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M.C. González-Guëreca [a], M. Soto-Hernández [b] & M. Martínez-Vázquez [c]

[a] Instituto Politécnico Nacional, Fraccionamiento 20 de Noviembre, 34220 Durango, Dgo, México

[b] Colegio de Postgraduados, Km 36.5 Carretera México-Texcoco 56230, Montecillo, Texcoco, Edo de México, México

[c] Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior, Ciudad Universitaria, Coyoacán 04510, México D.F., México

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Isolation of (−)(2S)-5,6,7,3′,5′-pentahydroxyflavanone-7-*O*-*β*-D-glucopyranoside, from *Lippia graveolens* H.B.K. var. *berlandieri* Schauer, a new anti-inflammatory and cytotoxic flavanone

M.C. González-Guëreca[a], M. Soto-Hernández[b] and M. Martínez-Vázquez[c]*

[a]*Instituto Politécnico Nacional, Fraccionamiento 20 de Noviembre, 34220 Durango, Dgo, México;* [b]*Colegio de Postgraduados, Km 36.5 Carretera México-Texcoco 56230, Montecillo, Texcoco, Edo de México, México;* [c]*Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior, Ciudad Universitaria, Coyoacán 04510, México D.F., México*

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A new flavanone glycoside, (−)(2S)-5,6,7,3′,5′-pentahydroxyflavanone-7-*O*-*β*-D-glucopyranoside (**1**), was isolated from the stems of *Lippia graveolens* H.B.K. (Verbenaceae). The structure of **1** was elucidated based on spectral analysis and chemical transformations. The treatment of **1** with acetic anhydride and pyridine afforded the corresponding per-acetylated derivative **2**, while an acid hydrolysis reaction of **1** afforded a 5,6,7,3′,5′-pentahydroxy flavanone (**3**). Additionally, the anti-inflammatory and cytotoxic activities of **1**, **2** and **3** were determined.

Keywords: *Lippia graveolens*; flavanone; anti-inflammatory; cytotoxic

1. Introduction

Lippia graveolens H.B.K. (syn. *Lippia berlandieri* Schauer., Verbenaceae) is an aromatic species and medicinal plant known as 'Mexican oregano'. In Mexican traditional medicine, the aerial parts of this species are used as an antiseptic, antipyretic, analgaesic, abortive, antispasmodic and anti-inflammatory agent and for the treatment of menstrual disorders and diabetes (Pascual, Slowing, Carretero, Sánchez-Mata, & Villar, 2001). The chemical composition of the essential oil of *L. graveolens* has been determined by gas chromatography mass spectroscopy (GC–MS). A high content of oxygenated monoterpenes was found, its major constituents being carvacrol, thymol and *p*-cymene. In addition, the essential oil demonstrated significant antimicrobial and antioxidant activity (Rocha-Guzmán et al., 2007). As far as other components are concerned, naringenin, pinocembrin, lapachenole and 10 iridoids have been isolated (Domínguez, Sánchez, Suárez, Baldas, & González, 1989; Rastrelli, Caceres, Morales, De Simone & Aquino, 1998).

Most of the studies of *L. graveolens* have focused on the chemical composition and biological activities of the essential oil, but few scientific reports regarding chemical characterisation of the polar extracts as well as their biological activities are found (Arcila-Lozano, Loarca-Piña, Lecona-Uribe, & González de Mejía, 2004).

*Corresponding author. Email: marvaz@servidor.unam.mx

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Despite its ethno-medical importance, the main use of this species is of its essential oils, which are popular condiments for food. Mexico is the largest exporter of *L. graveolens* in the world, with 35–40% of the international market. This high demand is due to the quality of essential oil present in the leaf (González-Guëreca, Soto-Hernández, Kite, Martínez-Vázquez, 2007). The collection of leaves for extraction of essential oils of *L. graveolens* is an economic activity which is complementary to the rain-fed agriculture in the arid and semi-arid zones of North Mexico. Approximately 4000 tons of leaves of *L. graveolens* are exported every year. As a result of this activity, large quantities of stems are produced as 'useless' residue. In spite of this abundance, chemical and biological evaluations of the stems of this species have not been carried out. Recently, we published a study on the presence of the flavonoids pilosin, cirsimartin, narigenin, kaempferol and isokaemferide, among other compounds, in the EtOAc extract of *L. graveolens* stems (González-Guëreca et al., 2007).

Now, as part of our systematic study of the isolation of anti-inflammatory and cytotoxic compounds from natural sources (Flores-Rosete & Martínez-Vázquez, 2008), we wish to report the isolation and structural elucidation of (−)(2S)-5,6,7,3′,5′-pentahydroxyflavanone-7-*O*-β-glucopyranoside (**1**), a new compound isolated from the EtOAc extract of *L. graveolens* stems. The treatment of **1** with acetic anhydride and pyridine afforded the per-acetylated derivative **2**, while an acid hydrolysis of **1** yielded the flavanone **3** (Figure 1). The anti-inflammatory and cytotoxic properties of **1** and its derivatives **2** and **3** are also reported.

2. Results and discussion

Compound **1** was obtained as yellow amorphous powder ($[\alpha]_D^{25}$–36.5°), m.p. 187–189°C. HRMS of **1** gave the quasimolecular ion $[M+H]^+$ at $m/z = 467.1188$ (Calcd 467.1190), corresponding to the molecular formula $C_{21}H_{22}O_{12}$. Its IR spectrum showed bands at 3394, 3001–2939, 1651 and 1605 cm^{-1}, which together with its UV absorptions at 215, 288 and 366 nm suggested that **1** could be a flavonoid compound. This proposal is supported by its ^{1}H-NMR spectrum, where the typical ABX three proton system signals of a flavanone nucleus were observed at 5.34 ppm (dd, $J = 3$, 13), 2.67 ppm (dd, $J = 3$, 17) and 3.21 ppm (dd, $J = 13$, 17), assigned to the H-2, H-3 eq. and H-3 ax., respectively. These signals correlated, in the HETCOR spectrum, with the signals at δ 78.65 (C-2) and at 42.64 (C-3), which were observed in the DEPT spectrum as methine and methylene signals, respectively. A survey of the literature revealed that the spectral data of **1** were very similar to those of (2S)-5,7,3′,5′-tetrahydroxyflavanone-7-*O*-β-D-glucopyranoside isolated from *Jasminium lanceolarium* (Sun, Yang, & Zhang, 2007). However, the molecular formula of the flavanone **1** showed 16 units of mass, in contrast to those found in *J. lanceolarium*, indicating the presence of an additional hydroxyl group in **1**. The position of the extra hydroxyl group at C-6 in **1** was deduced as follows. The impact electron mass spectroscopy (IEMS) spectrum of **1** showed the ion molecular at M 466. Loss of a glucose residue from **1** was indicated by the peak at $m/z = 303$. It also showed peaks at $m/z = 136$ and 168 assigned to fragments of a retro Diels–Alder breakup from the 303 m/z fragment.

Taking into account the published data of similar flavonoids, the signal of an aromatic methine atom carbon at 94.20 ppm, in the ^{13}C-NMR spectrum of **1**, was

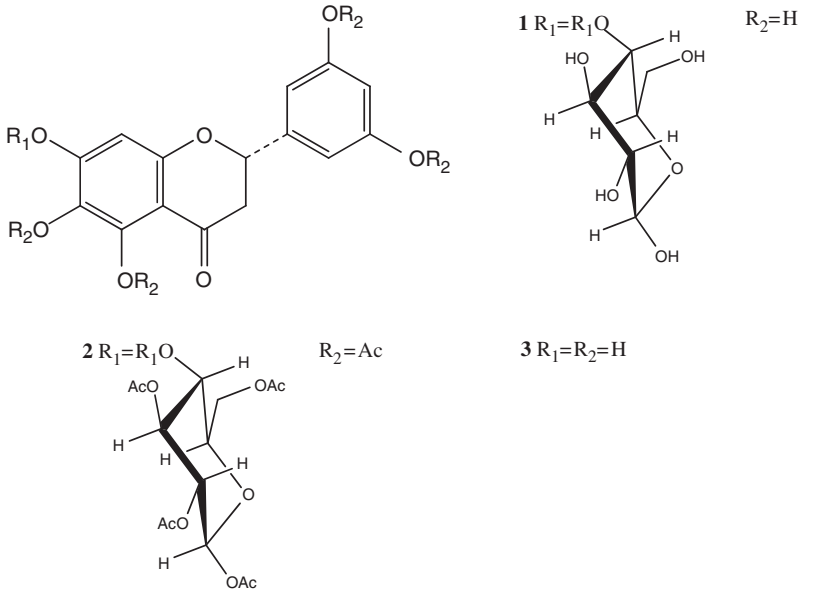
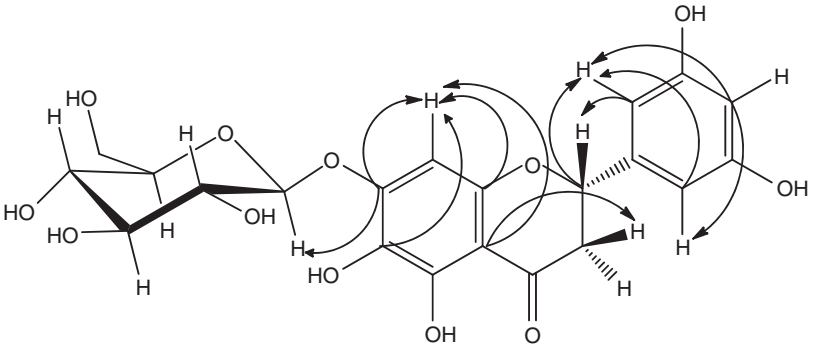


Figure 1. Structures of **1**–**3**.

assigned to C-8 (Kawabata et al., 2003; Saracoglu Varel, Sebnem-Harput, & Nagatsu, 2004). The signal of a quaternary carbon atom at 153.40 showed that a glucose residue could be in position C-7, as in all the flavonones presented in *L. graveolens* (Lin, Mukhopadhyay, Robbins, & Harnly, 2007). All of these assignments were confirmed by the correlations shown in the HMBC experiment (Figure 2). The circular dichroism (CD) spectrum showed a positive effect at 316 nm and a negative one at 289 nm, consistent with the *S*-configuration at C-2, as in all the flavanones isolated from *L. graveolens* (Flores-Rosete & Martínez-Vázquez, 2008). Then the compound **1** was identified as (−)(2S)-5,6,7,3′,5′-pentahydroxyflavanone-7-*O*-*β*-D-glucopyranoside, which, according to our knowledge, constituted a new compound isolated from a natural source.

The per-acetylated derivative **2** was obtained by treating **1** with pyridine and acetic anhydride. Although the molecular ion of **2** was not shown in its EI–MS spectrum, the peak at $m/z = 331$ (44%) revealed the presence of a tetra-acetylated–glucopyranoside residue in **2**, while the peak at $m/z = 169$ (100%) was due to the di-acetylated B ring fragment. The ^{1}H-NMR spectrum of **2** showed eight singlets (3H each) at δ 1.95, 2.02, 2.04, 2.06, 2.26, 2.31, 2.32 and 2.37 ppm assigned to the methyl groups of the acetate residues. All these signals correlated with a complex signal at 20.4–20.7 ppm in the HETCOR spectrum. The rest of the signals in the ^{1}H- and ^{13}C-NMR spectra as well as the bands in the IR spectrum support the structure of **2** (see Section 3).

When **1** was treated with H_2SO_4 diluted solution, the yellow-greenish solid aglycon (**3**) was obtained. The HRMS of this product showed a pseudo-molecular ion at 305.0661, corresponding to the molecular formula $C_{15}H_{13}O_7$ (Calcd 305.0661), which is in full agreement with the expected structure. Furthermore, the IEMS spectrum showed the molecular ion at $m/z = 304$ M^+ (85%), as well as the expected



1

Figure 2. Important HMBC correlations of **1**.

Table 1. Effect of ME and EAE in the TPA-induced oedema model.

Sample	Doses (mg per ear)	Oedema (mg)	IE (%)
TPA[a]		15.12 ± 0.34	
TPA + ME[a]	1	10.7 ± 0.84**	29.26
TPA[b]		13.60 ± 0.48	
TPA + EAE[b]	1	7.92 ± 1.20**	41.73

Notes: [a]Solvent used MeOH–H_2O (1 : 1). [b]Solvent used MeOH–EtOAc (1 : 1). **All data were analysed by ANOVA followed by Dunnett's test, and values of $p = 0.01$ are considered as statistically significant with respect to the control.

peaks at $m/z = 168$ (100%) and 136 (20%) of a retro Diels–Alder fragmentation of **3**. The ^{1}H-NMR spectrum of **3** showed the ABX system at 5.32 ppm (dd, $J = 2.5$, 12.8), 3.19 ppm (dd, $J = 17$, 12.8) and 2.61 ppm (dd, $J = 17$, 3), assigned to the protons H-2, H-3 ax. and H-3 eq., respectively. These signals correlated, in the HECTOR spectrum, with the methine carbon at 78.40 (C-2) and the methylene carbon at 42.35 ppm (C-3), respectively, while those singlets at 6.74 (2H) and 6.90 assigned to the protons H-2′, H-6′ and H-4′, respectively, correlated, in the HECTOR experiment, with the signals at 114.24, 117.80 and 115.25 ppm, respectively, and the singlet at 5.93 ppm was assigned to H-8; this signal correlated with the methine at 94.64 (C-8). The remaining signals at 101.66 (C-10), 126.21 (C-6), 155.68 (C-5), 155.14 (C-9) and 156.39 (C-7) were assigned by comparison with those spectral data of similar structures (Miyake et al., 2003).

It is well known that several flavonoids possess both cytotoxic and anti-inflammatory activities, probably by inhibition of the activation of the NF-κB factor (Liang et al., 1999). Then it was decided to evaluate the cytotoxic and anti-inflammatory properties of **1**–**3**. Also, both an aqueous extract and an EAE of the stems were tested. The results showed that both extracts and **1**–**3** showed anti-inflammatory activity in the TPA-induced oedema in mice model (Tables 1 and 2). The EtOAc extract was more active than the ME, while the compounds **1** and **2** showed an oedema inhibition effect (IE) of 34.32% and 30.40% at the doses of

Table 2. Effect of the compounds **1**, **2** and **3** in the TPA-induced oedema model.

Sample	Doses (mg per ear)	Doses (mmol per ear)	Oedema (mg)	IE (%)
TPA			14.23 ± 0.81	
TPA + **1**[a]	0.31	6.65×10^{-4}	11.63 ± 2.21*	34.32
TPA + **2**[a]	0.31	3.86×10^{-4}	9.90 ± 1.59*	30.40
TPA + **3**[a]	0.31	1.01×10^{-3}	9.18 ± 1.09*	35.50
TPA[b]			16.24 ± 0.86	
TPA + indomethacine[b]	0.46	1.28×10^{-3}	1.57 ± 0.33**	89.19

Notes: [a]Solvent used MeOH–CH_2Cl_2 (1 : 1). [b]Solvent used EtOH–acetone (1 : 1). The weight of ears was presented as the mean ± standard error of mean (SEM) of 3–4 male mice. IE (%) was calculated from the test sample group with reference to the TPA, and data were analysed using Student's *t*-test; *$p \leq 0.05$ and **$p \leq 0.01$ were considered significant with respect to the control.

Table 3. Effect of **1**–**3** ME and EAE on human tumour cell lines.

	Cell lines GI (%)				
Sample ($50\,\mu g\,mL^{-1}$)	HCT-15	MCF-7	U251	PC-3	K562
ME	NA	5.11	1.59	3.26	60.09
EAE	21.57	6.31	2.84	22.14	100.0
1	NA	NA	4.94	5.45	91.97
2	85.51	98.86	70.33	73.16	97.87
3	55.20	65.10	69.95	66.09	83.39

Notes: NA, not active; ME, methanol extract; EAE, EtOAc extract.

6.65×10^{-4} and 3.86×10^{-4} mmol by ear, respectively. The flavanone **3** and the reference drug indomethacine showed IE of 35.50% and 89.19% at the doses of 1.01×10^{-3} and 1.28×10^{-3} mmol by ear, respectively. These results indicated that **2** was the most active anti-inflammatory, probably because it possesses more lipophilic character than **1** and **3**, and it penetrates more easily through the skin of the test animals. On the other hand, the results of growth inhibition of human cancer lines showed that the EtOAc extract exhibits 100% of growth inhibition of the line K562 at the $50\,\mu g\,mL^{-1}$ dose. Again **2** was more active than **1** and **3** at the $50\,\mu g\,mL^{-1}$ dose level (Table 3). Taking into account these results, it was decided to establish the IC_{50} values of **2**, against several human cancer lines. The results were 47.36 ± 4.6 (HCT-15), 69.62 ± 2.6 (MCF-7), 53.26 ± 7.0 (U251) and 37.82 ± 4.3 (K562). Our results showed that **2** showed better anti-inflammatory and cytotoxic properties than the glycoside **1** and the flavanone **3**, although, as mentioned before, its lipophilic properties could account for this anti-inflammatory activity. However, it is not clear whether this property can account for the growth inhibition of cancer cells.

Although the anti-inflammatory and cytotoxic activities of **1** were relatively low, its potential abundance could account for the development of anti-inflammatory and cytotoxic drugs from this natural resource.

3. Experimental

3.1. *General*

Vacuum column chromatography (VCC) and thin-layer chromatography (TLC) were carried on silica gel 60 F254 (Merck 1.05554). The spots were observed under ultraviolet (UV) light at 366 and 254 nm, and the TLC plate was then sprayed with cerium sulphate tetrahydrate (1%) in sulphuric acid solution. Melting points were determined with a Fisher Johns apparatus and are uncorrected. The UV spectra were recorded on a Shimadzu U 160 model spectrophotometer. The infrared (IR) spectra were recorded on a Nicolet FT-IR5-SX spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian-Unity 300 (^{1}H at 300 and ^{13}C at 75.4 MHz) and a Varian-Inova 500 (^{1}H at 500 and ^{13}C at 12 MHz). Chemical shifts are expressed in δ (ppm) relative to tetramethylsilane (TMS) as the internal standard, and coupling constants, J, are in Hz. The solvent is indicated for each compound. Correlation spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY) and heteronuclear correlation (HETCOR) were carried out using the Varian-Unity 300 spectrometer, while the heteronuclear multiple bond correlation (HMBC) experiment was performed using the Varian-Inova 500 spectrometer. The electron ionisation mass spectroscopy (EI–MS) and high resolution mass spectrometry (HRMS) were recorded on a Jeol AH505HR mass spectrometer.

3.2. *Plant material*

The collection of stems was done in Mezquital, Durango, Mexico (at 2230 m altitude and 23°43′, 24.2″N; 104°24′, 0.79″W). A voucher of a complete (leaves and stem) specimen has been deposited at the Herbarium, CIIDIR-IPN, under register no. 16543.

3.3. *Extraction and isolation*

Dried and ground stems (1.7 kg) were successively extracted with hexane, EtOAc and MeOH at room temperature (9 times × 48 h each). Evaporation of the solvents under low pressure afforded 5.3, 37.5 and 184.5 g of the respective extracts. The aqueous extract was prepared by the addition of 10 g of stems to 90 mL of boiling water. The resulting solution was lyophilised, affording 1 g of the aqueous lyophilised extract (WE).

A fraction of the EtOAc extract (12 g) was dissolved in methanol and absorbed into 10 g of silica gel G (Alltech). Afterwards the solvent was evaporated at room temperature. The resulting dried mixture was put on the top of a chromatography column, packed with 130 g of silica gel and eluted with hexane and mixtures of hexane–EtOAc and EtOAc–MeOH of increasing polarity. A total of 198 fractions of 200 mL each were obtained. Fractions eluted with EtOAc 100% (162 and 163) were combined to afford 547 mg of 5,6,3′,4′-tetrahydroxy flavanone 7-*O*-β-D-glucose (**1**) (0.033% yield from dried material).

3.3.1. *5,6,7,3′,4′ pentahydroxy flavanone 7-O-β-d-glucopyranoside (**1**)*

5,6,7,3′,4′ pentahydroxy flavanone 7-O-β-D-glucopyranoside (**1**) was isolated as yellow-greenish solid with m.p. 187–189°C. Its HRMS showed a pseudo-molecular

ion at $m/z = 467.1188$ ($[M]^+ + 1$), Calcd for $C_{21}H_{23}O_{12}$. IEMS (70 eV) (rel. int.) $m/z = 466.40$, 304, 168 and 136. UV λ_{max} (nm) (EtOH) 215 ($\log \varepsilon = 1.52$), 288 ($\log \varepsilon = 2.1$) and 366 ($\log \varepsilon = 2.26$). IR (KBr) ν_{max} (cm^{-1}) 3394, 1651, 1497 and 1073. ^{1}H-NMR (dimethyl sulphoxide (DMSO)), 500 MHz): δ 2.67 (1H, dd, 2.5, 17.3 Hz, H-3 eq.), 3.12 (1H, m, H-4″), 3.21 (1H, dd, 12.8, 17.3 Hz, H-3 ax.), 3.25 (1H, m, H-2″), 3.26 (1H, m, H-5″), 3.50 (1H, m, H-3″), 3.42 (1H, m, H-6a″), 3.66 (1H, m, H-b″), 4.85 (1H, d, 7.5 Hz, H-1″), 5.34 (1H, dd, 2.5, 12.8 Hz, H-2), 6.28 (1H, s, H-8), 6.73 (2H, s, H-2′and H-6′) and 6.86 (1H, s, H-4′); ^{13}C-NMR (DMSO, 125 MHz): δ 78.72 (C-2), 42.66 (C-3), 197.89 (C-4), 149.19 (C-5), 127.74 (C-6), 153.40 (C-7), 94.20 (C-8), 154.51 (C-9), 103.36 (C-10), 129.50 (C-1′), 117.97 (C-2′), 145.60 (C-3′), 114.33 (C-4′), 145.10 (C-5′), 115.28 (C-6′), 100.58 (C-1″), 73.09 (C-2″), 77.14 (C-3″), 69.58 (C-4″), 75.64 (C-5″) and 60.56 (C-6″). HETCOR main correlations ^{1}H/^{13}C: 6.86/114.33, 6.73/117.91, 6.73/115.28, 6.28/94.20, 5.34/78.7, 4.85/110.58, 2.69 and 3.21/42.66.

3.3.2. *Per-acetylated compound (**2**)*

To a solution of **1** (100 mg) in of pyridine (1 mL), anhydride acetic (1 mL) was added. The resulting solution was heated at 60°C for 6 h. After the usual work up, 100 mg of the per-acetylated derivate **2** as a brown–red powder was obtained. It showed an m.p. of 114–115°C. IEMS (70 eV) m/z (rel. int.): 331 (45), 169 (100), 109 (40), 43 (44). IR (KBr) ν_{max} (cm^{-1}) 2969, 1622, 1216 and 1075. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 2.02 (3H, s, CH_3CO), 2.04 (3H, s, CH_3CO), 2.05 (3H, s, CH_3CO), 2.06 (3H, s, CH_3CO), 2.26 (3H, s, CH_3CO), 2.31(3H, s, CH_3CO), 2.32 (3H, s, CH_3CO), 2.37 (3H, s, CH_3CO), 2.74 (1H, dd, 3, 17 Hz, H-3 eq.), 2.98 (1H, dd, 12.8, 17.3 Hz, H-3 ax.), 3.89 (1H, m, H-5″), 4.19 (2H, m, H-6″), 5.08 (1H, d, 8 Hz, H-1″), 5.11 (1H, m, H-4″), 5.30 (2H, m, H-2″ and H-3″), 5.43 (1H, dd, 2.5, 13), 6.06 (1H, s, H-8), 7.26 (2H, brs, H-2′ and H-6′) and 7.29 (1H, brs, H-4″); ^{13}C-NMR ($CDCl_3$,75.4 MHz): δ 79.08 (C-2), 44.87 (C-3), 188.03 (C-4), 143.29 (C-5), 136.59 (C-6), 160.40 (C-7), 101.29 (C-8), 154.57 (C-9), 109.35 (C-10), 128.13 (C-1′), 124.36 (C-2′), 142.56 (C-3′), 123.98 (C-4′), 142.45 (C-5′), 121.54 (C-6′), 98.24 (C-1″), 70.81 (C-2″), 72.29 (C-3″), 67.99 (C-4″), 72.45 (C-5″), 61.63 (C-6″), 170.48 (CH_3CO), 169.99 (CH_3CO), 169.35 (CH_3CO), 168.33 (CH_3CO), 168.01(CH_3CO) and 20.67–19.90 (CH_3CO, complex signal).

3.3.3. *5,7,8,3′,5′Pentahydroxy flavanone (**3**)*

To 100 mg of **1** dissolved in MeOH (20 mL), a solution of H_2SO_4 4N (5 mL) was added. The mixture of reaction was refluxed for 24 h. After the usual work up, 22 mg of 5,7,8,3′,4′pentahydroxy flavanone (**3**), as a yellow-greenish powder, was obtained. It showed an m.p. of 189°C. HRMS showed a pseudo-molecular ion at 305.0661 for $C_{15}H_{13}O_7$, Calcd 305.0661. IEMS (70 eV) m/z (rel. int.)= 304 M^+ (85), 168 (100), 136 (20), 69 (18), 57 (19). ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 2.61 (1H, dd, 3, 17 Hz, H-3 eq.), 3.19 (1H, dd, 12.8, 17 Hz, H-3 ax.), 5.32 (1H, dd, 2.5, 12.8 Hz, H-2), 5.93 (1H, s, H-8), 6.74 (2H, s, H-2′and H-6′) and 6.90 (1H, s, H-4′); ^{13}C-NMR (DMSO, 75 MHz): δ 78.45 (C-2), 42.35 (C-3), 196.91 (C-4), 155.68 (C-5), 126.21 (C-6), 156.39 (C-7), 94.64 (C-8), 155.14 (C-9), 101.66 (C-10), 129.72 (C-1′), 114.24 (C-2′), 145.12 (C-3′), 115.25 (C-4′), 145.57 (C-5′) and 117.80 (C-6′).

3.4. *Anti-inflammatory activity*

The anti-inflammatory assay of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear oedema in mice was performed as described previously (De Young, Kheifets, Ballaron, & Young, 1989). Solutions of samples of the methanol extract (ME), ethyl acetate extract (EAE), **1–3** and indomethacin, dissolved in mixtures (water/MeOH 1 : 1, MeOH/EtOAc 1 : 1, MeOH–CH_2–Cl_2 1 : 1 and EtOH–CH_2–Cl_2 1 : 1), respectively, were evaluated.

3.5. *Cytotoxic activity*

The cytotoxic activity of the test compound was assayed in prostate cancer (PC-3), leukaemia (K562), central nervous system (U251), breast cancer (MCF-7) and colon cancer (HCT-15) human tumour cell lines using the protein-binding dye sulphorhodamine B (SRB), as reported previously (Monks et al., 1991).

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