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Antioxidant aryl-prenylcoumarin, flavan-3-ols and flavonoids from *Eysenhardtia subcoriacea*

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Abstract

Antioxidant activity (AOA) assay-guided chemical analysis, using a rat pancreas homogenate model, of aerial parts from *Eysenhardtia subcoriacea*, led to isolation of the new compound subcoriacin (3-(2′-hydroxy-4′,5′-methylendioxyphenyl)-6-(3″-hydroxymethyl-4″-hydroxybut-2″-enyl)-7-hydroxycoumarin) together with the known substances: (+)-catechin, (−)-epicatechin, (+)-afzelechin, eriodictyol, (+)-catechin 3-*O*-β-D-galactopyranoside and quercetin 3-*O*-β-D-galactopyranoside as bioactive constituents. The structure of the compound was determined from 1D and 2D NMR spectroscopic analyses. Additional known constituents were characterized. The bioactive compounds showed also moderate to strong radical scavenging properties against diphenylpicrylhydrazyl radical (DPPH). In addition, subcoriacin, (+)-catechin, (−)-epicatechin and (+)-afzelechin improved the reduced glutathione levels in rat pancreatic homogenate.

Keywords: *Eysenhardtia subcoriacea*; Fabaceae; Coumarins; Flavan-3-ols; 3-(2′-hydroxy-4′,5′-methylendioxyphenyl)-6-(3″-hydroxymethyl-4″-hydroxybut-2″-enyl)-7-hydroxycoumarin; Subcoriacin; Antioxidant activity; Reduced glutathione

1. Introduction

Eysenhardtia subcoriacea Pennell (Fabaceae) is a small tree located principally in central Mexico, where it is known as palo dulce (sweet wood), palo azul (blue wood) and taray. It has traditionally been used as a diuretic and for the treatment of kidney and bladder disorders developing during diabetes (Martínez, 1959). Several authors have highlighted the fact that hyperglycemia increases the concentration of oxygen-centered free radicals (OFR) and that these entities play a central role in the oxidative damage of tissues of several organs, such as pancreas, liver and kidney. Therefore, OFR have been implicated as the first cause of the onset and progression of diabetes (Baynes, 1991; Baynes and Thorpe, 1999). On the other hand, a number of enzymatic and nonenzymatic cellular antioxidative defenses protect cells from the damage of OFR (Buettner, 1993). One of the most important and versatile protectors is glutathione (GSH) (Li et al., 1997) which is both a substrate and a cofactor for a number of protective enzymes, such as GSH peroxidase, GSH *S*-transferase and glyoxalase (Meister and Anderson, 1983).

In this sense, it is recognized that natural antioxidants are important for the treatment of diabetes (Triggiani et al., 2006). Many studies have shown that phenolic compounds display antioxidant activity (AOA) as a result of their capacity to reduce free radicals (Seyoum et al., 2006). Phenolic compounds can also act as antioxidants by chelating metal ions, preventing radical formation, and improving the antioxidant endogenous system (Prasad, 2000; Soto et al., 2003; De Souza et al., 2004; Quine and Raghu, 2005; Whang et al., 2005; Al-Azzawie and Mohamed-Saiel, 2006; Vitor et al., 2004). Previous chemical analyses of some *Eysenhardtia* species have allowed the isolation of phenolic compounds such as flavonoids, methylprenylflavanones, flavan-3-ols (Narváez-Mastache et al., 2003,

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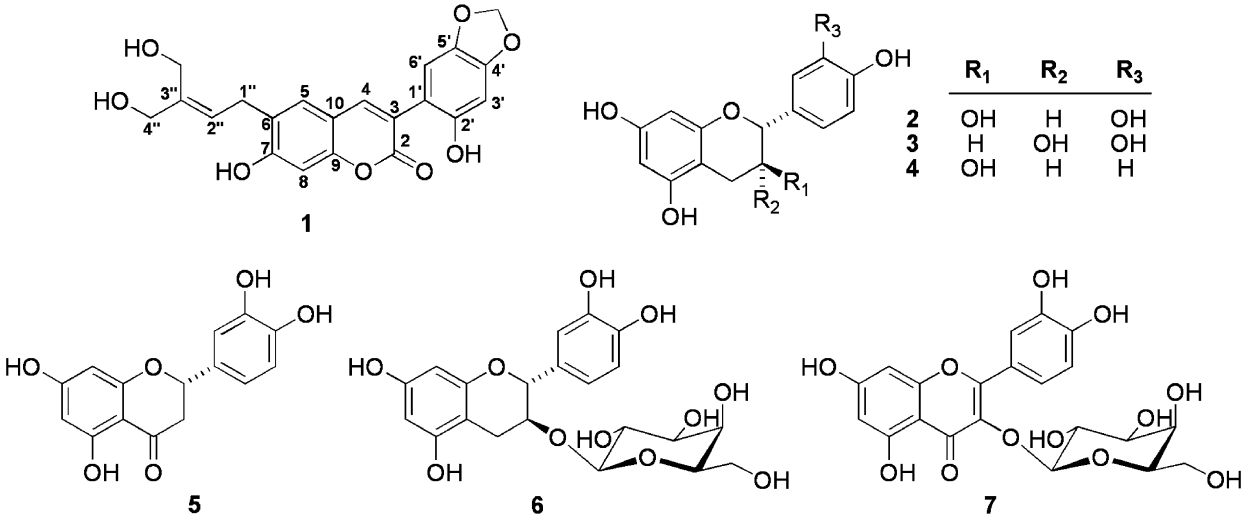
2006, 2007), pterocarpans, isoflavones, coumestans (Burns et al., 1984) and several flavonoids with antibacterial, insecticidal (Wächter et al., 1999; Alvarez and Delgado, 1999) and cytotoxic activities (Alvarez et al., 1998).

Here we report the isolation of a new coumarin, four flavan-3-ols and two flavonoids that displayed antioxidant activity (AOA) against 2,2′-azo-bis(2-amidinopropane)-dihydrochloride (AAPH)-induced damage in rat-pancreatic homogenate.

The EtOAc-soluble part of the methanolic extracts from bark and branches of *E. subcoriacea*, that displayed strong AOA, was composed of a complex mixture of phenolic compounds. The bioguided-chemical analysis afforded the new compound named subcoriacin (**1**), together with the known substances: (+)-catechin (**2**), (−)-epicatechin (**3**), (+)-afzelechin (**4**), eriodictyol (**5**), (+)-catechin 3-*O*-β-D-galactopyranoside (**6**) and quercetin 3-*O*-β-D-galactopyranoside (**7**) as bioactive constituents, together with several known natural products that did not display bioactivity. Some of these substances were previously isolated from *E. platycarpa* (Narváez-Mastache et al., 2003, 2006, 2007).



2. Results and discussion

2.1. Isolation and identification of compounds

Repeated column chromatography of the methanolic extracts from bark, branches and leaves, following the AOA against AAPH-induced damage in pancreatic homogenate, led to the isolation of the active compounds **1**–**7**. Quercetin was used as antioxidant positive control (Table 2). The bioguided-chemical analysis of the methanolic extract from bark afforded the new coumarin **1** and the known flavan-3-ols **2**, **4** and **6** as bioactive compounds, together with the inactive constituents: 3-*O*-acetyloleanolic acid, lupeol, betulinic acid, oleanolic acid, glabranin, 6-methyl-8-prenylflavanone, 8-methyl-6-prenylflavanone and 3-*O*-methyl-*chiro*-inositol. The fractionation of the methanolic extract from branches allowed the isolation of the bioactive compounds **1**, **2**, **3**, **5** and **6**, as well as the inactive compounds betulinic acid, oleanolic acid and 3-*O*-methyl-*chiro*-inositol. The bioactive quercetin 3-*O*-β-D-galactopyranoside (**7**), was isolated from the methanolic extract of leaves. Known compounds were identified by comparison of their spectroscopic data with those in the literature and by direct comparison with authentic samples. The structure of **1** was established on the evidence discussed below.

Compound **1**, obtained as a yellow solid from CH_2Cl_2-MeOH (m.p. 230–235 °C), possessed the molecular formula $C_{21}H_{18}O_8$ according to its HR-FAB-MS molecular ion peak observed at *m*/*z* 399.1078. The IR spectrum showed absorption bands for hydroxyl (3387 cm^{-1}), carbonyl (1693 cm^{-1}), and aromatic (1615 cm^{-1}) groups. The UV spectra showed a maximum of absorption at λ_{max} 348, 270 and 210 nm suggesting that **1** possessed a 3-arylcoumarin skeleton (Gordon and An, 1995). The 6,7-disubstitution of the coumarin followed from the aromatic singlets, showed in the ^{1}H NMR spectrum (Table 1), and assigned to H-5 (δ 7.36) and H-8 (δ 6.73), while the appearance of just one olefinic proton, assigned to H-4 (δ 7.82), indicated the presence of the substituent at C-3 (Wu et al., 2003). The sharp singlets observed at δ 5.88 (2H), δ 6.45 (1H) and δ 6.76 (1H) were in agreement with one dioxymethylene and two aromatic protons, assigned to H-3′ and H-6′ of the aryl group, respectively. In addition, the AB_2X system observed in the ^{1}H NMR spectrum [one triplet of doublet at δ 5.76 (2H, $J = 0.5$ and 7.5 Hz) being coupled with both a doublet at δ 3.49 (1H, $J = 7.5$ Hz) and a doublet at δ 4.14 (2H, $J = 0.5$ Hz)], together with one singlet at δ 4.29 (2H) established the presence of a 3-hydroxymethyl-4-hydroxybut-2-enyl moiety in the structure of **1**.

Table 1
^{13}C and ^{1}H NMR spectroscopic data for compound **1**, *J* (Hz) in parentheses

C	^{13}C	^{1}H
C-2	163.7	
C-3	122.4	
C-4	144.8	7.82 *s*
C-5	129.8	7.36 *s*
C-6	126.9	
C-7	160.3	
C-8	102.4	6.73 *s*
C-9	154.9	
C-10	113.7	
C-1′	115.5	
C-2′	151.4	
C-3′	99.12	6.45 *s*
C-4′	142.1	
C-5′	149.7	
C-6′	110.8	6.76 *s*
C-1″	28.3	3.49 *d*, (7.5)
C-2″	127.6	5.76 *t d*, (0.5, 7.5)
C-3″	140.4	
C-4″	58.4	4.29 *s*
C-5″	65.5	4.14 *d*, (0.5)
OCH_2O	102.5	5.88 *s*

In addition to the resonances of carbons belonging to the coumarin nucleus, the ^{13}C spectrum (Table 1) showed 12 other signals arising from one dioxymethylene, one aryl group, one sp^3 methylene, two oxymethylenes and two olefinic carbons. The analysis of the HSQC spectra and NOESY and HMBC correlations (Fig. 1) allowed us to establish all of the spectroscopic assignments for the structure **1**. The dioxymethylene group (δ 5.88) was placed at C-4′ and C-5′ since it exhibited HMBC correlations with δ 142.1 (C-4′) and δ 149.7 (C-5′), that in turn, showed a cross-peak with the two singlets at δ 6.76 (H-6′) and δ 6.45 (H-3′) (Fig. 1a). The NOESY interactions observed between H-4 (δ 7.82) and H-6′ protons (Fig. 1b), and the ^{3}J correlations of H-4 with C-1′ (δ 115.5), and H-6′ with C-3 (122.4) observed in the HMBC spectrum, confirmed that the aryl group was attached to C-3. In addition, the

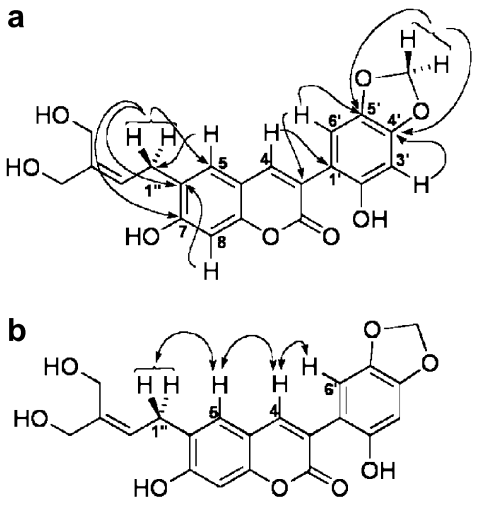


Fig. 1. Selected HMBC (a) and NOESY (b) correlations of **1**.

^{2}J, ^{3}J correlations of H-1″ (δ 3.49) with C-6 (δ 126.9), C-5 (δ 129.8) and C-7 (δ 160.3) and the ^{3}J correlations of H-8 (δ 6.73) with C-6 shown in the HMBC spectrum, together with the NOESY interactions of H-1″ with H-5 indicated that the 3-hydroxymethyl-4-hydroxybut-2-enyl moiety was connected to C-6. These spectroscopic assignments confirmed the structure of subcoriacin as **1**.

2.2. Antioxidant activity of methanolic extracts from bark, branches, leaves and pure compounds

The AOA displayed by methanolic extracts from the bark (EC_{50} 26.2 ± 5.8 ppm), branches (EC_{50} 20.8 ± 4.7 ppm) and leaves (EC_{50} 22.2 ± 3.7 ppm), together with the DPPH scavenger activity (EC_{50} 21.2 ± 0.1, 79.6 ± 3.5 and 102.3 ± 3.4 ppm, respectively), may provide evidence of the protective role of preparations of *E. subcoriacea* against the principal tissue damage related to diabetes.

The results of assays of the AOA of the pure compounds against AAPH-induced damage in a rat pancreas homogenate are summarized in Table 2. As expected, the phenolic compounds **1**–**7** showed strong AOA. We previously isolated compounds **2** and **6** from the bark of *E. platycarpa* and evaluated the protective effect in this same model (Narváez-Mastache et al., 2007). AAPH is an azo compound that to break up at 37 °C and produces two peroxyl radicals in water. The high production of this radical lead to an oxidative stress state in the pancreatic homogenate (Liégeois et al., 2000), inducing oxidative damage. The effect displayed by **1**–**7** against DPPH radical suggested that these compounds were able to neutralize the radicals formed during the peroxidation. However, **1** and **4** displayed strong AOA and moderate DPPH radical scavenging activity (Table 2), while quercetin displayed both strong AOA and DPPH radical scavenging effect (Seyoum et al., 2006). These effects suggested that exist other mechanisms implied in the AOA for **1** and **4**.

On the other hand, treatment of pancreatic homogenate with AAPH significantly decreased the GSH levels compared with the untreated group (Table 2). Li et al. (1997) indicated that GSH depletion may be related with cell death. The decreases of GSH levels in the pancreatic homogenate indicated the counteracting effect of the antioxidant systems against oxidative-induced damage. Compounds **1**, **3** and **4** significantly improved the pancreatic GSH levels alone or in combination with AAPH (compared with untreated and AAPH groups) while **5**–**7** and quercetin did not display any effect in this model. Quercetin possesses a double bond between C-2 and C-3 and a carbonyl group at C-4, suggesting that the saturation of these carbons was necessary for the effect on the GSH system. Compound **5** did not display effect in GSH levels, and it possesses a carbonyl group at C-4, supporting this proposal. Compound **3**, which increased the pancreatic GSH levels in our bioassay, has been reported to enhance the antioxidant system in diabetic rats, mainly related to the increase of GSH in liver and kidney (Quine and Raghu, 2005). Therefore, the enhance-

Table 2
Antioxidant activity of coumarin and flavan-3-ols

Compound	AOA $EC_{50} \pm$ SEM (μM)	DPPH $EC_{50} \pm$ SEM (μM)	GSH ± SEM (μg GSH mg protein^{-1})	
			Untreated (0.88 ± 0.05)	APPH (0.22 ± 0.06*)
1	9.11 ± 1.1	51.2 ± 2.3	0.99 ± 0.05*	0.92 ± 0.05*
3	11.4 ± 0.3	10.1 ± 2.5	1.02 ± 0.03*	1.09 ± 0.24*
4	11.2 ± 0.3	71.2 ± 1.3	1.15 ± 0.02*	1.01 ± 0.02*
5	37.1 ± 0.7	32.5 ± 1.2	0.87 ± 0.01	0.85 ± 0.02
7	7.3 ± 0.6	16.8 ± 2.3	0.86 ± 0.09	0.85 ± 0.06
Quercetin (reference)	18.1 ± 1.1	14.3 ± 1.1	0.88 ± 0.01	0.86 ± 0.02

Each value represents the mean of three independent assays ± standard error of the mean (SEM).
* Turkey's multiple comparison test showed a significant difference compared to the untreated group ($p < 0.001$).

ment of pancreatic GSH indicated an important additional mechanism for the AOA of **1**, **3** and **4**. In this sense, Hatano et al. (1989) observed that glycycoumarin, a 6-prenyl-3-arylcoumarin, inhibited the xanthine oxidase activity *in vitro*; this enzyme is responsible for the production of superoxide anion radical, indicating that several mechanisms are operating for the AOA of 6-prenyl-3-arylcoumarins. In addition, it has been demonstrated that umbelliferone (7-hydroxycoumarin) increases the concentration of nonezymatic and enzymatic antioxidants together with the decrease of the levels of biomarkers of lipid peroxidation in plasma and liver (Ramesh and Pugalendi, 2006).

2.3. Conclusion

The AOA bioguided-chemical analysis of several parts of *E. subcoriacea* allowed the isolation of six known flavonoids together with a new 6-prenyl-3-arylcoumarin, whose structure was elucidated by spectroscopic analysis. The number of natural 3-arylcoumarins having a prenyl moiety at C-6 is relatively small and these compounds has been suggested as important intermediates in the biosynthesis of coumestans (Martin and Dewick, 1978, 1980). Interestingly, Burns et al. (1984) previously reported the presence of 9-methoxy-2,3-methylendioxycoumestan from the bark of E. polystachya, and this is the first report of the isolation of a 6-(3″-hydroxymethyl-4″-hydroxybut-2″-enyl)-3-arylcoumarin from *Eysenhardtia* species, supporting the proposed biogenetic sequence. The phenolic compounds **1**–**7** displayed AOA against AAPH-induced damage in pancreatic homogenate. The bioassays indicated that only **1**, **3** and **4** improved the antioxidant system of the pancreatic homogenate.

The relatively high concentrations of subcoriacin (**1**), **6** and **7** found in the bark and branches of *E. subcoriacea* and their AOA observed in several assays, could explain the traditional medicinal use of this species for several ailments related to diabetes.

3. Experimental

3.1. General experimental procedures

Melting points were measured on a Fisher–Johns apparatus and are uncorrected. The ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Varian Unity Plus-500 instrument, chemical shifts are expressed in parts per million (δ) relative to TMS and the coupling constants are given in Hz. 2D NMR experiments (HSQC, HMBC, NOESY) were obtained using standard pulse sequences. Infrared spectra were recorded with a Perkin–Elmer 283B instrument. HR-FAB-MS spectral data were recorded with a JEOL JMS-AX 505 mass spectrometer. Vacuum chromatography was performed on Merck Kiesel gel 60 (0.040–0.863 mm) and the eluent is specified in each experiment. TLC analyses were performed on Alugram Sil G/UV_{254} silica gel plates. The chromatograms were examined under UV and by ceric ammonium sulfate/H_2SO_4 spray reagent. AAPH, DPPH, sodium dihydrogen phosphate monohydrate, anhydrous sodium hydrogen phosphate, and trichloroacetic acid (TCA), were purchased from Sigma–Aldrich Co. Double distilled water was used for all biochemical assays.

3.2. Plant material

Bark, branches and leaves of *E. subcoriacea* were collected in Tolantongo (7 km NE of Cardonal, Hidalgo and 22 km NE of Ixmiquilpan, Hidalgo, México) in august 2003. Voucher specimens (Ramiro Cruz 11322) were deposited in the Herbario de la Facultad de Ciencias de la UNAM and were authenticated by MSc Ramiro Cruz Durán.

3.3. Extraction and isolation

The dried-plant material [bark (550 g), branches (1300 g) and leaves (920 g)] of *E. subcoriacea* was dried and successively extracted (3 times) with *n*-hexane, CH_2Cl_2 and MeOH at room temperature. After the solvent were removed, the corresponding residues were obtained. An aliquot (80 g) of the methanolic extract from bark was suspended in H_2O (300 ml) and extracted with EtOAc, where 53 g of a brown solid (CMS1) was obtained, and extracted with *n*-BuOH. The EtOAc soluble fraction (8.2 g, EC_{50} 29.6 ± 1.2 ppm) was subjected to vacuum-liquid Kiesel gel cc chromatography column eluting with *n*-hexane and then increasing the polarity with EtOAc, and finally with

EtOAc/MeOH/H_2O. Eleven main fractions with similar TLC spots were collected as follows F_1 (1/7, 126 mg), F_2 (8/20, 425 mg), F_3 (21/24, 395 mg), F_4 (25/27, 512 mg), F_5 (28/31, 574 mg), F_6 (32/41, 225 mg), F_7 (42/48, 231 mg), F_8 (49/51, 384 mg), F_9 (52/58, 621 mg), F_{10} (59/64, 2.2 g) and F_{11} (65/80, 1.3 g), and were tested for AOA using the rat pancreas model. Further crystallization of the solids obtained from F_3 (EC_{50} 15.3 ± 2.1 ppm) and F_4 (EC_{50} 35.8 ± 1.13 ppm) afforded **2** (132 mg) (Narváez-Mastache et al., 2007) and **4** (115 mg) (Hillis and Inoue, 1967), respectively. The fractions F_9–F_{11} (4.121 g, EC_{50} 10.2 ± 1.1 ppm) were pooled and applied to a sephadex-LH-20-column [MeOH/H_2O (1:1)], to obtain **1** (2.3 g) and **6** (520 mg) (Narváez-Mastache et al., 2007). The known compounds lupeol (42 mg), glabranin (135 mg), 6-methyl-8-prenylflavanone (135 mg) and 8-methyl-6-prenylflavanone (119 mg) (Narváez-Mastache et al., 2006) were obtained from F_2. Fractions F_5 contained a mixture of betulinic and oleanolic acids (1.1 g). The *n*-BuOH residue (22.1 g, EC_{50} 15.8 ± 2.1 ppm) was subjected to cc and eluted first with EtOAc and then with EtOAc/MeOH/H_2O, affording 3-*O*-methyl-*chyro*-inositol (2.4 g) and **6** (12.5 g).

The methanolic extract from branches (210 g) was fractionated using cc and eluted with *n*-hexane, mixtures of EtOAc/*n*-hexane and finally with EtOAc/MeOH/H_2O, respectively to obtained eleven main fractions as follows F_1 [1/16, 3.5 g], F_2 [17/33, 4.32 g], F_3 [34/52, 5.12 g], F_4 [53/65, 512 mg], F_5 [66/79, 574 mg], F_6 [80/91, 225 mg], F_7 [92/104, 231 mg], F_8 [105/117, 6.25 g], F_9 [118/127, 4.71 g], F_{10} [128/136, 9.48 g] and F_{11} [137/157, 14.2 g]. The bioactive fractions F_{2-3} (9.44 g, EC_{50} 112.1 ± 1.9 ppm) was applied to a cc and eluted with *n*-hexane and EtOAc affording four fractions F_{2-3A}, F_{2-3B}, F_{2-3C} and F_{2-3D}. The purification of bioactive subfractions F_{2-3D} (2.3 g, EC_{50} 10.2 ± 0.7 ppm) with cc permitted isolation of (−)-epicatechin **3** (425 mg) (Hillis and Inoue, 1967) and eriodictyol **5** (221 mg) (Garo et al., 1996). The fractions $F_{8\text{-}11}$ were pooled (34.6 g) and suspended in water (200 ml). The suspension was successively extracted with EtOAc (EC_{50} 9.2 ± 1.1 ppm) and *n*-BuOH (EC_{50} 11.5 ± 3.1 ppm). The EtOAc soluble fraction was purified by cc to afford four main subfractions A (1/5), B (6/11), C (12/17) and D (18/22). The successive crystallization of solids obtained from B (EC_{50} 9.1 ± 2.0 ppm) and C (EC_{50} 18.1 ± 1.8 ppm) afforded **1** and **6,** respectively.

The methanolic extract from leaves (385 g) was suspended in water (600 ml). The suspension was successively extracted with EtOAc (EC_{50} 28.2 ± 2.4 ppm) and *n*-BuOH (EC_{50} 11.5 ± 1.8 ppm), obtaining two organic phases, one yellow solid (Solid A, 92 g, EC_{50} 9.8 ± 1.2 ppm) and the aqueous phase (EC_{50} > 100 ppm). The analysis of EtOAc soluble phase in TLC on silica gel showed a mixture of **2** and **3**. An aliquot of solid A (80 g) was purified using cc with EtOAc and then EtOAc/MeOH/H_2O mixtures as eluants to afford 3 main fractions: F_1 (1/5), F_2 (6/16) and F_3 (17/30). Successive crystallizations of the solid from F_2 afforded quercetin 3-*O*-β-D-galactopyranoside **7** (61 g) (Hillis and Inoue, 1967).

3.4. 3-(2′-hydroxy-4′,5′-methylendioxyphenyl)-6-(3″-hydroxymethyl-4″-hydroxybut-2′-enyl)-7-hydroxycoumarin (*1*)

Yellow solid; m.p. 230-235 °C (CH_2Cl_2:MeOH); UV $\lambda_{max}^{CH_3OH}$ nm (log ε) 348 (44.9), 270 (23.9) 210 (138.1) nm; IR ν_{max}^{KBr} 3387 (OH), 2928, 1693 (CO), 1615, 1231, 1037, 843 cm^{-1}; for ^1H NMR (500 MHz, CD_3OD, TMS) and ^{13}C NMR (125 MHz, CD_3OD, TMS) spectra, see Table 1; HR-FAB-MS *m/z* 399.1078 $[M+1]^+$ ($C_{21}H_{18}O_8$ requires 399.1080).

3.5. Antioxidant activity assay

3.5.1. Animals

Male Wistar rats (200–250 g body weight) were obtained from Instituto de Fisiología Celular of the Universidad Nacional Autónoma de México. Prior to the experiments its were fed with standard food and water *ad libitum*. All the procedures were carried out following the guidelines 'Principles of Laboratory Animal Care' (NIH publication #85-23, revised 1985) and the Mexican Official Norm "Norma Oficial Mexicana NOM-062-ZOO-1999".

3.5.2. Antioxidant activity (AOA) in pancreatic homogenate

The antioxidant activity was measured in a rat pancreas homogenate followed the method previously described (Narváez-Mastache et al., 2007). The degree of lipid peroxidation was determined according to method of Ohkawa et al. (1979). Quercetin was used as antioxidant positive control. The result was expressed in nmol of malondialdehyde (MDA) per mg of protein and the EC_{50} was determined. The extracts and natural products were tested at 1, 100 and 1000 ppm and 1, 3.1, 10, 31 and 100 μM, respectively.

3.5.3. Determination of reduced GSH

The concentration of GSH in the pancreas homogenate was established following the method of Sedlak and Lindsay (1968) and that we previously described (Narváez-Mastache et al., 2007). The results were expressed as μg GSH per mg of protein. The natural products were tested at 1, 3.1, 10, 31 and 100 μM on three independent experiments.

3.5.4. 1,2-Diphenyl-2-picrylhydrazyl (DPPH) radical assay

This assay was carried out according to the procedure previously described (Garduño-Ramírez and Delgado, 2003). Fifty microliters of samples at various concentrations (1, 31, 100, 310 and 1000 μg/ml for extracts and 1, 3.1, 10, 31 and 100 μM for pure substances), dissolved in DMSO and 150 μl of DPPH (133.33 μM in EtOH) in 96-well microliter plates, were incubated at 37 °C for 30 min, with absorbances were subsequently measured at 515 nm in a microplate reader ELx 808. Measurements were per-

formed in triplicate in at least three independent experiments. EC_{50} was determined following standard procedures.

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