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Flavonoid characterization and *in vitro* antioxidant activity of *Aconitum anthora* L. (Ranunculaceae)

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Abstract

In this paper, we report studies on morphological, phytochemical, and biological aspects of a population belonging to *Aconitum anthora* L. Two compounds, quercetin 3-*O*-((β-D-glucopyranosyl-(1→3)-(4-*O*-(*E*-*p*-coumaroyl))-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside))-7-*O*-α-L-rhamnopyranoside (**1**) and kaempferol 3-*O*-((β-D-glucopyranosyl-(1→3)-(4-*O*-(*E*-*p*-coumaroyl))-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside))-7-*O*-α-L-rhamnopyranoside (**2**), together with two known flavonol glycosides (**3–4**) were isolated and identified from *A. anthora*. The antioxidant activity of the four identified flavonoids was screened by three *in vitro* tests.

Keywords: *Aconitum anthora*; Ranunculaceae; Flavonoids; Antioxidant activity

1. Introduction

The *Aconitum* genus (Ranunculaceae) includes many Asian and European species, with yellow or blue-violet flowers. *Aconitum anthora* L. is a European species characterized by tuberous roots, yellowish flowers, equal dimension of height and width of helmet, terminal raceme, hairy follicles, and black seeds (Seitz, 1969; Hegi, 1974; Pignatti, 1982; Akeroyd and Charter, 1993). The interest on this genus is based on the presence of diterpene alkaloids, used in Oriental medicine (Bisset, 1981), and flavonoids, studied in the last ten years as chemotaxonomic markers (Lim et al., 1999; Fico et al., 2003) and for their pharmacological activities (Di Carlo et al., 1999; Braca et al., 2003; Williams et al., 2004).

Flavonoids are a class of low molecular weight phenolic compounds, widely distributed in the plant kingdom. They exhibit different biological functions that allow interactions between plants and their environment: they are involved in the plant-pathogen interaction, plant-plant interaction and plant-insect interaction (Treutter, 2005). In the flowers the flavonoids can act as a visual signal to attract pollinating animals. Besides anthocyanins, responsible of red or purple colorations of petals, flavonols or flavanones can form a complex with anthocyanins, a phenomenon termed co-pigmentation, to give, for example, strong blue colorations (Koes et al., 1994).

The photo protection from sunlight ultraviolet (UV) is a predominant role of flavonoids: the epidermal flavonoids are strongly UV-absorbing and protect the internal tissues of leaves and stems; moreover flavonoids are potent scavengers of reactive oxygen species and thus prevent peroxidation of lipids (Treutter, 2005).

These compounds often exhibit a strong antioxidant activity, notably due to the presence of hydroxyl groups and aromatic heterocyclic rings that provide these molecules with hydrophilicity and particular stability; these

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features permit the delocalization and the scavenging of free radicals present into the cells (Peng et al., 2003).

Recent studies have also indicated that flavonoids act as cellular modulators by interaction with enzymes, receptors, and transcription factors of intracellular signalling cascades: understanding the role of flavonoids as antioxidants and modulators of cell signalling could explain their potential as anticancer agents and neurodegeneration inhibitors (Williams et al., 2004; Visioli and Hagen, 2007).

During the last years the presence of alkaloids in the aerial parts of *A. anthora* has been verified (Mericli et al., 2000) and, for the first time, the flavonoid composition and their *in vitro* antioxidant activity has been studied in this work.

2. Results

2.1. Morphological analysis

The morphological analysis allowed for species determination. Three taxonomic keys (Seitz, 1969; Hegi, 1974; Pignatti, 1982; Akeroyd and Charter, 1993) have been used for species classification. In particular, nine characters were used as diacritic: the roots of the entities appear tuberous; the leaves are alternate and palmately divided with linear segments not more than 3 mm of wide; the plant stalk is straight; the inflorescence is simple or branched; the flowers are yellowish with a ratio height/width of helmet between 0.71 and 1; only eglandular hairs along the inflorescence are present; the nectaries appear to bend forward; the fruits are follicles covered with eglandular hairs; the seeds are black with four irregular sides and acute angles.

For each *A. anthora* character one photo has been taken (Fig. 1A–F): such images are useful tools for species identification.

On the basis of morphological evidence, the population of Piani di Bobbio (LC), Italy, can be ascribed to the species *A. anthora*.

2.2. Phytochemical study

The methanol extract of *A. anthora* aerial parts was chromatographed on Sephadex LH-20 and RP-HPLC (C-18) to yield pure compounds **1–4**.

Compound **1** was isolated as yellow amorphous powder. Its UV spectrum exhibited absorption bands characteristic for flavonols at 230sh, 262, and 318 nm. Its molecular formula was established as $C_{48}H_{56}O_{27}$ by means of ESI-MS ($[M–H]^-$ peak at *m/z* 1063) and elemental analysis. Its ESIMS spectrum showed peaks at *m/z* 917 $[M–H–146]^-$, 771 $[M–H–146–146]^-$, 609 $[M–H–146–146–162]^-$, and 301 $[M–H–146–146–162–146–162]^-$ due to the loss of two deoxyhexoses, two hexoses, and one *p*-coumaroyl residue. The ^1H NMR spectrum (Table 1) of **1** showed between δ 4.20 and δ 8.00 four anomeric protons and signals ascribable to a *p*-coumaroyl residue, in addition to resonances characteristic for a quercetin aglycone moiety (Agrawal, 1989). These data indicate that compound **1** is an acylated quercetin glycosides, which was confirmed by analysis of the ^{13}C NMR spectroscopic data (Table 1). All the ^1H and ^{13}C NMR signals of **1** were assigned using 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments. Complete assignments of proton and carbon chemical shifts of the sugar portion were accomplished by DQF-COSY and 1D-TOCSY experiments and allowed the identification of the sugars as two α-L-rhamnopyranosyl units, one terminal and one esterified, one inner β-D-galactopyranosyl, and one terminal β-D-glucopyranosyl moiety. The configurations of the sugar units were assigned after hydrolysis of **1** with 1 N HCl. The hydrolysate was trimethylsilylated and GC retention times were compared with those of authentic sugar samples prepared in the same manner. The lower field shifts of H-4 (δ 5.06) of one rhamnopyranosyl unit suggested the substitution site of the *p*-coumaroyl moiety. Unequivocal information could be obtained by 2D-NMR spectra; the HMBC experiment indicated correlations between δ 5.13 ($H\text{-}1_{gal}$) and 135.2 (C-3), δ 4.67 ($H\text{-}1_{rha}$) and 69.3 ($C\text{-}6_{gal}$), δ 4.25 ($H\text{-}1_{glc}$) and 79.1 ($H\text{-}3_{rhaI}$), δ 5.06 ($H\text{-}4_{rhaI}$) and 169.0 (COO), δ 5.55 ($H\text{-}1_{rhaII}$) and 163.7 (C-7). Thus, the structure of **1** was determined as quercetin 3-*O*-((β-D-glucopyranosyl-(1→3)-(4-*O*-(*E*-*p*-coumaroyl))-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside))-7-*O*-α-L-rhamnopyranoside (Fig. 2).

Compound **2** was obtained as a yellow powder with a molecular formula $C_{48}H_{56}O_{26}$ as determined by ESIMS ($[M–H]^-$ at *m/z* 1047]) and also supported by elemental analysis. The ESIMS fragments and UV spectra were similar to those of **1**, suggesting a flavonol glycoside structure. A comparison of its NMR spectroscopic data with those of **1** (Table 1) indicated that the only difference between **2** and **1** was the aglycone moiety, being a kaempferol instead of a quercetin in **2** (Agrawal, 1989). Consequently, **2** was identified as kaempferol 3-*O*-((β-D-glucopyranosyl-(1→3)-(4-*O*-(*E*-*p*-coumaroyl))-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside))-7-*O*-α-L-rhamnopyranoside (Fig. 2).

Two known flavonoids, quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside or clovin (**3**) and kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside or robinin (**4**) were also isolated and identified by spectral analysis and comparison of data with those reported in the literature (Yoshida et al., 2005; Zallocchi and Pomilio, 1994a; Schaufelberger et al., 1987; Wenkert and Gottlieb, 1977) (Fig. 2).

2.3. Antioxidant activity

The pure compounds isolated from *A. anthora* were subjected to *in vitro* tests to evaluate their antioxidant activity. In particular, we carried out three tests: the DPPH scavenging test, the assessment of total antioxidant capacity and a lipid peroxidation assay. The standard reference compound for all tests was quercetin.

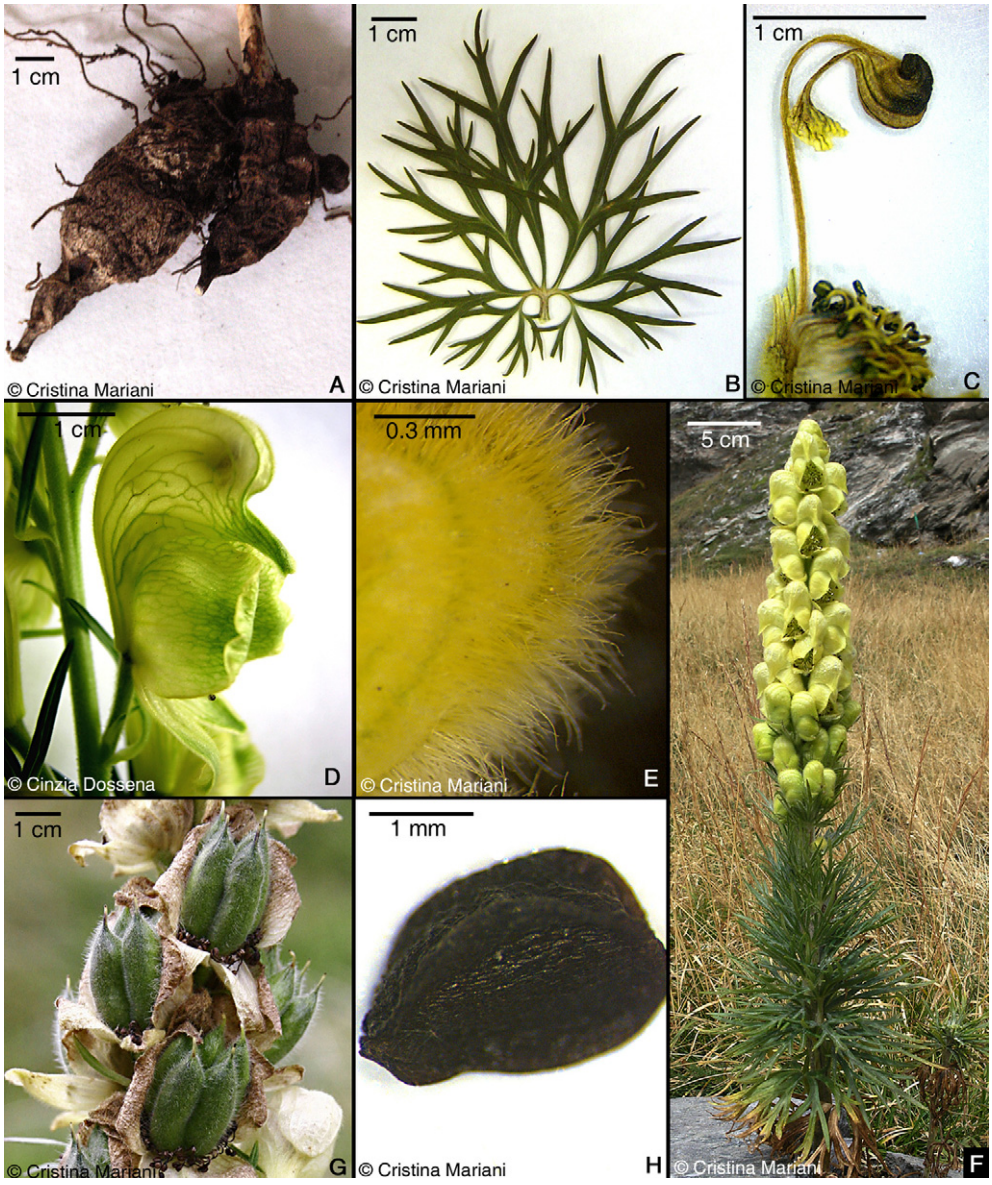

Fig. 1. Morphological characters used to identify *Aconitum anthora* species: roots (A); leaf (B); nectary (C); flower (D); eglandular hairs on helmet (E); straight plant stalk with a terminal inflorescence (F); hair follicles (G); black seed (H).

The DPPH test is a non-enzymatic method currently used to provide basic information on the ability of individual compounds or mixtures to scavenge free radicals. The analysis of the reduction of Cu^{2+} to Cu^{+} is useful to evaluate the total antioxidant capacity by reduction of metal ions that, at high concentrations, promote LDL oxidation (Gaut and Heinecke, 2001). As shown in Table 2, we tested flavonoids **1**, **2**, **3** and **4**. Concerning the first assay, each compound showed lower activity in comparison with quercetin; in particular quercetin derivatives (**1** and **3**) presented values higher of ten times than those of kaempferol derivatives (**2** and **4**); **1** and **3** showed values higher than **2** and **4** also in the second test. Compound **1** resulted to be the most active among the investigated flavonoids (1.31×10^{-5} M in the DPPH test; 0.92 mEq. uric acid in the total antioxidant capacity test). Oxidized low-density lipoprotein (LDL) is atherogenic *in vitro* and *in vivo* and appears to play a crucial role in the development of cardiovascular disease (Gaut and Heinecke, 2001); we assessed *in vitro* the oxidation of LDL by thiobarbituric acid-reactive substances (TBARS) formation and the four newly detected flavonoids were evaluated and compared.

As shown in Table 2 all analysed compounds exhibited modest activity towards lipid peroxidation; indeed, quercetin was more than ten-fold more active than the tested flavonoids.

3. Discussion

The phytochemical study of *A. anthora* allowed to identify four flavonol glycosides, of which two (**1** and **2**) have

Table 1
^{1}H and ^{13}C NMR data of compounds **1** and **2** (CD_3OD, 600 MHz)[a]

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
2		158.0		158.1
3		135.2		135.8
4		179.6		179.5
5		162.5		163.0
6	6.50 *d* (1.5)	100.6	6.50 *d* (1.5)	100.7
7		163.7		163.6
8	6.62 *d* (1.5)	95.6	6.61 *d* (1.5)	95.5
9		156.8		156.4
10		105.5		107.0
1′		122.5		120.8
2′	7.92 *d* (2.0)	117.9	8.22 *d* (8.5)	132.6
3′		145.8	6.89 *d* (8.5)	117.5
4′		149.0		160.2
5′	6.90 *d* (8.5)	116.2	6.89 *d* (8.5)	117.5
6′	7.63 *dd* (2.0, 8.5)	123.1	8.22 *d* (8.5)	132.6
3-*O*-Gal 1	5.13 *d* (7.0)	105.6	5.10 *d* (7.0)	105.5
2	3.61 *dd* (7.0, 8.0)	75.0	3.61 *dd* (7.0, 8.0)	75.0
3	3.87 *dd* (8.0, 4.0)	73.6	3.88 *dd* (8.0, 3.5)	73.6
4	3.81 *dd* (4.0, 2.5)	70.2	3.83 *dd* (3.5, 2.5)	70.4
5	3.82 *m*	75.4	3.82 *m*	75.4
6a	3.80 *dd* (4.5, 12.0)	69.3	3.78 *dd* (4.0, 12.0)	69.6
6b	3.76 *dd* (2.5, 12.0)		3.76 *dd* (2.5, 12.0)	
Rha 1	4.67 *d* (1.8)	102.4	4.67 *d* (1.8)	102.5
2	4.00 *dd* (3.0, 1.8)	71.8	3.98 *dd* (3.0, 1.8)	71.8
3	3.85 *dd* (9.0, 3.0)	79.1	3.84 *dd* (9.0, 3.0)	79.0
4	5.06 *t* (9.0)	73.6	5.06 *t* (9.0)	73.6
5	3.87 *m*	68.0	3.86 *m*	68.0
6	0.96 *d* (6.5)	18.0	0.96 *d* (6.0)	18.1
Glc 1	4.25 *d* (7.5)	105.5	4.25 *d* (7.5)	105.6
2	3.16 *dd* (7.5, 9.0)	74.8	3.15 *dd* (7.5, 9.0)	74.8
3	3.24 *t* (9.0)	77.5	3.26 *t* (9.0)	77.5
4	3.35 *t* (9.0)	71.0	3.35 *t* (9.0)	70.9
5	3.32 *m*	77.4	3.32 *m*	77.4
6a	3.80 *dd* (12.0, 5.0)	62.3	3.80 *dd* (12.0, 5.0)	62.1
6b	3.71 *dd* (12.0, 3.5)		3.72 *dd* (12.0, 3.5)	
p-coumaroyl 1		126.3		126.5
2,6	7.52 *d* (8.5)	131.4	7.53 *d* (8.5)	131.5
3,5	6.83 *d* (8.5)	117.5	6.83 *d* (8.5)	117.5
4		163.7		163.9
α	6.31 *d* (16.0)	117.0	6.31 *d* (16.0)	117.1
β	7.61 *d* (16.0)	147.2	7.60 *d* (16.0)	147.2
COO		169.0		169.2
7-*O*-Rha 1	5.55 *d* (1.5)	99.3	5.55 *d* (1.8)	99.4
2	4.03 *dd* (3.0, 1.5)	71.8	4.04 *dd* (3.0, 1.8)	71.6
3	3.85 *dd* (9.0, 3.0)	71.9	3.84 *dd* (9.0, 3.0)	71.8
4	3.51 *t* (9.0)	73.6	3.51 *t* (9.0)	73.7
5	3.62 *m*	71.0	3.60 *m*	71.2
6	1.29 *d* (6.0)	18.2	1.30 *d* (6.5)	18.2

[a] *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

been isolated for the first time. Similar flavonol 3,7-*O*-glycosides were identified from *A. chiisanense* Nakai (Jeong et al., 1997), *A. napellus* ssp. *tauricum* (Wulfer) Gáyer (Fico et al., 2001a), and *A. napellus* ssp. *neomontanum* (Wulfer) Gáyer (Fico et al., 2001b). Compounds **1** and **2** possess an acylated ramified trisaccharide moiety linked at C-3, which univocally characterizes the species *A. anthora*.

Compound **3**, known as clovin, and compound **4**, known as robinin, were isolated for the first time in *Melilotus alba* Medicus (Nicollier and Thompson, 1982) and in *Robinia pseudoacacia* L. (Farkas et al., 1976), respectively. Both compounds are present also in other Fabaceae species: *M. officinalis* (L.) Pallas (Sam Sik et al., 1988), *Macroptilium prostratum* (Benth.) Urban, *M. psammodes* (Lindman) Lackey, *M. fraternum* (Piper) Lackey, *Vigna luteola* Savi, *V. peduncularis* (H., B., K.) Fawcett and Rendle, *Phaseolus vulgaris* L., *P. lumnatus* L., and *P. augustii* Harms (Zallocchi and Pomilio, 1994a, 1994b, 1994c). Clovin was isolated also in other plants such as *Coutoubea spicata* Aubl. (Gentianaceae) (Schaufelberger et al., 1987) and *Alternanthera brasiliana* Kuntze (Amaranthaceae) (de O. Brochado et al., 2003), while robinin in *Paeonia albiflora* Pall. (Paeoniaceae) (Egger, 1961), *Alternanthera brasiliana* Kuntze (Amaranthaceae) (de O. Brochado et al., 2003), *Randia hebecarpa* Benth. (Rubiaceae) (Nazari et al., 2006), and *Rhodiola rosea* L. (Crassulaceae) (Petsalo et al., 2006). These flavonoids have been isolated for the first time in the Ranunculaceae family.

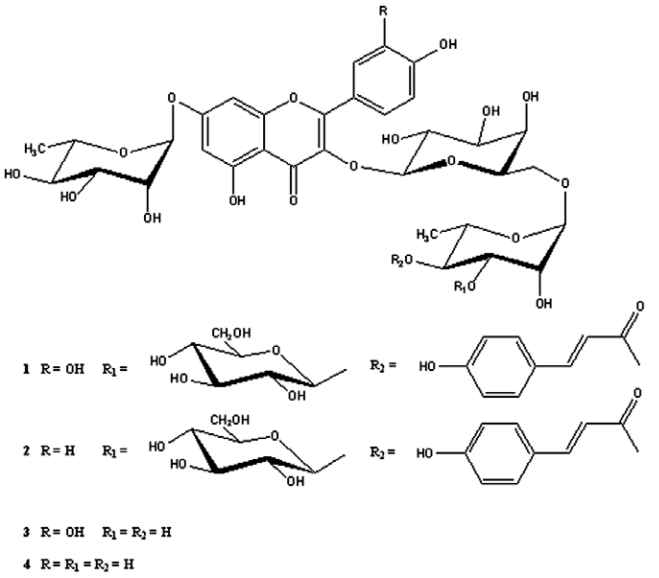

Fig. 2. Chemical structures of *A. anthora* flavonols.

The four flavonoids were tested to verify their *in vitro* potential antioxidant activity: the glycosylation of the 3-hydroxyl group could be correlated to lower activity of compounds **1** and **3** in comparison with quercetin; while the presence of a catechol group in the ring B could be responsible for the increase of antioxidant activity of compounds **1** and **3** compared with compounds **2** and **4** that show only one hydroxyl group in the ring B (Pietta, 2000). Furthermore, the comparison of our data with previous work on flavonols of *Aconitum* species (Braca et al., 2003) has shown that the highest antioxidant power could be ascribed to some flavonols isolated from *A. napellus* subspecies.

Concerning robinin, Nazari et al. (2006) reported results obtained on a hydromethanolic fraction containing robinin, suggesting a modest DPPH activity of this fraction which confirms our results.

Table 2
Antioxidant activity of *A. anthora* flavonols

Compounds	DPPH (IC_{50})	Antioxidant capacity (mEq uric acid)		TBARS (nmol TBARS/mg LDL)	
		10^{-6} M	10^{-5} M	10^{-6} M	10^{-5} M
Quercetin	4.40×10^{-6} M	0.75 ± 0.06	2.17 ± 1.35	3.85 ± 0.5	0.75 ± 0.04
1	2.30×10^{-5} M	0.05 ± 0.003	0.75 ± 0.06	55.24 ± 4.41	43.48 ± 3.47
2	2.80×10^{-4} M	0.01 ± 0.001	0.01 ± 0.001	49.68 ± 3.97	49.19 ± 0.73
3	1.31×10^{-5} M	0.06 ± 0.004	0.92 ± 0.07	48.11 ± 3.84	39.09 ± 3.12
4	3.50×10^{-4} M	0.01 ± 0.001	0.08 ± 0.002	52.78 ± 4.22	48.18 ± 3.85

IC_{50}: concentration of sample required to scavenge 50% DPPH free radicals; mEq of uric acid: unit of measurement of the antioxidant capacity to reduce Cu^{2+} to Cu; nmol TBARS/mg; LDL: unit of measurement of the assay of lipid peroxidation are expressed; TBARS: thiobarbituric acid-reacting substances, measured in $CuSO_4$ – challenged human LDL. Experiments were done in triplicate and the standard reference for all tests is the quercetin; results are mean ± SD.

As far as lipid peroxidation is concerned, in comparison with a reference antioxidant, the compounds we investigated showed a modest activity at concentrations and of 10^{-6} and of 10^{-5} M (TBARS production by oxidized controls was 55.98 ± 3.24 nmol/mg LDL; Table 2). Both glycosylation of 3-hydroxyl and 7-hydroxyl groups and a possible steric hindrance might explain the low activity of each tested flavonoid (Pietta, 2000).

Lacking *in vivo* data on the absorption and metabolism of the above mentioned flavonoids, we cannot infer on their *in vivo* antioxidant potential. Hence, further studies are necessary to assess the effectiveness of these flavonoids in other biological models, focusing the attention on the quercetin derivative compounds.

4. Experimental

4.1. General

Optical rotations were measured on a Perkin–Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. All the 2D NMR spectra were acquired in CD_3OD in the phase-sensitive mode with the transmitter set at the solvent resonance and TPPI (Time Proportional Phase Increment) used to achieve frequency discrimination in the ω_1 dimension. The standard pulse sequence and phase cycling were used for DQF-COSY, TOCSY, HSQC, HMBC, and ROESY experiments. The NMR data were processed on a Silicon Graphic Indigo2 Workstation using UXNMR software. ESIMS (positive and negative mode) were obtained from a LCQ Advantage ThermoFinnigan spectrometer, equipped with a Xcalibur software. HPLC separation was performed on a Merck Hitachi L-6200 Intelligent Pumping system, with a Merck Hitachi L-4200 UV–vis detector, with a Waters μ-Bondapak C_{18} column and with LC Organizer injector. TLC was conducting on silica 60 F254 gel-coated aluminium sheets (Merck). GC analyses were performed using a Dani GC 1000 instrument on a L-CP-Chirasil-Val column (0.32 mm × 25 m).

4.2. Plant material and morphological analysis

The plants of *A. anthora* were collected at Piani di Bobbio, Lecco, at 1700 m above sea level during the summer of 2005.

Ten herbal samples, 30 flowers and 30 leaves collected from different individuals were analysed. The voucher specimens (Aa 101–Aa 110) were deposited at the Department of Biology of University of Milan.

To identify the entities under study nine morphological characters were chosen on the basis of literature data (Seitz, 1969; Pignatti, 1982; Akeroyd and Charter, 1993). Each leaf and flower was subjected to measurements and observations by stereomicroscope (LEICA MZ6). The investigations were performed on *in vivo* and *exsiccata* samples.

4.3. Extraction and isolation

Dried and powdered *A. anthora* aerial parts (25 g) were defatted with *n*-hexane and successively exhaustively extracted with $CHCl_3$, $CHCl_3$:MeOH (9:1) and MeOH.

3.5 g of methanolic extract were chromatographed on Sephadex LH-20, using MeOH as eluent, to obtain one-hundred fractions of 12 ml, combined together into 20 groups, according to TLC separations [Silica 60 F_{254}-gel coated aluminium sheets; eluent: *n*-BuOH–CH_3COOH–H_2O (60:15:25)]. The group 13 was submitted to RP-HPLC on C_{18} μ-Bondapak column (300 × 7.8 mm, flow rate 2.5 ml min^{-1}) with MeOH–H_2O (40:60) to yield four flavonoid glycosides: **1** (7 mg) ($t_R = 54.0$ min), **2** (11 mg) ($t_R = 96.0$ min), **3** (9 mg) ($t_R = 18.5$ min), **4** (3 mg) ($t_R = 25.5$ min).

4.3.1. Quercetin 3-O-((β-D-glucopyranosyl-(1→3)-(4-O-(E-p-coumaroyl))-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside))-7-O-α-L-rhamnopyranoside (*1*)

Yellow amorphous powder, $[\alpha]_D$: −47° (*c* 0.1, MeOH); UV λ_{max} (MeOH) nm (log ε): 230sh (3.15), 262 (4.01), 318 (3.77); ^1H and ^{13}C NMR (600 MHz, CD_3OD): see Table 1; ESIMS: *m/z* 1063 $[M–H]^-$, 917 $[M–H–146]^-$, 771 $[M–H–146–146]^-$, 609 $[M–H–146–146–162]^-$, 301 $[M–H–146–146–162–146–162]^-$; Anal. Calc. for $C_{48}H_{56}O_{27}$: C, 54.14; H, 5.30. Found C, 54.10; H 5.32.

*4.3.2. Kaempferol 3-O-((β-D-glucopyranosyl-(1→3)-(4-O-(E-p-coumaroyl))-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside))-7-O-α-L-rhamnopyranoside (**2**)*

Yellow amorphous powder, $[\alpha]_D$: −54° (*c* 0.1, MeOH); UV λ_{max} (MeOH) nm (log ε): 229sh (3.05), 250sh (3.65), 268 (3.89), 319 (3.97); ^1H and ^{13}C NMR (600 MHz, CD_3OD): see Table 1; ESIMS: *m/z* 1047 $[M–H]^-$, 901 $[M–H–146]^-$, 755 $[M–H–146–146]^-$; Anal. Calc. for $C_{48}H_{56}O_{26}$: C, 54.96; H, 5.38. Found C, 54.98; H 5.35.

*4.3.3. Acid hydrolysis of compounds **1**–**2***

A solution of each compound (2.0 mg) in 1 N HCl (1 ml) was stirred at 80 °C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. Each residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 ml), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using a L-CP-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Peaks of the hydrolysate were detected by comparison with retention times of authentic samples of D-glucose (14.74 min) D-galactose (14.97 min), and L-rhamnose (10.71 min) (Sigma–Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

4.4. Antioxidant activity

4.4.1. DPPH scavenging test

Five decreasing concentrations (from 10^{-4} to 10^{-6} M) of each pure compound, dissolved in MeOH, were added to a 15 μM EtOH solution of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The absorbance at 517 nm, against a control of MeOH without DPPH, was read after 15 min of incubation in the dark (Visioli and Galli, 1998).

The IC_{50} was calculated by employing Prism® 4 (GraphPad Software Inc.) as the software. The experiment was run in triplicate.

4.4.2. Total antioxidant capacity

The total antioxidant capacity of the compounds under investigation was evaluated by a validated assay based upon the reduction of Cu^{2+} to Cu^+ (BIOXYTECH® AOP-490™, Oxis Research™, Portland, OR) (Visioli et al., 2001). The samples were tested at concentrations of 10^{-5} and 10^{-6} M. The results were shown as mEq uric acid.

4.4.3. Assay of lipid peroxidation

After isolation of human low density lipoprotein (LDL) from plasma of healthy volunteers by sequential ultracentrifugation (Havel et al., 1995), the total protein content was determined by the Bradford method (Bradford, 1976). Subsequently LDL was diluted to a concentration of 200 μg protein/ml in PBS 10 mM. The thiobarbituric acid-reactive substance (TBARS) content of lipoproteins was employed as a measure of lipid peroxidation. Five-hundred microliters of LDL, containing 100 μg lipoprotein, were treated by the addition of the pure compounds at concentrations of 10^{-5} and 10^{-6} M and were incubated for 15 min at 37 °C. Oxidation was triggered by the addition of $CuSO_4$ (5 μM) and samples were incubated at 37 °C for 3 h again (Perugini et al., 2000). Three-hundred microliters of samples were then assayed by the addition of 600 μl thiobarbituric acid reagent (0.375 g thiobarbituric acid, 2.08 ml 12 N HCl, 15 ml trichloroacetic acid 100% and distilled water to a final volume of 100 ml) (Buege and Aust, 1978).

After heating at 100 °C for 15 min, the samples were cooled to room temperature and centrifuged at 10,000*g* for 10 min at 4 °C. The clear supernatants were analysed spectrophotometrically at 532 nm and the results are shown as nanomoles of thiobarbituric acid-reactive substances/mg of LDL protein (Balla et al., 1991).

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