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Flavonoid characterization and antioxidant activity of hydroalcoholic extracts from *Achillea ligustica* All.

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ABSTRACT

Ethanolic extracts of *Achillea ligustica* All. (Asteraceae) flowering tops were evaluated. High-performance liquid chromatography–electrospray ionisation-mass spectrometry was used for the identification and quantification of phenolic compounds. 6-Hydroxykaempferol-3,6,4′-trimethyl ether, apigenin-6-*C*-glucoside-8-*C*-arabinoside, luteolin, and apigenin were the most abundant flavonoids. For the first time *C*-glycosylflavones were detected in *A. ligustica* with apigenin-6-*C*-glucoside-8-*C*-arabinoside being the most representative. The radical scavenging activity of the extracts was determined by DPPH test and ranged between 4.18 and 12.3 mM. The ability of these extracts to inhibit non-enzymatic lipid peroxidation was studied using the simple *in vitro* system of linoleic acid oxidation: five of the nine extracts exerted a protective effect at the lower amount tested (5 μg). Protection on CaCo-2 intestinal cells against TBH-induced toxicity was also investigated: the results showed that two of the extracts tested in this cell system had the ability to protect against oxidative stress induced by TBH starting from concentrations as low as 10 μg/ml.

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1. Introduction

Phenolic compounds are important for plant physiology as they are involved in growth and development pathways; in defense mechanisms and also influence the color of flowers and fruits [1]. Phenols are able to affect several biological activities in human beings, who cannot synthesize them, but can introduce them through the food chain [2,3]. Flavonoids represent one of the most studied classes of phenolic compounds and lately they have been of particular interest owing to their role in contributing to human health [4]. Extracts from *Achillea* spp. (Asteraceae) have been extensively studied and antimicrobial [5–7], antihypertensive and antihyperlipidemic effects [8], antispasmodic [9,10], antidiabetic [11], antispermatogenic–antifertility [12], and immunosuppressive activities [13] have been reported. Moreover, flavonoids like casticin are known to show anti-tumor activity [14], centaureidin is a cytotoxic compound [15], while apigenin and luteolin have important estrogenic [16], and antispasmodic activities [10]. Total flavonoid content is also linked to the antioxidant activity exerted by *Achillea* extracts. During the past five years, studies have been conducted to investigate the antioxidant properties of different *Achillea* species. The antioxidant potential of the different fractions obtained from methanolic extracts of *Achillea alexandri-regis* [17] and *Achillea biebersteini* [18], which contain flavonoids and phenolic acids, has been evaluated. Both extracts and fractions showed scavenging activity towards hydroxyl radicals in different *in vitro* systems [17,18]. Also infusions obtained from 15 *Achillea* species have antioxidant activity: they show scavenging activity towards hydroxyl radicals and protect from oxidative damage induced by H_2O_2 [19]. This is the consequence of their ability to modulate some antioxidant enzymes activity (CAT, GPx and SOD), as was confirmed by the presence of high levels of GSH, and correlates to the flavonoids and total phenols content of the infusions [19]. By employing different *in vitro* assays (DPPH, LDL oxidation), it has been shown that methanol extracts obtained from aerial parts of *Achillea distans* and *Achillea moschata* possess antioxidant activity [20]. Only one study evaluated the antioxidant activity of *Achillea ligustica* showing that methanolic extracts from flowered parts of the plant possess antioxidant activity in the *in vitro* DPPH assay [11]. Antioxidant activity of the phenolic fraction and of the *n*-hexane fraction was tested using the lipid peroxidation of liposomes assay, and it was shown that the phenolic extract exerted the highest antioxidant activity [11].

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Table 1
Characteristics of the *Achillea* samples and related hydroalcoholic extracts.

Sample		Site	Dry weight[a] (g/100 g)		Dry matter[a] (g/l)		Total phenols[a] GAE[b] (mg/l)		TEAC[a,c] (mM)	
			Mean	±S.D.	Mean	±S.D.	Mean	±S.D.	Mean	±S.D.
1L	*A. ligustica* All.	San Nicolò Gerrei	43.11	2.79	17.2	0.3	851.9	22.0	6.10	0.36
2L	*A. ligustica* All.	Burcei (Monte Forrà)	43.69	1.63	16.5	0.1	1136.8	19.1	9.50	0.58
3L	*A. ligustica* All.	Serpeddì (Genna Manunga)	52.02	1.54	17.4	0.2	1280.8	53.8	6.85	0.48
4L	*A. ligustica* All.	Fluminimaggiore (passo Bidderdi)	43.10	2.93	17.4	0.0	1811.0	51.7	12.53	0.34
5L	*A. ligustica* All.	Fonni-Desulo (Tascusì)	31.97	2.32	13.6	0.0	1021.9	36.2	4.18	0.73
6L	*A. ligustica* All.	Aritzo (monte Texile)	39.48	1.45	16.7	0.1	1371.2	3.3	6.15	0.54
7L	*A. ligustica* All.	Dolianova	44.39	2.03	20.3	0.2	1693.1	28.8	10.50	0.61
8L	*A. ligustica* All.	Iglesias (lago Corsi)	39.06	1.65	16.7	0.0	1236.2	26.1	6.37	0.69
9M	*A. millefolium* L.	Villamassargia	52.58	1.39	18.2	0.1	1131.2	23.7	6.20	0.41

[a] Mean value of triplicate data.
[b] GAE gallic acid equivalent.
[c] TEAC is the millimolar concentration of a Trolox solution having an antioxidant capacity equivalent to that of the dilution of the hydroalcoholic extract.

Most of the species belonging to the *Achillea* genus contain flavonoids: flavonols and flavones and their derivatives. In different species of *Achillea* aglycons (apigenin, luteolin, quercetin), monoglycosides (mainly *O*-glucosides, *C*-glucosides, and *O*-glucuronides), diglycosides (*O*-diglucosides, *C*-diglucosides, *O*-rutinosides, 6-C-glucosyl-8-*C*-arabinosyl, 6-*C*-arabinosyl-8-*C*-glucosil, luteolin-6-*C*-apiofuranosyl-(1 → 2)-glucoside, 3-*O*-arabinosyl-(1 → 6)-glucoside), and methyl derivatives were found [6,7,15,17,21–30]. The phenolic content in *A. ligustica* was investigated in a few papers: quercetin, kaempferol, patuletin, 6-hydroxykaempferol-6-methyl ether 3-*O* glycosides [31], nevadensin, 3,6-dimethoxy-5,7,4′-trihydroxy flavone and quercetagetin-3,6,7-trimethyl ether [32], apigenin, luteolin, apigenin-7-*O*-glucoside, 6-hydroxykaempferol-3,6-dimethyl ether, 6-hydroxykaempferol-3,6,4′-trimethyl ether [33] were detected. None of these studies found *C*-glycosylflavones, although they are typical within the genus *Achillea*.

The aims of the present paper were (a) to characterize the flavonoidic composition of hydroalcoholic extracts of wild Ligurian yarrow from Sardinia, and (b) to evaluate their *in vitro* antioxidant activity. A first screening was made by using two simple chemical tests: DPPH and linoleic acid autoxidation assays. The most powerful extracts were then tested in a more biologically relevant experimental system. Because *Achillea* species have been used for centuries in folk medicine for the treatment of gastrointestinal disorders [34], we tested the ability of our extracts to counteract oxidative stress induced by tert-butylhydroperoxide in differentiated small intestine enterocytes (CaCo-2 cells).

2. Experimental

2.1. Plant material and alcoholic extracts preparation

Eight wild samples of *A. ligustica* All. and one sample of cultivated *A. millefolium* L. were collected in June 2005 in different areas of Sardinia. Blooming state, harvest and preliminary treatments were as previously described [35]. The harvest involved a random sampling with three different samples (1.0 kg) collected in each area. The specimens were identified and deposited in the Herbarium of the University Botanical Garden of Cagliari (Italy) (Table 1).

Alcoholic extracts were prepared on a laboratory scale as follows: 200 g of fresh vegetable material, 700 g of ethanol 95% (v/v), and 300 g of water [36]. Each sample was left to macerate for 28 days in the dark at 20 °C and shaken every 4 days. At the end of the maceration period, vegetable residues were separated, pressed out gently and the obtained liquid was combined with the macerate. *Achillea* samples dry weight and hydroalcoholic extracts dry matter were determined as reported by Tuberoso et al. [37].

2.2. Reagents and chemicals

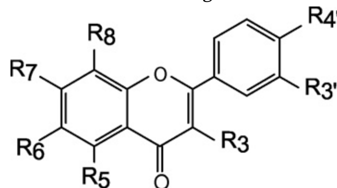
Standards of flavonoids luteolin, quercetin, apigenin, kaempferol, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-rhamnoside, apigenin-6-*C*-glucoside-8-*C*-arabinoside (shaftoside), apigenin-6-*C*-arabinoside-8-*C*-glucoside (isoshaftoside) were purchased from Extrasynthese (Genay, France). Folin-Ciocalteu reagent, Na_2CO_3, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylcromano-2-carboxylic acid (Trolox), linoleic acid, tert-butyl hydroperoxide (TBH), 2-thiobarbituric acid (TBA), 1,1,3,3,-tetraethoxypropane (TEP) and trichloroacetic acid (TCA) were purchased from Sigma–Aldrich (Milan, Italy). Cis,trans-13-hydroperoxy-octadeca-dienoic acid (c,t-13-HPODE) and cis,trans-9-hydroperoxy-octadeca-dienoic acid (c,t-9-HPODE) were purchased from Cascade (Cascade Biochem. Ltd., London).

The CaCo-2 cell line was purchased from ECACC (Salisbury, Wiltshire, UK). Cell culture media and supplements were purchased from Life Technologies (Milano, Italy). Alamarblue was purchased from Biosource Europe (Nivelles, Belgium). HPLC grade methanol, acetonitrile and trifluoroacetic acid were purchased from J.T. Baker (Baker Mallinckrodt, Phillipsburg, NJ, USA). Ethanol (95% v/v) for food use was from Silvio Carta srl (Baratili S. Pietro, Sassari, Italy). All solvents used for the antioxidant activity assays were of the highest available purity and were purchased from Merck (Darmstadt, Germany). HPLC grade water (18 mΩ) was prepared using a Millipore (Bedford, MA, USA) Milli-Q purification system. All other chemicals used in this study were of analytical grade.

2.3. Qualitative LC–ES/MS and LC–ES/MS/MS

Qualitative on-line LC–ES/MS analyses of extracts were performed using the Thermo Finnigan Spectra System HPLC coupled with the LCQ Deca IT (Thermo Electron, San Josè, CA, USA) with the following chromatographic conditions. Analyses were carried out using a Waters Symmetry shield C18 column (150 mm × 2.0 mm i.d.; 5 μm particle size) eluted with mixtures of 0.05% trifluoroacetic acid (TFA, solvent A) and acetonitrile containing 0.05% TFA (solvent B) at a flow rate of 0.25 ml/min. Elution was by step gradient from 90:10 (A:B) isocratic for 5 min, then the gradient raised to 60:40 (A:B) in 40 min, then from 60:40 (A:B) to 5:95 (A:B) in 15 min. Under these chromatographic conditions flavonoids could be well separated (Table 2). Electrospray ion source worked at the temperature of 280 °C. The parameters were optimized for the compounds, and they were the following: capillary voltage 5 kV, spray voltage 5 kV, tune lens offset +30. Nitrogen was supplied at the flow of 80 (arbitrary units). Ions were acquired in positive ion mode the range 150–1000 amu (atomic mass units). Reconstructed ion chromatograms were elaborated in order to identify flavonoids by their

Table 2
Flavonoids found in *A. ligustica* All. and *A. millefolium* L.



	Rt (min)	Compounds name	Formula	MW	3	5	6	7	8	3′	4′
1	20.34	Apigenin-6,8-*C*-diglucoside (vicenin 2)	$C_{27}H_{30}O_{15}$	594.53	H	OH	G	H	G	H	OH
2	20.84	Quercetin-3-*O*-rutinoside (rutin)	$C_{27}H_{30}O_{16}$	610.52	R	OH	H	OH	H	OH	OH
3	21.25	Luteolin-7-*O*-glucoside	$C_{21}H_{20}O_{11}$	448.38	H	OH	H	G	H	OH	OH
4	23.68	Apigenin-7-*O*-rutinoside	$C_{27}H_{30}O_{14}$	578.53	H	OH	H	R	H	H	OH
5	25.21	Apigenin-7-*O*-glucoside (cosmosiin—apigetrin)	$C_{21}H_{20}O_{10}$	432.38	H	OH	H	G	H	H	OH
6	33.82	Apigenina-6-*C*-arabinoside-8-*C*-glucoside (isoschaftoside)	$C_{26}H_{28}O_{14}$	564.50	H	OH	A	OH	G	H	OH
7	34.35	Apigenin-6-*C*-glucoside-8-*C*-arabinoside (schaftoside)	$C_{26}H_{28}O_{14}$	564.50	H	OH	G	OH	A	H	OH
8	35.50	6-Hydroxykaempferol-3,6-dimethyl ether	$C_{17}H_{14}O_{7}$	330.29	M	OH	M	OH	H	H	OH
9	38.25	Luteolin	$C_{15}H_{10}O_{6}$	286.24	H	OH	H	OH	H	OH	OH
10	42.87	Quercetin	$C_{15}H_{10}O_{7}$	302.24	OH	OH	H	OH	H	OH	OH
11	42.91	6-Hydroxykaempferol-3,6,4′ trimethyl ether (santin)	$C_{18}H_{16}O_{7}$	344.32	M	OH	M	OH	H	H	M
12	44.11	Apigenin	$C_{15}H_{10}O_{5}$	270.24	OH	H	H	OH	H	H	OH
13	48.73	6-Hydroxykaempferol-3,6,7,4′ tetramethyl ether	$C_{19}H_{18}O_{7}$	358.35	M	OH	M	M	H	H	M

G = glucoside; A = arabinoside; R = rutinoside; M = OCH_3.

protonated molecular ions. In LC–ESI–MS/MS data were acquired using the dependent scanning mode.

2.4. ES/MS, ES/MS/MS and quantitative LC–ESI–MS/MS

Full ES/MS and CID ES/MS/MS analyses of standards were performed on an Applied Biosystems (Foster City, CA, USA) API2000 ES mass spectrometer. Parameters were optimized by infusing a standard solution of quercetin-3-*O*-rutinoside (1 μg/ml in methanol) into the source at a flow rate of 5 μl/min. The optimized parameters were: declustering potential 160 eV, focusing potential 200 eV, and entrance potential 8 eV. Experiments were run in the Q1 MS mode in order to obtain ES/MS spectra, and in the product ion scan mode in order to carry out MS/MS experiments: in the product ion scan mode the collision energy was 30 eV, and the collision cell exit potential was 3 eV.

Quantitative on-line LC–ESI–MS/MS analyses of extracts were performed using the Agilent 1100 HPLC system interfaced to the Applied Biosystems API2000 instrument with chromatographic conditions as described for the LC–ESI–MS experiments. The instrument was used in the tandem MS mode with multiple reaction monitoring (MRM). The selected fragmentation reaction for each flavonoid is described below. The API 2000 ES source was tuned by infusing a standard solution of quercetin (1 μg/ml in methanol) into the source at a flow rate of 10 μl/min. The optimized parameters were: declustering potential 160 eV, focusing potential 200 eV, entrance potential 8 eV, collision energy 30 eV, and collision cell exit potential 3 eV.

Calibration and quantification of flavonoids was performed with the internal and external standard method. A sample (10 mg) of each flavonoid standard was accurately weighed into a 10 ml volumetric flask and dissolved in methanol and the volume made up to the mark with methanol. The resulting stock solutions were diluted with methanol in order to obtain reference solutions containing 2, 5, 15 and 25 μg/ml, and to each reference standard solution an appropriate amount of IS (quercetin-3-*O*-rhamnoside) was added in order to give a final concentration of 10 μg/ml. Calibration curves for each of the reference standards were obtained by injecting the standard solutions at each concentration level in triplicate. The ratios of the peak areas of the external standard (at each concentration) to those of the IS were calculated and plotted against the corresponding standard concentration using weighted linear regression to generate standard curves. All quantitative data were elaborated by Analyst software.

2.5. Method validation

Validation of the method was realised in agreement with EMEA note guidance on validation of analytical methods [38]. Validation of the LC/MS/MS method included intra and inter-day precision and accuracy studies on three days. Precision was evaluated by intra- and inter-day assays at four concentration levels for each compound.

The specificity is the non interference with other substances detected in the region of interest and the LC–MS/MS method, developed by using a characteristic fragmentation of flavonoids-*O*-glycosides, flavonoids-*C*-glycosides and flavonoids aglycons, resulted to be specific with no any other peak interfering at the retention times of the three marker compounds in the MS/MS detection mode.

Recoveries were determined by the addition of known quantities (5 mg/g of dried extract) of the investigated compounds to known amount of *A. ligustica* samples. Quantities were calculated by subtracting the total amount of each compound before spiking to the total amount after spiking. Ratio between detected amount and spiked amount was used to calculate the recovery.

Quantification limit was measured to establish the sensitivity of the method. In the present study it was determined using the signal to noise ratio, by injection of a series of solutions until the signal to noise ratio 10 for LOQ.

Five aliquots of each extract of *A. ligustica* were analyzed in order to quantify their flavonoidic content. An internal standard was introduced into both extracts and standards before the extraction, improving precision and accuracy of the quantitative analysis.

2.6. Determination of total phenols content

Total phenols content of hydroalcoholic extracts was determined by the Folin–Ciocalteu method [39], using a calibration curve of a freshly prepared gallic acid standard solution and results were expressed as mg/l of gallic acid. Briefly, 1 ml of diluted extract (1:10, v/v) was assayed with 1 ml Folin–Ciocalteu's reagent and, after 5 min, 4 ml sodium carbonate (10%, w/v) was added. The mixture was shaken and diluted with water to a final volume of 20 ml. After

a 90 min period incubation at room temperature, the absorbance was determined at 760 nm against a blank using a 10 mm quartz cuvette.

2.7. Antiradical activity

A spectrophotometric analysis using DPPH and comparison with the Trolox calibration curve was performed [40]. A 50 μl aliquot of hydroalcoholic macerate (diluted 1:50 with water) was dissolved in 2.00 ml of 0.04 mM DPPH in methanol. A calibration curve in the range 0.05/0.1/0.2/0.5/1.0 mM was used for the Trolox, and data were expressed in Trolox equivalent antioxidant capacity (TEAC, mM). Spectrophotometric readings were carried out with a Cary 50 spectrophotometer (Varian, Milan, Italy) at 517 nm using a 10 mm quartz cuvette.

2.8. Linoleic acid autoxidation assay

Linoleic acid autoxidation was conducted in dry state as previously described [41]. Samples of 0.5 ml of linoleic acid solution (2 mg/ml in methanol) were dried under vacuum. The samples were incubated in a water bath at 37 °C for 32 h. Different concentrations (5–100 μg) of the methanol solution of each extract were incubated, in dry state, with linoleic acid, before its autoxidation as described above. Autoxidation was stopped by cooling and adding 1 ml of CH_3CN–CH_3COOH (100:0.14, v/v). Aliquots of the samples were injected into the HPLC system. Analyses of linoleic acid and its oxidation products, conjugated diene linoleic acid hydroperoxides (c,t- and t,t-HPODEs), were carried out with an HPLC 1100 (Agilent Technologies, Palo Alto, USA) equipped with a diode array detector. An Inertsil 5 ODS-2 column (150 mm × 4.6 mm, Chrompack, Middelburg, The Netherlands) was used with a mobile phase of CH_3CN–H_2O–CH_3COOH (70:30:0.12, v/v/v) at a flow rate of 1.5 ml/min. Linoleic acid was detected at 200 nm and the four HPODE isomers (c,t-9-HPODE, t,t-9-HPODE, c,t-13-HPODE, and t,t-13-HPODE) were detected at 234 nm. The identification of the fatty acid and HPODEs was made using pure standard compounds to compare the UV spectra, generated using the Agilent Chemstation A.10.02 software.

2.9. Cell culture, cytotoxicity and protective effects of the extracts and TBARS test

Sub-cultures of the CaCo-2 cells were grown in T-75 culture flasks and passaged using a trypsin-versene solution. Cells were cultured in MEM medium supplemented with 20% FBS, 1% non-essential amino acids, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in 5% CO_2. For all experiments, CaCo-2 cells were seeded in 24 well plates at a density of 5×10^4 cells/ml (0.5 ml/well). Culture medium was replaced three times a week for 21 days, until complete differentiation of cells.

The cytotoxicity of alcoholic extracts from 2L and 4L samples was evaluated. Prior to the addition of the extracts the old media was removed, CaCo-2 cells were washed with PBS containing Ca^{2+} and Mg^{2+} and then fresh media was added (490 μl). 10 μl of extract dissolved in methanol were added to each well (1, 5, 10, 50 and 100 μg/ml final concentration). After 24 h incubation cells viability was evaluated by alamarblue test [42] collecting alamarblue/media solution and measuring absorbance at 570 and 600 nm. The percentage of alamarblue reduction was calculated and compared to the controls as detailed in the instructions. Data are presented as % of cell viability.

The protective effect of the extracts from 2L and 4L samples against tert-butyl hydroperoxide (TBH) induced cell damage in CaCo-2 cells was investigated. Prior to the treatment media was removed, CaCo-2 cells were washed with PBS containing Ca^{2+} and Mg^{2+} and then fresh PBS was added. Cell damage was induced by incubating the cells in presence of 5 mM TBH for 2 h. In order to evaluate the protective effect of our extracts, cells where preincubated for 30 min with the extracts (1–100 μg/ml) and then TBH was added. The supernatant was collected, stored at −20 °C to determine malonyl dialdehyde (MDA) production and the alamarblue test was carried out to determine cells viability as previously detailed.

MDA levels in supernatants from treated cells were measured with the TBARS test with HPLC quantification, using the method described by Templar et al. [43]. A standard curve was prepared with the samples using a 1,1,3,3,-tetraethoxypropane (TEP) solution (0.05–10 μM). MDA-TBA adduct quantification was obtained by HPLC-DAD analysis. Samples aliquots (50 μl) were injected and analysis were carried out using the Inertsil ODS-2 column previously described; the mobile phase was a mixture of 50 mM KH_2PO_4 pH 7-methanol (65:35, v/v) at a flow rate of 1 ml/min. The adduct MDA-TBA was revealed at 532 nm.

2.10. Statistical analyses

Data are presented as mean ± standard deviation of triplicate values obtained in two independent experiments ($n = 6$). Statistical significance within sets of data was determined by one-way analysis of variance ANOVA using the Graph Pad INSTAT Software (GraphPad Software, San Diego, CA, USA) and the Bonferroni post test.

3. Results and discussion

3.1. Dry residues and total phenolic content of A. ligustica extracts

A. ligustica extracts showed strong differences concerning the dry matter (ranging between 13.6 g/l in sample 5L and 20.3 g/l in sample 7L), and total phenols content (going from 851.9 to 1811.0 GAE mg/l, in samples 1L and 4L respectively).

3.2. LC–MS and LC–MS/MS analysis of A. ligustica extracts

The use of a Symmetry Shield C18 column allows to obtain a good separation of the flavonoids from the extracts of *A. ligustica*. The identification of each compound was performed by co-chromatography and by MS spectra evaluation. In LC–MS the flavonoids and flavonoids glycosides displayed the pseudomolecular ion $[M+H]^+$. The MS spectra in ES positive mode were useful for the detection of molecular weight. Positive ion electrospray LC/MS analysis of hydroalcoholic extract of *A. ligustica* and *A. millefolium* samples is shown in Fig. 1. This figure shows preliminary qualitative fingerprints obtained by LC–MS, useful to qualitatively compare the samples collected in different geographical areas of Sardinia. In addition, MS/MS experiments, performed by using Dependent Data Scan instrumental option with parameters optimized without variability, were diagnostic for the identification of specific fragmentation patterns, i.e. sugar loss for flavonoid *O*-glycosides or peculiar sugar fragmentation for flavonoid *C*-glycosides. Further investigations are needed for confirm the structure of these compounds. Nevertheless LC–MS analysis can be used as a fingerprint comparative analysis in order to individuate differences in the qualitative content of flavonoids from samples collected in different geographical areas (Fig. 1), although more quantitative than qualitative differences in flavonoids content are present.

Using LC–MS experiments, we have identified flavonoids and flavonoid glycosides reported in literature for other *Achillea* species in the extracts without time-consuming pre-purification steps or optimization of chromatographic procedures. Identifi-

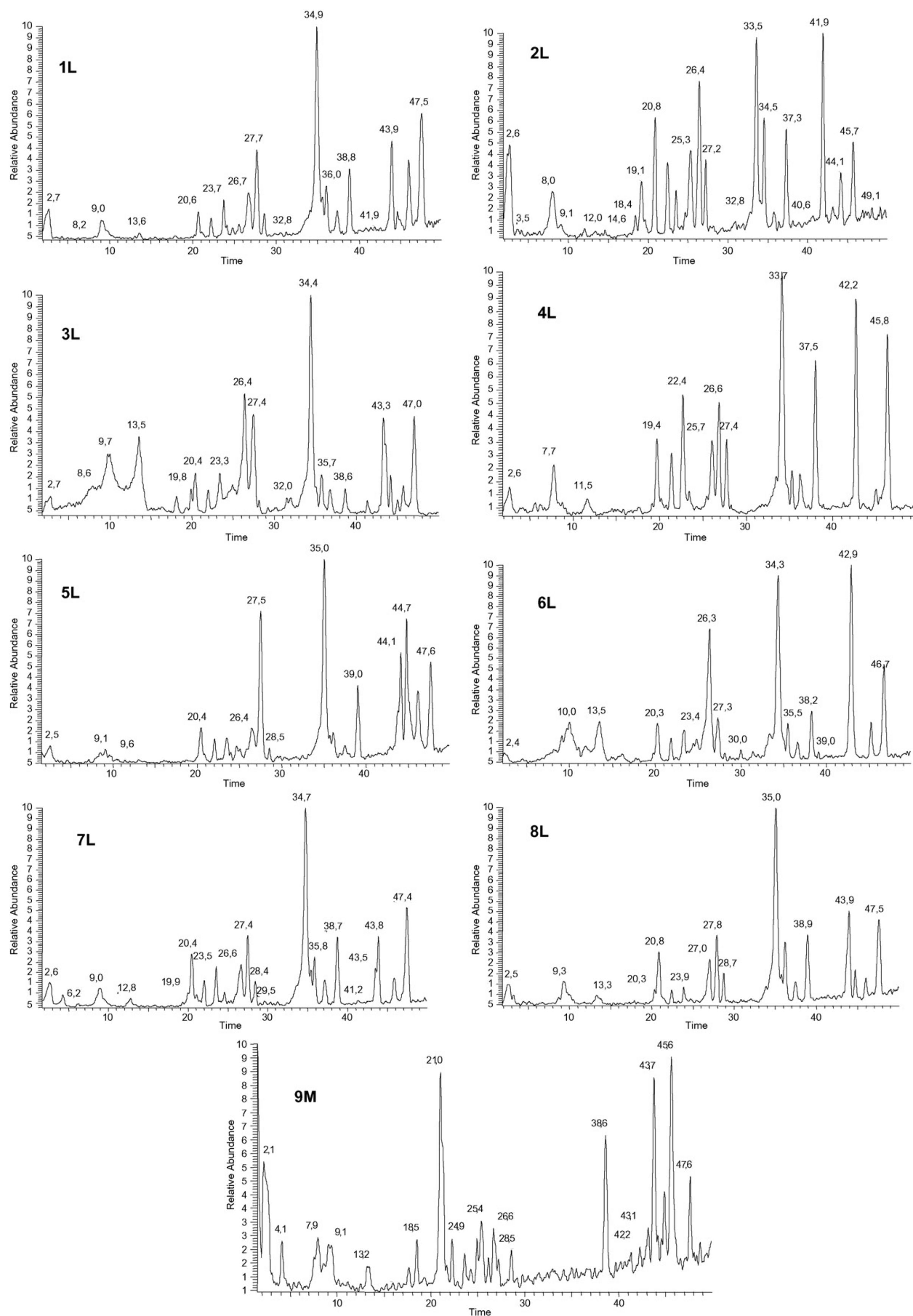


Fig. 1. Comparison among LC–ESI–MS fingerprints obtained by hydroalcoholic extracts from *Achillea* samples collected in different areas of Sardinia. Column: Waters Symmetry shield C18 (150 mm × 2.0 mm i.d.; 5 μm particle size) eluted with mixtures of 0.05% trifluoroacetic acid (TFA; solvent A) and acetonitrile containing 0.05% TFA (solvent B) at a flow rate of 0.25 ml/min. Elution was by step gradient from 90:10 (A:B) isocratic for 5 minutes, then the gradient was raised to 60:40 (A:B) in 40 min, and from 60:40 (A:B) to 5:95 (A:B) in 15 min.

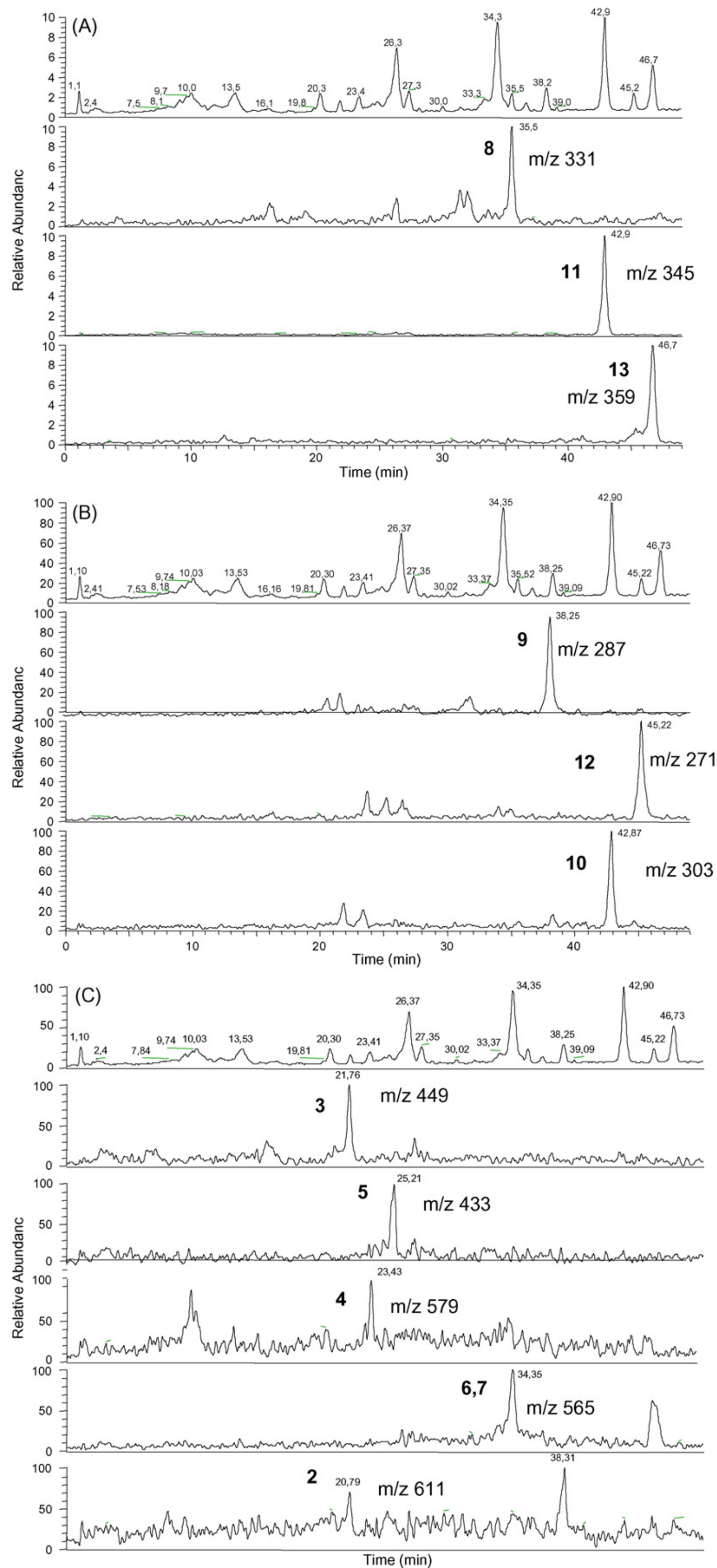


Fig. 2. Reconstructed ion chromatograms relative to flavonoids pseudomolecular ions in 6L sample: (A) 6-hydroxykaempferol derivatives, (B) flavonoids, (C) flavonoids glycosides. HPLC conditions as described in Fig. 1.

Table 3
LC–ES–MS/MS selected fragmentations for MRM analysis.

	Compounds	Precursor ion	Product ion
ES[a]	Luteolin	287	153
	Apigenin	271	153
	Kaempferol	287	153
	Quercetin	303	153
	Luteolin-7-*O*-glucoside	449	287
	Apigenin-7-*O*-glucoside	433	271
	Quercetin-3-*O*-rutinoside	611	303
	Apigenin-6-*C*-glucoside-8-*C*-arabinoside	565	445
IS[b]	Quercetin-3-*O*-rhamnoside	465	303

[a] ES: external standard.
[b] IS: internal standard. Collision Energy 30 eV.

cation was confirmed by standard's analysis and retention time comparison, and with the support of MS/MS fragmentation pattern of each compound. Fig. 2 shows LC–MS reconstructed ion chromatograms. Panel A reports the mass chromatograms for compounds 8, 11 and 13, respectively 6-hydroxykaempferol-3,6-dimethyl ether, 6-hydroxykaempferol-3,6,4′-trimethyl ether, and 6-hydroxykaempferol-3,6,4′,7-tetramethyl ether. Panel B reports the mass chromatogram for flavonoids aglycon, and specifically luteolin, apigenin and quercetin. Panel C shows reconstructed ion chromatograms for flavonoid glycosides, namely luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, apigenin-7-*O*-rutinoside, apigenin-6-*C*-arabinoside-8-*C*-glucoside, apigenin-6-*C*-glucoside-8-*C*-arabinoside and quercetin-3-*O*-rutinoside.

Comparison between extracts obtained from *A. ligustica* samples collected in different areas of Sardinia, showed that quantitative differences are more consistent than qualitative. Thus a quantitative method was developed in order to perform a quantitative comparison. For this purpose an accurate method on a mass spectrometer equipped with a triple quadrupole analyzer was developed for the analysis of flavonoids in *A. ligustica* extracts. Fragmentation patterns were studied by analyzing a standard solution of 0.1 μg/ml for each investigated compound using ES–QqQ–MS. An MRM method was developed selecting different fragmentation reactions for different groups of compounds (Table 3). The loss of sugar was selected as the specific reaction through which flavonoid-*O*-glycosides were monitorized. In flavonoid aglycones cleavage of two C–C bonds of the C-ring, generate informative $^{i,j}A^+$ and $^{i,j}B^+$ ions [44]. For flavonoid-*C*-glycosides, a characteristic product ion formed by cross-ring cleavages in the sugar residue was chosen [45]. Quercetin-3-*O*-rhamnoside was selected as internal standard, on the basis of its chromatographic and spectrometric features, and the reaction selected for its pseudomolecular ion was the sugar loss. The chromatographic profile obtained by MRM analysis exhibited all of the peaks corresponding to the compounds under investigation, and with sufficient intensity for quantitative purposes.

The calibration curves obtained by plotting the ratio of the area between the external and internal standards versus the known concentration of each compound were linear in the range of 2–25 μg/ml for all compounds. Analyzing replicate standard concentration with the same method in the same day and day-to-day it was shown that standard deviation was not higher then ± 2.00%. The mean recovery of the method was 100 ± 2%. LOQ values for each compound are enclosed in the range 10–55 ng/ml. This analytical method resulted to be precise and reliable.

Table 4 reports quantitative analysis results. *A. ligustica* samples are characterized by 6-hydroxykaempferol-3,6,4′-trimethylether (0.98–2.91 mg/g), apigenin-6-*C*-glucoside-8-*C*-arabinoside (1.34–1.84 mg/g), luteolin (0.79–1.20 mg/g) and apigenin (0.77–1.16 mg/g). Sample 6L is the only one in which apigenin-6-*C*-arabinoside-8-*C*-glucoside has been detected, and in which quercetin-3-*O*-rutinoside is not present. *A. millefolium* is characterized by high amounts of luteolin and apigenin-6-*C*-glucoside-8-*C*-arabinoside (1.70 and 1.41 mg/g, respectively) and it lacks both in tri- and tetramethyl ether derivatives of 6-hydroxykaempferol. Previous works on *A. ligustica* extracts did not detect *C*-glycosylflavones [31–33], but this can be due to the methodology and analytical methods used. In fact, flavonoids structure confirmation was obtained by traditional analytical approaches, like TLC, UV and ^{1}H NMR spectral data comparison with pure flavonoids and no direct analysis like HPLC were performed. Moreover, only a few samples were studied and this can be misleading because *A. ligustica* belongs to the *A. nobilis* group that is characterized by remarkable biochemical variability within its species. For this reason, only a correct botanical attribution of samples, a significant number of them and a proper analytical method can result in reliable quali-quantitative results.

3.3. DPPH radical scavenging activity

Table 1 shows the antioxidant activity of *Achillea* extracts expressed in TEAC. DPPH radical scavenging activity of the hydroalcoholic extracts shows TEAC values ranging between 4.18 and 12.53 mM, with samples 4L and 7L showing the highest activity. A

Table 4
Quantitative analysis of flavonoids in *A. ligustica* All. and *A. millefolium* L.

	Rt (min)	Compounds	Flavonoids content[a,b] (mg/g of dried extract)								
			1L	2L	3L	4L	5L	6L	7L	8L	9M
1	20.34	Apigenin-6,8-*C*-diglucoside	0.18	–	0.20	–	0.24	–	0.21	0.24	0.16
2	20.84	Quercetin-3-*O*-rutinoside	0.33	0.84	0.45	0.65	0.58	–	0.57	0.51	0.21
3	21.25	Luteolin-7-*O*-glucoside	0.31	0.43	0.23	0.48	0.44	0.45	0.53	0.47	–
4	23.68	Apigenin-7-*O*-rutinoside	0.11	0.09	0.07	0.07	0.10	0.08	–	–	–
5	25.21	Apigenin-7-*O*-glucoside	0.12	0.18	0.14	–	–	0.14	0.11	–	0.17
6	33.82	Apigenin-6-*C*-arabinoside-8-*C*-glucoside	–	–	–	–	–	0.21	–	–	–
7	34.35	Apigenin-6-*C*-glucoside-8-*C*-arabinoside	1.34	1.71	1.54	1.84	1.72	1.52	1.57	1.77	1.41
8	35.50	6-Hydroxykaempferol-3,6-dimethylether	0.76	1.10	1.21	1.45	0.92	0.86	1.34	1.14	1.11
9	38.25	Luteolin	1.20	1.05	0.93	1.05	0.81	1.02	0.79	0.89	1.70
10	42.87	Quercetin	0.31	0.24	0.25	0.22	0.25	0.21	0.31	0.21	0.14
11	42.91	6-Hydroxykaempferol-3,6,4′-trimethylether	2.11	0.98	2.56	0.97	1.97	2.91	1.99	2.34	–
12	44.11	Apigenin	0.99	0.99	0.77	1.11	1.08	1.08	1.16	0.85	1.01
13	48.73	6-Hydroxykaempferol-3,6,7,4′-tetramethylether	0.41	0.87	0.56	0.75	0.67	1.012	0.77	0.34	–

[a] Values are means (n = 5): the relative standard deviations for all compounds were <2%.
[b] Obtained applying calibration curves for the following flavonoids: luteolin, quercetin and apigenin for luteolin, quercetin and apigenin; kaempferol for hydroxykaempferol derivatives; luteolin-7-*O*-glucoside for luteolin-7-*O*-glucoside; apigenin-7-*O*-glucoside for apigenin-7-*O*-glucoside and apigenin-7-*O*-rutinoside; apigenin-6-*C*-glucoside-8-*C*-arabinoside for apigenin-6-*C*-glucoside-8-*C*-arabinoside, apigenin-6-*C*-arabinoside-8-*C*-glucoside and apigenin-6,8-*C*-diglucoside, quercetin-3-*O*-rutinoside for quercetin-3-*O*-rutinoside.

significant correlation (r = +0.80, p < 0.01) between antiradical activity and total phenol amount was found.

3.4. Linoleic acid autoxidation assay

Fig. 3 shows the results obtained during the autoxidation of linoleic acid in the presence of different amounts of the extracts (5–100 μg). All the extracts tested could completely inhibit the oxidative process at the highest concentrations tested (50 and 100 μg). The protection showed by all extracts was extremely significant starting from 10 μg and at the lower concentration tested (5 μg) only 5 extracts exerted a statistically significant protective effect (1L, 2L, 3L, 4L and 8L), with the highest values for 4L, which contained the highest amount of total phenols (1811 mg/ml), and surprisingly 2L, an extract containing less total phenols (1136.8 mg/ml) than other extracts which showed a lower activity (6L and 7L). This suggests that the protective effect of the extract could depend on its total phenolic content, but also on its composition and in particular on a synergistic effect of its components. The relative concentration of the major linoleic-derived HPODEs isomers (c,t-9-HPODE, t,t-9-HPODE, c,t-13-HPODE and t,t-13-HPODE) was measured. The two c,t isomers were added up as well as the t,t isomers and their ratio was calculated. These data are presented in Fig. 3B: all the extracts gave an amount-dependent shift of the c,t/t,t ratio versus the c,t isomers, with an higher value for the 4L extract, suggesting a mechanism of action/system involving an hydrogen atom donating mechanism [46].

3.5. Protective effect of the extracts against TBH-induced cell damage

To investigate the potential cytoprotective effects of 2L and 4L *Achillea* extracts against TBH-induced toxicity, we initially carried out control experiments to assess their cytotoxicity on CaCo-2 small intestinal cells. Data from the literature show that the treatment with TBH induces lipid peroxidation in differentiated CaCo-2 cells [47], and it has been used previously as a model to test the antioxidant activity of plant phenolic compounds [48]. Fig. 4A shows the % of cell viability after a 24 h treatment of CaCo-2 cells with the extracts. Cell viability remained unchanged in the presence of extracts at concentrations up to 100 μg/ml, demonstrating that the extract by itself does not induce significant toxicity. Enterocyte cultures were exposed to 5 mM TBH with or without pretreatment with *Achillea* extracts (1–100 μg/ml). Pretreatment with *Achillea* extracts resulted in a statistically significant protection against TBH-induced toxicity (Fig. 4B) at concentrations starting from 25 μg/ml and higher for both extracts. The 4L extract exerted a significant protection at 10 μg/ml. Concentration-dependent protective effects observed with the cell viability test were correlated to a reduction in the production of MDA (Fig. 4C). In fact the TBARS test results showed that pretreating the cells with our extracts a significant reduction of the MDA levels was observed for both extracts starting with 25 μg/ml and higher. Again the 4L extract was the most active, significantly reducing MDA levels even at the lower concentration tested (10 μg/ml).

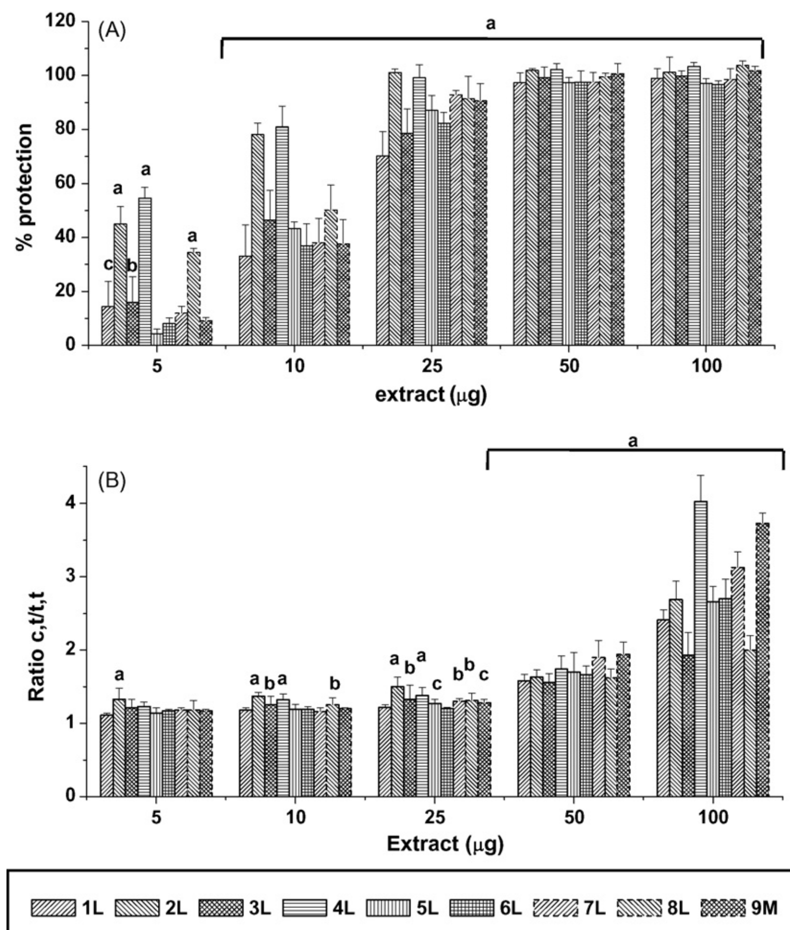


Fig. 3. Autoxidation of linoleic acid at 37 °C for 32 h in the presence of different amounts (5–100 μg) of *Achillea* samples. (A) % of protection of linoleic acid. (B) Ratio of the hydroperoxy-octadeca-dienoic acid isomers, c,t-HPODE and t,t-HPODE (c,t/t,t) formed during the autoxidation of linoleic acid. Reference values obtained for initial linoleic acid (18:2) and after linoleic acid autoxidation (18:2ox) are: 18:2 c,t/t,t = 3.42 ± 0.20; 18:2ox c,t/t,t = 1.04 ± 0.08. a = p < 0.001; b = p < 0.01; c = p < 0.05 vs. controls.

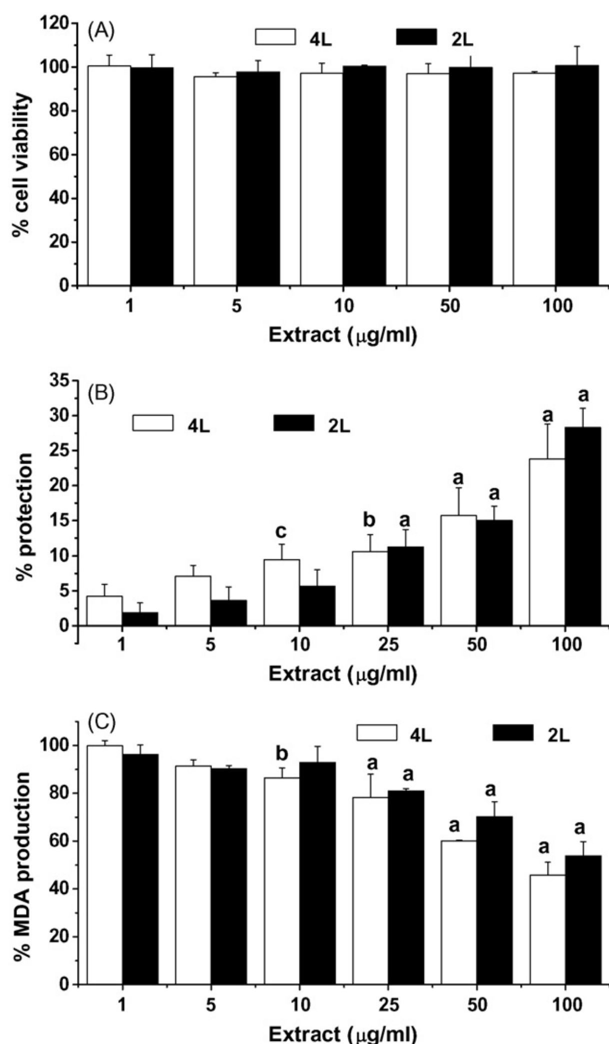


Fig. 4. Effects of the exposure to different amounts (1–100 μg/ml) of extracts obtained from 2L and 4L *A. ligustica* All. samples on CaCo-2 cells. (A) % of cell viability, measured with the alamarblue assay, after 24 h exposure to the extract. (B) % of protection, measured with the alamarblue assay, exerted by extracts pretreatment against TBH (5 mM) induced cell damage. (C) % of MDA production measured in the cells supernatants, pretreated with the extracts and treated with TBH 5 mM. a = $p < 0.001$; b = $p < 0.01$; c = $p < 0.05$ vs. controls.

In this paper a qualitative and quantitative analysis of flavonoids in *A. ligustica* was described. Since all the flavonoids reported are present as major bioactive constituents in *A. ligustica* the proposed LC–MS/MS method may be considered suitable for routine quantitative determinations on either plant material or its derivate products, in quality control protocols. Interestingly, the antioxidant activity in both DPPH and linoleic acid autoxidation assays and the cytoprotective effect of the extracts on CaCo-2 cell line, suggest the potential oral use of ethanolic *A. ligustica* extracts as a basic component of dietary supplements.

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