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# The protective ability of Mediterranean dietary plants against the oxidative damage: The role of radical oxygen species in inflammation and the polyphenol, flavonoid and sterol contents

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ABSTRACT

Ten hydroalcoholic extracts of edible plants from the Calabria region (Italy) were evaluated for their *in vitro* antioxidant and antiradical properties and *in vivo* topical anti-inflammatory activity. All the extracts had radical-scavenging and/or antioxidant properties, the most active plants being hawkweed oxtongue and viper's bugloss. The best free radical (DPPH·)-scavenging activity was found in hawkweed oxtongue and chicory leaves extracts ($IC_{50}$ = 25 and 26 μg/ml, respectively). Hawkweed oxtongue, poppy and viper's bugloss extracts showed the greatest inhibition of linoleic acid oxidation ($IC_{50}$ = 3 μg/ml). Viper's bugloss and hawkweed oxtongue extracts had the greatest antioxidant effect on bovine brain peroxidation ($IC_{50}$ = 11 and 22 μg/ml). All the extracts also showed an anti-inflammatory effect: 300 μg/cm$^2$ provoked oedema reductions ranging from 18% to 43%. Cress was the most active plant. Chicory leaves contained the highest amount of phenolics (190 mg/g) whilst Rush crimps contained the highest amount of flavonoids (32.9 mg/g), followed by hawkweed oxtongue (15.8 mg/g). Cress contained the highest number of sterols. Among them, γ-sitosterol (12.2%) and ergost-5-en-3-ol (3β) (4.5%) were found to be the major constituents. Moreover, three of the identified molecules (stigmasta-5,23-dien-3β-ol, stigmasta-5,24(28)-dien-3-ol (3β,22E) and 9,19-cyclolanost-24-en-3-ol (3β)) were found in this plant only.


## 1. Introduction

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. It is commonly accepted that, in a situation of oxidative stress, reactive oxygen species (ROS), such as superoxide ($O_2^{-\circ}$, OOH°), hydroxyl (OH°) and peroxyl (ROO°) radicals, are generated. The ROS plays 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). 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 (Lin & Huang, 2002; Perry, Pickering, Wang, Houghton, & Perru, 1999; Repetto & 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 (Gutteridge, 1995; Winrow, Winyard, Morris, & Blake, 1993). In addition, ROS propagate inflammation by stimulating release of cytokines, such as interleukin-1, tumour 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 neutralisation by antioxidants and radical scavengers can attenuate inflammation (Delaporte, Sánchez, Cuellar, Giuliani, & Palazzo de Mello, 2002; Geronikaki & Gavalas, 2006).

Most clinically important medicines are steroidal or non-steroidal anti-inflammatory chemical therapeutics, for treatment of inflammation-related diseases. Though these have potent activity, long-term administration is required for the treatment of the chronic disease. Furthermore, these drugs have various and severe adverse effects. Therefore, naturally occurring agents with very few side-effects are required to substitute the 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

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diseases, arthritis, chronic inflammation and cancers (Chen et al., 2005; Prior, 2003; Saleem, Husheem, Harkonen, & Pihlaja, 2002; Zhang, Vareed, & Nair, 2005). These physiological functions of fruits and vegetables may be partly attributed to their abundance of phenolics.

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

In the search for sources of natural antioxidants, in recent years some medicinal plants have been extensively studied for their antioxidant activity (AA) and radical-scavenging activity (De las Heras et al., 1998; Desmarchelier, Ciccia, & Coussio, 2000; Schinella, Tournier, Prieto, Mordujovich de Buschiazzo, & Rios, 2002; Vander-Jagt, Ghattas, Vanderjagt, Crossey, & Glew, 2002). Recent studies also showed the antioxidant potential of phytosterols. Plant sterols are distributed in various parts of plants, including seeds, nuts, fruits and vegetable oils. Phytosterols and their derivatives are essential components of plant biomembranes and they are biogenetic precursors of numerous metabolics such as plant steroid hormones.

Plant sterols have been investigated as an alternative for lowering plasma cholesterol levels, and several studies have shown that they significantly reduce plasma total and LDL cholesterol. Antiatherosclerotic effects of plant sterols are well documented in apo E-KO mice. The anti-atherogenic effects may be due, not only to their cholesterol-lowering activities, but also to other properties, such as effects on the coagulation system, antioxidant system, and hepatic and lipoprotein lipase activities. Moreover, plant sterols have been shown to have other metabolic effects. For example, several epidemiological and animal studies suggest that phytosterols suppress the growth of colon tumours.

Humans are not able to synthesise phytosterols, and dietary consumption is the only source of these compounds. Thus, human intake of phytosterols is governed by eating habits and availability of the source of plant sterols (Moghadasian, 2000).

The present paper, deals with a preliminary screening of the following Italian plants: italian thistle (*Carduus pycnocephalus* L.), chicory (*Cichorium intybus* L.), wild artichoke (*Cynara cardunculus* L. ssp. *cardunculus*), viper's bugloss (*Echium vulgare* L.), wild fennel (*Foeniculum vulgare* Miller ssp. *piperitum*), cress (*Lepidium sativum* L.), poppy (*Papaver rhoeas* L. ssp. *rhoeas*), hawkweed oxtongue (*Picris hieracioides* L.) and rush crimps (*Sonchus oleraceus* L). They are spontaneous edible plants present in the area of Alto Ionio cosentino, a territory characterised by different vegetation belts from sea level to the highest peak of Pollino Mt. (about 2000 m a.s.l.), mostly rich in Mediterranean elements.

In this work, 10 extracts obtained from the Italian plants listed before were studied to assess their radical-scavenging, antioxidant activities. Moreover, considering that antioxidants and free radical-scavengers can also exert an anti-inflammatory effect (Geronikaki & Gavalas, 2006), these extracts were also evaluated for their *in vivo* anti-inflammatory activity which, to the best of our knowledge, is not reported for these plants so far. The radical-scavenging activity was assessed with the 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-aluminium. Moreover, information on the traditional uses of these plants was collected through structured interviews for a comparison of the folk information and experimental data.

The aim of the present study was also to evaluate, for the first time, the chemical properties of the extracts of plants concerning the composition of sterols.

## 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, site of collection, and selected medicinal uses. The present research site (Cassano allo Ionio) is located in the Cosenza district, north of Calabria, Italy (Fig. 1). The investigated area includes plane, hilly and sub-mountainous belts of the east sector of Massiccio del Pollino complex, characterised by calcareous substrata with Mediterranean vegetation. Field data were collected during the periods December, 2004, and May, 2005, in the area of Cassano allo Ionio. Ethnobotanical 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 from old people, since most young interviewed persons knew nothing about this aspect of local traditions.

The collected plants were authenticated by Dr. Uzunov from the 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, $H_2SO_4$, 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, $FeCl_3$, ascorbic acid, butylated hydroxytoluene (BHT), propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH?), β-carotene, linoleic acid, Tween 20, Folin–Ciocalteu reagent, chlorogenic acid, aluminium chloride, potassium acetate, quercetin, 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 to dryness at room temperature, cut into small pieces (0.5–1 cm), and then extracted with 70% aqueous EtOH (3 l) through maceration (48 h × 3) at room temperature. The resultant total extracts were filtered and dried under reduced pressure to determine the weight (Table 2). Total phenolic and flavonoid contents were determined for each total extract. Extracts were then partitioned between 100 ml 90% MeOH and *n*-hexane (100 ml × 3), and the *n*-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 standards (Singleton & Rossi, 1965). About 50 mg of the extracts was weighed

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

| Sample | Scientific name (family) | Voucher specimen | Common name | Plant part used | Collection site | Collection date | Local traditional uses |
|---|---|---|---|---|---|---|---|
| CP | *Carduus pycnocephalus* L. (Asteraceae) | CLU 18061 | Italian Thistle | Stems | Monte di Cassano Ionio | May 2005 | Diuretic |
| Cil | *Cichorium intybus* L. (Asteraceae) | CLU 18051 | Chicory | Leaves | Lauropoli | April 2005 | Laxative, hypoglycaemic, diuretic, depurative, febrifuge, disinfectant of urinary tract, hepatoprotective, and appetiser |
| Cir | *Cichorium intybus* L. (Asteraceae) | CLU 18051 | Chicory | Roots | Lauropoli | April 2005 | Appetiser, cholagogue, hyperglycaemic, digestive, and antidiabetic |
| CC | *Cynara cardunculus* L. ssp. *cardunculus* (Asteraceae) | CLU 18059 | Wild Artichoke | Leaves, capitula | Monte di Cassano Ionio | December 2004 | Liver disease |
| EV | *Echium vulgare* L. (Boraginaceae) | CLU 18058 | Viper's Bugloss | Leaves, flowers | Giastreta | May 2005 | Diuretic |
| FV | *Foeniculum vulgare* Miller ssp. *piperitum* (Ucria) Bég (Umbelliferae) | CLU 18057 | Wild Fennel | Leaves | Monte di Cassano Ionio | May 2005 | Mouth ulcers |
| LS | *Lepidium sativum* L. (Brassicaceae) | CLU 18053 | Cress | Leaves | Cassano Ionio | April 2005 | Antiscorbutic, depurative and stimulant |
| PR | *Papaver rhoeas* L. ssp. *rhoeas* (Papaveraceae) | CLU 18054 | Poppy | Leaves | Madonna della catena | April 2005 | Measles treatment |
| PH | *Picris hieracioides* L. (Asteraceae) | CLU 18052 | Hawkweed oxtongue | Leaves | Lappicello | April 2005 | Febrifuge |
| SO | *Sonchus oleraceus* L. (Asteraceae) | CLU 18049 | Rush crimps | Leaves | Madonna della catena | March 2005 | Depurative, pyrosis, diuretic contusions, and burns |

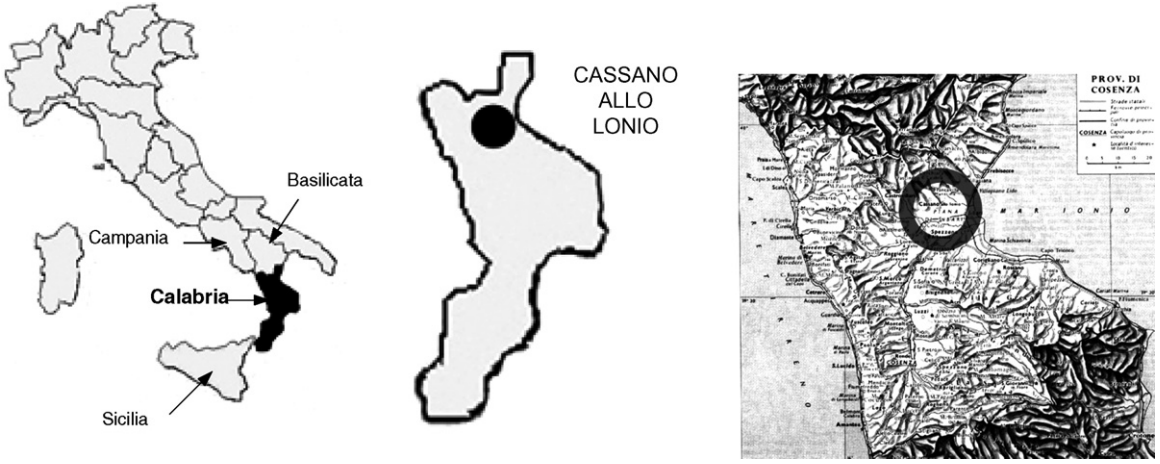


**Fig. 1.** Geographical location of the field research area in the Calabria region, Italy (latitude 39°44′1″ and longitude 34°2′18″).

into 50 ml plastic extraction tubes 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 homogenised for 30 s with a sonicator. About 200 μl (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 mg of chlorogenic acid equivalents per g of dry material.

**Table 2**
Yield of the plant materials extraction

| Plant | Weight of plant (g) | Hydroalcoholic extract (g) | Yield (%) |
|---|---|---|---|
| *Carduus pycnocephalus* L. | 50 | 6.47 | 13.06 |
| *Cichorium intybus* L. leaves | 50 | 8.63 | 17.26 |
| *Cichorium intybus* L. roots | 50 | 8.98 | 17.95 |
| *Cynara cardunculus* L. ssp. *cardunculus* | 50 | 6.47 | 13.06 |
| *Echium vulgare* L. | 50 | 3.75 | 7.50 |
| *Foeniculum vulgare* Miller ssp. *piperitum* (Ucria) Bég | 50 | 7.34 | 14.68 |
| *Lepidium sativum* L. | 50 | 11.24 | 22.48 |
| *Papaver rhoeas* L. ssp. *rhoeas* | 50 | 15.25 | 30.49 |
| *Picris hieracioides* L. | 50 | 6.40 | 12.81 |
| *Sonchus oleraceus* L. | 50 | 11.11 | 22.22 |

### 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-aluminium, having the absorptivity maximum at 430 nm (Quettier-Deleu et al., 2000). All determinations were done in triplicate and values were calculated from a calibration curve obtained with quercetin. Final results were expressed as milligrammes of quercetin equivalents per gramme 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 × 0.25 mm, static phase SE30, using programed temperature from 60 to 280 °C (rate 16°/min); the detector and the injector was set to temperatures of 280 and 250 °C, respectively (split vent flow 1 ml $min^{-1}$). Injection volume for the samples was 1 μl (10 mg/ml). Compounds identification was verified according to relative retention factor and mass spectra compared with those of the 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). To an ethanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH?) (final concentration $1.0 \times 10^{-4}$ 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?. Decrease of DPPH? solution absorbance indicates an increase of DPPH radical-scavenging activity. This activity is given as % DPPH radical-scavenging that is calculated by

$$\%\ \text{DPPH radical-scavenging} = \text{sample absorbance/control absorbance} \times 100. \quad (1)$$

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 thiobarbituric acid (TBA) test described by Fernandez, Perez-Alvarez, and Fernandez-Lopez (1997), modified as reported by Conforti, Statti, Tundis, Menichini, and Houghton (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, Hinrichsen, Web, & Witte, 1999). The inhibition of lipid peroxidation (in %) was calculated by

$$\%\ \text{inhibition} = [(\text{FRM} - \text{B}) - (\text{Et} - \text{B} - \text{EA})/(\text{FRM} - \text{B})] \times 100, \quad (2)$$

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 into account.

#### 2.7.3. β-Carotene bleaching test

Antioxidant activity was determined by using the β-carotene bleaching test (Amin, Zamaliah, & Chin, 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. About 5 ml of the emulsion were transferred into different test tubes containing 0.2 ml of samples in 70% ethanol at different concentrations (100, 50, 25, 10, 5 and 1 μg/ml). About 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 absorbances of the samples, standard and control were 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

$$\text{AA} = \left(1 - \frac{A_0 - A_t}{A_0^\circ - A_t^\circ}\right) \times 100, \quad (3)$$

where $A_0$ and $A_0^\circ$ are the absorbance values measured at the initial incubation time for samples/standard and control, respectively, whilst $A_t$ and $A_t^\circ$ are the absorbance values measured 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, Dri, Delbello, Zilli, & Della Loggia, 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 one 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 always induced 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 application 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 (6 mm Ø) 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 ± S.E. Statistical analysis was performed by using the 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 $p \leqslant 0.05$. 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 Fig. 2. The model for scavenging stable DPPH free radicals can be used to evaluate the antioxidative

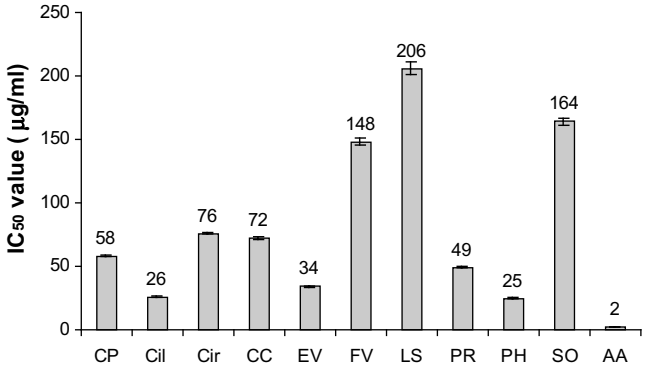


**Fig. 2.** Comparison of DPPH radical-scavenging activity. Ascorbic acid (AA) was used as positive control. ±S.E.M. ($n = 3$).

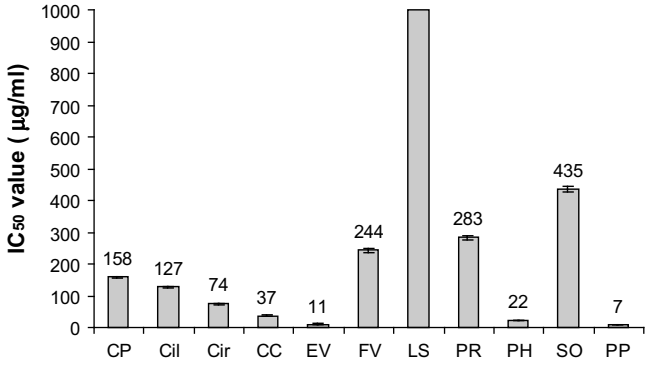


**Fig. 4.** Comparison of bovine brain peroxidation activity. Propyl gallate (PP) was used as positive control. ±S.E.M. ($n = 3$).

activities in a relatively short time. The absorbance decreases as a result of a colour change from purple to yellow as the radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H molecule (Gadow, Joubert, & Hansmann, 1997). 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 activities were exerted by *Picris hieracioides* (PH) and *Cichorium intybus* leaves (Cil) extracts ($IC_{50} = 25$ and 26 μg/ml, respectively). The lowest radical-scavenging activity was exhibited by *Lepidium sativum* (LS) ($IC_{50} = 206$ μg/ml). As reference, the $IC_{50}$ value of ascorbic acid was 2 μg/ml.

### 3.2. Antioxidant activity

The antioxidant activity was determined by the β-carotene bleaching method and bovine brain peroxidation assay. Inhibition of the breakdown of lipid hydroperoxides to unwanted volatile products allowed us to determine secondary antioxidants in related mechanisms. In the absence of antioxidants, oxidation products (lipid hydroperoxides, conjugated dienes and volatile byproducts) of linoleic acid simultaneously attack β-carotene, resulting in bleaching of its characteristic yellow colour in ethanolic solution. In the presence of the total extracts, oxidation products were scavenged and bleaching was prevented.

In the β-carotene bleaching test, after 30 min of incubation, *Picris hieracioides* (PH), *Papaver rhoeas* ssp. *rhoeas* (PR) and *Echium vulgare* (EV) extracts showed the greatest inhibition of linoleic acid oxidation ($IC_{50} = 3$ μg/ml). The lowest activity was exhibited by *Cichorium intybus* leaves (Cil) extract ($IC_{50} = 50$ μg/ml). The antioxidant activity of the extracts decreased during the reaction time.

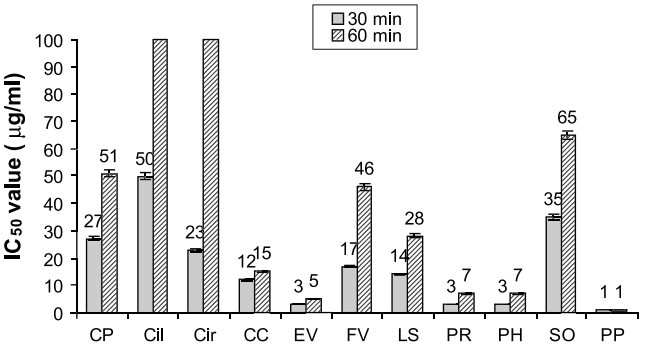


**Fig. 3.** Comparison of β-carotene bleaching activity after 30 and 60 min of incubation. Propyl gallate (PP) was used as positive control. ±S.E.M. ($n = 3$).

After 60 min of incubation, the $IC_{50}$ values of most active ones [*Picris hieracioides* (PH), *Papaver rhoeas* ssp. *rhoeas* (PR) and *Echium vulgare* (EV)] were 7, 7 and 5 μg/ml, respectively, whereas the $IC_{50}$ value of *Cichorium intybus* leaves (Cil) extract was >100 μg/ml, similar to *Cichorium intybus* roots (Cir) extracts. As reference, $IC_{50}$ of propyl gallate was 1 μg/ml, after both 30 and 60 min of incubation (Fig. 3).

Using liposomes prepared from bovine brain, *Echium vulgare* (EV) and *Picris hieracioides* (PH) extracts, the most active ones in the previous assay, had the highest antioxidant effect ($IC_{50} = 11$ and 22 μg/ml), whereas *Papaver rhoeas* ssp. *rhoeas* (PR) was less active ($IC_{50} = 283$ μg/ml). *Lepidium sativum* (LS) extract showed the lowest activity ($IC_{50} > 1$ mg/ml). As reference, the $IC_{50}$ of propyl gallate was 7 μg/ml (Fig. 4).

### 3.3. Topical anti-inflammatory activity

Results on the topical anti-inflammatory activity of the plant extracts, administered at the dose of 300 μg/cm$^2$, are shown in Table 3. All the extracts reduced the oedematous response to a certain extent. The most active was *Lepidium sativum* (LS) extract, which induced 43% oedema inhibition, followed by extracts of *Papaver rhoeas* ssp. *rhoeas* (PR), *Cichorium intybus* leaves (Cil), *Echium vulgare* (EV), *Picris hieracioides* (PH), *Cardus pycnocephalus* (CP) and *Sonchus oleraceus* (SO), which provoked oedema reductions ranging from 30% to 39%. The other plant extracts induced oedema inhibitions of 18% to 22%. As reference, the non-steroidal anti-inflammatory drug, indomethacin, reduced the oedematous response by 57% at the dose of 100 μg/cm$^2$.

**Table 3**
Topical anti-inflammatory activity of the hydroalcoholic extracts

| Hydroalcoholic extract | Dose (μg/cm$^2$) | No. an. | Oedema (mg) mean ± S.E. | % Oedema reduction |
|---|---|---|---|---|
| Control | – | 10 | 6.7 ± 0.3 | – |
| *Carduus pycnocephalus* L. | 300 | 10 | 4.6 ± 0.2* | 31 |
| *Cichorium intybus* L. leaves | 300 | 10 | 4.3 ± 0.4* | 36 |
| *Cichorium intybus* L. roots | 300 | 10 | 5.2 ± 0.3* | 22 |
| *Cynara cardunculus* L. ssp. *cardunculus* | 300 | 10 | 5.2 ± 0.3* | 22 |
| *Echium vulgare* L. | 300 | 10 | 4.5 ± 0.3* | 33 |
| *Foeniculum vulgare* Miller ssp. *piperitum* (Ucria) Bég | 300 | 10 | 5.5 ± 0.4* | 18 |
| *Lepidium sativum* L. | 300 | 10 | 3.8 ± 0.3* | 43 |
| *Papaver rhoeas* L. ssp. *rhoeas* | 300 | 10 | 4.1 ± 0.3* | 39 |
| *Picris hieracioides* L. | 300 | 10 | 4.5 ± 0.2* | 33 |
| *Sonchus oleraceus* L. | 300 | 10 | 4.7 ± 0.3* | 30 |
| Indomethacin | 100 | 10 | 2.9 ± 0.3* | 57 |

* $p < 0.05$ in the analysis of variance, as compared with controls.

### 3.4. Total phenolic and flavonoid contents

Table 4 shows the results of total phenolics and total flavonoid analyses. It should be emphasised that these results are estimations of total phenolic and total flavonoids as their chemical equivalents (chlorogenic acid and quercetin, respectively), since different phenolic compounds contribute differently to the readings, using the Folin–Ciocalteu reagent, for total phenolic results, and complex flavonoid-aluminium for total flavonoids results.

Total soluble phenolic constituents were measured by the Folin–Ciocalteu method. Folin–Ciocalteu reagent, a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, is reduced to blue oxides of tungsten ($W_8O_{23}$) and molybdenum ($Mo_8O_{23}$) during phenol oxidation. This reaction, occurring under alkaline conditions, is carried out in the presence of sodium carbonate. Blue colouration is monitored at 726 nm and reflects the quantity of phenols, usually expressed as gallic acid or chlorogenic acid equivalents.

The amounts of total phenolics varied widely in the different analysed extracts and ranged from 30 to 190 mg/g of extract (Table 4). 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, *Cichorium intybus* leaves contained the highest amount of phenolics (190 mg/g), followed by *Carduus pycnocephalus* (155 mg/g) and *Cynara cardunculus* ssp. cardunculus (105 mg/g), whereas the lowest level was found in *Lepidium sativum* (30 mg/g). The amount of phenolics in *Cichorium intybus* roots was similar to that of *Sonchus oleraceus* (60 and 61 mg/g, respectively), as well as *Foeniculum vulgare* ssp. *piperitum* and *Picris hieracioides* (80 and 85 mg/g, respectively).

**Table 4**
Total phenolic and flavonoid contents of the hydroalcoholic extracts

| Hydroalcoholic extract | Total phenolic content (mg/g)[a] | Total flavonoid content (mg/g)[a] |
|---|---|---|
| *Carduus pycnocephalus* L. | 155 ± 1.98 | 7.49 ± 0.08 |
| *Cichorium intybus* L. leaves | 190 ± 2.03 | 10.0 ± 0.12 |
| *Cichorium intybus* L. roots | 60 ± 0.89 | 0.94 ± 0.01 |
| *Cynara cardunculus* L. ssp. *cardunculus* | 105 ± 0.83 | 9.01 ± 0.09 |
| *Echium vulgare* L. | 68 ± 0.73 | 10.5 ± 0.13 |
| *Foeniculum vulgare* Miller ssp. *piperitum* (Ucria) Bég | 80 ± 0.95 | 12.3 ± 0.18 |
| *Lepidium sativum* L. | 30 ± 0.37 | 5.88 ± 0.09 |
| *Papaver rhoeas* L. ssp. *rhoeas* | 72 ± 0.76 | 4.68 ± 0.08 |
| *Picris hieracioides* L. | 85 ± 0.81 | 15.8 ± 0.27 |
| *Sonchus oleraceus* L. | 61 ± 0.65 | 32.9 ± 0.36 |

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

Phenols were found in higher concentrations in *Cichorium intybus* leaves (190 mg/g) than roots (60 mg/g), confirming that the biosynthesis of phenols is accelerated by light exposure and serves as a filtration mechanism against UV–B radiation (Harborne & Williams, 2000).

Among plant extracts, *Sonchus oleraceus* contained the highest amount of flavonoids (32.9 mg/g), followed by *Picris hieracioides* (15.8 mg/g), whereas the lowest level was found in *Cichorium intybus* roots (0.94 mg/ml).

Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the total antioxidant activity of many fruits and vegetables (Luo, Basile, & Kennelly, 2002). But, in general, we 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 summarises the sterol composition of each plant determined by GC–MS. Ten compounds were identified. Results showed that *Lepidium sativum* (LS) contained the highest number of sterols. Among them, γ-sitosterol (12.2%) and ergost-5-en-3-ol (3β) (4.5%) were found to be the major constituents. Moreover, three of the identified molecules [stigmasta-5,23-dien-3β-ol, stigmasta-5,24(28)-dien-3-ol (3β,22E) and 9,19-cyclolanost-24-en-3-ol (3β)] were found in this plant only.

*Picris hieracioides* (PH), *Foenicum vulgare* (FV), *Cichorium intybus* leaves (Cil) and *Cynara cardunculus* (CC) contained fewer sterols. *P. hieracioides* contained only γ-sitosterol.

γ-Sitosterol and stigmasterol were the predominant sterols since they were widely distributed. The first one was identified in every plant, exception *Foenicum vulgare* (FV) and it was mainly contained in *Lepidium sativum* (LS), *Cichorium intybus* leaves (Cil) and *Sonchus oleraceus* (SO) (12.2%, 9.5% and 6.5%, respectively). Stigmasterol was identified in seven extracts. The amount was particularly high for *Cichorium intybus* extracts (4.3% in root extract and 3.6% in leaves). Ergost-5-en-3-ol (3β) and campesterol were less widely distributed since they were identified in three extracts. The other phytosterols were found in just one of the examined plants.

## 4. Discussion and conclusions

The data presented in this study have demonstrated that almost all of the reported species possess antioxidant and free radical-scavenging activity. Indeed, their hydroalcoholic extracts inhibited linoleic acid oxidation, liposome peroxidation and/or scavenged

**Table 5**
Sterols composition of dietary plants

| Sterol[a] | Rf[b] | RAP[c] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CP | Cil | Cir | CC | EV | FV | LS | PR | PH | SO |
| Ergosta-5,24-dien-3-ol (3β) | 31.9 | – | – | – | – | 2.0 | – | – | – | – | – |
| Ergost-5-en-3-ol (3β) | 32.0 | – | – | – | – | 4.1 | – | 4.5 | 2.3 | – | – |
| Campesterol | 32.1 | 0.7 | – | 1.1 | – | – | – | – | – | – | 0.3 |
| Stigmasterol | 32.8 | – | 3.6 | 4.3 | 1.3 | 0.8 | 3.5 | – | 0.3 | – | 3.3 |
| (24S)-24-methyl-26,26-dimethyl-27-norcholesta-5,22-dien-3β-ol | 32.8 | 1.4 | – | – | – | – | – | – | – | – | – |
| Stigmasta -5,23-dien-3β-ol | 32.9 | – | – | – | – | – | – | 1.6 | – | – | – |
| γ-Sitosterol | 34.3 | 3.2 | 9.5 | 5.8 | 3.4 | 5.5 | – | 12.2 | 2.7 | 1.7 | 6.5 |
| (22R,24S)-22,24-dimethylcholesterol | 34.3 | – | – | – | – | – | 2.0 | – | – | – | – |
| Stigmasta-5,24(28)-dien-3-ol (3β,22E) | 34.6 | – | – | – | – | – | – | 0.6 | – | – | – |
| 9,19-Cyclolanost-24-en-3-ol (3β) | 36.6 | – | – | – | – | – | – | 0.5 | – | – | – |

[a] Compounds listed in order of elution from SE30 MS column.
[b] Retention factor.
[c] Relative area percentage (peak area relative to total peak area %).

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 the oxidation process. Because the antioxidant activity measured by an individual assay reflects only the chemical reactivity under specific conditions applied in that assay, it is inappropriate and misleading to use a single method to evaluate antioxidant capacity of a sample (Huang, Ou, & Prior, 2005).

The observed *in vitro* activities suggest that the investigated plant extracts could also exert protective effects *in vivo* against oxidative and free radical injuries occurring in different pathological conditions. In this respect, the most promising plants appear to be *Echium vulgare* and *Picris hieracioides*.

Several studies have evaluated the relationships between antioxidant activities of plant products and their phenolic contents. Some authors found a correlation between the phenolic content and the antioxidant activity, whilst others found no relationship. Velioglu, Mazza, Gao, and Oomah (1998) reported a high correspondence between total phenolic content and antioxidant activity (using the β-carotene bleaching method) in selected fruits, vegetables and grain products. On the other hand, no correlation between antioxidant activity (using methyl linoleate oxidation assay) 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. Extracts with high radical-scavenging and antioxidant activities did not show a high phenolic content. For example, *Picris hieracioides*, which showed a medium phenolic content, exhibited a high radical-scavenging and antioxidant activity. The relatively high antioxidant and free radical-scavenger activity of extracts with low phenolic content suggests that the type of phenolics is a determinant of 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 compositions 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, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005).

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

Considering that antioxidants can also exert anti-inflammatory effects (Geronikaki & Gavalas, 2006), the plant extracts were also evaluated for their *in vivo* anti-inflammatory activity. The obtained data reveal that almost 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 & Gavalas, 2006). In the present study the highest anti-inflammatory activity was exhibited by *Lepidium sativum* which has the highest content of sterols. Previous studies have demonstrated that phytosterols exert topical anti-inflammatory activity and that some of them inhibit 12-*O*-tetradecanoylphorbol acetate (TPA)-induced oedema (Garcìa, Sáenz, Gómez, & Fernández, 1999; Kimura, Yasukawa, Takido, Akihisa, & Tamura, 1995).

One of the species under study is reported to contain toxic secondary metabolites. *Echium vulgare* contains small amounts of pyrrolizidine alkaloids (Bruneton, 1999; El-Shazly et al., 1996). 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, including the species *Echium vulgare*, *Lepidium sativum* and *Picris hieracioides*, are interesting sources of anti-inflammatory and antioxidant principles, with potential use in different fields (food, cosmetics, and pharmaceutical).

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