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

Rita Aquino,*,† Armando Cáceres,‡ Silvana Morelli,† and Luca Rastrelli†

Dipartimento di Scienze Farmaceutiche and Scuola di Specializzazione in Scienza e Tecnologia Cosmetiche, Università di Salerno, Invariante 11/C, 84084 Fisciano, Salerno, Italy, and Departamento de Citohistología, Escuela de Química Biológica, Universidad de San Carlos de Guatemala, Zona 12, 01002 Guatemala

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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° 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° 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-Ciocalteau method[5] 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 EC_{50} value was calculated as 6.4 *μ*g with respect to α-tocopherol (EC_{50} 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 (EC_{50} 170.7 *μ*g) and showed a minor total phenolic content (23.9 *μ*g/mg), whereas fractions II−IV showed EC_{50} 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-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 ether[13] from fraction III; caffeic acid[14] 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 acid[16] 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 EC_{50} 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

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)]
n-BuOH extract	279.3 ± 2.44	6.4 (5.45−7.59)[c]
fraction I	23.9 ± 3.15	170.7 (146.50−198.89)[c]
fraction II	243.3 ± 3.69	5.3 (4.57−6.11)[c]
fraction III	358.6 ± 3.88	2.8 (2.31−3.33)[c]
fraction IV	365.4 ± 3.94	4.9 (4.37−5.42)[c]
quercetagenin-7-*O*-glucoside		4.6 (4.06−5.28)[c]
6-hydroxykaempferol 7-*O*-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 ± SD of three determinations. [b] Caffeic acid equivalents. [c] 95% confidence limits. [d] Positive control.

* To whom correspondence should be addressed. Tel: ++39 89 962814. Fax: ++39 89 962828. E-mail: aquinorp@unisa.it.
† Università di Salerno.
‡ Universidad de San Carlos de Guatemala.

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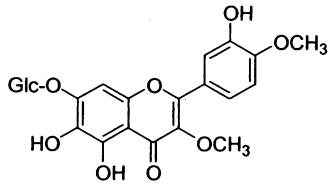
Table 2. ^{13}C NMR and ^{1}H NMR of Compounds **1**−**3** in CD_3OD[a]

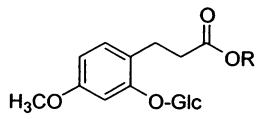
	1			**2**		**3**		**2** and **3**
position[b]	δ_C	δ_H (J_{HH} in Hz)[c]	position[b]	δ_C	δ_H (J_{HH} in Hz)[c]	δ_C	δ_H (J_{HH} in Hz)[c]	cross-peaks (δ_C) in HMBC spectrum
2	157.0		1	176.6		174.9		
3	138.8		2	39.1	2.47 m	35.4	2.64 m	C-1, C-1′
4	179.1		3	27.5	2.92 m	26.1	2.92 m	C-1, C-1′, C-2′, C-6′
5	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] $^{1}H-^{1}H$ coupling constants.

phenolic acids free-radical-scavenging ability[16] 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 = CH_3

Glc = β-D-glucopyranosyl

The negative FABMS of compound **1** showed an $[M - H]^-$ ion at *m*/*z* 507, consistent with the molecular formula $C_{23}H_{24}O_{13}$, which was also deduced using ^{13}C and DEPT NMR analysis. The MS and NMR spectra (Table 2) suggested that quercetagenin 3,4′-dimethyl ether[11] (−OMe signals at δ_H 3.82 and 3.92 and δ_C 58.8 and 60.2) was the aglycon and β-D-glucopyranosyl[5] was the sugar residue linked to C-7.[17] The 3-*O*-methyl etherification[17] 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 $C_{16}H_{22}O_9$, 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 ^{1}H NMR data indicated a β-configuration at the anomeric position ($J_{H-1-H-2}$ = 7.5 Hz); a 1D TOCSY[18] 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 H_2-6′, 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** ($C_{17}H_{24}O_9$) 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 ^{1}H and 150.9 for ^{13}C and using the UXNMR software package; DEPT, ^{1}H−^{1}H DFQ-COSY (double-quantum filtered COSY), ^{1}H−^{13}C HSQC, and HMBC experiments were obtained using conventional pulse sequences. 1D TOCSY[20] (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 CD_3OD. 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 $CHCl_3$ and then extracted with MeOH to give 11.2 g of residue, which was partitioned between *n*-BuOH and H_2O 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 × 5 cm) using MeOH as eluent. Fractions (9 mL) were collected and checked by TLC [Si gel, *n*-BuOH−HOAc−H_2O (60:15:25)]. Fractions 11−25 (I) (340.1 mg) were submitted to RP-HPLC on a C_{18} *μ*-Bondapack column (30 cm × 7.8 mm, flow rate 2.5 mL min^{-1}) using MeOH−H_2O (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−H_2O (4:6) to give caffeic acid[13] (18.1 mg, t_R 7.8 min) and a small amount of quercetagenin derivatives from II, and quercetagenin 7-*O*-*β*-D-glucopyranoside[10] (16 mg, t_R 20.5 min), quercetagenin 3-methyl ether 7-*O*-*β*-D-glucopyranoside[10] (4.1 mg, t_R 30.1 min), 6-hydroxykaempferol-7-*O*-*β*-D-glucopyranoside[11] (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 ether[12] (4.5 mg, t_R 39.4 min) from III. Fractions 80−95 (40.2 mg), separated using MeOH−H_2O (3:7), gave 6-*O*-caffeoyl-*β*-D-glucopyranoside[14] (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 acid[15] (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; $[\alpha]^{20}_D$ −43.5 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 353 (1.00), 270 (0.83), 258 (0.93) nm; ^{1}H and ^{13}C 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 $C_{23}H_{24}O_{13}$, C 54.34, H 4.76.

Compound 2: amorphous powder; mp 195−197 °C; $[\alpha]^{20}_D$ −38.2 (*c* 0.5, MeOH); UV (MeOH) λ_{max} 326 (4.40), 273 (1.50) nm; ^{1}H and ^{13}C 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 $C_{16}H_{22}O_9$, C 53.63, H 6.19.

Compound 3: amorphous powder; mp 88−90 °C; $[\alpha]^{20}_D$ −37.1 (*c* 0.2, MeOH); UV (MeOH) λ_{max} 328 (4.38), 272 (1.45) nm; ^{1}H and ^{13}C 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 $C_{17}H_{24}O_9$, C 54.84, H 6.50.

Quercetagenin-7-*O*-*β*-D-glucopyranoside: ^{13}C NMR (CD_3-OD) 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); ^{13}C NMR data for the sugar were almost superimposable on those of compound **1**.

Quercetagenin-3-methyl ether 7-*O*-*β*-D-glucopyranoside: ^{13}C NMR (CD_3OD) 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); ^{13}C 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 (*μ*g/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 *μ*L) 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 *μ*g/mL. An equal volume (37.5 *μ*L) 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:

$$\%\ \mathrm{DPPH}^\circ_{REM} = [\mathrm{DPPH}^\circ]_T/[\mathrm{DPPH}^\circ]_0 \times 100$$

where *T* is the experimental duration time (20 min). All experiments were carried out in triplicate, and the mean effective scavenging concentrations (EC_{50}) were calculated by using the Litchfield & Wilcoxon[21] test. Results are reported in Table 1.

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