

An Extract of *Tagetes lucida* and Its Phenolic Constituents as Antioxidants

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Received January 18, 2002

Analysis of a methanolic extract of *Tagetes lucida* leaves has resulted in the isolation of a new flavonol glycoside, quercetagenin 3,4'-dimethyl ether 7-*O*- β -D-glucopyranoside (**1**), two new phenolic acids, 3-(2-*O*- β -D-glucopyranosyl-4-methoxyphenyl)propanoic acid (**2**) and its methylester (**3**), and known flavonols, aromatic acids, and 7-methoxycoumarin. Using the DPPH^o test, the extract and some of its constituents showed a significant free-radical-scavenging effect in comparison to α -tocopherol and standard flavonols.

Plants as sources of antioxidants can be used both for food quality preservation and for medicinal and cosmetic purposes. The antioxidant capacity of vegetables is due to vitamins, carotenoids, sterols, and, particularly, polyphenols, which have the capacity to reduce free-radical formation by either chelating trace elements or scavenging free radicals and protecting antioxidant defenses.^{1–5} In our continuous search for antioxidative plant extracts and constituents from Central and South American medicinal plants,⁵ *Tagetes lucida* Cav. (Asteraceae), “perigon” and “Mexican mint marigold”, was investigated phytochemically and biologically. *T. lucida* is a medicinal plant native to Mexico and Guatemala used by the Aztec and Maya and, actually, as a condiment due to its anise-scented foliage. Its extracts are reported to possess bactericidal and platelet antiaggregant activity,^{6,7} as well as inhibitory effect on smooth-muscle contraction.⁸ In the present work the in vitro antioxidant effect of a polar extract of *T. lucida* leaves in homogeneous solution was tested employing the DPPH^o test (the bleaching of the stable 1,1-diphenyl-2-picrylhydrazyl radical). The total phenolic content of the extract was determined, and the major constituents were isolated, characterized, and tested.

Results and Discussion

The dried leaves of *T. lucida*, defatted with petroleum ether and chloroform, were extracted with MeOH, and this extract was partitioned between water and *n*-BuOH. The *n*-BuOH extract had a total phenolic content, determined by the Folin-Ciocalteu method⁵ and expressed as caffeic acid equivalents, equal to 279.3 μ g/mg (Table 1). As to the DPPH test,⁹ the free-radical-scavenging effect elicited by this extract was concentration-dependent, so that the EC₅₀ value was calculated as 6.4 μ g with respect to α -tocopherol (EC₅₀ 10.1 μ g), used as positive control. The *n*-BuOH extract gave four main fractions, I–IV, by gel filtration on a Sephadex LH-20 column. In comparison to the parent extract, fraction I was less potent in the DPPH test (EC₅₀ 170.7 μ g) and showed a minor total phenolic content (23.9 μ g/mg), whereas fractions II–IV showed EC₅₀ values comparable to that of the extract and high levels of total phenols (Table 1).

Thus, with the aim to isolate and characterize the constituents of *T. lucida* responsible for the observed free-

Table 1. Total Phenol Content and Free-Radical-Scavenging Activity of the *n*-BuOH Extract, Fractions I–IV, and Pure Compounds from *T. lucida*

extract and fractions	phenol content ^a (μ g/mg extract) ^b	DPPH test [EC ₅₀ (μ g of extract)]
<i>n</i> -BuOH extract	279.3 \pm 2.44	6.4 (5.45–7.59) ^c
fraction I	23.9 \pm 3.15	170.7 (146.50–198.89) ^c
fraction II	243.3 \pm 3.69	5.3 (4.57–6.11) ^c
fraction III	358.6 \pm 3.88	2.8 (2.31–3.33) ^c
fraction IV	365.4 \pm 3.94	4.9 (4.37–5.42) ^c
quercetagenin-7- <i>O</i> - glucoside		4.6 (4.06–5.28) ^c
6-hydroxykaempferol 7- <i>O</i> -glucoside		6.9 (5.92–7.98) ^c
caffeic acid		3.0 (2.49–3.61) ^c
gallic acid		1.2 (1.01–1.33) ^c
quercetin ^d		2.3 (1.86–2.84) ^c
rutin ^d		4.8 (4.23–5.39) ^c
kaempferol ^d		4.4 (3.97–4.87) ^c
α -tocopherol ^d		10.1 (8.8–11.4) ^c

^a Mean \pm SD of three determinations. ^b Caffeic acid equivalents. ^c 95% confidence limits. ^d Positive control.

radical scavenging activity, fractions II–IV were chromatographed by HPLC, giving, as major constituents, a new flavonol glycoside **1**, as well as quercetagenin 7-*O*- β -D-glucopyranoside,¹⁰ quercetagenin 3-methyl ether 7-*O*- β -D-glucopyranoside,¹¹ 6-hydroxykaempferol-7-*O*- β -D-glucopyranoside,¹² and quercetagenin 3,3'-dimethyl ether¹³ from fraction III; caffeic acid¹⁴ and a small amount of quercetagenin derivatives from fraction II; and 6-*O*-caffeoyl- β -D-glucopyranoside,¹⁵ 4-(β -D-glucopyranosyloxy)benzoic acid, and gallic acid¹⁶ from fraction IV. The separation of the less active fraction I gave two new phenylpropanoid glycosides, 3-(2-*O*- β -D-glucopyranosyl-4-methoxyphenyl)propanoic acid (**2**) and methyl 3-(2-*O*- β -D-glucopyranosyl-4-methoxyphenyl)propanoate (**3**), and 7-methoxycoumarin. The EC₅₀ values (Table 1) found for the major components of the *T. lucida* extract with respect to standard flavonols (quercetin, rutin, and kaempferol) used as controls are consistent with the reported hierarchy of flavonoids and

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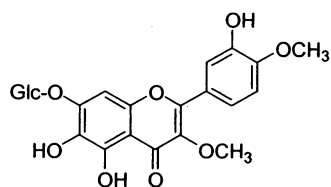
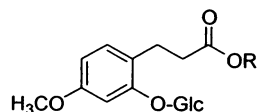
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Table 2. ^{13}C NMR and ^1H NMR of Compounds **1–3** in CD_3OD^a

1			2			3		2 and 3
position ^b	δ_{C}	δ_{H} (J_{HH} in Hz) ^c	position ^b	δ_{C}	δ_{H} (J_{HH} in Hz) ^c	δ_{C}	δ_{H} (J_{HH} in Hz) ^c	cross-peaks (δ_{C}) in HMBC spectrum
2	157.0		1	176.6		174.9		
3	138.8		2	39.1	2.47 m	35.4	2.64 m	C-1, C-1'
4	179.1		3	27.5	2.92 m	26.1	2.92 m	C-1, C-1', C-2', C-6'
5	150.2		1'	123.0		123.0		
6	130.9		2'	157.5		157.7		
7	152.8		3'	102.2	6.80 d (2.0)	102.9	6.80 d (2.0)	C-1', C-2', C-4'
8	95.2	6.97 s	4'	161.0		161.2		
9	150.6		5'	107.8	6.53 dd (8.5, 2.0)	108.3	6.53 dd (8.5, 2.0)	C-1'
10	106.5		6'	130.9	7.04 d (8.5)	131.3	7.05 d (8.5)	C-3, C-2', C-4'
1'	123.6		–OMe at C-4'	55.6	3.78 s	55.7	3.78 s	C-4'
2'	115.3	7.69 d (1.5)	–OMe at C-1			51.8	3.66 s	
3'	147.2		Glc-1	102.7	4.87 d (7.5)	102.5	4.90 d (7.5)	C-2', Glc-2
4'	150.8		Glc-2	74.8	3.55 dd (8.5, 7.5)	75.0	3.50 dd (8.5, 7.5)	Glc-1
5'	112.1	6.95 d (8.0)	Glc-3	78.1	3.48 t (8.5)	78.3	3.49 t (8.5)	
6'	121.0	7.60 dd (8.0, 1.5)	Glc-4	71.6	3.42 t (8.5)	71.5	3.41 t (8.5)	
–OMe at C-3	58.8	3.82 s	Glc-5	78.1	3.49 m	78.3	3.49 m	
–OMe at C-4'	60.2	3.92 s	Glc-6	62.6	3.73 dd (12.0, 4.5)	62.6	3.72 dd (12.0, 4.5)	
					3.94 dd (12.0, 3.0)		3.93 dd (12.0, 3.0)	
Glc-1	102.8	5.08 d (7.5)						
Glc-2	74.6	3.58 dd (8.5, 7.5)						
Glc-3	77.3	3.54 t (8.5)						
Glc-4	71.1	3.44 t (8.5)						
Glc-5	78.4	3.60 m						
Glc-6	62.3	3.74 dd (12.0, 4.5)						
		3.98 dd (12.0, 3.0)						

^a Assignments confirmed by 1D TOCSY and 2D COSY, HSQC, and HMBC experiments. ^b Glc = β -D-glucopyranosyl. ^c ^1H – ^1H coupling constants.

phenolic acids free-radical-scavenging ability¹⁶ dependent on structural features.^{1,2} As could be anticipated, compounds **2** and **3** showed undetectable activity. The structures of the known compounds were determined by NMR and MS.^{10–15,17} The ^{13}C NMR data for quercetagenin-7- O - β -D-glucopyranoside and quercetagenin 3-methyl ether 7- O - β -D-glucopyranoside are reported for the first time in the Experimental Section. The structure identification of compounds **1–3** was based on the evidence outlined below.

**1****2** R = H**3** R = CH₃Glc = β -D-glucopyranosyl

The negative FABMS of compound **1** showed an $[\text{M} - \text{H}]^-$ ion at m/z 507, consistent with the molecular formula $\text{C}_{23}\text{H}_{24}\text{O}_{13}$, which was also deduced using ^{13}C and DEPT NMR analysis. The MS and NMR spectra (Table 2) suggested that quercetagenin 3,4'-dimethyl ether¹¹ (–OMe signals at δ_{H} 3.82 and 3.92 and δ_{C} 58.8 and 60.2) was the aglycon and β -D-glucopyranosyl⁵ was the sugar residue linked to C-7.¹⁷ The 3- O -methyl etherification¹⁷ was indicated by the ^{13}C NMR chemical shift of C-3 (δ_{C} 138.8); the 4'- O -methyl etherification was suggested by the downfield shift of C-4' (from about δ_{C} 148.8 to 150.8) as well as by

the concurrent upfield shift of C-5' (from about δ_{C} 116.1 in quercetagenin-7- O -glucoside to 112.1) and then confirmed by the cross-peaks between the –OMe (δ_{H} 3.92), H-2' (δ_{H} 7.69), H-5' (δ_{H} 6.95), and H-6' (δ_{H} 7.60) signals and C-4' (δ_{C} 150.8) in the heteronuclear multiple bond correlation (HMBC) spectrum. HMBC correlations between the anomeric signal (δ_{H} 5.08, HSQC δ_{C} 102.8) and C-7 (δ_{C} 152.8), C-6 (δ_{C} 130.9), and C-8 (δ_{C} 95.2) confirmed the position of the sugar attachment. Accordingly, compound **1** was determined as quercetagenin 3,4'-dimethyl ether 7- O - β -D-glucopyranoside (**1**).

Compound **2** was assigned a molecular formula of $\text{C}_{16}\text{H}_{22}\text{O}_9$, as deduced by a combination of FABMS and ^{13}C NMR and DEPT analysis. The ^{13}C NMR spectra of **2** indicated a phenyl propanoic acid aglycon showing nine signals comprising one –COOH (δ 176.6, C-1), two methylenes (C-2 and C-3), three methine, and three quaternary aromatic carbon signals. In addition signals for an –OMe group (δ_{H} 3.78, δ_{C} 55.6) and a glucopyranosyloxy unit linked to the aglycon (Table 2) were observed. For the sugar unit, the ^1H NMR data indicated a β -configuration at the anomeric position ($J_{\text{H}-1-\text{H}-2} = 7.5$ Hz); a 1D TOCSY¹⁸ subspectrum obtained by irradiating at the well-resolved anomeric proton at δ 4.87 showed a set of coupled protons at δ 3.42, 3.48, 3.49, 3.55 (all CH), and 3.73 and 3.94 (CH_2); the DQF-COSY spectrum established the proton sequence within this monosaccharide as H-1' to H₂-6', and analysis of the correlated ^{13}C NMR signals in the HSQC spectrum led to the identification of a β -D-glucopyranosyl unit.⁵ Analysis of the resonances of the aromatic hydrogen signals (δ 7.04, d, $J = 8.5$ Hz, 6.53 dd, $J = 8.5$ and 2.0 Hz, and 6.80 d, $J = 2.0$ Hz) and of the correlated ^{13}C NMR signals in the HSQC spectrum (Table 2) suggested a 2,4-dihydroxy-substituted phenyl residue. This unusual substitution^{19,20} on the aromatic ring was confirmed by the long-range correlations observed in the HMBC spectrum (Table 2). The relative position of –OMe and –OGlc at C-4' and C-2' was established unambiguously by the HMBC correlations

observed between the anomeric proton signal at δ_{H} 4.87 (H-1) and C-2' (δ_{C} 157.5) and between the -OMe signal (δ_{H} 3.78) and C-4' (δ_{C} 161.0). Therefore, the structure of **2** was determined as 3-(2-*O*- β -D-glucopyranosyl-4-methoxyphenyl)propanoic acid. Compound **3** (C₁₇H₂₄O₉) showed NMR spectra almost superimposable on those of compound **2** except for the presence of signals ascribable to a -COOMe (-OMe δ_{H} 3.66, δ_{C} 51.8; -CO δ_{C} 174.9) instead of -COOH group in **2** (Table 2). All the connectivity information inferred by the HMBC spectrum was compatible with the structure methyl-3-(2-*O*- β -D-glucopyranosyl-4-methoxyphenyl)propanoate. Because compound **3** was also isolated from an EtOH extract of *T. lucida* leaves, it seems to be a native product and not an artifact formed from compound **2** by MeOH extraction.

Experimental Section

General Experimental Procedures. Melting points are uncorrected. UV spectra were obtained with a Perkin-Elmer 550 SE spectrophotometer. Optical rotations were measured on a Perkin-Elmer 141 polarimeter using a sodium lamp operating at 589 nm in MeOH solutions. For NMR experiments, a Bruker DRX-600 spectrometer was used, operating at 599.2 MHz for ¹H and 150.9 for ¹³C and using the UXNMR software package; DEPT, ¹H-¹H DFQ-COSY (double-quantum filtered COSY), ¹H-¹³C HSQC, and HMBC experiments were obtained using conventional pulse sequences. 1D TOCSY²⁰ (selective excitation spectra) were acquired as previously reported.⁵ Chemical shifts are expressed in δ (ppm) referring to the following solvent center peaks: δ_{H} 3.34 and δ_{C} 40.0 for CD₃OD. The FABMS were recorded in a glycerol matrix in the negative-ion mode on a VG ZAB instrument (XE atoms of energy 2–6 kV). Semipreparative HPLC separations were carried out on a Waters model 6000A pump equipped with a U6K injector and a Model 401 refractive index detector. 1,1-Diphenyl-2-picrylhydrazyl radical, rutin, quercetin, kaempferol, and α -tocopherol were purchased from Sigma-Aldrich (Milan, Italy).

Plant Material. The leaves of *T. lucida* Cav. (Asteraceae) were collected in Cabricán, Qetzaltenango, Guatemala, in February 1997 and identified by Dr. E. de Poll, Universidad del Valle de Guatemala. A specimen of the plant (TAG. 119, 1997) used in this study has been deposited at the Herbarium of the Farmaya Laboratory, Guatemala.

Extraction and Isolation. The powdered, air-dried leaves (200 g) were defatted at room temperature with hexane and CHCl₃ and then extracted with MeOH to give 11.2 g of residue, which was partitioned between *n*-BuOH and H₂O to afford an *n*-BuOH-soluble portion (8.06 g). An aliquot (2.0 g) of this was chromatographed over a Sephadex LH-20 column (100 \times 5 cm) using MeOH as eluent. Fractions (9 mL) were collected and checked by TLC [Si gel, *n*-BuOH-HOAc-H₂O (60:15:25)]. Fractions 11–25 (I) (340.1 mg) were submitted to RP-HPLC on a C₁₈ μ -Bondapak column (30 cm \times 7.8 mm, flow rate 2.5 mL min⁻¹) using MeOH-H₂O (35:65) as the eluent to yield 3-(2-*O*- β -D-glucopyranosyl-4-methoxyphenyl)propanoic acid (**2**) (7.9 mg, *t*_R 13.8 min), methyl 3-(2-*O*- β -D-glucopyranosyl-4-methoxyphenyl)propanoate (**3**) (6.1 mg, *t*_R 23.1 min), and 7-methoxycoumarin (3.5 mg, *t*_R 38 min).¹⁷ Fractions 26–40 (II) (151.4 mg) and 41–79 (III) (221.6 mg) were separated using MeOH-H₂O (4:6) to give caffeic acid¹³ (18.1 mg, *t*_R 7.8 min) and a small amount of quercetagenin derivatives from II, and quercetagenin 7-*O*- β -D-glucopyranoside¹⁰ (16 mg, *t*_R 20.5 min), quercetagenin 3-methyl ether 7-*O*- β -D-glucopyranoside¹⁰ (4.1 mg, *t*_R 30.1 min), 6-hydroxykaempferol-7-*O*- β -D-glucopyranoside¹¹ (11.5 mg, *t*_R 32.1 min), quercetagenin 3,4'-dimethyl ether 7-*O*- β -D-glucopyranoside (**1**) (6.5 mg, *t*_R 34.3 min), and quercetagenin 3,3'-dimethyl ether¹² (4.5 mg, *t*_R 39.4 min) from III. Fractions 80–95 (40.2 mg), separated using MeOH-H₂O (3:7), gave 6-*O*-caffeoyl- β -D-glucopyranoside¹⁴ (5.5 mg, *t*_R 18.8 min), 4-(β -D-glucopyranosyloxy)benzoic acid (5.2 mg, *t*_R 9.5 min),¹⁵ and gallic acid¹⁵ (12 mg, *t*_R 12.7 min). To establish if

compound **3** was an artifact of MeOH extraction, part of the dried plant material (50 g) was extracted with EtOH. Compound **3** (1.2 mg) was isolated from the EtOH extract (2.2 g) following the same procedure described for the MeOH extract.

Compound 1: amorphous powder; mp 200–202 °C; [α]_D²⁰ -43.5 (c 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 353 (1.00), 270 (0.83), 258 (0.93) nm; ¹H and ¹³C NMR, see Table 2; FABMS *m/z* 507 [M - H]⁻; 345 [(M - H) - 162]⁻, 330 [(M - H) - (162 + 15)]⁻, 298 [320 - 32]⁻; *anal.* C 54.26, H 4.80, calcd for C₂₃H₂₄O₁₃, C 54.34, H 4.76.

Compound 2: amorphous powder; mp 195–197 °C; [α]_D²⁰ -38.2 (c 0.5, MeOH); UV (MeOH) λ_{max} 326 (4.40), 273 (1.50) nm; ¹H and ¹³C NMR, see Table 2; FABMS *m/z* 357 [M - H]⁻; 313 [(M - H) - 44]⁻, 195 [(M - H) - 162]⁻, 151 [(M - H) - (44 + 162)]⁻; *anal.* C 53.10, H 6.30, calcd for C₁₆H₂₂O₉, C 53.63, H 6.19.

Compound 3: amorphous powder; mp 88–90 °C; [α]_D²⁰ -37.1 (c 0.2, MeOH); UV (MeOH) λ_{max} 328 (4.38), 272 (1.45) nm; ¹H and ¹³C NMR, see Table 2; FABMS *m/z* 371 [M - H]⁻, 312 [(M - H) - COOMe]⁻, 209 [(M - H) - 162]⁻; *anal.* C 54.75, H 6.55, calcd for C₁₇H₂₄O₉, C 54.84, H 6.50.

Quercetagenin-7-*O*- β -D-glucopyranoside: ¹³C NMR (CD₃OD) aglycon δ 177.4 (C-4), 157.2 (C-2), 152.9 (C-7), 150.6 (double signal C-5 and C-9), 148.8 (C-4'), 146.1 (C-3'), 137.0 (C-3), 130.8 (C-6), 123.8 (C-1'), 121.8 (C-6'), 116.1 (C-5'), 115.9 (C-2'), 106.4 (C-10); ¹³C NMR data for the sugar were almost superimposable on those of compound **1**.

Quercetagenin-3-methyl ether 7-*O*- β -D-glucopyranoside: ¹³C NMR (CD₃OD) aglycon δ 179.0 (C-4), 157.0 (C-2), 152.9 (C-7), 150.6 (C-9), 150.0 (C-5), 148.0 (C-4'), 145.4 (C-3'), 138.8 (C-3), 130.4 (C-6), 122.8 (C-1'), 122.0 (C-6'), 116.4 (C-5'), 116.2 (C-2'), 106.8 (C-10), 58.9 (-OMe); ¹³C NMR data for the sugar were almost superimposable on those of compound **1**.

Quantitative Determination of Total Phenols. The *T. lucida* dried *n*-BuOH extract and fractions I–IV, dissolved in MeOH, were analyzed for their total phenolic content according to the Folin-Ciocalteu colorimetric method.⁴ Total phenols were expressed as caffeic acid equivalents ($\mu\text{g}/\text{mg}$ extract). Results are reported in Table 1.

Bleaching of the Free Radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH Test). The antiradical activities of the *T. lucida* extract, fractions, compounds, and positive controls (rutin, quercetin, kaempferol, and α -tocopherol) were determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH[•]) and the procedures described by Saija et al.⁹ DPPH[•] has an absorption band at 515 nm, which disappears upon reduction by an antiradical compound. An aliquot (37.5 μL) of the MeOH solution containing different amounts of the *n*-BuOH extract, or of fractions I–IV, or of pure compounds from *T. lucida* and controls was added to 1.5 mL of freshly prepared DPPH[•] solution (0.025 g/L in methanol); the maximum concentration employed was 100 $\mu\text{g}/\text{mL}$. An equal volume (37.5 μL) of MeOH was added to control tubes. Absorbance at 515 nm was measured on a Shimadzu UV-1601 UV-visible spectrophotometer 20 min after starting the reaction. The DPPH[•] concentration in the reaction medium was calculated from a calibration curve analyzed by linear regression. The percentage of remaining DPPH[•] (% DPPH[•]_{REM}) was calculated as follows:

$$\% \text{DPPH}^{\bullet}_{\text{REM}} = [\text{DPPH}^{\bullet}]_T / [\text{DPPH}^{\bullet}]_0 \times 100$$

where *T* is the experimental duration time (20 min). All experiments were carried out in triplicate, and the mean effective scavenging concentrations (EC₅₀) were calculated by using the Litchfield & Wilcoxon²¹ test. Results are reported in Table 1.

Acknowledgment. This work was supported by a grant of the Italian Ministry of Education, University and Scientific Research (Project "Fitoterapici: ottimizzazione delle caratteristiche tecnologiche e biofarmaceutiche").

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NP020018I