

Phenolic compounds from *Achillea millefolium* L. and their bioactivity

Sara Vitalini^{1,7}, Giangiacomo Beretta², Marcello Iriti¹✉, Simone Orsenigo³, Nicoletta Basilico⁴, Stefano Dall'Acqua⁵, Maria Iorizzi⁶ and Gelsomina Fico^{3,7}

¹Dipartimento di Produzione Vegetale, ²Dipartimento di Scienze Farmaceutiche "Pietro Pratesi", ³Dipartimento di Biologia, ⁴Dipartimento di Sanità Pubblica - Microbiologia - Virologia, Università degli Studi di Milano, Milano, Italy; ⁵Dipartimento di Scienze Farmaceutiche, Università degli Studi di Padova, Padova, Italy; ⁶Dipartimento di Scienze e Tecnologie per l'Ambiente e il Territorio, Università degli Studi del Molise, Pesche (Isernia), Italy; ⁷Orto Botanico 'G. E. Ghirardi', Dipartimento di Biologia, Università degli Studi di Milano, Toscolano Maderno (Brescia), Italy

Since antiquity, *Achillea millefolium* L. (Asteraceae) has been used in traditional medicine of several cultures, from Europe to Asia. Its richness in bioactive compounds contributes to a wide range of medicinal properties. In this study, we assessed *A. millefolium* methanolic extract and its isolated components for free radical scavenging activity against 2,2-diphenyl-picrylhydrazyl, total antioxidant capacity (based on the reduction of Cu⁺⁺ to Cu⁺), and ability to inhibit lipid peroxidation. The activity against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* was also tested. Chlorogenic acid, its derivatives and some flavonoids isolated by semipreparative HPLC and identified by NMR and spectrometric techniques were the major bioactive constituents of the methanolic extract. The latter exhibited significant antioxidant properties, as well as its flavonol glycosides and chlorogenic acids. With regard to the antiplasmodial activity, apigenin 7-glucoside was the most effective compound, followed by luteolin 7-glucoside, whereas chlorogenic acids were completely inactive. On the whole, our results confirmed *A. millefolium* as an important source of bioactive metabolites, justifying its pharmaceutical and ethnobotanical use.

Keywords: *Achillea millefolium*, Asteraceae, dicaffeoylquinic acids, flavonol glycosides, antioxidant power, antiplasmodial activity

Received: 21 October, 2010; **revised:** 24 January, 2011; **accepted:** 08 March, 2011; **available on-line:** 19 April, 2011

INTRODUCTION

Achillea millefolium L. (Asteraceae) grows wild all around Europe, Asia, North Africa and North America and it is widely used in Italian folk medicine (Pieroni & Quave, 2005; Passalacqua *et al.*, 2007; Vitalini *et al.*, 2009). Its properties have been known since antiquity and its use is diffused in many cultures from Europe to Asia: in Greece, in the region of Thessaloniki, for instance, *A. millefolium* is recommended for the treatment of many different ailments (Kokkini *et al.*, 2004); in West Azerbaijan, Iran, the infusion of dried flowers is considered suitable for the treatment of hemorrhoids, dyspepsia, dysmenorrhoea and gastritis (Miraldi *et al.*, 2001); in the Parvati valley, west Himalaya, India, leaves and flowers are used for gastric problems and fever (Sharma *et al.*, 2004).

Since 1975, several studies on the phytochemical composition of *A. millefolium* have been reported and led to

the identification of flavonoids and caffeic acid derivatives (Falk *et al.*, 1975; Guédon *et al.*, 1993; Glasl *et al.*, 2002; Benedek *et al.*, 2007; Innocenti *et al.*, 2007). All these studies increased the knowledge on the chemical composition of this species but, to date, a complete characteristics of its phenolic compounds is not yet available.

Concerning the bioactivity of this plant, recent studies reported antimicrobial, antiphlogistic, hepatoprotective, antispasmodic and calcium antagonist activities of its polar extracts (Stojanović *et al.*, 2005; Yaeesh *et al.*, 2006), and a protective effect of its infusions against H₂O₂-induced oxidative damage in human erythrocytes and leucocytes (Konyalioglu & Karamenderes, 2005). Some articles have described antimalarial activity of flavonoids from plant sources (Schwickard & van Heerden, 2002; Saxena *et al.*, 2003; Lehane & Saliba, 2008; Kaur *et al.*, 2009) and, particularly, Murnigsih and colleagues (2005) screened the activity of water extract of *A. millefolium* against *Plasmodium falciparum* with positive results, stimulating our interest to study the activity of methanolic extract from *A. millefolium* and of its pure compounds.

Hence, the first aim of the present work was to achieve a comprehensive characterization of phenolic bioactive compounds present in this species; subsequently, the crude extract and pure compounds were tested in different models for antioxidant and antiplasmodial activities.

MATERIALS AND METHODS

Chemicals. Ascorbic acid (99%), chlorogenic acid (95%), gallic acid (98%) and quercetin (98%) were from Sigma-Aldrich (Milan, Italy). The organic solvents were all of analytical grade (Sigma-Aldrich, Milan, Italy). Deuterated dimethylsulphoxide (dms_o-d₆) was from Sigma-Aldrich (Milan, Italy).

✉ e-mail: marcello.iriti@unimi.it

Abbreviations: CQ, chloroquine; DCQA, dicaffeoyl-quinic acid; DPPH, 2,2-diphenyl-picrylhydrazyl; ESI-MS/MS, electrospray ionization tandem mass spectrometry; HPLC, high performance liquid chromatography; IC₅₀, 50% inhibitory concentration; LDL, low density lipoproteins; *m*, molecular mass; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; RP, reverse phase; R_t, retention time; S.D., standard deviation; TAC, total antioxidant capacity; TBARS, thiobarbituric acid-reactive substance; TLC, thin layer chromatography; TMS, tetramethylsilane; t_r, relative retention time

Plant material. The aerial parts of *A. millefolium* were collected during summer 2007, in Oro di Morondo, Varallo Sesia (700 m) (Vercelli, Italy). A voucher specimen (no. Am 310) has been deposited in the Department of Biology of Milan University after their identification by an expert local botanist (Dr. Gianfranco Rotti), according to "Flora d'Italia" (Pignatti, 1982).

Extraction, isolation and identification. Air-dried, powdered aerial parts of *A. millefolium* (112 g) were extracted exhaustively with *n*-hexane, CHCl₃, CHCl₃/MeOH (9:1, v/v) and MeOH in a Soxhlet apparatus. MeOH extract (4.5 g) was chromatographed on Sephadex LH-20 (Pharmacia, 100×2.5 cm, flow rate 3.0 mL/min) using MeOH as eluent, giving 254 fractions of 3 mL, combined together into 20 subfractions according to TLC separations (Silica 60 F₂₅₄-gel coated aluminium sheets; eluent: *n*-BuOH/CH₃COOH/H₂O (60:15:25, by vol.)).

Subfractions 4–5, 6–7 and 10–11 were further combined together, according to their chromatographic (TLC) chemical pattern, and submitted to RP-HPLC on C₁₈ μ -Bondapak column (300 mm×7.8 mm, flow rate 2.5 mL/min) with MeOH/H₂O (40:60, v/v) to yield compounds **1** (5 mg) (*t*_R= 8.27 min), **2** (4 mg) (*t*_R= 23.17 min), **3**, **5**, **6**, **7** and **8** (3.5 mg) (*t*_R= 29.24 min). The purity of all substances was between 95 and 98% based on ¹H NMR and HPLC analysis. Compound **4** was identified by comparison with a sample previously isolated (Innocenti *et al.*, 2007).

¹H NMR spectra were recorded at 303 K in Fourier transform mode at 300 MHz on a Varian Mercury VX instrument (Varian, Torino, Italy) equipped with a broad band 20-mm probe, using a spectral width of 20 p.p.m. and TMS as internal standard. HPLC-ESI-MS analysis was performed with a Thermo Finnigan LCQ Advantage ion trap mass spectrometer (Thermoquest, Milan, Italy). The ESI/MS source was set as follows: capillary temperature 220 °C; spray voltage 4.5 kV; capillary voltage 10 V (positive ion mode) or –3 (negative ion mode); sheath gas flow rate 2 L/min; auxiliary gas flow rate 5 L/min. Spectra were detected in positive and negative ion mode (100–1000 *m/z*, 0.5 scan/s). Components were separated on a Phenomenex Synergy RP80 A column (150 mm×2 mm i.d., particle size 4 μ m) protected with a Max-RP guard column (4 mm×2 mm i.d., particle size 4 μ m). Gradient elution: 100% solvent A (H₂O, 0.1% HCOOH) to 60% B (CH₃CN, 0.1% HCOOH) in 60 min, followed by re-equilibration. Flow rate 0.2 mL/min.

Parasite cultures and drug susceptibility assay. *P. falciparum* cultures were carried out according to Trager and Jensen (1976) with minor modifications. Briefly, the CQ-sensitive (D10) and CQ-resistant (W2) strains were maintained at 5% hematocrit (human type A-positive red blood cells) in RPMI 1640 (EuroClone, Celbio) medium with the addition of 1% AlbuMaxII (lipid-rich bovine serum albumin), 0.01% hypoxanthine, 20 mM Hepes, 2 mM glutamine. All the cultures were maintained at 37 °C in a standard gas mixture consisting of 1% O₂, 5% CO₂, 94% N₂. Compounds were dissolved in either H₂O or EtOH and then diluted with medium to achieve the required concentrations (final EtOH concentration <1%, which is non-toxic to the parasite). Samples were placed in 96-well flat-bottom microplates (COSTAR) after serial dilutions. Asynchronous cultures with parasitaemia of 1–1.5% and 1% final hematocrit were aliquoted into the plates and incubated at 37 °C for 72 h. Parasite growth was determined spectrophotometrically (OD₆₅₀) by measuring the activity of the parasite lactate dehydrogenase (pLDH), according to a modified version of

the method of Makler *et al.* (1993), in control and treated cultures. The antimalarial activity is expressed as IC₅₀; each IC₅₀ value is the mean \pm S.D. of at least three separate experiments performed in duplicate.

Determination of polyphenolic content. Total polyphenols were quantified colorimetrically by the Folin-Ciocalteu assay using gallic acid as reference standard (Vitalini *et al.*, 2006). An aliquot of the samples was combined with 50 μ L of Folin-Ciocalteu reagent; after 3 min, 100 μ L of a saturated sodium carbonate solution was added and then distilled water to reach a final volume of 2.5 mL. After 1 h of incubation in the dark at room temperature, the absorbance was read at 725 nm. Results were reported as mEq gallic acid.

DPPH scavenging test. The DPPH assay was performed as previously described (Vitalini *et al.*, 2006). Briefly, aliquots of the MeOH extract and pure compounds, at five different concentrations (from 1 to 100 μ M), were added to a 15 μ M EtOH solution of DPPH free radical. Absorbance at 517 nm was read after 15 min of incubation in the dark. The IC₅₀ was calculated with Prism[®] 4 (GraphPad Software Inc.). Each IC₅₀ value is the mean \pm S.D. of at least three separate experiments performed in duplicate.

Total antioxidant capacity. Total antioxidant capacity (TAC) of the samples (at two concentrations: 1 and 10 μ M) was measured by a validated assay based on copper (II) reduction. (BIOXYTECH[®] AOP-490[™], Oxis Research[™], Portland, OR, USA) (Vitalini *et al.*, 2006). Results were reported as mEq uric acid.

Lipid peroxidation measurement. The lipid peroxidation analysis was carried out according to a procedure previously reported (Vitalini *et al.*, 2006). After isolation of human LDL by sequential ultracentrifugation, the total protein content was determined by the Bradford method. Subsequently, LDL fraction was diluted to 200 μ g protein/mL in 10 mM PBS. The content of TBARS was employed as a measure of lipid peroxidation. LDL fraction (500 μ L), containing 100 μ g of lipoprotein was treated by the addition of MeOH extract or pure compounds at concentrations of 10 μ M or 1 μ M and then incubated for 15 min at 37 °C. Oxidation was triggered by the addition of CuSO₄ (5 μ M) and samples were incubated at 37 °C for 3 h. Then, 300 μ L of each sample was assayed by the addition of 600 μ L of thiobarbituric acid reagent (0.375 g thiobarbituric acid, 2.08 mL 12 M HCl, 15 mL trichloroacetic acid 100% and distilled water to a final volume of 100 mL) and boiled for 15 min. After centrifugation (10000×*g* for 10 min at 4 °C), supernatants were analysed spectrophotometrically at 532 nm. Results are expressed as nmol of TBARS/mg of LDL protein.

Statistical analyses. Results are expressed as mean \pm S.D. of three independent determinations. All statistical analyses were performed using the SPSS ver. 17.0 software for Windows (SPSS, Chicago, IL, USA). Relationships between variables were examined by Spearman rank nonparametric correlation analysis. Multivariable linear regression was used to identify variables that influence the antiplasmodial activity, and conducted using a stepwise algorithm.

RESULTS AND DISCUSSION

Phytochemical study

Table 1 shows chemical structures of ten compounds identified in the MeOH extract of the aerial parts of

Table 1. Compounds identified in methanol extract of *A. millefolium* L.

Peak	R _t (min)	m (Da)	Compound	Structure
1	6.22	354.3	Chlorogenic acid	
2	16.48	610.5	Rutin	
3	17.53	448.4	Luteolin 7-O-glucoside	
4	18.32	516.4	1,3-dicaffeoylquinic acid	
5	19.44	516.4	1,4-dicaffeoylquinic acid	
6	21.05	516.4	3,4-dicaffeoylquinic acid	
7	21.38	432.4	Apigenin 4'-O-glucoside	
8	21.20	432.4	Apigenin 7-O-glucoside	
9	22.32	448.4	Luteolin 4'-O-glucoside	
10	22.91	516.4	3,5-dicaffeoylquinic acid	

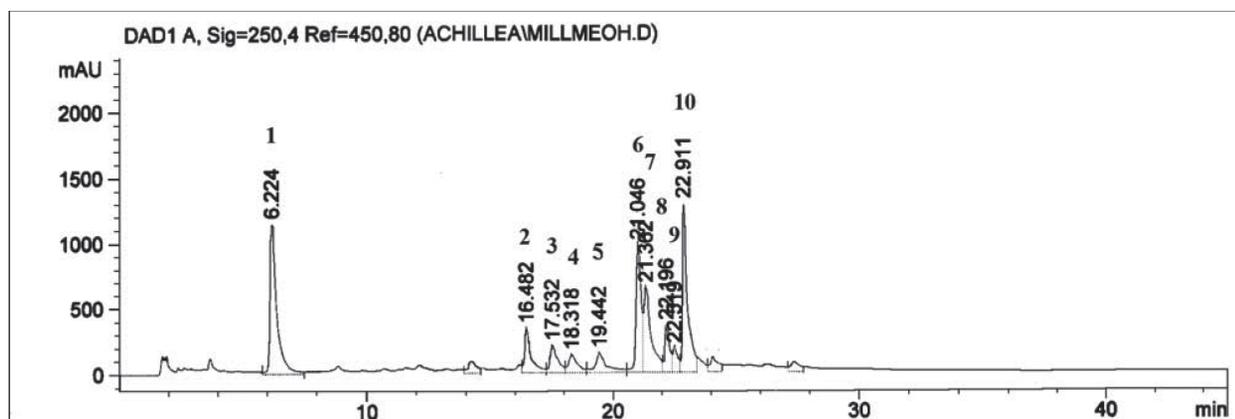


Figure 1. HPLC profile ($\lambda=250$ nm) of methanolic extract from *A. millefolium*

1, Chlorogenic acid; **2**, rutin; **3**, luteolin 7-*O*-glucoside; **4**, 1,3-dicaffeoylquinic acid; **5**, 1,4-dicaffeoylquinic acid; **6**, 3,4-dicaffeoylquinic acid; **7**, apigenin 4'-*O*-glucoside; **8**, apigenin 7-*O*-glucoside; **9**, luteolin 4'-*O*-glucoside; **10**, 3,5-dicaffeoylquinic acid.

A. millefolium. These compounds accounted for over 90% of the total area of the HPLC chromatogram ($\lambda=250$ nm). Three major peaks, detected at $R_t=6.22$ min, $R_t=21.05$ min and $R_t=22.91$ min (**1**, **6** and **10** respectively) and two minor peaks detected at $R_t=18.32$ min and at $R_t=19.44$ min (**4** and **5**) were tentatively attributed to five caffeic acid derivatives, whereas minor peaks **2**, **3**, **7**, **8** and **9** were identified as flavonoid glycosides on the basis of their UV spectra (not shown).

In accordance with the results from previous studies (Benedek *et al.*, 2007; Innocenti *et al.*, 2007), compounds **1**, **2**, **3**, **8** and **9** were identified as chlorogenic acid, rutin, luteolin 7-*O*-glucoside, apigenin 7-*O*-glucoside, and luteolin 4'-*O*-glucoside, by comparison of their chromatographic retention times and spectral data with those of pure commercially available compounds.

The peaks corresponding to compounds **6**, **7**, and **10** were isolated by semi-preparative HPLC and identified by NMR and HPLC-MS techniques.

The HPLC-DAD chromatogram ($\lambda=250$ nm, Fig. 1) showed the presence of two main peaks ($R_t=21.04$ min and $R_t=21.36$ min) corresponding to compounds **6** and **10** characterized by identical UV spectra, with two main peaks at λ_{max} 217 nm and 329 nm and shoulders at 239 nm and 300 nm (not shown), typical of the chlorogenic acid chromophore. The presence of two chlorogenic acid moieties in these compounds was confirmed by the HPLC-ESI-MS experiments that evidenced, in all cases, protonated pseudo-molecular ions at m/z 517 $[M+H]^+$ (m 516 Da). The presence of a fragment ion at m/z 355 $[M\text{-caffeoyl}+H]^+$, in the ESI-MS² spectra of both compounds (not shown) indicated the structure of two isomeric dicaffeoyl derivatives of quinic acid never reported before in *A. millefolium*. Unequivocal confirmation of these structures was achieved by ¹H NMR analyses. In accordance with the NMR data previously reported (Wang & Liu, 2007), these two major isomeric species were identified as 3,4-DCQA (compound **6**) and 3,5-DCQA (compound **10**).

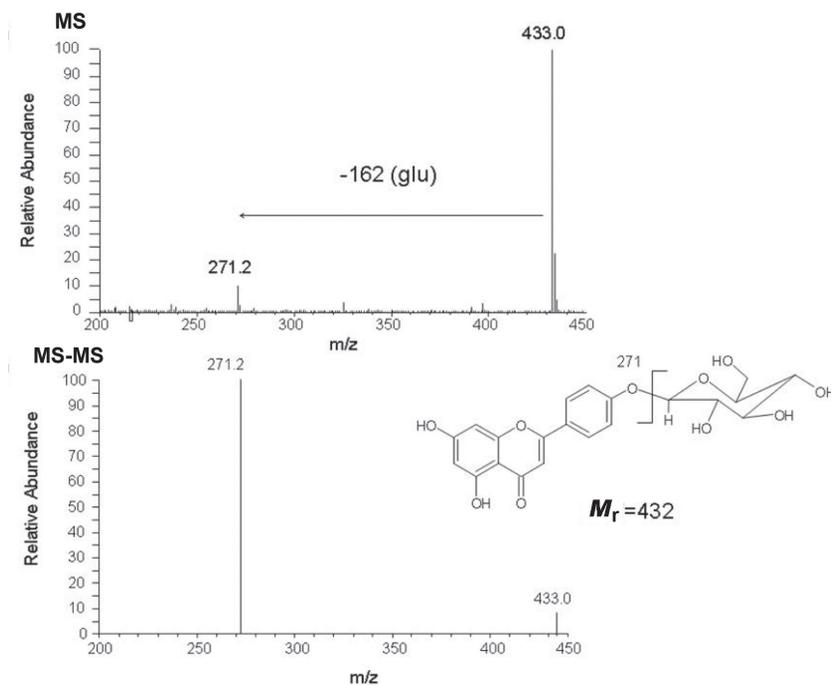


Figure 2. HPLC-ESI-MS and HPLC-ESI-MS² spectra of pseudo-molecular ion of compound **7** at $[M+H]^+$ m/z 433

Table 2. *In vitro* antiplasmodial activity against D10 and W2 strains of *P. falciparum*

Compound	D10 IC ₅₀ (µg/mL)	W2 IC ₅₀ (µg/mL)
Chloroquine	0.010±0.03	0.18±0.07
Luteolin	6.1±0.8	5.0±1.1
Apigenin	25.4±7.9	20.2±6.4
1	>100	>100
2	68.5±22.9	76.4±7.7
3	26.2±13.5	26.8±3.6
4		
5		
6	>100 ^a	>100 ^a
10		
7	71.4±11	58.7±11.2
8	10.1±1.3	6.1±3.8
9	>100	>100
Methanolic extract	>100	44.6±8.8

^aCompounds **4–6** and **10** in the same fraction tested before their identification. The results are expressed as IC₅₀ ± S.D. of three different experiments each performed in duplicate.

The identity of compound **7** was established on the basis of (i) its protonated [M+H]⁺ pseudo-molecular ion at *m/z* 433 and of a fragment at *m/z* 271 in the ESI-MS/MS spectrum (Fig. 2), and of (ii) its ¹H and ¹³C NMR data (dms_o-d₆, 300 MHz) that, according to the ¹H NMR data reported by Teng and colleagues (2002), were consistent with the structure of apigenin 4'-*O*-α-glucopyranoside **7**, an unusual derivative of apigenin, identified in this study for the first time in *A. millefolium*.

The results of this part of the work have demonstrated that the phytochemical profile of *A. millefolium* is mainly characterized by the presence of chlorogenic acid and its caffeoylquinic derivatives, besides luteolin, rutin, and apigenin flavonoid glycosides, some of which never reported before in this important plant species.

Antiplasmodial activity

The crude MeOH extract and its isolated components were tested for antiplasmodial activity in CQ-sensitive (D10) and CQ-resistant (W2) strains of *P. falciparum*, using CQ as a positive control. The results (Table 2) showed that the crude MeOH extract did not induce 50% mortality in the D10 strain of the parasite even at the highest concentration tested, but showed a measurable activity against the CQ-resistant W2 strain, with an IC₅₀ value of 44.6 (± 8.8) µg/mL.

Among the isolated compounds, apigenin 7-*O*-glucoside (**8**) and luteolin 7-*O*-glucoside (**3**) were the most active against both strains of *P. falciparum* (Table 2), in accordance with the findings from a previous study, in which both luteolin and apigenin inhibited the growth of other strains (3D7 and 7G8) of *P. falciparum* (Lehane

Table 3. Antioxidant activity of methanolic extract from *A. millefolium* and its isolated pure constituents in different model systems

Samples	DPPH (IC ₅₀) ^a		Antioxidant capacity (mEq uric acid) ^b		TBARS (nmol TBARS/mg LDL) ^c	
	1 µM	1 µM	10 µM	1 µM	10 µM	
Ascorbic acid	1.31±0.12	0.33±0.02	0.36±0.02	70.00±2.10	4.62±0.54	
Chlorogenic acid	5.70±0.24	0.41±0.06	1.48±0.15	11.03±0.77	3.52±0.49	
Quercetin	4.37±0.21	0.75±0.06	2.17±0.17	3.85±0.50	0.75±0.04	
1	1.58±0.11	0.30±0.05	1.40±0.38	71.49±1.44	4.47±0.62	
2	1.50±0.11	0.35±0.07	1.84±0.47	70.54±1.09	4.94±0.71	
3	1.10±0.09	0.11±0.03	1.61±0.52	10.04±0.55	4.27±0.46	
4						
5						
6	3.78±0.33 ^d	0.34±0.04 ^d	1.56±0.63 ^d	69.59±1.12 ^d	3.04±0.39 ^d	
10						
7	3.83±0.46	0.39±0.05	1.72±0.56	64.00±0.96	3.66±0.40	
8	2.70±0.17	0.21±0.03	1.08±0.34	10.79±0.48	4.27±0.62	
9	2.68±0.13	0.09±0.01	0.20±0.06	72.11±1.56	12.84±0.99	
MeOH extract ^e	1.18±0.10	0.17±0.05	1.07±0.29	50.40±1.79	2.47±0.51	

^aIC₅₀ = concentration of sample needed to achieve 50% scavenging of DPPH free radical; ^bmEq uric acid = unit of antioxidant capacity for copper reduction; ^cTBARS (thiobarbituric acid-reacting substances) in control samples were 71.14 (± 1.03) nmol/mg LDL; ^dCompounds **4–6** and **10** in the same fraction tested before their identification; ^eThe concentration of MeOH extract was 184 mg/ml; Ascorbic acid, chlorogenic acid and quercetin are reference compounds. Experiments were performed in triplicate; results are mean ± S.D.

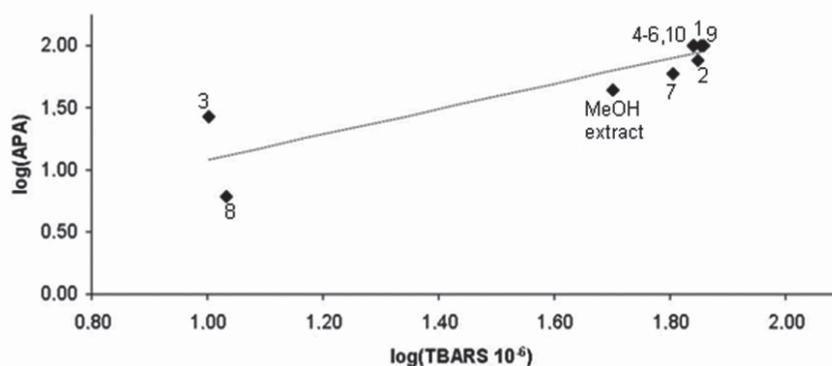


Figure 3. Comparison of antiliperoxidant and antiplasmodial activities of compounds isolated from *A. millefolium* and its methanolic extract

The antiplasmodial activity was determined in a chloroquine resistant strain of *P. falciparum* (W2).

& Saliba, 2008). These results suggest that the presence of the 7-*O*-glycoside in the flavonoid moiety does not inhibit their antiplasmodial activity or that the aglycone becomes active after enzymatic hydrolysis of the glycoside bond.

Apigenin 4'-*O*-glucoside (**7**) and rutin (**2**) showed moderate activity against the both strains, while the other components were completely inactive.

Antioxidant activity

The antioxidant activity of the MeOH extract and its components were evaluated using different *in vitro* assays. The radical scavenging activity was evaluated by the DPPH test, the TAC by the copper reducing power assay and the anti-liperoxidant activity in LDL against Cu^{2+} insult by the TBARS assay. Ascorbic acid, chlorogenic acid and quercetin were used as reference compounds and the results are summarized in Table 3. Noticeably, for TAC, ascorbic acid did not exhibit significant differences between 10^{-5} and 10^{-6} concentrations. We speculatively attribute this to a plateau effect at low concentrations associated with the method.

The MeOH extract, whose polyphenolic content determined by the Folin-Ciocalteu method was 281.7 mg/g, exhibited significant activities in all the models used, comparable to those of the control antioxidants. Concerning pure compounds, on the whole they displayed a rather high degree of activities. In particular rutin (**2**), chlorogenic acid (**1**) and its derivatives **4**, **5**, **6** and **10** (in the same fraction not further separated because of its low amount), and the apigenin derivatives **7** and **8** showed values similar to those of the reference standards, both in terms of scavenging ability (DPPH) and TAC.

The results from the TBARS assay showed that, among the compounds isolated, only **3** and **8** displayed an activity somewhat comparable to that of chlorogenic acid, even at the lowest concentration tested ($1 \mu\text{M}$); all the other compounds were able to inhibit the TBARS formation only at the highest concentration tested ($10 \mu\text{M}$).

Statistics

The correlation and multivariate regression analyses carried out on the antiplasmodial and antioxidant data of compounds **1–10** at $1 \mu\text{M}$ concentration evidenced a significant correlation (Fig. 3) between their activity against TBARS formation and growth inhibition of the CQ re-

sistant strain of *P. falciparum* ($R_{\text{Spearman}} = 0.786$, $P < 0.005$; regression: $\beta_{\text{TBARS}} = 0.776$, $P < 0.01$), suggesting that the antiliperoxidant compounds were the same as those responsible for the inhibition of the parasite. As shown in Fig. 3, this correlation was mainly due to compounds **3** and **8** in both tests suggesting luteolin 7-*O*-glucoside (**3**) and apigenin 7-*O*-glucoside (**8**) as the components putatively responsible for both the antiliperoxidant and antiplasmodial activities of the MeOH extract. To some extent, these results are in accordance with those of other studies (Kirmizibekmez *et al.*, 2004; Tasdemir, 2006; Tasdemir *et al.*, 2006) that reported luteolin 7-*O*-glucoside (**3**) as an inhibitor of *P. falciparum* growth. Most importantly, they provided evidence that inhibition of enzymes involved in the plasmodial type II fatty acid biosynthesis is a potential biochemical target for the *in vitro* inhibitory activity of flavonoids against the parasite. It is interesting to observe that the isomeric forms of **3** and **8**, in which glycosylation occurs at the 4'-*O*-position (compounds **7** and **9**), are much less active in both tests, confirming that the availability of ring B phenol groups is an important factor for the definition of the structure–activity relationships of these compounds.

CONCLUSIONS

The results of this work contribute to the definition of the phytochemical profile of the MeOH extract of *A. millefolium*. Evaluation of the antioxidant and antiplasmodial activities of the isolated pure compounds suggests that flavonoid glycosides **3** and **8** are the main components responsible for both investigated activities and, to the best of our knowledge, antiplasmodial activity of apigenin 7-*O*-glucoside is reported here for the first time, as well as the correlation between the antioxidant power and the antiplasmodial activity of the isolated compounds.

Acknowledgements

The authors are grateful to Dr. G. Rotti for plant identification. This work was supported by MIUR grant PRIN 2004038183/004.

REFERENCES

- Benedek B, Gjoncaj N, Saukel J, Kopp B (2007) Distribution of phenolic compounds in Middle European taxa of the *Achillea millefolium* L. aggregate. *Chem Biodivers* **4**: 849–857.

- Glasl S, Mucaji P, Werner I, Presser A, Jurenitsch J (2002) Sesquiterpenes and flavonoid aglycones from a Hungarian taxon of the *Achillea millefolium* group. *Z Naturforsch* **57c**: 976–982.
- Guédon D, Abbé P, Lamaison JL (1993) Leaf and flower head flavonoids of *Achillea millefolium* L. subspecies. *Biochem Syst Ecol* **21**: 607–611.
- Innocenti G, Vegeto E, Dall'Acqua S, Ciana P, Giorgetti M, Agradi E, Sozzi A, Fico G, Tomè F (2007) *In vitro* estrogenic activity of *Achillea millefolium* L. *Phytomedicine* **14**: 147–152.
- Kaur K, Jain M, Kaur T, Jain R (2009) Antimalarials from nature. *Bioorg Med Chem* **17**: 3229–3256.
- Kirmizibekmez H, Çalis I, Perozzo R, Brun R, Dönmez AA, Linden A, Rüedi P, Tasdemir D (2004) Inhibiting activities of secondary metabolites of *Phlomis brunneogaleata* against parasitic protozoa and plasmoidal enoyl-ACP reductase, a crucial enzyme in fatty acid biosynthesis. *Planta Med* **70**: 711–717.
- Kokkini S, Kleftoyanni V, Hanlidou E, Karousou R (2004) The herbal market of Thessaloniki (N Greece) and its relation to the ethnobotanical tradition. *J Ethnopharmacol* **91**: 281–299.
- Konyalioglu S, Karamenderes C (2005) The protective effects of *Achillea* L. species native in Turkey against H₂O₂-induced oxidative damage in human erythrocytes and leucocytes. *J Ethnopharmacol* **102**: 221–227.
- Lehane AM, Saliba KJ (2008) Common dietary flavonoids inhibit the growth of the intraerythrocytic malaria parasite. *BMC Res Notes* **1**: 26.
- Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL, Hinrichs DJ (1993) Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *Am J Trop Med Hyg* **48**: 739–741.
- Miraldi E, Ferri S, Mostaghimi V (2001) Botanical drugs and preparations in the traditional medicine of West Azerbaijan (Iran). *J Ethnopharmacol* **75**: 77–87.
- Murnigsih T, Matsuura SH, Takahashi K, Yamasaki M, Yamato O, Maede Y, Katakura K, Suzuki M, Kobayashi S, Yoshihara C, Yoshihara T (2005) Evaluation of the inhibitory activities of the extracts of Indonesian traditional medicinal plants against *Plasmodium falciparum* and *Babesia gibsoni*. *J Vet Med Sci* **67**: 829–831.
- Passalacqua NG, Guarrera PM, De Fine G (2007) Contribution to the knowledge of the folk plant medicine in Calabria region (Southern Italy). *Fitoterapia* **78**: 52–68.
- Pieroni A, Quave CL (2005) Traditional pharmacopoeias and medicines among Albanians and Italians in southern Italy: a comparison. *J Ethnopharmacol* **101**: 258–270.
- Pignatti S (1982) *Flora d'Italia* **3**: 77–85. Edagricole, Bologna.
- Saxena S, Pant N, Jain DC, Bhakuni RS (2003) Antimalarial agents from plant sources. *Curr Sci* **85**: 1314–1329.
- Schwikkard S, van Heerden FR (2002) Antimalarial activity of plant metabolites. *Nat Prod Rep* **19**: 675–692.
- Sharma PK, Chauhan NS, Lal B (2004) Observations on the traditional phytotherapy among the inhabitants of Parvati valley in western Himalaya, India. *J Ethnopharmacol* **92**: 167–176.
- Stojanović G, Radulović N, Hashimoto T, Palić R (2005) *In vitro* antimicrobial activity of extracts of four *Achillea* species: the composition of *Achillea clavennae* L. (Asteraceae) extract. *J Ethnopharmacol* **101**: 185–190.
- Tasdemir D (2006) Type II fatty acid biosynthesis, a new approach in antimalarial natural product discovery. *Phytochem Rep* **5**: 99–108.
- Tasdemir D, Lack G, Brun R, Rüedi P, Scapoza L, Perozzo R (2006) Inhibition of *Plasmodium falciparum* fatty acid biosynthesis: evaluation of FabG, FabZ, and FabI as drugs targets for flavonoids. *J Med Chem* **49**: 3345–3353.
- Teng R, Xie H, Li HZ, Liu X, Wang D, Yang C (2002) Two new acylated flavonoid glycosides from *Morina nepalensis* var. *alba* Hand-Mazz. *Magn Reson Chem* **40**: 415–420.
- Trager W, Jensen JB (1976) Human malaria parasites in continuous culture. *Science* **193**: 673–675.
- Vitalini S, Grande S, Visioli F, Agradi E, Fico G, Tomè F (2006) Antioxidant activity of wild plants collected in Valsesia, an alpine region of northern Italy. *Phytother Res* **20**: 576–580.
- Vitalini S, Tomè F, Fico G (2009) Traditional uses of medicinal plants in Valvestino (Italy). *J Ethnopharmacol* **12**: 106–116.
- Yaesh S, Jamal Q, Khan AU, Gilani AH (2006) Studies on hepatoprotective, antispasmodic and calcium antagonist activities of the aqueous-methanol extract of *Achillea millefolium*. *Phytother Res* **20**: 546–551.
- Wang Y, Liu B (2007) Preparative isolation and purification of dicaffeoylquinic acids from the *Ainsliaea fragrans* champ by high-speed counter-current chromatography. *Phytochem Anal* **18**: 436–440.