Free Radical Scavengers and Antioxidants from *Tagetes mendocina*

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Tagetes mendocina (Asteraceae) is a medicinal plant widely used in the Andean provinces of Argentina. Preliminary assays showed free radical scavenging activity in the methanol extract of the aerial parts, measured by the decoloration of a methanolic solution of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and scavenging of the superoxide anion. Assay-guided isolation led to 4′-hydroxyacetophenone (**1**), protocatechuic acid (**2**), syringic acid (**3**), patuletin (**4**), quercetagetin 7-*O*-β-D-glucoside (**5**), patuletin 7-*O*-β-D-glucoside (**6**) and axillarin 7-*O*-β-D-glucoside (**7**) as the free radical scavengers and antioxidant compounds from *Tagetes mendocina.* On the basis of dry starting material, the total phenolic content of the crude drug was 3.00% with 0.372% of flavonoids. The content of compounds **1**–**7** in the crude drug was 0.008, 0.015, 0.010, 0.029, 0.238, 0.058 and 0.017%, respectively. Quercetagetin 7-*O*-β-D-glucoside proved to be the main free radical scavenger of the extracts measured by the DPPH decoloration test as well as for quenching the superoxide anion and inhibition of lipoperoxidation in erythrocytes. In the lipid peroxidation assay the percentual inhibition was related with the number of methoxy groups in the molecule, ranging from 86% for the quercetagetin glucoside to 67% for the monomethoxylated and 31% for the dimethoxylated derivative. The compounds showed low cytotoxicity towards human lung fibroblasts with $IC_{50} > 1$ mM for compounds **1**–**3** and 0.24 to 0.52 mM for the flavonoids **4**–**7**.

Key words: Tagetes mendocina, Phenolics, Antioxidant Activity

Introduction

In the past decade, it has been shown that antioxidants and free radical scavengers are relevant in the prevention of pathologies like arteriosclerosis, heart diseases, cancer and arthritis, in which reactive oxygen species or free radicals are implicated (Middleton *et al.*, 2000). Flavonoids and phenolics from plants have been reported as potent free radical scavengers and frequently occur in medicinal and aromatic plants. Recently, the strong antioxidant activity of *Tagetes lucida* from Guatemala has been reported (Aquino *et al.*, 2002).

Some *Tagetes* species are used in South American traditional medicine to treat several conditions including gastrointestinal diseases and skin infections, as digestive and vermifuge as well as a spice in traditional recipes (Aquino *et al.*, 2002; Tereschuk *et al.*, 1997). An infusion of the aerial parts of *Tagetes mendocina* is recommended as digestive and diuretic in the traditional medicine of the San Juan Province in Argentina (Feresin *et al.*, 2000). The genus *Tagetes* (Asteraceae) comprises 12 species in Argentina, distributed from the northwestern part of the country to the southwest in the Province of San Juan (Abdala, 1999). Previous studies on Argentinian *Tagetes* have been focused on the chemotaxonomy and distribution of flavonoids in the genus but little has been done on the identification of the bioactive metabolites in the medicinal and aromatic species (Abdala *et al.*, 1991; Abdala, 1999). Since a crude methanolic extract of *Tagetes mendocina* showed a strong free radical scavenging effect measured by decoloration of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), a study was undertaken to isolate and identify the free radical scavengers and antioxi-

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dant metabolites from this plant. Following our ethnopharmacological studies on South American medicinal plants we now report the assay-guided isolation of free radical scavengers and antioxidants from *Tagetes mendocina.*

Materials and Methods

Plant material

The plant was collected in December 2001 and January 2002 in the Bauchazeta district, San Juan Province, Argentina, with assistance of local traditional healers. It was identified by Dr. L. Ariza-Espinar at IMBIV (CONICET), Universidad Nacional de Córdoba, Argentina, and has been kept at the Instituto Multidisciplinario de Biología Vegetal, Universidad de Córdoba, Argentina.

Total phenolic content

The total phenolic content of the plant was determined by the Folin-Ciocalteau technique using a calibration curve with tannic acid. The total flavonoid content was estimated by the aluminium chloride method (Heldrich, 1990).

Isolation

The air-dried plant material (2.0 kg) was extracted successively with hexane (H), dichloromethane (DCM) and methanol (MeOH) at room temperature to afford an H, DCM and MeOH extract, respectively. The w/w yields in terms of dry starting material for the H, DCM and MeOH extract were 1.28% (22.56 g), 0.59% (11.86 g) and 6.0% (120.17 g), respectively. Each fraction was tested for free radical scavenging effect by the DPPH decoloration test as well as by superoxide anion and lipid peroxidation assay. The active freeze-dried MeOH extract (96 g) was resuspended in water (1 l) and partitioned with diethyl ether (Et_2O, 3×500 ml) and ethyl acetate (EtOAc, 3×500 ml) to yield an Et_2O (18 g) and an EtOAc extract (3.28 g). The remaining aqueous phase was lyophilized (57.48 g). Chromatographic isolation of the compounds was guided by the TLC autographic assay described above.

The MeOH-soluble fraction from the Et_2O extract (5 g) was applied to a Sephadex LH-20 column (column length 33 cm, diameter 5.5 cm, equilibrated with MeOH). Some 24 fractions of 50 ml each were obtained. The fractions were pooled according to their TLC profiles (cellulose; glacial acetic acid/water 20% as the mobile phase). The combined fractions 7 and 8 (345 mg) were submitted to preparative HPLC (column: Lichrospher RP-18, 250 mm × 25 mm, 7 μm) using a 40 min linear gradient from 40 to 60% acetonitrile in water adjusted to pH 2.5 with formic acid at a flow rate of 5 ml/min. The monitoring wavelength was 280 nm. Three phenolic compounds were obtained with the following retention times: 25 min (10 mg, compound **2**), 28 min (5 mg, compound **3**) and 32 min (6 mg, compound **1**). Further purification of fractions 17 and 18 on Sephadex afforded 40 mg of the flavonoid aglycone **4**.

The EtOAc extract (3 g) was dissolved in MeOH and was applied to a Sephadex LH-20 column (column length 33 cm, diameter 5.5 cm, equilibrated with MeOH). Some 26 fractions of 10 ml each were obtained. After TLC comparison [silica gel, ethyl acetate/formic acid/glacial acetic acid/water (10:1:1:3) as the mobile phase, detection under UV light and after spraying with diphenyl boric acid/ethanolamine complex in methanol (Wagner *et al.*, 1984)] fractions with similar TLC patterns were combined. The flavonoid glycoside **5** (100 mg) eluted in fractions 13–15. Further purification of fractions 7–10 on Sephadex LH-20 with hexane/$CHCl_3$/MeOH (2:1:1) afforded 12 mg of compound **7**.

Repeated permeation of a representative sample of the lyophilized aqueous phase (8 g) on Sephadex LH-20 with MeOH afforded 12 mg of the flavonoid **6**.

Assays

TLC-autographic assay

After developing and drying, TLC plates were sprayed with a 0.2% 1,1-diphenyl-2-picrylhydrazyl radical solution in MeOH (DPPH, Aldrich). The plates were examined after 30 min. After spraying free radical scavenger, compounds appear like yellow spots against a purple background (Cavin *et al.*, 1998).

DPPH decoloration assay

The free radical scavenging effect of the samples was assessed by the decoloration of a methanolic solution of 1,1-diphenyl-2-picrylhydrazyl radical as previously reported (Schmeda-Hirschmann *et al.*, 2003). Crude extracts were assayed at 100, 50 and 10 μg/ml while pure compounds at 100, 50, 10 and

1 μg/ml. Values are presented as mean ± standard deviation of three determinations.

The quenching of free radicals by extracts and compounds was evaluated spectrophotometrically at 517 nm against the absorbance of the DPPH radical (Schmeda-Hirschmann *et al.*, 2003). A freshly prepared DPPH solution (20 mg/l) was used for the assays. Samples were dissolved in MeOH and the methanolic solution of DPPH served as a control. The degree of decoloration indicates the free radical scavenging efficiency of the substances. Catechin was used as a free radical scavenger reference. The percentage of DPPH decoloration was calculated as previously described (Schmeda-Hirschmann *et al.*, 2003).

Superoxide anion

The enzyme xanthine oxidase (XO) is able to generate $O_2^{\cdot-}$ *in vivo* by oxidation of reduced products from intracellular ATP metabolism. The superoxide anion generated in this reaction sequence reduces the nitro blue tetrazolium dye (NBT), leading to a chromophore with a maximum of absorption at 560 nm. Superoxide anion scavengers reduce the generation of the chromophore. The activity was measured spectrophotometrically as reported previously (Paya *et al.*, 1992). Extracts and products were evaluated at 50 μg/ml. Values are presented as mean ± standard deviation of three determinations.

Xanthine oxidase activity

Xanthine oxidase (XO) derived from cow's milk, xanthine and the standard inhibitor allopurinol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The XO activities with xanthine as substrate were measured spectrophotometrically as previously reported using a Shimadzu UV-160A equipment (Schmeda-Hirschmann *et al.*, 2003). Extracts and products were evaluated at 50 μg/ml. Results are presented as mean ± standard deviation of three determinations.

Lipoperoxidation in erythrocytes

Studies on erythrocyte lipid peroxidation were carried out as described by De Azevedo *et al.* (2000) with slight modifications. Human red blood cells obtained from healthy donors were washed three times in cold phosphate buffered saline (PBS) by centrifugation at 3500 rpm. After the last washing cells were suspended in PBS and its density adjusted to 1 mM haemoglobin in each reaction tube. The final cell suspensions were incubated with different concentrations of the test compounds dissolved in DMSO and PBS during 5 min at 37 °C. The final mass fraction of DMSO in the samples and controls was 1%. After incubation cells were exposed to *tert*-butylhydroperoxide (1 mM) during 15 min at 37 °C under vigorous shaking. After treatment lipid peroxidation was determined indirectly by the TBAR formation as described previously by De Azevedo *et al.* (2000). Results are expressed as percentage of inhibition compared to controls. Each determination was repeated four times.

Cytotoxicity assay

The cytotoxic effect of the compounds, expressed as cell viability, was assessed on a permanent fibroblast cell line derived from human lung (MRC-5) (ATCC Nr CCL-171). MRC-5 fibroblasts were grown as monolayers in minimum essential Eagle medium (MEM), with Earle's salts, 2 mM L-glutamine and 1.5 g/l sodium bicarbonate, supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 in air at 37° C. Cells were plated at a density of 2.5×10^3 per well in 96-well plates. Confluent cultures of MRC-5 cells were treated with medium containing the compounds at concentrations ranging from 80 up to 1000 μM. The substance was firstly dissolved in DMSO and then in MEM. The final mass fraction of DMSO in the test medium and controls was 1%. Cells were exposed for 24 h to test medium with or without the compound (control). Each drug concentration was tested in quadruplicate and repeated three times in separate experiments. At the end of incubation, the neutral red uptake (NRU) assay was carried out as described by Rodríguez and Haun (1999). To calculate the IC_{50} values the results were transformed to percentage of controls and the IC_{50} values were graphically obtained from the dose-response curves.

HPLC analysis of phenolics

HPLC analyses were performed using a Merck-Hitachi equipment consisting of a L-6200 pump, a L-4000 UV detector and D-2500 chromato-integrator. Screening of flavonoids and phenolic acids

in the sample was performed as reported by Feresin *et al.* (2002) using a 250 mm × 4 mm Lichrospher RP 18 column. The solvent system used to assess the presence of gallic acid, catechin, ellagic acid and quercetin was as follows: solvent A: 50 mM ammonium dihydrogen phosphate, pH 2.6; solvent B: 0.20 mM *ortho*-phosphoric acid, pH 2.0; solvent C: 20% solvent A in 80% acetonitrile. The gradient elution program was as reported previously (Schmeda-Hirschmann *et al.*, 2003). Under our experimental conditions, the retention time (min) of the active compounds was as follows: compound **1**, 50.55; compound **2**, 30.27; compound **3**, 47.32; compound **4**, 63.15; compound **5**, 55.49; compound **6**, 57.91; compound **7**, 59.33.

4′-Hydroxyacetophenone (**1**): MS (EI): *m/z* = 136.148 (calcd. for $C_8H_8O_2$: 136.148). – ^{1}H NMR (400 MHz, MeOH-d_4): δ = 7.87 (d, J = 8.78 Hz, H-2, H-6), 6.83 (d, J = 8.78 Hz, H-3, H-5), 2.51 (s, CH_3).

3,4-Dihydroxybenzoic acid (protocatechuic acid) (**2**): MS (EI): *m/z* = 154.120 (calcd. for $C_7H_6O_4$: 154.120). – ^{1}H NMR (400 MHz, MeOH-d_4): δ = 7.41 (brs, H-2), 6.77 (d, J = 7.8 Hz, H-5), 7.40 (d, J = 7.8 Hz, H-6).

4-Hydroxy-3,5-dimethoxybenzoic acid (syringic acid) (**3**): MS (EI): *m/z* = 198.173 (calcd. for $C_9H_{10}O_5$: 198.173). – ^{1}H NMR (400 MHz, MeOH-d_4): δ = 7.31 (s, H-2, H-6), 3.86 (s, 2 × OCH_3).

Patuletin (**4**): MS (EI): *m/z* = 332.262 (calcd. for $C_{16}H_{12}O_8$: 332.262). – ^{1}H NMR: see Table I.

3,3′,4′,5,6-Pentahydroxyflavone 7-O-β-D-glucoside (quercetagetin 7-O-β-D-glucoside) (**5**): Molecular formula $C_{21}H_{20}O_{13}$, 480.376. – Spot appearance: UV: yellow; UV/NH_3: yellow. – R_f values: *n*-BuOH/HOAc/H_2O (3:1:1): 0.24; HOAc (15%): 0.09. – ^{1}H and ^{13}C NMR: see Tables I and II.

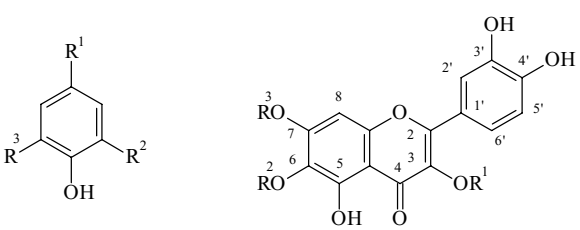
3,3′,4′,5-Tetrahydroxy-6-methoxyflavone 7-O-β-D-glucoside (patuletin 7-O-β-D-glucoside; patulitrin) (**6**): Molecular formula $C_{22}H_{22}O_{13}$, 494.403. – Spot appearance: UV: yellow; UV/NH_3: yellow. – R_f values (after hydrolysis): *n*-BuOH/HOAc/H_2O (3:1:1): 0.83; HOAc (15%): 0.22. – ^{1}H and ^{13}C NMR: see Tables I and II.

3′,4′,5-Trihydroxy-3,6-dimethoxyflavone 7-O-β-D-glucoside (axillarin 7-O-β-D-glucoside) (**7**): Molecular formula $C_{23}H_{24}O_{13}$, 508.430. – Spot appearance: UV: deep purple; UV/NH_3: deep purple. – ^{1}H and ^{13}C NMR: see Tables I and II.

Results and Discussion

The aerial parts of *Tagetes mendocina* (Asteraceae) are used in infusion or decoction as digestive and diuretic in the San Juan Province, Argentina. Preliminary tests showed free radical scavenging activity in the methanol extract of the plant, measured by decoloration of a methanolic solution of the DPPH radical and scavenging of the superoxide anion. Assay-guided isolation led to 4′-hydroxyacetophenone (**1**), protocatechuic acid (**2**), syringic acid (**3**), patuletin (**4**), quercetagetin 7-*O*-β-D-glucoside (**5**), patuletin 7-*O*-β-D-glucoside (**6**) and axillarin 7-*O*-β-D-glucoside (**7**) as the free radical scavengers and antioxidant compounds from *Tagetes mendocina* (Fig. 1). The spectroscopic data of the isolated compounds are in agreement with those reported in literature. The ^{13}C NMR spectral data of the aglycones have been published by D'Agostino *et al.* (1997) for quercetagetin, by Agrawal (1989) for patuletin and by Al-Yahya *et al.* (1988) for axillarin. The ^{1}H and ^{13}C NMR data of compounds **4**–**7** are presented in Tables I and II.

On the basis of dry starting material, the total phenolics content of the crude drug was 3.00% with 0.372% of flavonoids. The content of compounds **1**–**7** was 0.008, 0.015, 0.010, 0.029, 0.238, 0.058 and 0.017%, respectively. The compounds showed low cytotoxicity towards human lung fibroblasts with IC_{50} values > 1 mM for the phenolics **1**–**3** and 0.24–0.52 mM for the flavonoids **4**–**7** (Table III).



1–3 **4–7**

	R^1	R^2	R^3
4′-Hydroxyacetophenone (**1**)	$COCH_3$	H	H
Protocatechuic acid (**2**)	COOH	OH	H
Syringic acid (**3**)	COOH	OCH_3	OCH_3
Patuletin (**4**)	H	CH_3	H
Quercetagetin 7-*O*-β-D-glucoside (**5**)	H	H	Glucose
Patuletin 7-*O*-β-D-glucoside (**6**)	H	CH_3	Glucose
Axillarin 7-*O*-β-D-glucoside (**7**)	CH_3	CH_3	Glucose

Fig. 1. Free radical scavengers and antioxidant compounds isolated from *Tagetes mendocina*.

Table I. ^{1}H NMR data of compounds **4**–**7** (400 MHz; MeOH-d_4 for **4**, **5** and **7**, DMSO-d_6 for compound **6**).

H	**4**	**5**	**6**	**7**
8	6.43 s	6.97 s	6.94 s	6.89 s
2′	7.70 d (2.0)	7.79 d (2.2)	7.72 d (2.1)	7.66 d (2.1)
5′	6.86 d (8.08)	6.93 d (8.5)	6.91 d (8.5)	6.92 d (8.5)
6′	7.59 dd (8.6; 2)	7.69 dd (8.5; 2.1)	7.56 dd (8.5; 2.1)	7.58 dd (8.5; 2.1)
Sugar				
1	–	5.09 d (7.4)	5.12 d (7.3)	5.10 d (7.4)
2	–	3.58 dd (8.5; 7.5)	3.35–3.80 m	3.55 dd (9.1; 8.5)
3	–	3.44 dd (9.3; 8.9)		3.44 dd (9.2; 9)
4	–			
5	–	3.61 m		3.59 m
6	–	3.98 dd (12.1; 2)		3.97 dd (12.2; 2)
		3.75 dd (12.1; 6)		3.73 dd (12.2; 6)
OMe	3.85 s	–	3.78 s	3.90 s
OMe	–	–	–	3.82 s

Table II. ^{13}C NMR data of compounds **4**–**7** (100 MHz; MeOH-d_4 for **4**, **5** and **7**, DMSO-d_6 for **6**).

C	**4**	**5**	**6**	**7**
2	147.22 s	149.32 s	148.61 s	154.0 s
3	135.90 s	130.11 s	136.62 s	139.1 s
4	176.48 s	176.64 s	176.97 s	178.1 s
5	151.90 s	145.89 s	151.91 s	149.15 s
6	131.16 s	136.30 s	132.65 s	132.78 s
7	157.43 s	152.01 s	157.18 s	156.81 s
8	93.75 d	94.45 d	94.75 d	94.57 d
9	152.55 s	149.30 s	152.23 s	157.76 s
10	103.92 s	105.78 s	105.83 s	105.1
1′	120.76 s	123.16 s	122.67 s	121.76 s
2′	115.04 d	115.48 d	116.27 d	115.47 d
3′	145.16 s	145.37 s	145.95 s	145.47 s
4′	147.77 s	148.07 s	148.83 s	148.9 s
5′	115.23 d	115.58 d	116.51 d	115.64 d
6′	123.13 s	121.01 d	120.99 d	121.59 d
Sugar				
1	–	101.78 d	101.00 d	100.98 d
2	–	73.79 d	74.06 d	73.75 d
3	–	76.57 d	77.49 d	76.95 d
4	–	70.45 d	70.44 d	70.31 d
5	–	77.68 d	78.04 d	77.48 d
6	–	61.55 t	61.46 t	61.55 t
OMe	60.10 q	–	61.23 q	60.54 q
	–	–	–	59.53 q

Three phenolic acid derivatives, namely 4′-hydroxyacetophenone (**1**), protocatechuic acid (**2**) and syringic acid (**3**) were identified as constituents of the plant extract. 4′-Hydroxyacetophenone displays anti-inflammatory effects in the carrageenan-induced paw edema in mouse (Alvarez *et al.*, 2000).

Syringic acid was identified as one of the free radical scavengers from *Coix lachryma-jobi* hulls (Kuo *et al.*, 2002) while protocatechuic and syringic acid were two of the antioxidant phenolics isolated from the most active fraction of the plant *Mesona procumbens* (Hung and Yen, 2002). The free radical scavenging effect of syringic acid towards DPPH has been reported by Hirota *et al.* (2000). Furthermore, it has been recently shown that protocatechuic acid at concentrations $\geq$ 10 mM acts as an oxidative stress inducer (Babich *et al.*, 2002). Protocatechuic acid presents antioxidant effects *in vitro* and *in vivo* as well as antitumor activity and protection against hepatic damage induced by lipopolysaccharides in rats (Lin *et al.*, 2003). The compound was also active in the *tert*-butylhydroperoxide induced oxidative damage

Table III. Percentage of activity relative to the corresponding control induced by *Tagetes mendocina* extracts and compounds on free radical and superoxide anion scavenging, xanthine oxidase, lipid peroxidation inhibition and cytotoxicity in human fibroblasts. Results are presented as mean ± s.d.

Extract and compounds	Superoxide anion	XO	Cytotoxicity IC_{50} [μM (μg/ml)]	Inhibition of lipoperoxidation in erythrocytes		DPPH decoloration			
Sample concentration [μg/ml]	50	50		500	100	100	50	10	1
H	0	42.5 ± 1	–	67 ± 2	–	0	0	0	–
DCM	0	0	–	74 ± 3	–	16 ± 2	14 ± 2	6 ± 2	–
MeOH	71 ± 2	0	–	83 ± 2	–	98 ± 3	95 ± 3	41 ± 3	–
Ethyl ether	0	0	–	54 ± 2	–	58 ± 2	28 ± 2	9 ± 2	–
EtOAc	84 ± 2	48.5 ± 2	–	65 ± 1	–	96 ± 3	95 ± 3	94 ± 3	–
Aqueous	37 ± 2	0	–	60 ± 2	–	96 ± 2	93 ± 2	30 ± 4	–
Compound **1**	20 ± 2	–	> 1000 (> 136)	–	59 ± 3	96 ± 2	85 ± 2	22 ± 2	2 ± 2
Compound **2**	64 ± 2	–	> 1000 (> 154)	–	46 ± 2	95 ± 3	96 ± 3	92 ± 3	24 ± 2
Compound **3**	37 ± 3	–	> 1000 (> 198)		96 ± 2	93 ± 2	94 ± 2	76 ± 3	17 ± 2
Compound **4**	61 ± 2	31 ± 2	516 (171)	–	69 ± 2	96 ± 2	96 ± 2	97 ± 2	54 ± 3
Compound **5**	74 ± 3	5 ± 1	239 (115)	–	86 ± 2	98 ± 4	95 ± 4	92 ± 2	58 ± 2
Compound **6**	73 ± 2	0	496 (245)	–	67 ± 3	94 ± 3	90 ± 3	89 ± 3	16 ± 3
Compound **7**	71 ± 2	0	254 (129)	–	31 ± 1	90 ± 2	89 ± 4	55 ± 2	10 ± 3
Catechin	72 ± 2	–	–	–	73 ± 4	98 ± 3	97 ± 2	69 ± 2	13 ± 2
Quercetin	88 ± 3	–	–	–	69 ± 2	100 ± 3	98 ± 3	97 ± 3	30 ± 3

H, Hexane extract; DCM, dichloromethane extract; MeOH, methanol extract; EtOAc, ethyl acetate extract.
–: Not determined.

in rat primary hepatocytes by its antioxidant and anti-inflammatory effects and blocked stress signal transduction (Liu *et al.*, 2002). Protocatechuic acid inhibits oxidation of low-density lipoprotein induced by nitric oxide donor or copper ion (Lee *et al.*, 2002) and interact with Fe(II) showing a strong inhibitory effect on iron-induced oxidative DNA damage (Lodovici *et al.*, 2001). Syringic and protocatechuic acid have been reported as inhibitors of both forms of the enzyme phenolsulfotransferase (Yeh and Yen, 2003). The cytotoxicity of both phenolic acids is low using different cell lines (Babich and Visioli, 2003).

Quercetagetin 7-*O*-β-D-glucoside (**5**) proved to be the main free radical scavenger of the extracts measured in the DPPH decoloration test as well as towards the superoxide anion and in the inhibition of lipoperoxidation in erythrocytes (Table III and IV). In the lipid peroxidation assay the inhibition ranged from 86% for the quercetagetin glucoside to 67% for the monomethoxylated and 31% for the dimethoxylated derivative and was related with the number of methoxy groups in the molecule.

Biological activities described for quercetagetin and its derivatives comprises inhibition of β-glu-

Table IV. Percentage of activity relative to the corresponding control induced by compounds **1**–**7** from *Tagetes mendocina* on the free radical DPPH. Results are presented as mean ± s.d. in μg/ml. The concentrations in μM at 100, 50, 10 and 1 μg/ml are included.

Compound	μg/ml 100	μM	μg/ml 50	μM	μg/ml 10	μM	μg/ml 1	μM
1	96 ± 2	735.2	85 ± 2	367.6	22 ± 2	73.5	2 ± 2	7.35
2	95 ± 3	649.3	96 ± 3	324.6	92 ± 3	64.9	24 ± 2	6.49
3	93 ± 2	505.0	94 ± 2	252.5	76 ± 3	50.5	17 ± 2	5.05
4	96 ± 2	301.2	96 ± 2	150.6	97 ± 2	30.1	54 ± 3	3.01
5	98 ± 4	208.3	95 ± 4	104.1	92 ± 2	20.8	58 ± 2	2.08
6	94 ± 3	202.4	90 ± 3	101.2	89 ± 3	20.2	16 ± 3	2.02
7	90 ± 2	196.9	89 ± 4	98.4	55 ± 2	19.7	10 ± 3	1.96

curonidase and lysozyme release by quercetagetin-7-*O*-glucoside (Tordera *et al.*, 1994), protection of endothelial cells against linoleic acid hydroperoxide (Kaneko and Baba, 1999), inhibition of tomato bushy stunt virus infection (Rusak *et al.*, 1997), and antimicrobial effect of extracts and quercetagetin-7-arabinosyl-galactoside isolated from *Tagetes minuta* (Tereschuk *et al.*, 1997). Quercetagetin has been proved to interact with the mitochondrial electron transport chain (Hodnick *et al.*, 1998) and to be a powerful inhibitor of the reverse transcriptase from Rauscher murine leukemia virus at 1 μg/ml and HIV at 1 μg/ml (Wang *et al.*, 1998). Furthermore, quercetagetin was a strong inhibitor of DNA polymerase beta and DNA polymerase I. The antioxidant and pro-oxidant effect of flavonoids including quercetagetin towards DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion has been reported (Ohshima *et al.*, 1998).

The quercetagetin glycosides 4′-methylquercetagetin 7-*O*-(6″-*E*-caffeoyl-β-D-glucopyranoside), quercetagetin 7-*O*-(6-acetyl-β-D-glucopyranoside) and quercetagetin 7-*O*-β-D-glucoside have been reported by Nair *et al.* (1995) from the leaves of *Eupatorium glandulosum* while quercetagetin 6-*O*-β-D-glucopyranoside was first reported from the aerial parts of *Tagetes mandonii* (D'Agostino *et al.*, 1997). Other quercetagetin derivatives recenty reported include the 7-methyl ether glycosides from the Eriocaulaceae *Paepalanthus vellozioides* and *Paepalanthus latipes* (Vilegas *et al.*, 1999). In a chemotaxonomic study of Argentinian *Tagetes* species, the main flavonoids identified were quercetagetin (6-hydroxyquercetin), patuletin (6-methoxyquercetin) and its glycosides (Abdala, 1999). The study was based on voucher herbarium specimens and the following compounds were reported from *Tagetes mendocina*: quercetagetin 3-glucoside, quercetagetin 7-diglucoside, patuletin 7-glucoside, luteolin 7-glucoside and the corresponding aglycones.

The IC_{50} value of patuletin 7-*O*-β-D-glucoside (**6**) in the DPPH assay was shown to be 10.3 μg/ml (20.8 μM) and **6** was also active in the cytochrome-c reduction assay using HL-60 cell culture system with an IC_{50} value of 8.1 μg/ml (16.4 μM) (Park *et al.*, 2000). Under our assay conditions, however, the IC_{50} value of the compound in the DPPH assay was in the range 1–10 μg/ml.

The aglycone of **7**, known as axillarin, has been found to decrease the metabolism of benzo[α]pyrene by 61.3% at 25 μg/ml (Al-Yahya *et al.*, 1988) and to be one of the most powerful inhibitors of the enzyme aldose reductase from rat and bovine lenses (Okuda *et al.*, 1982). The xanthine oxidase inhibitory activity of axillarin isolated from *Ajana fruticulosa* has been reported (Li *et al.*, 1999) with an IC_{50} value of 36 μM. In a recent study on the antioxidant activity of flavonoids upon cell cultures (Kim *et al.*, 2002), it was found that axillarin protected primary cultures of rat cortical cells from oxidative stress induced by glutamate.

In Table III, the compounds **1**, **2** and **3** showed the lower cytotoxicity with IC_{50} values higher than 136, 154 and 198 μg/ml, respectively. At 10 μg/ml, compound **2** produced a 92% decoloration of the free radical DPPH, a concentration which is at least fifteen times less than its cytotoxicity IC_{50} value towards human fibroblasts. Similar results were observed for compound **3**.

In the superoxide anion assay at 50 μg/ml, compounds **4**–**7** showed similar scavenging activities ranging from 61 to 74%. Compound **6** was the less cytotoxic active metabolite presenting cytotoxic effect at 5-fold concentration compared to those of the superoxide anion scavenging assay. Compound **6** also proved to have good inhibitory effect against induced lipoperoxidation in erythrocytes with a reduced cytotoxic effect compared to the other compounds. Similar results were obtained for compound **3**. The low cytotoxicity values (greater than 1 mM) of compounds **1**, **2** and **3** do not allow to determine their IC_{50} values suggesting that the best toxicity/activity relationships should be expected for this compounds.

In the report of Abdala (1999) three flavonol and a flavone glycoside were identified from *Tagetes mendocina* by paper chromatography and TLC comparison with standards. In the present work, three simple phenolics and four flavonols including three glycosides were isolated and identified by spectroscopic methods. Five out of the seven compounds are reported for the first time from *Tagetes mendocina*. Only patuletin (**4**) and its 7-*O*-glucoside **6** were previously identified by Abdala in a plant sample collected in the Provincia de Mendoza, Argentina. The free radical scavenging and antioxidant effect of the phenolics from *Tagetes mendocina* and their relative concentration support the beneficial effects adscribed to the plant and suggests that other close related species will also present antioxidant properties. This points out a positive effect of the crude drug

in the traditional medicine of the Andean provinces in Argentina. As the main compounds in the related species seem to be similar at least at a qualitative level (Abdala, 1999), a quantitative study of the phenolic profiles combined with selected assays will give a better picture of the potential of South American *Tagetes* spp. as a source of free radical scavenging and antioxidant compounds.

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