanus raphanistrum* L. subsp. *raphanistrum*. They are spontaneous edible plants present in the area of Alto Ionio cosentino, a territory characterized by different vegetation belt from the sea level to the highest peak of Pollino Mt. (about 2000 m a.s.l.), mostly rich in Mediterranean elements.

Plants selected for this study were chosen because of their use in local traditional medicine for the treatment of external inflammation and inflammation-related diseases such as rheumatism (Li et al., 2003; Lee et al., 2006). Information on the traditional uses of these plants was collected through structured interviews for a comparison of the folk information and experimental data.

In this work, five extracts obtained from the Italian plants listed before were studied to assess their *in vivo* anti-

inflammatory activity which, to the best of our knowledge, is not reported for these plants so far. Moreover, considering that antioxidants and free radical scavengers can exert also an anti-inflammatory effect (Geronikaki and Gavalas, 2006), these extracts were evaluated also for these activities. The radical scavenging activity was assessed with DPPH test (2,2-diphenyl-1-picrylhydrazyl). The β -carotene bleaching test and the bovine brain peroxidation assay were used to evaluate the antioxidant activity. The results were compared with those obtained with reference products: ascorbic acid and propyl gallate. Anti-inflammatory activity was evaluated as inhibition of the croton oil-induced ear oedema in mice, after topical application. Furthermore, the total phenolic content was determined by the Folin-Ciocalteu method and total flavonoid content by a method based on the formation of complex flavonoid-aluminum. Moreover, the aim of the present study was to evaluate the chemical properties of the extracts of plants concerning the composition of sterols. Plant sterols have been investigated as an alternative method in lowering plasma cholesterol levels, and several studies have shown that they significantly reduce plasma total and LDL cholesterol. Thus, human intake of phytosterols is governed by eating habits and availability of the source of plant sterols (Moghadasian, 2000). In this study the content and the composition of sterols in edible plants were assessed for the first time.

2. Materials and methods

2.1. Plant materials

The different botanical taxa studied in this work are shown in Table 1, together with some information about their common name, date and site of collection, and selected medicinal uses. The present research site (Cassano allo Ionio) is located in the Cosenza district, north of Calabria, Italy. The investigated area includes plane, hilly and sub-mountainous belts of the East sector of Massiccio del Pollino complex, characterized by calcareous substrata with Mediterranean vegetation. Field data were collected during the periods of March 2005 and July 2005 in the area of Cassano allo Ionio. Ethnobotanical

Table 1
Latin names, common name, plant part used, collection site and data, selected uses and yield of extraction of the studied plants

Scientific name (family)	Voucher specimen	Common name	Plant part used	Collection site	Collection date	Local traditional uses	Yield (%)
<i>Borago officinalis</i> L. (Boraginaceae)	CLU 18048	Borage	Leaves	Madonna della catena	March 2005	Diuretic, cough, external inflammations, diaphoretic	15.74
<i>Capparis sicula</i> Veill. subsp. <i>sicula</i> (Capparidaceae)	CLU 18060	Caper	Leaves, fruits	Madonna della catena	July 2005	Anti-rheumatic, hepatic stimulants and protectors	15.05
<i>Malva sylvestris</i> L. (Malvaceae)	CLU 18056	Mallow	Leaves	Madonna delle grazie	May 2005	Abscesses, anti-inflammatory, mild laxative	10.23
<i>Mentha aquatica</i> L. (Lamiaceae)	CLU 18055	Mint	Leaves	Lappicello	April 2005	Anti-rheumatic, vermifuge	16.12
<i>Raphanus raphanistrum</i> L. subsp. <i>raphanistrum</i> (Brassicaceae)	CLU 18050	Radish	Leaves	Giastreta	May 2005	Anti-rheumatic	14.32

information on the uses of wild plants was gathered through structured interviews. Prior informed consent was obtained for all interviews conducted. The most useful information came by old people, since most of young interviewed persons do not know anything about this aspect of local traditions.

The collected plants were authenticated by Dr. Uzunov from Botanic Garden, University of Calabria, Italy, and the plants were deposited at the Natural History Museum of Calabria. Voucher numbers are also indicated in Table 1.

2.2. Chemicals

Methanol, ethanol, ethyl acetate, petroleum ether, diethyl ether, H₂SO₄, chloroform, HCl, KOH, butanol, hexane, silica gel 70–230 mesh and thin-layer chromatography plates (TLC) were obtained from VWR International s.r.l. (Milan, Italy). Thiobarbituric acid (TBA), phosphate-buffered saline (PBS), bovine brain extract, FeCl₃, ascorbic acid, butylated hydroxytoluene (BHT), propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), β -carotene, linoleic acid, Tween 20, Folin-Ciocalteu reagent, chlorogenic acid, croton oil and indomethacin were obtained from Sigma–Aldrich S.p.A. (Milan, Italy). Ketamine hydrochloride was purchased from Virbac s.r.l. (Milan, Italy). All other reagents, of analytical grade, were Carlo Erba products (Milan, Italy).

2.3. Preparation of samples

The plant material was air dried until dryness at room temperature, cut into small pieces, and then extracted with 70% aqueous EtOH through maceration (48 h for three times) at room temperature. The resultant total extracts were dried under reduced pressure to determine the weight (Table 1). Total phenolic and flavonoid content was determined for each total extract. Extracts were then partitioned between 90% MeOH and *n*-hexane, and the hexane fractions were analysed by gas chromatography–mass spectrometry (GC–MS). The content and the composition of sterols were assessed.

2.4. Determination of total phenolic content

Total phenolic content of the total extracts was determined using Folin-Ciocalteu reagent and chlorogenic acid as standard (Singleton and Rossi, 1965). Fifty milligrams of the extracts were weighed into 50 ml plastic extraction tube and vortexed with 25 ml of the extraction solvent (40 ml acetone:40 ml methanol:20 ml water:0.1 ml acetic acid). Then, the samples with the extraction solvent were heated at 60 °C (water bath) for 1 h, allowed to cool to room temperature, and homogenized for 30 s with a sonicator. Two hundred microliters (three replicates) were introduced into screw cap test tubes; 1.0 ml of Folin-Ciocalteu's reagent and 1.0 ml of sodium carbonate (7.5%) were added. The tubes were vortexed and allowed to stand for 2 h. The absorption at 726 nm was measured (Perkin-Elmer Lambda 40 UV/VIS spectrophotometer) and the total phenolic content was expressed as milligram of chlorogenic acid equivalents per gram dry material.

2.5. Determination of total flavonoid content

Total flavonoids were estimated in the plant extracts using a colorimetric method based on the formation of a complex flavonoid-aluminum, having the absorbivity maximum at 430 nm (Quettier-Deleu et al., 2000). All determinations were made in triplicate and values were calculated from a calibration curve obtained with quercetin. Final results were expressed as milligram of quercetin equivalent per gram of dried weight.

2.6. Determination of sterol content and composition

The *n*-hexane fraction analysis was performed using a Hewlett-Packard gas chromatograph, model 5890 equipped with a mass spectrometer, model 5972 series II, and controlled by HP software equipped with capillary column 30 m \times 0.25 mm, static phase SE30, using programmed temperature from 60 to 280 °C (rate 16°/min); the detector and the injector have been set to a temperature of 280 and 250 °C, respectively (split vent flow 1 ml min⁻¹). Compounds identification was verified according to relative retention time and mass spectra with those of Wiley 138 library data of the GC–MS system (Hewlett-Packard Co.).

2.7. Antioxidant and free radical scavenging activity assays

2.7.1. DPPH assay

This experimental procedure was adapted from Wang et al. (1998). In an ethanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (final concentration was 1.0 \times 10⁻⁴ M), test extracts at different concentrations were added. The reaction mixtures were shaken vigorously and then kept in the dark for 30 min. The absorbance of the resulting solutions was measured in 1 cm cuvettes, using a Perkin-Elmer Lambda 40 UV/VIS spectrophotometer at 517 nm, against blank without DPPH. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. This activity is given as % DPPH radical scavenging that is calculated in the equation:

$$\% \text{ DPPH radical scavenging} = \frac{\text{sample absorbance}}{\text{control absorbance}} \times 100$$

The DPPH solution without sample solution was used as control. All tests were run in triplicate and averaged. Ascorbic acid was used as positive control.

2.7.2. Bovine brain peroxidation assay

The lipid peroxidation activity was evaluated using the TBA test described by Fernandez et al. (1997) modified as reported in Conforti et al. (2002). The TBA reaction is based on the fact that peroxidation of most membrane systems leads to formation of small amounts of free malonaldehyde (MDA). Absorbance at 532 nm was determined on a Perkin-Elmer Lambda 40 UV/VIS spectrophotometer. Total extracts were tested for their antioxidant activity against liposomes which were prepared from bovine brain extract in phosphate-buffered saline (5 mg/ml). Propyl gallate was used as a positive control (Jacobi et al., 1999). The inhibition of lipid peroxidation in % was calculated by the

following equation:

$$\% \text{ inhibition} = \left[\frac{(\text{FRM} - B) - (\text{Et} - B - \text{EA})}{\text{FRM} - B} \right] \times 100$$

where FRM is the absorbance of the control reaction and ET is the absorbance in the presence of the sample. The absorbance of liposomes only (*B*) and extract alone (EA) were also taken in account.

2.7.3. β -Carotene bleaching test

Antioxidant activity was determined using β -carotene bleaching test (Amin et al., 2004). Briefly, 1 ml of β -carotene solution (0.2 mg/ml in chloroform) was added to 0.02 ml of linoleic acid and 0.2 ml of 100% Tween 20. The mixture was then evaporated at 40 °C for 10 min by means of a rotary evaporator to remove chloroform and immediately diluted with 100 ml of distilled water. The water was added slowly to the mixture and agitated vigorously to form an emulsion. Five milliliters of the emulsion was transferred into different test tubes containing 0.2 ml of samples in 70% ethanol at different concentrations (100, 50, 25, 10, 5 and 1 $\mu\text{g/ml}$). 0.2 ml of 70% ethanol in 5 ml of the above emulsion was used as control. Standard (propyl gallate) at the same concentration as samples was used for comparison. The tubes were then gently shaken and placed at 45 °C in a water bath for 60 min. The absorbance of the samples, standard and control was measured at 470 nm using a Perkin-Elmer Lambda 40 UV/VIS spectrophotometer against a blank, consisting of an emulsion without β -carotene. The measurement was carried out at initial time ($t=0$) and successively at 30 and 60 min. All samples were assayed in triplicate and averaged.

The antioxidant activity (AA) was measured in terms of successful bleaching β -carotene by using the following equation:

$$\text{AA} = \left(1 - \frac{A_0 - A_t}{A_0^\circ - A_t^\circ} \right) \times 100$$

where A_0 and A_0° are the absorbance values measured at the initial incubation time for samples/standard and control, respectively, while A_t and A_t° are the absorbance values measure in the samples/standard and control, respectively, at $t=30$ and 60 min.

2.8. Topical anti-inflammatory activity

The topical anti-inflammatory activity was evaluated as inhibition of the croton oil-induced ear oedema in mice (Tubaro et al., 1985). All animal experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609 ECC).

Male CD-1 mice (28–32 g; Harlan-Italy, Udine, Italy) were kept for 1 week before the experiment, at constant conditions of temperature (21 ± 1 °C) and humidity (60–70%), and a fixed artificial light cycle (07.00–19.00 h). Inflammation was induced always in the late morning (10.00–12.00 h). Mice were anaesthetised with ketamine hydrochloride (145 mg kg^{-1} , intraperitoneally) and inflammatory response was induced on the inner surface of the right ear (surface: about 1 cm^2) by applica-

tion of 80 μg of croton oil suspended in 42% aqueous ethanol. Control animals received only the irritant, whereas other animals received the irritant together with the tested substances. At the maximum of the oedematous response, 6 h later, mice were sacrificed and a plug ($\varnothing 6$ mm) was removed from both the treated (right) and the untreated (left) ears. Oedema was measured as the weight difference between the two plugs. The anti-inflammatory activity was expressed as percentage of the oedema reduction in treated mice compared to the control mice. As reference, the non-steroidal anti-inflammatory drug (NSAID) indomethacin was used.

2.9. Statistical analysis

Data were expressed as means \pm S.E. Statistical analysis was performed by using Student's *t*-test or by one-way analysis of variance followed by the Dunnett's test for multiple comparisons of unpaired data. Differences were considered significant at $P \leq 0.05$. The inhibitory concentration 50% (IC_{50}) was calculated from the Prism dose–response curve (statistical programme) obtained by plotting the percentage of inhibition versus the concentrations.

3. Results

3.1. Radical scavenging activity

The results on the free radical scavenging activity of the different extracts are shown in Table 2. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability. The preparations were able to reduce the stable free radical DPPH to the yellow-coloured 1,1-diphenyl-2-picrylhydrazyl. The best free radical (DPPH) scavenging activity was exerted by *Mentha aquatica* extract ($\text{IC}_{50} = 29$ $\mu\text{g/ml}$). The lowest radical scavenging activity was exhibited by *Malva sylvestris* ($\text{IC}_{50} = 606$ $\mu\text{g/ml}$). As reference, the IC_{50} value of ascorbic acid was 2 $\mu\text{g/ml}$.

3.2. Antioxidant activity

At the β -carotene bleaching test after 30-min incubation, *Borago officinalis* and *Capparis sicula* subsp. *sicula* extracts showed the highest inhibition of linoleic acid oxidation ($\text{IC}_{50} = 2$ and 9 $\mu\text{g/ml}$, respectively). The lowest activity was exhibited by *Raphanus raphanistrum* subsp. *raphanistrum* extract ($\text{IC}_{50} = 57$ $\mu\text{g/ml}$). The antioxidant activity of the extracts decreased during the reaction time. Anyway, after 60-min incubation, the IC_{50} values of most active ones (*Borago officinalis* and *Capparis sicula* Veill. subsp. *sicula*) were 4 and 16 $\mu\text{g/ml}$, respectively, whereas IC_{50} value of *Raphanus raphanistrum* subsp. *raphanistrum* extract was >100 $\mu\text{g/ml}$. As reference, IC_{50} of propyl gallate was 1 $\mu\text{g/ml}$, both after 30- and 60-min incubation (Table 2).

Using liposomes prepared from bovine brain, the highest antioxidant activity was observed for *Mentha aquatica* extract ($\text{IC}_{50} = 9$ $\mu\text{g/ml}$). *Malva sylvestris* extract showed the lowest activity ($\text{IC}_{50} > 1$ mg/ml). As reference, the IC_{50} of propyl gallate was 7 $\mu\text{g/ml}$ (Table 2).

Table 2
IC₅₀ values of antioxidant activities of plant extracts

Hydroalcoholic extract	IC ₅₀ (µg/ml) ^a			
	DPPH	Lipid peroxidation	β-Carotene bleaching test	
			30 min of incubation	60 min of incubation
<i>Borago officinalis</i>	58 ± 0.11	38 ± 0.10	2 ± 0.03	4 ± 0.03
<i>Capparis sicula</i> subsp. <i>sicula</i>	114 ± 0.23	86 ± 0.13	9 ± 0.07	16 ± 0.06
<i>Malva sylvestris</i>	606 ± 0.57	>1000	13 ± 0.08	28 ± 0.09
<i>Mentha aquatica</i>	29 ± 0.09	9 ± 0.06	31 ± 0.09	>100
<i>Raphanus raphanistrum</i> subsp. <i>raphanistrum</i>	121 ± 0.28	176 ± 0.31	57 ± 0.12	>100
Ascorbic acid ^b	2 ± 0.03	–	–	–
Propyl gallate ^b	–	7 ± 0.05	1 ± 0.01	1 ± 0.01

^a ±S.E.M. (n = 3).

^b Propyl gallate and ascorbic acid were used as positive control.

Table 3
Topical anti-inflammatory activity of the hydroalcoholic extracts

Hydroalcoholic extract	Dose (µg/cm ²)	Oedema (mg) mean ± S.E.	% oedema reduction
Controls	–	6.7 ± 0.3	–
<i>Borago officinalis</i>	300	5.3 ± 0.4	21
<i>Capparis sicula</i> subsp. <i>sicula</i>	300	5.1 ± 0.3	24
<i>Malva sylvestris</i>	300	5.3 ± 0.3	21
<i>Mentha aquatica</i>	300	4.9 ± 0.4	27
<i>Raphanus raphanistrum</i> subsp. <i>raphanistrum</i>	300	5.0 ± 0.3	25
Indomethacin	100	2.9 ± 0.3	57

p < 0.05 at the analysis of variance, as compared with controls. No. animals = 10.

3.3. Topical anti-inflammatory activity

Results on the topical anti-inflammatory activity of the plant extracts, administered at the dose of 300 µg/cm², are reported in Table 3. All the extracts reduced the oedematous response to a certain extent. The most active was *Mentha aquatica* extract, which induced 27% oedema inhibition. The other plant extracts induced oedema inhibitions between 25 and 21%. As reference, the non-steroidal anti-inflammatory drug indomethacin reduced the oedematous response by 57% at the dose of 100 µg/cm².

3.4. Total phenolic and flavonoid content

Table 4 reports the results of total phenolics and total flavonoids analyses. The amounts of total phenolics varied widely in the different analysed extracts and ranged from 28 to 337 mg/g of extract. This variation can be expected for plant extracts due to the presence of other constituents and/or the presence of different types of phenols. Among plant extracts, *Mentha aquatica* contained the highest amount of phenolics

(337 mg/g) followed by *Capparis sicula* Veill. subsp. *sicula* (113 mg/g), *Borago officinalis* (97 mg/g), whereas the lowest level was found in *Raphanus raphanistrum* subsp. *raphanistrum* and *Malva sylvestris* (28 mg/g).

Among plant extracts, *Mentha aquatica* contained the highest amount of flavonoids (15.75 mg/g), whereas the lowest level was found in *Capparis sicula* Veill. subsp. *sicula* (2.52 mg/g).

Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the total antioxidant activity of many fruits and vegetables (Luo et al., 2002). But, in general, we have found no correlation between antioxidant activity and total phenol/flavonoid content as determined by square regression coefficient ($r^2 = 0.24$). Some plants have high phenol/flavonoid contents but low antioxidant activity.

3.5. Sterol content

Table 5 summarizes the sterol composition of each plants determined by GC–MS. Nine compounds were identified. Examination of the results showed that *Borago officinalis*

Table 4
Total phenolic content of the hydroalcoholic extracts using Folin-Ciocalteu method

Hydroalcoholic extract	Total phenolic content (mg/g) ^a	Total flavonoid content (mg/g) ^a
<i>Borago officinalis</i>	97 ± 1.03	8.53 ± 0.02
<i>Capparis sicula</i> subsp. <i>sicula</i>	113 ± 1.41	2.52 ± 0.06
<i>Malva sylvestris</i>	28 ± 0.35	4.77 ± 0.07
<i>Mentha aquatica</i>	337 ± 2.15	15.75 ± 0.25
<i>Raphanus raphanistrum</i> subsp. <i>raphanistrum</i>	28 ± 0.31	7.95 ± 0.11

^a Values expressed as chlorogenic acid equivalents/g of extract.

Table 5
Sterols composition of dietary plants

Sterol ^a	RT ^b	RAP ^c				
		<i>Borago officinalis</i>	<i>Capparis sicula</i> subsp. <i>sicula</i>	<i>Malva sylvestris</i>	<i>Mentha aquatica</i>	<i>Raphanus raphanistrum</i> subsp. <i>raphanistrum</i>
Cholesterol	29.74	0.4	–	–	–	–
Ergosta-5,24-dien-3-ol (3 β)	31.94	0.8	–	–	–	–
Ergost-5-en-3-ol (3 β)	31.96	–	1.3	–	–	2.7
Campesterol	32.12	6.4	–	0.3	–	–
Stigmasta-5,23-dien-3 β -ol	32.81	–	Tr	–	0.5	–
Stigmasterol	32.83	1.2	–	1.1	–	–
γ -Sitosterol	34.44	6.3	19.4	3.9	11.5	11.9
Stigmasta-5,24(28)-dien-3-ol (3 β ,22E)	34.89	2.1	–	–	–	–
4 α -Methyl-5 α -ergosta-7,24(28)-dien-3 β -ol	35.06	0.4	–	–	–	–

^a Compounds listed in order of elution from SE30 MS column.

^b Retention time (as min).

^c Relative area percentage (peak area relative to total peak area %).

contained the highest number of sterols. Among them, campesterol (6.4% of total extract) and γ -sitosterol (6.3%) were found to be the major constituents. *Mentha aquatica* contained the lowest number of sterols. γ -Sitosterol was the predominant sterol since it was largely distributed. This sterol was identified in each plant and it was mainly contained in *Capparis sicula* Veill. subsp. *sicula*, *Mentha aquatica* and *Raphanus raphanistrum* ssp. *raphanistrum* (19.4, 11.5 and 11.9%, respectively). Cholesterol and other three sterols were found in *Borago officinalis* only.

4. Discussion and conclusions

The data presented in this study demonstrate that almost all the reported species possess antioxidant and free radical scavenging activity. Indeed, their hydroalcoholic extracts inhibited linoleic acid oxidation, liposomes peroxidation and/or scavenged DPPH radical *in vitro*. They showed different behaviour in the three *in vitro* assays, probably due to the different mechanisms involved in the steps of oxidation process. The observed *in vitro* activities suggest that the investigated plant extracts could exert protective effects also *in vivo* against oxidative and free radical injuries occurring in different pathological conditions. With this respect, the most promising plants appear to be *Mentha aquatica* which is actually used by the local people against arthritic diseases which pathogenesis is at least partly attributed to ROS (Hitchon and El-Gabalawy, 2004). Previous studies shown that *Mentha* extracts appear to be able to prevent the propagation of the lipid peroxidation process in a complex lipid matrix, such as a foodstuff or biological membrane (Dorman et al., 2003; Mimica-Dukic et al., 2003).

Several studies evaluated the relationships between antioxidant activity of plant products and their phenolic content. Some authors found a correlation between the phenolic content and the antioxidant activity, while others found no relationship. Velioglu et al. (1998) reported a high correspondence between total phenolic content and antioxidant activity in selected fruits, vegetables and grain products. On the other hand, no correlation between antioxidant activity and phenolic content was observed

by Kähkönen et al. (1999) on some plant extracts containing phenolic compounds.

In this study, the findings do not show any relationship between antioxidant activity and total phenolic content. Among all the extracts analysed, a significant total phenolic content and radical scavenging and antioxidant activities were found only for *Mentha aquatica*. On the other hand, extracts with higher radical scavenging and antioxidant activities did not show a high phenolic content.

The relatively high antioxidant and free radical scavenger activity of extracts containing low phenolic content suggests that the type of phenolics is determinant for these activities rather than their amounts. These results agree with those of Kähkönen et al. (1999) and Shahidi and Marian (2003) who reported that differences in antioxidant activities of plant extracts could be due to different qualitative and quantitative composition of their phenolic constituents, from phenolic acids to flavonoids and their derivatives. For instance, the antioxidant activities of phenolic acids and their derivatives, such as esters, depend on the number of hydroxy groups in the molecules (Soobrattee et al., 2005).

Several findings suggest that the phytosterols such as β -sitosterol are responsible, at least in part, for preventive effects on the development of diseases due to reactive oxygen species (Vivacons and Moreno, 2005). Moreover, Yoshida and Niki (2003) showed the antioxidant effects of the phytosterols β -sitosterol, stigmasterol, and campesterol, against lipid peroxidation.

The obtained data reveal that all the reported species possess topical anti-inflammatory properties, since their extracts inhibited the Croton oil-induced ear oedema in mice. This activity could be due to the presence of antioxidants, according to previous studies (Geronikaki and Gavalas, 2006). Previous studies demonstrated that stigmasterol exert topical anti-inflammatory activity and that some phytosterols inhibit 12-*O*-tetradecanoylphorbol acetate-induced oedema (Kimura et al., 1995; Garcia et al., 1999).

The ethnobotanical inquiry revealed that studied species are used in the local folk medicine against inflammatory-based diseases or related conditions, such as rheumatisms. The obtained

results support the validity of the traditional uses of these species against inflammatory disorders.

Anyway, one of the species under study is reported to contain toxic secondary metabolites. *Borago officinalis* contains small amounts of pyrrolizidine alkaloids (El-Shazly et al., 1996; Bruneton, 1999). Therefore, an excessive or prolonged use of this plant should be avoided even if its content of the toxic principles is low.

In conclusion, this work reveals that the Italian flora, among which the species *Mentha aquatica*, can be an interesting source of anti-inflammatory and antioxidant principles, with a potential use in different fields (food, cosmetics and pharmaceutical).

References

- Amin, I., Zamaliah, M.M., Chin, W.F., 2004. Total antioxidant activity and phenolic content in selected vegetables. *Food Chemistry* 87, 581–586.
- Aruoma, O.I., 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. *Journal of the American Oil Chemists Society* 75, 199–212.
- Bruneton, J., 1999. *Toxic Plants Dangerous to Humans and Animals*. Lavoisier Publishing, Paris, p. 206.
- Chen, C.C., Liu, L.K., Hsu, J.D., Huang, H.P., Yang, M.Y., Wang, C.J., 2005. Mulberry extract inhibits the development of atherosclerosis in cholesterol-fed rabbits. *Food Chemistry* 91, 601–607.
- Chen, P.N., Chu, S.C., Chiou, H.L., Kuo, W.H., Chiang, C.L., Hsieh, Y.S., 2006. Mulberry anthocyanins, cyaniding 3-rutinoside and cyaniding-3-glucoside, exhibited and inhibitory effect on the migration and invasion of a human lung cancer cell line. *Cancer Letters* 28, 248–259.
- Conforti, F., Statti, G.A., Tundis, R., Menichini, F., Houghton, P.J., 2002. Antioxidant activity of methanolic extract of *Hypericum triquetrifolium* Turra aerial part. *Fitoterapia* 73, 479–483.
- Delaporte, R.H., Sánchez, G.M., Cuellar, A.C., Giuliani, A., Palazzo de Mello, J.C., 2002. Anti-inflammatory activity and lipid peroxidation inhibition of iridoid lamiide isolated from *Bouchea fluminensis* (Vell.) Mold. (Verbenaceae). *Journal of Ethnopharmacology* 82, 127–130.
- De las Heras, B., Slowing, K., Benedi, J., Carretero, E., Ortega, T., Toledo, C., Bermejo, P., Iglesias, I., Abad, M.J., Gómez-Serranillos, P., Liso, P.A., Villar, A., Chiriboga, X., 1998. Antiinflammatory and antioxidant activity of plants used in traditional medicine in Ecuador. *Journal of Ethnopharmacology* 61, 161–166.
- Desmarchelier, C., Ciccía, G., Coussio, J., 2000. Recent advances in the search for antioxidant activity in South American plants. In: Atta-ur-Rahman (Ed.), *Studies in Natural Products Chemistry*, vol. 22. Elsevier, Amsterdam, pp. 343–367.
- Dorman, H.J., Kosar, M., Kahlos, K., Holm, Y., Hiltunen, R., 2003. Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties, and cultivars. *Journal of Agricultural and Food Chemistry* 51, 4563–4569.
- El-Shazly, A., Sargm, T., Ateya, A., Abdel Aziz, A., El-Dahmy, S., Witte, L., Wink, K., 1996. Pyrrolizidine alkaloids from *Echium setosum* and *Echium vulgare*. *Journal of Natural Product* 59, 310–313.
- Fernandez, J., Perez-Alvarez, J., Fernandez-Lopez, J., 1997. Thiobarbituric acid test for monitoring lipid oxidation in meat. *Food Chemistry* 59, 345–353.
- García, M.D., Sáenz, M.T., Gómez, M.A., Fernández, M.A., 1999. Topical anti-inflammatory activity of phytosterols isolated from *Eryngium foetidum* on chronic and acute inflammation models. *Phytotherapy Research* 13, 78–80.
- Geronikaki, A.A., Gavalas, A.M., 2006. Antioxidants and anti-inflammatory diseases: synthetic and natural antioxidants with anti-inflammatory activity. *Combinatorial Chemistry & High Throughput Screening* 9, 425–442.
- Gutteridge, J.M., 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical Chemistry* 41, 1819–1828.
- Hitchon, C.A., El-Gabalawy, H.S., 2004. Oxidation in rheumatoid arthritis. *Arthritis Research & Therapy* 6, 265–278.
- Jacobi, H., Hinrichsen, M., Web, D., Witte, I., 1999. Induction of lipid peroxidation in human fibroblasts by the antioxidant propyl gallate in combination with copper (II). *Toxicology Letters* 110, 183–190.
- Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S., Heinonen, M., 1999. Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry* 47, 3954–3962.
- Kimura, Y., Yasukawa, K., Takido, M., Akihisa, T., Tamura, T., 1995. Inhibitory effect of some oxygenated stigmastane-type sterols on 12-teradecanoylphorbol-13-acetate-induced inflammation in mice. *Biological & Pharmaceutical Bulletin* 18, 617–619.
- Kris-Etherton, P.M., Lefevre, M., Beecher, G.R., Gross, M.D., Keen, C.L., Etherton, T.D., 2004. Bioactive compounds in nutrition and health-research methodologies for establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis. *Annual Review of Nutrition* 24, 511–538.
- Lee, H.J., Hyun, E.A., Yoon, W.J., Kim, B.H., Rhee, M.H., Kang, H.K., Cho, J.Y., Yoo, E.S., 2006. *In vitro* anti-inflammatory and anti-oxidative effects of *Cinnamomum camphora* extracts. *Journal of Ethnopharmacology* 103, 208–216.
- Li, R.W., Myers, S.P., Leach, D.N., Lin, G.D., Leach, G., 2003. A cross-cultural study: anti-inflammatory activity of Australian and Chinese plants. *Journal of Ethnopharmacology* 85, 25–32.
- Lin, C.C., Huang, P.C., 2002. Antioxidant and hepatoprotective effects of *Acatopanax senticosus*. *Phytotherapy Research* 14, 489–494.
- Luo, X.D., Basile, M.J., Kennelly, E.J., 2002. Polyphenolic antioxidants from the fruits of *Chrysophyllum crinito* L. (star apple). *Journal of Agricultural and Food Chemistry* 50, 1379–1382.
- Mimica-Dukic, N., Bozin, B., Sokovic, M., Mihajlovic, B., Matavulj, M., 2003. Antimicrobial and antioxidant activities of three *Mentha* species essential oils. *Planta Medica* 69, 413–419.
- Moghadasian, M.H., 2000. Minireview. Pharmacological properties of plant sterols *in vivo* and *in vitro* observations. *Life Sciences* 67, 605–615.
- Perry, E.K., Pickering, A.T., Wang, W.W., Houghton, P.J., Perru, N.S., 1999. Medicinal plants and Alzheimer's disease: from ethnobotany to phytotherapy. *Journal of Pharmacy and Pharmacology* 51, 527–534.
- Prior, R.L., 2003. Fruits and vegetables in the prevention of cellular oxidative damage. *American Journal of Clinical Nutrition* 78, 570S–578S.
- Qian, J.Y., Liu, D., Huang, A.G., 2004. The efficiency of flavonoids in polar extracts of Lycium chinese Mill fruits as free radical scavenger. *Food Chemistry* 87, 283–288.
- Quettier-Deleu, C., Gressier, B., Vasseur, J., Dine, T., Brunet, C., Luyckx, M., Cazin, M., Cazin, J.C., Bailleul, F., Trotin, F., 2000. Phenolic compounds and antioxidant activities of buckwheat hulls and flour. *Journal of Ethnopharmacology* 72, 35–42.
- Repetto, M.G., Llesuy, S.F., 2002. Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. *Brazilian Journal of Medicine and Biological Research* 35, 523–534.
- Saleem, A., Husheem, M., Harkonen, P., Pihlaja, K., 2002. Inhibition of cancer cell growth by crude extract and the phenolics of *Terminalia chebula* retz. *Fruit. Journal of Ethnopharmacology* 81, 327–336.
- Schinella, G.R., Tournier, H.A., Prieto, J.M., Mordujovich de Buschiazzo, P., Rios, J.L., 2002. Antioxidant activity of anti-inflammatory plant extracts. *Life Sciences* 70, 1023–1033.
- Shahidi, F., Marian, N., 2003. *Phenolics in Food and Nutraceuticals*, vol. 1. CRS Press LLC, Boca Raton, FL, pp. 144–150.
- Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture* 16, 144–158.
- Soobrattee, M.A., Neergheen, V.S., Luximon-Ramma, A., Aruoma, O.I., Bahorun, T., 2005. Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutation Research* 579, 200–213.
- Tubaro, A., Dri, P., Delbello, G., Zilli, C., Della Loggia, R., 1985. The croton oil ear test revisited. *Agents and Actions* 17, 347–349.
- VanderJagt, T.J., Ghattas, R., Vanderjagt, D.J., Crossey, M., Glew, R.H., 2002. Comparison of the total antioxidant content of 30 widely used medicinal plants of New Mexico. *Life Sciences* 70, 1035–1040.

- Velioglu, Y.S., Mazza, G., Gao, L., Oomah, B.D., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry* 46, 4113–4117.
- Vivacons, M., Moreno, J.J., 2005. Beta-sitosterol modulates antioxidant enzyme response in RAW 264.7 macrophages. *Free Radical Biology & Medicine* 39, 91–97.
- Wang, M., Li, J., Rangarajan, M., Shao, Y., La Voie, E.J., Huang, T.-C., Ho, C.-T., 1998. Antioxidative phenolic compounds from sage (*Salvia officinalis*). *Journal of Agricultural and Food Chemistry* 46, 4869–4873.
- Winrow, V.R., Winyard, P.G., Morris, C.J., Blake, D.R., 1993. Free radicals in inflammation: second messengers and mediators of tissue destruction. *British Medical Bulletin* 49, 506–522.
- Yoshida, Y., Niki, E., 2003. Antioxidant effects of phytosterol and its components. *Journal of Nutritional Science and Vitaminology* 49, 277–280.
- You-dim, K.A., McDonald, J., Kalt, W., Joseph, J.A., 2002. Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. *Journal of Nutritional Biochemistry* 13, 282–288.
- Zhang, Y., Vareed, S.K., Nair, M.G., 2005. Human tumor cell growth inhibition by non-toxic anthocyanidins, the pigments in fruits and vegetables. *Life Sciences* 76, 1465–1472.