Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 66 (2005) 2356–2362
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Acylated quercetagetin glycosides with antioxidant activity from *Tagetes maxima*

Irene Parejo, Jaume Bastida, Francesc Viladomat, Carles Codina *

Departament de Productes Naturals, Biologia Vegetal i Edafologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Catalonia, Spain

Received 21 January 2005; received in revised form 7 July 2005
Available online 13 September 2005

Abstract

The fractionation of a methanolic extract of *Tagetes maxima* guided for antioxidant activity resulted in the isolation of three acylated quercetagetin glycosides, quercetagetin-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside), quercetagetin-7-*O*-(6-*O*-*p*-coumaroyl-β-D-glucopyranoside) and quercetagetin-7-*O*-(6-*O*-galloyl-β-D-glucopyranoside), as well as four known flavonoid glycosides. The structural elucidation was accomplished by spectroscopic methods (ESI-MS/MS and NMR). The antioxidant activity of fractions and isolated compounds was determined by checking the scavenging activity against three different radicals: 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH$^{\bullet}$), hydroxyl ($^{\bullet}$OH), and superoxide ($O_2^{\bullet-}$). The three isolated compounds exhibited a high radical scavenging activity in comparison with reference compounds.

Keywords: *Tagetes maxima*; Asteraceae; Antioxidant; Radical scavenging activity; Quercetagetin; Acylated flavonoid glycosides

1. Introduction

The genus *Tagetes* (Asteraceae) includes many species which have been reported to be used in traditional medicine. Thus, for example, infusions of leaves from different species of *Tagetes* have been used to treat stomach and intestinal diseases (Cáceres et al., 1993), and others have been found to possess different biological activities, among others, antimicrobial, antiinflammatory, antioxidant and antiviral (Tereschuk et al., 1997; De las Heras et al., 1998; Abad et al., 1999; Lorenzo et al., 2002).

Regarding the chemical composition of plants of the genus *Tagetes*, most of the studies have been focused to the chemotaxonomy and distribution of flavonoids within this genus (Bohm and Stuessy, 2001; Abadala, 2001, 2003), but a few works have been carried out to look for bioactive constituents. Thus, to our knowledge only one work dealing with the bioguided isolation of antioxidant flavonoids from *Tagetes lucida* has been reported up to now (Aquino et al., 2002). The main phenolic substances occurring in *Tagetes* plants include kaempferol, isorhamnetin, quercetin, patuletin, quercetagetin, myricetin and luteolin glycosides as well as their respective aglycones. Quercetagetin and its derivatives are flavonoids characteristic of these plants, as they have been found to occur in all the hitherto studied species of this genus (Bohm and Stuessy, 2001; Abadala, 2001, 2003).

In a previous screening for antioxidant activity of Bolivian plants, *Tagetes maxima* was found to exhibit a strong radical scavenging and antioxidant activities (Parejo et al., 2003). No other biological activities are known for this plant species. From the chemical point of view, it was formerly studied only for essential oils

* Corresponding author. Tel.: +34 93 4024493; fax: +34 93 4029043.
E-mail address: carlescodina@ub.edu (C. Codina).

0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.07.004

(Lorenzo et al., 2002), but there is no information on its polar constituents. *T. maxima* Kuntze (vernacular name, "suico alto" or "alk'o suico") is a plant specie native in the region of Cochabamba (Bolivia). The leaves and stems are commonly used by the indigenous people of the region ("comunarios") in food as seasoning, beverage and also as a medicine for stomach ailments (Lorenzo et al., 2002).

This work deals with the isolation of the antioxidative compounds of *T. maxima*. The antioxidant activity of extracts and fractions, as well as that of the isolated compounds, was evaluated by checking the scavenging activity against three different radicals: 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH$^{\bullet}$), hydroxyl ($^{\bullet}$OH), and superoxide $O_2^{\bullet-}$. Moreover, the total phenolic content was also determined by the Folin–Ciocalteu method. Once the major constituents were chemically characterized, they were tested for their radical scavenging activity, and compared with that of different reference compounds.

2. Results and discussion

2.1. Fractionation for antioxidant activity

The results of both the total phenolic content and radical scavenging activity of the first crude extracts and fractions are shown in Table 1. The ethyl acetate fraction exhibited the highest total phenolic content (392.6 GAE/mg extract), as well as a high radical scavenging activity, comparable to that of the green tea extract. The ethyl acetate fraction was chromatographed by Sephadex LH-20 thus obtaining four active fractions (from A to D), which were also evaluated for their antioxidant activity. Fraction A exhibited the lowest total phenolic content and radical scavenging activity, whereas fractions C and D were found to contain the highest amount of phenolics (665.9 and 725.3 GAE/mg dry extract, respectively), even higher than that of both the rosemary and green tea extracts (that of grape seeds is a purified extract), and the highest radical scavenging activity in the three methods used (Table 1). Fraction B also exhibited a moderate antioxidant activity and total phenolic content.

2.2. Characterization of the phenolic compounds

The LC/DAD analysis of fractions A–D revealed the presence of seven major flavonoids (**1**–**7**). The use of the diode-array detector showed that the UV spectra of four of them (compounds **1**–**4**) suggested the acylation of the sugar moiety with hydroxycinnamic acid derivatives (Ferreres et al., 1997). The ESI-MS/MS spectra showed the pseudomolecular ions as well as characteristic fragmentations due to the cleavage of the glycosidic bond, which suggested the presence of both quercetagetin (*m*/*z* 317) and methylated quercetagetin glycosides (*m*/*z* 331 and 359). The structures of the new compounds were determined by ^{1}H, ^{13}C and 2D NMR, and ESI-MS/MS methods (Table 2).

Compounds **1**, **2** and **3** appeared to be new natural acylated quercetagetin glycosides. Their ^{1}H and ^{13}C NMR spectra (Table 2) were very similar to those of **6** (Aquino et al., 2002; Heilmann et al., 1999), but showing in the aromatic region the additional and characteristic shift values of caffeoyl, coumaroyl and galloyl moieties, respectively. In the MS, the parent peaks, the ions at *m*/*z*

Table 1
Radical scavenging activity of *Tagetes maxima* extracts and fractions

	Dry weight (g)	TPH[a]	DPPH[b]	CL[b]	SO[c]
Extract/fraction					
Crude extract	157.9	188.02 ± 13.01	27.17 ± 1.69	34.70 ± 2.24	36.79 ± 0.29
Defatted extract	80.26	272.64 ± 24.38	13.11 ± 1.11	11.24 ± 0.70	60.23 ± 2.48
Hexane	30.02	123.03 ± 11.36	46.63 ± 1.16	53.60 ± 2.36	20.67 ± 1.46
Ethyl acetate	25.62	392.58 ± 20.01	14.45 ± 0.27	4.11 ± 0.10	68.36 ± 0.95
Aqueous	35.33	199.54 ± 14.48	19.08 ± 0.85	11.84 ± 0.95	53.70 ± 1.38
Ethyl acetate fraction					
A	1.56	175.51 ± 4.02	124.53 ± 3.06	13.81 ± 0.14	39.36 ± 1.48
B	2.56	383.50 ± 10.3	13.55 ± 0.28	9.20 ± 0.37	73.43 ± 3.19
C	1.74	665.93 ± 6.60	7.42 ± 0.57	4.81 ± 0.40	74.73 ± 5.67
D	2.38	725.28 ± 1.28	5.40 ± 0.28	3.92 ± 0.01	79.82 ± 3.44
Reference extract					
Grape seeds extract		851.32 ± 12.83	6.91 ± 0.20	29.53 ± 1.41	88.31 ± 0.52
Rosemary extract		132.04 ± 5.66	35.08 ± 0.15	46.86 ± 1.60	38.56 ± 0.96
Green tea extract		387.25 ± 7.73	11.38 ± 0.58	13.34 ± 0.47	92.19 ± 4.87

Values are the mean of three determinations ± standard deviation (SD). See text for the identification of extracts and fractions.
[a] Total phenolic content. Values are expressed as equivalents of gallic acid (GAE)/mg dry extract.
[b] Values expressed as IC_{50} (μg/ml).
[c] Percentage of inhibition of the superoxide anion (at 50 μg/ml).

Table 2
^{1}H-NMR and ^{13}C-NMR data of compounds **1**, **2** and **3**

Position	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Quercetagetin						
2	–	148.74 *s*	–	148.61 *s*	–	149.22 *s*
3	–	137.09 *s*	–	137.16 *s*	–	137.06 *s*
4	–	177.25 *s*	–	177.27 *s*	–	177.43 *s*
5	–	146.79 *s*	–	146.73 *s*	–	148.71 *s*
6	–	130.75 *s*	–	130.71 *s*	–	130.82 *s*
7	–	152.49 *s*	–	152.51 *s*	–	152.59 *s*
8	6.73 *s*	94.91 *d*	6.79 *s*	94.84 *d*	6.82 *s*	95.01 *d*
9	–	150.10 *s*	–	150.09 *s*	–	150.30 *s*
10	–	106.55 *s*	–	106.48 *s*	–	106.71 *s*
1′	–	124.01 *s*	–	124.08 *s*	–	123.99 *s*
2′	7.69 *d* (2.0)[a]	116.10 *d*	7.74 *d* (2.0)	116.03 *d*	7.73 *d* (2.0)	116.37 *d*
3′	–	145.96 *s*	–	146.07 *s*	–	145.88 *s*
4′	–	148.74 *s*	–	148.81 *s*	–	149.22 *s*
5′	6.81 *d* (8.5)	116.10 *d*	6.84 *d* (8.5)	116.14 *d*	6.80 *d* (8.5)	116.20 *d*
6′	7.56 *dd* (2.0, 8.5)	121.82 *d*	7.64 *dd* (2.0, 8.5)	121.79 *d*	7.43 dd (2.0, 8.5)	121.67 *d*
β-D-Glucopyranoside						
1″	5.06 *d* (7.5)	102.01 *d*	5.06 *d* (7.5)	101.90 *d*	5.10 *d* (7.5)	102.32 *d*
2″	3.63 *dd* (7.5, 9.0)	74.50 *d*	3.61 *m*	74.45 *d*	3.61 *m*	74.61 *d*
3″	3.59 *t* (9.0)	77.34 *d*	3.57 *m*	77.37 *d*	3.59 *m*	77.22 *d*
4″	3.47 *dd* (9.0, 9.5)	72.04 *d*	3.43 *m*	72.28 *d*	3.57 *m*	71.31 *d*
5″	3.84 *ddd* (2.5, 7.5, 9.5)	75.53 *d*	3.88 *m*	75.48 *d*	3.85 *m*	75.81 *d*
6″a	4.64 *dd* (2.5, 12.0)	64.58 *t*	4.62 *brd* (11.5)	64.84 *t*	4.68 *dd* (2.0, 12.0)	64.20 *t*
6″b	4.32 *dd* (7.5, 12.0)	64.58 *t*	4.32 *dd* (7.5, 11.5)	64.84 *t*	4.45 *dd* (5.0, 12.0)	64.20 *t*
Caffeoyl			Coumaroyl		Galloyl	
1‴	–	127.29 *s*	–	126.62 *s*	–	121.19 *s*
2‴	6.72 *d* (2.0)	115.55 *d*	7.03 *d* (8.5)	130.86 *d*	7.03 *s*	110.07 *d*
3‴	–	146.36 *s*	6.53 *d* (8.5)	116.56 *d*	–	146.34 *s*
4‴	–	149.25 *s*	–	160.93 *s*	–	139.76 *s*
5‴	6.50 *d* (8.5)	116.37 *d*	6.53 *d* (8.5)	116.56 *d*	–	146.34 *s*
6‴	6.54 *dd* (2.0, 8.5)	122.16 *d*	7.03 *d* (8.5)	130.86 *d*	7.03 *s*	110.07 *d*
7‴	7.41 *d* (16.0)	147.48 *d*	7.47 *d* (16.0)	147.16 *d*	–	168.22 *s*
8‴	6.15 *d* (16.0)	114.37 *d*	6.20 *d* (16.0)	114.21 *d*	–	
9‴	–	168.97 *s*	–	168.99 *s*	–	

C-multiplicities were determined by DEPT data.
[a] Chemical shifts in ppm. Coupling constants (*J*, Hz).

479, obtained after the lost of the acyl moiety, and the intensive fragments at *m*/*z* 317 derived from the aglycone, were in accordance with the proposed structures (Heilmann et al., 1999). The observed upfield shift of the C-7 carbon resonances (Table 2), in comparison with the free aglycone, as well as a long-range connectivity between H-1″ and C-7 in the HMBC spectrum, and a NOESY spatial proximity between the same proton and H-8, confirmed that the sugar was attached to C-7 of the aglycone (Aquino et al., 2002; Heilmann et al., 1999). Assignment of the remaining ^{1}H and ^{13}C resonances of the sugar and the signal of the anomeric glucose proton (H-1″) appearing as a doublet ($J_{\text{H-1}'',\text{H-2}''} = 7.5$ Hz) indicated that the sugar residue was a β-D-glucopyranosyl. The downfield-shifted resonances of 6″a and 6″b protons and C-6″ of the β-glucopyranosyl residue, in comparison with resonances of the corresponding spectra of the β-glucopyranosyl residue of **6**, indicated that the acyl groups were linked to C-6″ position (Aquino et al., 2002; Heilmann et al., 1999). This was confirmed by the long-range connectivity observed between both the 6″a and the 6″b protons and the carbonyl groups of compounds **1** (C-9‴), **2** (C-9‴) and **3** (C-7‴), respectively. The identity of the acyl groups was established by ^{1}H and ^{13}C NMR spectroscopic analysis (Table 2), whose assignments were facilitated by COSY, HSQC and HMBC experiments and were in agreement with the values of caffeoyl (Nair et al., 1993), coumaroyl (Ferreres et al., 1997) and galloyl (Brakat et al., 1999) moieties of different compounds reported in the literature. Consequently, the identity of **1**, **2** and **3** was confirmed as quercetagetin-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside), quercetagetin-7-*O*-(6-*O*-*p*-coumaroyl-β-D-glucopyranoside) and quercetagetin-7-*O*-(6-*O*-galloyl-β-D-glucopyranoside), respectively. This is the first report on acylated quercetagetin glycosides

in the plant kingdom. Compound **4** was identified as 6-hydroxykaempferol-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside) by comparison of its MS, ^{1}H and ^{13}C NMR spectra with those reported in the literature (Nair et al., 1993). Compounds **5**–**7** were identified as 3,6,4′-tri-*O*-methylquercetagetin-7-*O*-β-D-glucopyranoside (centaureidin-7-*O*-β-D-glucopyranoside) (**5**) (Long et al., 2003), quercetagetin-7-*O*-β-D-glucopyranoside (**6**) (Heilmann et al., 1999) and patuletin-7-*O*-β-D-glucopyranoside (**7**) (Park et al., 2000) by comparison of their MS, ^{1}H and ^{13}C NMR spectra with those reported in the literature. Moreover, the NOESY experiment detected spatial proximity between the glucose anomeric proton (H-1″) and H-8, which confirmed the attachment of the sugar to the hydroxy group of C-7 position of the aglycone.

2.3. Antioxidant and radical scavenging activity of isolated compounds

Compounds **1**–**7** were evaluated for their radical scavenging activity and the results compared with those of some standard compounds (Table 3). The new compound quercetagetin-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside) (**1**) was found to exhibit the highest antioxidant activity, even higher than that of the four standards used as reference. In general, all the quercetagetin type flavonoids, as well as the 6-hydroxykaempferol derivative (**4**), exhibited a high radical scavenging activity in all the tests, comparable to that of some of the standards used (Table 3). Of all these flavonoids, that acylated with gallic acid (**3**) was the less active.

It is generally assumed that the maximal radical scavenging and/or antioxidant activity of flavonoids is mainly due to the occurrence of the 2,3-double bond in conjugation with a 4-oxo function, and to the additional presence of hydroxyl groups in positions 3′, 4′ and 7. These sites can be considered as the active centers or the prerequisite factors for the scavenging of free radicals (Heim et al., 2002). In our study, the additional presence of an hydroxyl group in position 6 of the quercetagetin derivatives has resulted, in general, in an increase of the radical scavenging activity in comparison with the C-6 non-hydroxylated reference compounds isoquercitrin (quercetin glucoside), rutin (quercetin rutinoside) and quercetin (Table 3). On the other hand, the acylated C-6″ position of the glucose with a hydroxycinnamic acid increased the radical scavenging activities measured in comparison with the non-acylated quercetagetin-7-*O*-β-D-glucopyranoside (**6**). On the contrary, methoxylation of quercetagetin (compounds **5** and **7**) decreased the activity, in agreement with Heim et al. (2002). In summary, the acylation of these flavonoids could have important in vivo effects, since after consumption they are probably hydrolized thus affording free hydroxycinnamic acids, which can act as antioxidant by themselves (Chen et al., 1999).

Many activities have been attributed to flavonoids owing to their antioxidant activity, such as antiinflammatory, antiviral, antimicrobial, antiallergic, antiespasmodic, antihepatotoxic, and antiulcerogenic activities (Pietta, 2000). The isolation of antioxidative acylated quercetagetin glycosides from *T. maxima* could explain, at least partially, the beneficial effects of this plant species and its traditional use.

3. Experimental

3.1. General experimental procedures

^{1}H, ^{13}C NMR, DEPT, ^{1}H COSY, NOESY, HSQC and HMBC spectra were recorded on a Varian

Table 3
Radical scavenging activity of the compounds isolated from *Tagetes maxima* and that of standard compounds

Compound	DPPH[a]	CL[a]	SO[b]
Quercetagetin-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside) (**1**)	2.73 ± 0.04	1.10 ± 0.08	89.28 ± 0.61
Quercetagetin-7-*O*-(6-*O p*-coumaroyl-β-D-glucopyranoside) (**2**)	3.29 ± 0.05	1.54 ± 0.06	85.38 ± 1.56
Quercetagetin-7-*O*-((6-*O*-galloyl)-β-D-glucopyranoside) (**3**)	6.91 ± 0.02	8.30 ± 0.22	81.41 ± 3.03
6-Hydroxykaemferol-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside) (**4**)	5.27 ± 0.12	1.28 ± 0.07	90.23 ± 1.23
Centaurein-7-*O*-β-D-glucopyranoside (**5**)	115.30 ± 6.18	120.84 ± 4.98	15.62 ± 1.23
Quercetagetin-7-*O*-β-D-glucopyranoside (**6**)	5.08 ± 0.03	3.92 ± 0.13	82.85 ± 2.34
Patuletin-7-*O*-D-glucopyranoside (**7**)	23.34 ± 0.10	25.63 ± 0.15	45.69 ± 0.87
Standard			
Isoquercitrin	7.40 ± 0.02	26.21 ± 1.52	75.90 ± 4.69
Quercetin	6.11 ± 0.53	5.13 ± 0.12	97.45 ± 3.25
BHA	34.12 ± 0.63	2.14 ± 0.01	67.51 ± 0.29
Rutin	9.85 ± 0.23	30.04 ± 0.36	21.66 ± 1.72
Chlorogenic acid	3.82 ± 0.33	28.36 ± 0.42	84.30 ± 1.68

Values expressed as the mean of three determinations ± standard deviation (SD).
[a] Values expressed as IC_{50} (μg/ml).
[b] Percentage of inhibition of the superoxide anion (at 50 μg/ml).

Gemini-300 or Inova 500 NMR spectrometer operating at 500 and 125 MHz, respectively, for ^{1}H and ^{13}C NMR measurements. The chemical shifts are reported as part per million (ppm) units relative to TMS. CD_3OD was used as a solvent. IR spectra were recorded on a Nicolet Avantar 320 FT-IR spectrometer. Only noteworthy IR absorptions (cm^{-1}) are listed. Melting points were determined in a capillary tube and are uncorrected. Optical rotations were performed in a Perkin–Elmer 241 instrument, using a 1-cm cuvette (V total 1 ml). High resolution mass spectra (HRMS) were performed in a mass spectrometer Autospec Micromass using ESI in negative mode. ESI-MS/MS analyses were carried out using an API 3000 triple quadrupole mass spectrometer-Turbo Ionspray source (Perkin–Elmer Sciex, Concord, Ont., Canada) in negative mode. The compounds were directly infused by a flow syringe pump at 400 μl min^{-1}, and analyzed in full-scan and product ion scan modes, scanning from m/z 100 to 800 u. Capillary voltage: 3500 V; nebulizer gas (N_2): 10 (arbitrary units); curtain gas (N_2): 12 (arbitrary units); collision gas (N_2): 10 (arbitrary units); focusing potential: 200 V; entrance potential: 10 V; and declustering potential (DP): 75; collision energy (CE): 45. To avoid carryovers among the compounds, the system was rinsed with 100% ACN during 15 min between two analyses. Preliminary analytical LC/DAD analyses were performed on a Hewlett–Packard HP Series 1050 chromatograph equipped with an automatic injector, vacuum degasser and a diode-array detector (DAD). A C_{18} Nucleosil 120 column (250 × 0.46 mm, 5 μm) (Tecknokroma, Spain) was used at a flow rate of 1 ml/min. The mobile phase consisted on H_2O–0.1% HCO_2H (solvent A) and ACN–0.1% HCO_2H (solvent B), using an increasing linear gradient (v/v) of solvent B ranging from 15% to 26% in 40 min. The chromatograms were carried out at 280 nm, with peak scanning between 200 and 600 nm. Semi-preparative HPLC analyses were performed using a C_{18} Nucleosil 120 column (250 × 10 mm, 10 μm) (Tecknokroma, Spain) at a flow rate of 3 ml/min, using the same conditions as those described above for the analytical LC/DAD method.

3.2. Plant material

The aerial parts (stems and leaves) of *T. maxima* were collected in Sivingani, Province of Ayopaya (Cochabamba, Bolivia), at an altitude of 2780 m, in November 2002. The plant was authenticated by Lic. Modesto Zárate and Lic. Magaly Mercado, from the Unidad de Biodiversidad y Genética, of the Universidad Mayor de San Simón, and the plant was deposited at the Herbario Nacional Martín Cárdenas of Cochabamba (Bolivia), where a voucher specimen was registered with No. MM 1837.

3.3. Chemicals

All chemicals and reagents used the for total phenolic content and for the radical scavenging assays were purchased from Sigma–Aldrich (MO, USA), with the exception of the Folin–Ciocalteu's reagent, which was purchased from Panreac (Barcelona, Spain). All the chemicals and reagents were of analytical grade: ACN (HPLC-grade, SDS, Peypin, France), HCO_2H (analytical grade, Probus, Badalona, Spain) and ultrapure water (Milli-Q, Waters, Milford, USA) were used for the mobile phase preparation in the high-performance liquid chromatographic analysis. CD_3OD was purchased to SDS (Peypin, France). The standards were also purchased from Sigma–Aldrich, and the reference extracts were kindly provided by RAPS, Germany (green tea and rosemary) and EUROMED, Spain (grape seeds).

3.4. Extraction and isolation

The powdered dry stems and leaves (750 g) were extracted at room temperature by maceration with MeOH (5 l × 5) to give a dry extract of 157.9 g. This crude extract was redissolved in H_2O (1 l) and defatted by partitioning with hexane until the organic solvent was colourless, thus affording an hexane soluble fraction (30.02 g) and a defatted crude extract (80.26 g). Then, the defatted crude extract was partitioned with EtOAc affording an EtOAc soluble fraction (25.62 g) and a water soluble fraction (35.33 g). The ethyl acetate fraction was chromatographed on a Sephadex LH-20 column (Pharmacia, 5 × 50 cm, 1 ml/min, MeOH) to afford 226 fractions (10 ml each). The fractions were monitored by thin-layer chromatography (TLC: Alugram® silicagel plates, Macherey-Nagel, EtOAc:HOAc:H_2O 10:2:3). After developing and drying, the plates were sprayed with 1% diphenylboric acid in MeOH for UV enhancement of phenolic compounds and visualized under UV light at 254 and 365 nm. The TLC plates were also sprayed with a DPPH methanolic solution (20 mg/ml) and examined 20 min after spraying. Active compounds appeared as yellow spots against a purple background. Fractions were combined to obtain four active fractions (from A to D). The same TLC systems were used for the monitoring of the subfractions obtained in further fractionations. Fractions A–D were subjected to LC/DAD analysis and to a subsequent fractionation process guided for antioxidant activity to isolate the major active compounds. Fractions A and B were further cleaned-up on Sephadex LH-20 (2 × 50 cm, MeOH, 0.5 ml/min) and subfractions of 2 ml were collected. The eluates were combined on the basis of the TLC behavior as described above, and the most active subfractions (aliquotes of 200 mg) were then subjected to a purification process by semi-preparative HPLC to yield the major antioxidant constituents. Fractions C

and D were directly purified by semi-preparative HPLC as described before.

Chromatographic separation by preparative HPLC of the obtained fractions afforded seven major constituents: 3,6,4′-tri-*O*-methylquercetagetin-7-*O*-β-D-glucopyranoside (centaureidin-7-*O*-β-D-glucopyranoside) (5) (16 mg, t_R28.9 min) in fraction A, quercetagetin-7-*O*-β-D-glucopyranoside (6) (18 mg, t_R 19.9 min) and 6-*O*-methylquercetagetin-7-*O*-β-D-glucopyranoside (patuletin-7-*O*-glucoside) (7) (7 mg, t_R 22.9 min) in fraction B, quercetagetin-7-*O*-β-D-glucopyranoside (6) (5 mg, t_R19.9 min), quercetagetin-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside) (1) (5 mg, t_R 25.7 min), 6-hydroxykaempferol-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside) (4) (13 mg, t_R 28.6 min), and quercetagetin-7-*O*-(6-*O*-*p*-coumaroyl-β-D-glucopyranoside) (2) (5 mg, t_R 29.2 min) in fraction C, and quercetagetin-7-*O*-(6-*O*-galloyl-β-D-glucopyranoside) (3) (8 mg, t_R 21.7 min) and quercetagetin-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside) (1) (15 mg, t_R 25.7 min) in fraction D. Their chemical structures are shown in Fig. 1. Compound **1** was further purified by crystallization in methanol, giving yellow crystals.

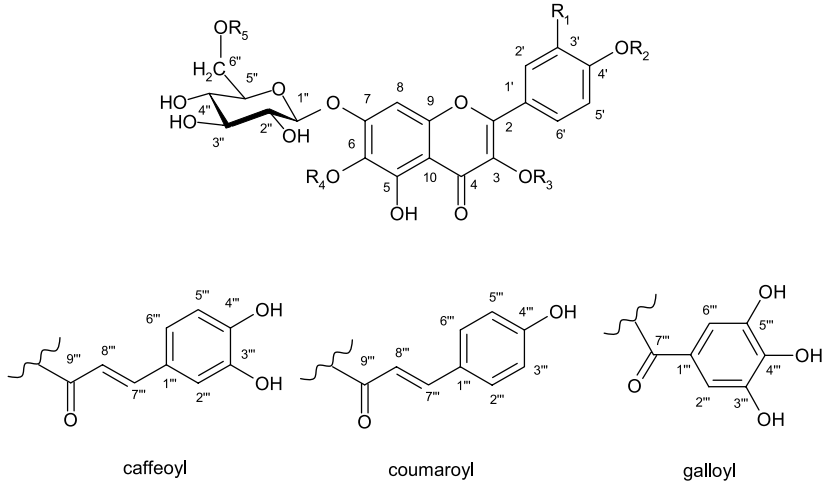
Compound **1**: yellow crystals; m.p. 198–200 °C; $[\alpha]_D$ −248° (*c* 0.501, MeOH); UV λ_{max} (MeOH): 235, 275, 337; IR (KBr) ν_{max}: 3401 (OH), 1662 (ester C=O), 1600 (α,β-unsaturated C=O) 1559, 1490, 1445, 1322, 1282, 1181, 1047 (C–O); LC–ESI-MS: *m*/*z* 641 $[M - H]^-$; HR-ESI-FABS: *m*/*z* 641.1121 $[M - H]^-$ ($C_{30}H_{25}O_{16}$, requires 641.1143). See Table 2 for NMR data.

Compound **2**: yellow powder; m.p. 224–226 °C; $[\alpha]_D$ −193° (*c* 0.321, MeOH); UV λ_{max} (MeOH): 276, 316, 365; IR (KBr) ν_{max}: 3395 (OH), 1663 (ester C=O), 1602 (α,β-unsaturated C=O) 1559, 1491, 1364, 1275, 1179, 1072 (C–O); LC–ESI-MS: *m*/*z* 631 $[M - H]^-$; HR-ESI-FABS: *m*/*z* 631.0931 $[M - H]^-$ ($C_{28}H_{23}O_{17}$, requires 631.0935). See Table 2 for NMR data.

Compound **3**: yellow amorphous powder; $[\alpha]_D$ −235° (*c* 0.085, MeOH); UV λ_{max} (MeOH): 263, 365; LC–ESI-MS: *m*/*z* 625 $[M - H]^-$; HR-ESI-FABS: *m*/*z* 625.1183 $[M - H]^-$($C_{30}H_{25}O_{15}$, requires 625.1193). See Table 2 for NMR data.

3.5. Total phenolic content and radical scavenging assays

The determination of the total phenolic content (TPH), as well as the DPPH· free radical, superoxide-nitro-blue tetrazolium hypoxanthine/xanthine oxidase (SO), and ·OH/luminol chemiluminescence (CL) radical scavenging activity was carried out according to methodology previously described (Parejo et al., 2003). Values are expressed as inhibitory concentration, IC_{50} (μg/ml), for both the DPPH· and CL methods, and as percentage of inhibition for the SO method. The total phenolic content, determined by the Folin–Ciocalteu



Compound	R_1	R_2	R_3	R_4	R_5
Quercetagetin-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside) (**1**)	OH	H	H	H	caffeoyl
Quercetagetin-7-*O*-(6-*O*-*p*-coumaroyl-β-D-glucopyranoside) (**2**)	OH	H	H	H	coumaroyl
Quercetagetin-7-*O*-(6-*O*-galloyl-β-D-glucopyranoside) (**3**)	OH	H	H	H	galloyl
6-Hydroxykaempferol-7-*O*-(6-*O*-caffeoyl-β-D-glucopyranoside) (**4**)	H	H	H	H	caffeoyl
Centaureidin-7-*O*-β-D-glucopyranoside (**5**)	OH	CH_3	CH_3	CH_3	H
Quercetagetin-7-*O*-β-D-glucopyranoside (**6**)	OH	H	H	H	H
Patuletin-7-*O*-β-D-glucopyranoside (**7**)	OH	H	H	CH_3	H

Fig. 1. Chemical structures of the compounds isolated from *Tagetes maxima*.

method, was expressed as equivalents of gallic acid (GAE)/mg of dry extract.

Acknowledgements

This work was financed by the Generalitat de Catalunya (Ref. 2001SGR-00124) and was carried out within the framework of the Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo (CYTED, project IV.11). The authors thank Dr. Olga Jáuregui, from the Serveis Cientificotècnics de la Universitat de Barcelona, for her technical assistance. I.P. is grateful to the Generalitat de Catalunya for providing a research fellowship.

References

Abad, M.J., Bernejo, P., Palomino, S.S., Chiriboga, X., Carrasco, L., 1999. Antiviral activity of some South American medicinal plants. Phytother. Res. 13, 142–146.

Abadala, L.R., 2001. *Tagetes tenuifolia* Cav. (Asteraceae): some chemosystematic implications of their flavonoids. Biochem. Syst. Ecol. 29, 861–863.

Abadala, L.R., 2003. Chemosystematic interpretations of the flavonoids identified in *Tagetes gracilis* (Asteraceae). Biochem. Syst. Ecol. 31, 323–325.

Aquino, R., Cáceres, A., Morelli, S., Rastrelli, L., 2002. An extract of *Tagetes lucida* and its phenolic constituents. J. Nat. Prod. 65, 1773–1776.

Bohm, B.A., Stuessy, T.F., 2001. Flavonoids of the Sunflower Family (Asteraceae). Springer-Verlag, Wien.

Brakat, H.H., Souleman, A.M., Hussein, S.A.M., Ibrahiem, O.A., Nawwar, M.A.M., 1999. Flavonoid galloyl glucosides from the pods of *Acacia farnesiana*. Phytochemistry 51, 139–142.

Cáceres, A., Fletes, L., Aguilar, L., Ramírez, O., Figueroa, L., Tarcena, A.M., Samayoa, B., 1993. Plants used in Guatemala for the treatment of gastrointestinal disorders. J. Ethnopharmacol. 38, 31–38.

Chen, C.P., Yokozawa, T., Chung, H.Y., 1999. Inhibitory effect of caffeic acid analogues isolated from *Salviae Miltiorrhizae* radix against 1,1-diphenyl-2-picrylhydrazyl radical. Exp. Toxicol. Pathol. 51, 59–63.

De las Heras, B., Slowing, K., Benedí, J., Carretero, E., Ortega, T., Toledo, C., Bermejo, P., Iglesias, I., Abad, M.J., Gómez-Serranillos, P., Liso, P.A., Villar, A., Chiriboga, X., 1998. Antiinflammatory and antioxidant activity of plants used in traditional medicine in Ecuador. J. Ethnopharmacol. 61, 161–166.

Ferreres, F., Castañer, M., Tomás-Barberán, F.A., 1997. Acylated flavonol glycosides from spinach leaves (*Spinacia oleracea*). Phytochemistry 45, 1701–1705.

Heilmann, J., Müller, E., Merfort, I., 1999. Flavonoid glucosides and dicaffeoylquinic acids from flowerheads of *Buphthalmum salicifolium*. Phytochemistry 51, 713–718.

Heim, E.H., Tagliaferro, A.R., Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. J. Nutr. Biochem. 13, 572–584.

Long, C., Sauleau, P., David, B., Lavaud, C., Cassabois, V., Ausseil, F., Massiot, G., 2003. Bioactive flavonoids of *Tanacetum parthenium* revisited. Phytochemistry 64, 567–569.

Lorenzo, D., Loayza, I., Dellacassa, E., 2002. Composition of the essential oil of *Tagetes maxima* Kuntze from Bolivia. Flavour Fragr. J. 17, 115–118.

Nair, A.G.R., Jayaprakasam, R., Gunasegan, R., Bayet, C., Voirin, B., 1993. 6-Hydroxykaempferol-7-(6″-caffeoylglucoside) from *Eupatorium grandulosum*. Phytochemistry 33, 1275–1276.

Parejo, I., Viladomat, F., Bastida, J., Rosas-Romero, A., Saavedra, G., Murcia, M.A., Jiménez, A.M., Codina, C., 2003. Investigation of Bolivian plant extracts for their radical scavenging activity and antioxidant activity. Life Sci. 73, 1667–1681.

Park, E.U., Kim, Y., Kim, J., 2000. Acylated flavonol glycosides from flower of *Inula britanica*. J. Nat. Prod. 63, 34–36.

Pietta, P.G., 2000. Flavonoids as antioxidants. J. Nat. Prod. 63, 1035–1042.

Tereschuk, M.L., Riera, M.V.Q., Castro, G.R., Abdala, L.R., 1997. Antimicrobial activity of flavonoids from leaves of *Tagetes minuta*. J. Ethnopharmacol. 56, 227–232.