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Flavonoids and Phenolic Compounds from *Rosmarinus officinalis*

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A new flavonoid, 6″-*O*-(*E*)-feruloylhomoplantaginin (**1**), and 14 known compounds, 6″-*O*-(*E*)-feruloylnepitrin (**2**), 6″-*O*-(*E*)-*p*-coumaroylnepitrin (**3**), 6-methoxyluteolin 7-glucopyranoside (**4**), luteolin 3′-*O*-β-D-glucuronide (**5**), luteolin 3′-*O*-(3″-*O*-acetyl)-β-D-glucuronide (**6**), kaempferol (**7**), luteolin (**8**), genkwanin (**9**), and ladanein (**10**), together with 1-*O*-feruloyl-β-D-glucopyranose (**11**), 1-*O*-(4-hydroxybenzoyl)-β-D-glucopyranose (**12**), rosmarinic acid (**13**), carnosic acid (**14**), and carnosol (**15**), were isolated from the leaves of *Rosmarinus officinalis*. The structures were established on the basis of NMR spectroscopic methods supported by HRMS. All isolated compounds were tested for cytotoxicity in human cancer cell lines (HepG2, COLO 205, and HL-60) and for anti-inflammatory activities in lipopolysaccharide (LPS)-treated RAW 264.7 macrophage cells. Among them, compounds **14** and **15** were modestly active in the inhibition of nitrite production in macrophages, followed by compounds **8** and **5**. Compounds **14** and **15** were more effective as an antiproliferative agent in HL-60 cells with IC_{50} values of 1.7 and 5.5 μM, followed by compounds **8** and **7** with IC_{50} of 39.6 and 82.0 μM, respectively. In addition, compounds **14** and **15** showed potent antiproliferative effects on COLO 205 cells with IC_{50} values of 32.8 and 29.9 μM, respectively.

KEYWORDS: *Rosmarinus officinalis*; flavonoids; 6″-*O*-(*E*)-feruloylhomoplantaginin; human cancer cell lines (HepG2, COLO 205, HL-60); anti-inflammatory

INTRODUCTION

Rosemary, *Rosmarinus officinalis* L. (Lamiaceae), is a well-known culinary spice often used to adjust flavor in cooking and widely employed in the food industry as a natural antioxidant for food conservation (*1*). The topical anti-inflammatory property of rosemary extracts (RA) in mice was reported, where a dose-dependent activity from the lipophilic fractions of RA was shown (*2*). Interest was also generated due to the anticarcinogenic activity of RA for cancer chemopreventative potential (*3*, *4*). Rosmarinic acid, carnosic acid, and carnosol are the major bioactive constituents in rosemary leaves responsible for the antioxidant, anti-inflammatory, and anticarcinogenic effects (*5*−*8*). A number of flavonoids, including eriocitrin, luteolin 3′-*O*-β-D-glucuronide, luteolin 3′-*O*-(4″-*O*-acetyl)-β-D-glucuronide, luteolin 3′-*O*-(3″-*O*-acetyl)-β-D-glucuronide, hesperidin, diosmin, isoscutellarein 7-*O*-β-D-glucoside, homoplantaginin, and genkwanin, have been identified from rosemary (*1*). It has been demonstrated that nitric oxide (NO) is involved in many inflammatory diseases when NO is produced in large amounts. Overproduction of NO and its more reactive N-nitrosating agents, such as peroxynitrite, may also represent an essential link between inflammation and carcinogenesis (*9*, *10*). Therefore, it is our purpose to isolate and characterize compounds that are responsible for the bioactivities on the basis of the reported anti-inflammatory and cancer preventive characteristics of rosemary. As a result, we report herein the isolation of 10 flavonoids and 5 phenolic compounds, as well as the biological testing results on LPS-induced nitrite production in RAW 264.7 macrophage and human cancer cell lines of these compounds.

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MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. FT-IR was performed on a Perkin-Elmer spectrum BX system (Perkin-Elmer Instruments, Norwalk, CT) with a MIRacle ATR accessory (Pike Technologies, Madison, WI). UV spectra were acquired on a Shimadzu UV-1700 UV−visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). The ^{1}H and ^{13}C NMR spectra were recorded on an Inova-400 (^{1}H at 400 MHz) instrument (Varian Inc., Palo Alto, CA) with methanol-d_4 (reference 3.30 ppm in ^{1}H NMR and 49.0 ppm in ^{13}C NMR) as the solvent (Aldrich Chemical Co., Allentown, PA). The 2D correlation spectra were obtained using standard gradient pulse sequences of Varian VNMR software and performed on 4-nuclei PFG AutoSwitchable or PFG Indirect Detection probes. HRESIMS was run on Waters Micromass LCT (Waters Corp., Milford, MA) or Thermo Scientific LTQ Orbitrap mass spectrometers (Thermo-Finnigan, San Jose, CA). ESIMS was obtained on an LCQ ion trap. GC-MS analysis was carried out on an Agilent HP 6890 series gas chromatograph system and Agilent HP 5973 mass spectrometer

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(Santa Clara, CA) with an Rxi-1 ms capillary GC column (60 m × 0.25 mm i.d. × 1.0 μm). HPLC analysis was performed on an Agilent 1100 LC series, and the column used was a 250 mm × 4.6 mm i.d., 5 μm, Luna C-8 (Phenomenex, Torrance, CA) with a flow rate of 1.0 mL/min. The solvent system consisted of a linear gradient that started with 5% (v/v) MeCN in 0.1% TFA/H_2O, increased to 95% MeCN over 40 min, and then increased to 100% MeCN over 5 min. At the end of the run, 100% MeCN was allowed to flush the column for 5 min, and an additional 10 min of post run time was set to allow for equilibration of the column. The UV detector was set at 280 nm wavelength, and column temperature was ambient.

Plant Material. Leaves of *R. officinalis* were collected in September 2007 in Morocco. The species was identified by chromatographic comparisons (TLC and HPLC) with authentic rosemary samples. A voucher specimen (RM10305) was deposited in the Herbarium of Naturex, Inc.

Extraction and Isolation of *R. officinalis* Constituents. The dry leaf (15 kg) of *R. officinalis* was extracted three times with 95% EtOH (50 L × 3) at room temperature. After concentration, the extract (2.1 kg) was extracted by 50% EtOH (5 L × 3) successively, and the obtained EtOH extracts were combined and concentrated. The soluble parts (320 g) were chromatographed on an Amberlite FPX 66 resin (Rohm and Haas) (2.5 L, 12 cm ×50 cm) column and eluted with a gradient of H_2O/EtOH (1:0, 3:1, 1:1, 1:3, 0:1). In each gradient step, 5.0 L of eluent was collected as a fraction. Eluates (55.6 g) from the 25% EtOH fraction were chromatographed on a polyamide resin (Sigma Chemical Co., St. Louis, MO) column (0.4 L, 3.5 cm × 60 cm) and eluted with H_2O/EtOH (1:0, 10:1, 5:1, 3:1, 2:1, 1:1, 1:2, 0:1). A total of 1.5 L of eluent was used in each step, and 0.5 L was collected as one fraction (labeled I-fractions). Fractions I3−I6 and I7−I9 were separately combined and concentrated. The two new fractions were separately and repeatedly subjected to column chromatography over MCI GEL CHP-20P (Mitsubishi Kasei Co.) and/or Sephadex LH-20 (Sigma Chemical Co.) (0.1 L, 2.5 cm × 40 cm) and eluted with a H_2O/MeOH gradient system to yield **4** (56 mg from I7−9, t_R = 22.3 min in HPLC), **5** (167 mg from I7−9, t_R = 20.8 min), **11** (51 mg from I3−6, t_R = 21.6 min), **12** (55 mg from I 3−6, t_R = 19.5 min), and **13** (2.38 g from I3−6, t_R = 13.9 min).

The 50 and 75% EtOH eluates from the FPX 66 column were combined and concentrated (38.2 g). The solids were column chromatographed on polyamide resin using the same conditions described above to produce II-fractions. These fractions were separately and repeatedly subjected to column chromatography over MCI GEL CHP-20P and/or Sephadex LH-20 with a H_2O/MeOH solvent system to yield **1** (33 mg from II10−12, t_R = 23.6 min), **2** (32 mg from II10−12, t_R = 23.5 min), **3** (28 mg from II7−9, t_R = 22.4 min), **6** (53 mg from II7−9, t_R = 21.7 min), **7** (18 mg from II17−19, t_R = 26.6 min), **8** (26 mg from II17−19, t_R = 24.8 min), and **9** (16 mg from II19−21, t_R = 25.1 min). The III-fractions were produced by 100% EtOH eluates (23.5 g) from the FPX 66 column in the same manner as fractions I and II. These fractions were separately and repeatedly subjected to column chromatography over silica gel (0.1 L, 2.5 cm × 40 cm) and eluted with a CH_3Cl/MeOH gradient system to yield **10** (135 mg from III9−10, t_R = 30.8 min), **14** (236 mg from III5−6, t_R = 34.8 min), and **15** (185 mg from III7−8, t_R = 32.5 min).

6″-*O*-(*E*)-Feruloylhomoplantaginin (1): yellow amorphous powder; $[\alpha]^{25}_D$ −138.2 (*c* 2.20, MeOH); UV (MeOH) λ_{max} 217, 232, 275, 329 nm; IR ν_{max} 3323, 1599, 1512, 1456, 1351, 1252, 1171 cm^{-1}; ^1H and ^{13}C NMR data (**Table 1**); HRESIMS *m*/*z* 639.1701 $[M + H]^+$ (calcd for $C_{32}H_{31}O_{14}$, 639.1714).

6″-*O*-(*E*)-Feruloylnepitrin (2): yellow amorphous powder; $[\alpha]^{25}_D$ −142.8 (*c* 2.00, MeOH); UV (MeOH) λ_{max} 215, 232, 277, 322 nm; IR ν_{max} 3320, 1599, 1513, 1457, 1350, 1266, 1165 cm^{-1}; ^1H and ^{13}C NMR data (**Table 1**); HRESIMS *m*/*z* 655.1673 $[M + H]^+$ (calcd for $C_{32}H_{31}O_{15}$, 655.1663).

6″-*O*-(*E*)-*p*-Coumaroylnepitrin (3): yellow amorphous powder; $[\alpha]^{25}_D$ −176.4 (*c* 1.60, MeOH); UV (MeOH) λ_{max} 215, 235, 278, 325 nm; IR ν_{max} 3272, 1653, 1599, 1512, 1455, 1349, 1266, 1166 cm^{-1}; ^1H and ^{13}C NMR data (**Table 1**); HRESIMS *m*/*z* 625.1548 $[M + H]^+$ (calcd for $C_{31}H_{29}O_{14}$, 625.1557).

Acid Hydrolysis of Compounds 1−3 and Sugar Analysis. A solution of compounds **1**−**3** (2.0 mg each) in 1 N HCl (1 mL) was stirred at 85 °C for 3 h. The solution was evaporated under a stream of N_2. The residue was dissolved in 0.1 mL of Tri-Sil Z (*N*-trimethylsilylimidazole/pyridine, 1:4; Pierce Biotechnology, Rockford, IL), and the mixture was allowed to react at 60 °C for 15 min. After drying under a stream of N_2, the residue was dissolved in 1 mL of water and partitioned with 1 mL of CH_2Cl_2. The CH_2Cl_2 layer was analyzed by GC-MS (Rxi-1 ms GC column; temperatures for inlet injection, 200 °C; temperature gradient system was used for the oven, starting at 120 °C for 1 min and then rising to 280 °C at rate of 40 °C/min). D-Glucose was identified for **1**−**3** by comparison with the retention time of authentic D-glucose (t_R = 9.75 min) after treatment in the same manner with Tri-Sil Z.

Cell Culture and Chemicals. The COLO 205 cell lines were isolated from human colon adenocarcinoma (ATCC CCL-222); human promyelocytic leukemia (HL-60) cells were obtained from American Type Culture Collection (Rockville, MD). The human HepG2 hepatocellular carcinoma cell lines (BCRC 60025) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). COLO 205 and HL-60 cell lines were grown at 37 °C in a 5% CO_2 atmosphere in RPMI. HepG2 cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, Grand Island, NY), 100 units/mL of penicillin, and 100 μg/mL of streptomycin and kept at 37 °C in a humidified atmosphere of 5% CO_2 in air. Selected compounds were dissolved in dimethyl sulfoxide (DMSO). Propidium iodide was obtained from Sigma Chemical Co.

Determination of Cell Viability. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (*11*). Briefly, human cancer cells were plated at a density of 1×10^5 cells/mL into 24-well plates. After overnight growth, cells were pretreated with series of concentrations of test compounds for 24 h. The final concentration of DMSO in the culture medium was < 0.05%. At the end of treatment, 30 μL of MTT was added, and the cells were incubated for a further 4 h. Cell viability was determined by scanning with an enzyme-linked immunosorbent assay reader with a 570 nm filter.

Nitrite Assay. The RAW 264.7 cells were treated with selected compounds and LPS (*Escherichia coli* O127:E8, molecular weight, 60 kDa, Sigma Chemical Co.) or LPS alone. The supernatants were harvested, and the amount of nitrite, an indicator of NO synthesis, was measured by use of the Griess reaction. Briefly, supernatants (100 μL) were mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) in duplicate on 96-well plates. After incubation at room temperature for 10 min, absorbance at 570 nm was measured with an ELISA reader (Thermo Labsystems Multiskan Ascent, Finland).

RESULTS AND DISCUSSION

Chromatography of the ethanolic extract of *R. officinalis* yielded a total of 15 compounds, including 10 flavonoids, 6″-*O*-(*E*)-feruloylhomoplantaginin (**1**), 6″-*O*-(*E*)-feruloylnepitrin (**2**), 6″-*O*-(*E*)-*p*-coumaroylnepitrin (**3**) (*12*), 6-methoxyluteolin 7-*O*-β-D-glucopyranoside (**4**) (*13*), luteolin 3′-*O*-β-D-glucuronide (**5**) (*14*), luteolin 3′-*O*-(3″-*O*-acetyl)-β-D-glucuronide (**6**) (*14*), kaempferol (**7**), luteolin (**8**), genkwanin (**9**) (*15*), and ladanein (**10**) (*16*), together with 1-*O*-feruloyl-β-D-glucopyranose (**11**) (*17*), 1-*O*-(4-hydroxybenzoyl)-β-D-glucopyranose (**12**) (*18*), rosmarinic acid (**13**), carnosic acid (**14**), and carnosol (**15**). Compound **1** is a new flavonoid, and **10**−**12** were isolated for the first time from this plant. The chemical structures were established by spectroscopic methods and comparison to literature data. Kaempferol, luteolin, rosmarinic acid, and carnosic acid were identified by a direct comparison with commercial standards (ChromaDex, Irvine, CA).

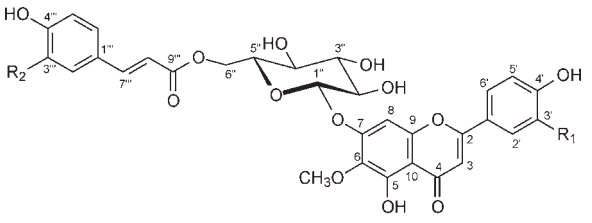
Compound **1** (structure given in **Figure 1**) was obtained as a yellow amorphous powder. The molecular formula, $C_{32}H_{30}O_{14}$, was established on the basis of its HRESIMS (*m*/*z* 639.1701 $[M + H]^+$) and supported by ^1H and ^{13}C NMR data (**Table 1**). The UV spectrum displayed a flavone derivative with maximum absorptions at 217.0, 232.0, 275.0, and 329.0 nm. The ^{13}C NMR spectrum displayed 32 carbons, including moieties of one feruloyl, one glucopyranosyl (δ 101.1, 74.5, 77.7, 72.3, 75.6, 64.7), and one hispidulin aglycone by comparison with literature data (*19*). The hispidulin unit was confirmed by the presence of the signals from a 4′-hydroxy-substituted B-ring with an AA′XX′ spin

Table 1. NMR Data for **1**−**3** (Methanol-d_4)

	1			**2**			**3**		
no.	δ_H	δ_C	HMBC (H to C) (NOE)	δ_H	δ_C	HMBC (H to C) (NOE)	δ_H	δ_C	HMBC (H to C) (NOE)
2		166.5			166.3			166.5	
3	6.40 s	103.3	2, 4, 9, 10, 1′ (2′, 6′)[a]	6.26 s	103.3	2, 4, 10, 1′ (2′, 6′)	6.40 s	103.5	2, 4, 9, 10, 1′ (2′, 6′)
4		184.2			183.9			184.1	
5		154.1			153.9			154.0	
6		134.2			134.0			134.1	
7		157.5			157.3			157.4	
8	6.79 s	95.5	4, 6, 7, 9, 10 (1″)	6.66 s	95.4	4, 6, 7, 9, 10 (1″)	6.75 s	95.4	4, 6, 7, 9, 10 (1″)
9		154.0			153.8			153.9	
10		107.5			107.4			107.5	
6-OCH_3	3.89 s	61.5	6	3.87 s	61.5	6	3.86 s	61.5	6
1′		122.7			123.0			123.3	
2′	7.67 d (8.8)	129.4	2, 1′, 3′, 4′, 6′	7.16 br s	114.0	2, 1′, 3′, 4′, 6′	7.29 br s	114.2	2, 1′, 3′, 4′, 6′
3′	6.82 d (8.8)	116.8	1′, 4′, 5′		146.7			146.9	
4′		162.8			151.0			151.1	
5′	6.82 d (8.8)	116.8	1′, 3′, 4′	6.76 d (8.4)	116.6	1′, 3′, 4′	6.83 d (8.8)	116.7	1′, 3′, 4′
6′	7.67 d (8.8)	129.4	2, 1′, 2′, 4′, 5′	7.15 dd (8.4,2.0)	120.3	2, 1′, 2′, 4′, 5′	7.28 d (8.8)	120.4	2, 1′, 2′, 4′, 5′
1″	5.14 d (7.2)	101.1	7, 2″	5.09 d (7.2)	101.1	7, 2″	5.11 d (7.2)	101.3	7, 2″
2″	3.62 m	74.5	1″, 3″	3.59 m	74.5	1″, 3″	3.59 m	74.5	1″, 3″
3″	3.56 m	77.7	2″, 4″	3.57 m	77.7	2″, 4″	3.57 m	77.8	2″, 4″
4″	3.39 m	72.3	3″, 5″	3.40 m	72.4	3″, 5″	3.41 m	72.2	3″, 5″
5″	3.80 m	75.6		3.83 m	75.4		3.83 m	75.5	
6″	4.74 dd (11.6,2.0)	64.7	5″, 9‴	4.71 dd (12.0,2.4)	64.6	5″, 9‴	4.62 dd (11.6,2.0)	64.8	5″, 9‴
	4.24 dd (11.6,8.8)			4.26 dd (12.0,8.4)			4.31 dd (11.6,8.0)		
1‴		127.2			127.1			126.6	
2‴	6.69 d (1.6)	110.9	3‴, 4‴, 6‴, 7‴	6.64 d (2.0)	110.9	3‴, 4‴, 6‴, 7‴	6.97 d (8.4)	130.8	3‴, 4‴, 6‴, 7‴
3‴		148.9			148.8		6.48 d (8.4)	116.6	1‴, 4‴, 5‴
4‴		150.4			150.2			161.0	
5‴	6.47 d (8.0)	116.2	1‴, 3‴, 4‴	6.45 d (8.0)	116.1	1‴, 3‴, 4‴	6.48 d (8.4)	116.6	1‴, 3‴, 4‴
6‴	6.54 dd (8.0,1.6)	123.7	2‴, 4‴, 7‴	6.48 dd (8.0,2.0)	123.7	2‴, 4‴, 7‴	6.97 d (8.4)	130.8	2‴, 3‴, 4‴, 7‴
7‴	7.39 d (15.6)	147.2	1‴, 2‴, 6‴, 8‴, 9‴ (2‴, 6‴)	7.34 d (16.0)	147.3	1‴, 2‴, 6‴, 8‴, 9‴ (2‴, 6‴)	7.42 d (16.0)	147.0	1‴, 2‴, 6‴, 8‴, 9‴
8‴	6.17 d (15.6)	114.7	1‴, 9‴ (2‴, 6‴)	6.13 d (16.0)	114.7	1‴, 9‴ (2‴, 6‴)	6.15 d (16.0)	114.4	1‴, 9‴ (2‴, 6‴)
9‴		168.7			168.8			169.0	
3‴-OCH_3	3.62 s	55.9	3‴ (2‴)	3.59 s	55.9	3‴ (2‴)			

[a] Figures in parentheses are the positions of NOE correlations.

system (δ 7.67 (2H, d, J = 8.8 Hz, H-2′, 6′; δ 6.82, 2H, d, J = 8.8 Hz, H-3′, 5′) in the ^{1}H NMR of **1** and a 6-methoxy-substituted A-ring due to the observed long-range correlation of *O*-methyl protons at δ 3.89 (3H, s, OCH_3) to C-6 in the gHMBC. The presence of a feruloyl moiety was deduced from an ABX spin system at δ 6.47 (1H, d, J = 8.0 Hz, H-5‴), δ 6.54 (1H, dd, J = 8.0, 1.6 Hz, H-6‴), and δ 6.69 (1H, d, J = 1.6 Hz, H-2‴). The *E*-configuration of the double bond was recognized by the large coupling constant of the coupled olefinic protons at δ 7.39 (1H, d, J = 15.6 Hz, H-7‴) and δ 6.17 (1H, d, J = 15.6 Hz, H-8‴). The methoxy group at δ 3.62 (3H, s, OCH_3) was determined at C-3‴ of the feruloyl, which was evidenced by the cross-peaks of the *O*-methyl hydrogens to C-3‴ (δ 148.9) in gHMBC and the NOE correlation of the methyl with H-2‴ (δ 6.69) in ROESY, respectively. A glucopyranosyl group was deduced by ^{1}H and ^{13}C NMR data (**Table 1**), where the coupling constant of H-1″ (J = 7.2 Hz) suggested a β-anomeric configuration for the glucosyl and its D-configuration was determined via acid hydrolysis comparing with standard D-glucose. The glycosidic site of the glucosyl moiety was attached to C-7 because the HMBC cross-peak of H-1″ to C-7 (δ 157.5) was observed. The position of glycosidation was also confirmed by the NOE correlation between H-1″ and H-8 in the ROESY spectrum. The connection of the glucopyranosyl group through C-6″ to the carbonyl group of *trans*-feruloyl at C-9‴ was determined by the HMBC correlations, where the cross-peaks of H-6″ (δ 4.74 and 4.24) to C-9‴ (δ 168.7) were presented. The ^{1}H and ^{13}C NMR data assignments (**Table 1**) of **1** were completed on the basis of DEPT and homo- and heteronuclear 2D NMR spectroscopic analyses. Thus, the structure of **1** was elucidated to be 7-*O*-(6″-*O*-*trans*-feruloyl)-β-D-glucopyranosyl-6-methoxy-5,4′-dihydroxyflavone [6″-*O*-(*E*)-feruloylhomoplantaginin], and it is a new compound.



1: R_1 = H, R_2 = OMe

2: R_1 = OH, R_2 = OMe

3: R_1 = OH, R_2 = H

Figure 1. Chemical structures of **1**−**3** from *Rosmarinus officinalis*.

Compound **2** (structure given in **Figure 1**) was isolated as a yellow amorphous powder, and the molecular formula, $C_{32}H_{30}O_{15}$, was inferred from the positive HRESIMS (m/z 655.1673 $[M + H]^+$). Its IR, UV, and NMR spectroscopic data showed patterns very similar to those of **1**, except for a 3′, 4′-di-*O*-substituted B ring as indicated by the ^{1}H NMR signals of δ 7.16 (1H, br s, H-2′), δ 6.76 (1H, d, J = 8.4 Hz, H-5′), and δ 7.15 (1H, dd, J = 8.4, 2.0 Hz, H-6′). For the two methoxy groups, one at δ 3.87 (3H, s, OCH_3) was assigned at C-6 by observed long-range correlation between the *O*-methyl hydrogens and C-6 (δ 134.0) in the gHMBC spectrum. The other at δ 3.59 (3H, s, OCH_3) was determined at C-3‴ for a feruloyl, where the cross-peaks of the *O*-methyl hydrogens to C-3‴ (δ 148.8) in gHMBC and the NOE correlation of the methyl with H-2‴ (δ 6.64) in ROESY were observed. The presence of a β-glucopyranosyl group was confirmed by ^{13}C NMR (**Table 1**). The glycosidic site and the position of the feruloyl group were the same as for **1** and were determined by the HMBC in which the correlations of H-1″ to C-7 (δ 157.3) and H-6″ (δ 4.71, 4.26) to C-9‴ (δ 168.8) were observed. On the basis of the NMR analysis (**Table 1**), **2** was identified as 7-*O*-(6″-*O*-*trans*-feruloyl)-β-D-glucopyranosyl-6-methoxy-5,3′,4′-trihydroxyflavone [6″-*O*-(*E*)-feruloylnepitrin]. Compound **3** (structure given in **Figure 1**) was obtained as a yellow amorphous powder. The molecular formula was determined as $C_{31}H_{28}O_{14}$ by HRESIMS (m/z 625.1548 $[M + H]^+$). It displayed ^{1}H and ^{13}C NMR (**Table 1**) spectra similar to those observed with **2**, but a *p*-coumaroyl moiety instead of a feruloyl substituted at C-6″ of the glucopranosyl group. The presence of a *p*-coumaroyl was deduced from a pair of AB coupling signals at δ 6.97 (2H, d, J = 8.4 Hz, H-2‴ and 6‴) and δ 6.48 (2H, d, J = 8.4 Hz, H-3‴ and 5‴). Thus, **3** was identified as 7-*O*-(6″-*O*-*trans*-*p*-coumaroyl)-β-D-glucopyranosyl-6-methoxy-5,3′,4′-trihydroxyflavone [6″-*O*-(*E*)-*p*-coumaroylnepitrin]. The structures of **2** and **3** from rosemary were presented by Offord-Cavin et al. in a published patent (*12*), but no NMR data were reported. Therefore, we report herein the completed NMR assignments of the two compounds (**Table 1**).

Compounds **1**−**15** were tested with regard to their effect on nitrite production in LPS-activated macrophages for anti-inflammatory screening. When RAW 264.7 cells were treated with test compounds at 40 μg/mL and LPS (100 ng/mL), respectively, the

Table 2. Effect of **1**−**15** on LPS-Induced Nitrite Production in RAW 264.7 Macrophages

	1	**2**	**3**	**4**	**5**
control	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6
LPS[a]	27.2 ± 3.0	27.2 ± 3.0	27.2 ± 3.0	28.1 ± 2.2	28.1 ± 2.2
20 μg/mL	27.3 ± 0.9	24.8 ± 0.4	26.5 ± 0.3	25.2 ± 0.5	23.6 ± 1.2
40 μg/mL	25.7 ± 1.6	19.8 ± 1.5	30.9 ± 2.0	23.8 ± 2.7	18.7 ± 2.7
	6	**7**	**8**	**9**	**10**
control	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6
LPS	24.7 ± 2.6	28.1 ± 2.2	24.7 ± 2.6	28.1 ± 2.2	28.1 ± 2.2
20 μg/mL	20.8 ± 0.3	19.2 ± 0.2	19.6 ± 2.0	21.5 ± 2.3	22.4 ± 0.9
40 μg/mL	21.5 ± 2.1	22.4 ± 2.4	11.9 ± 2.9	20.2 ± 2.0	22.4 ± 1.8
	11	**12**	**13**	**14**	**15**
control	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6
LPS	24.7 ± 2.6	24.7 ± 2.6	22.8 ± 1.5	24.7 ± 2.6	22.8 ± 1.5
20 μg/mL	22.7 ± 0.7	22.0 ± 0.4	21.8 ± 1.0	12.7 ± 0.5	1.2 ± 0.9
40 μg/mL	22.6 ± 1.2	22.7 ± 2.5	20.9 ± 2.2	2.8 ± 0.3	0.3 ± 0.6

[a] LPS: 100 ng/mL.

Table 3. Effect of **1**−**15** on the Growth of Various Human Cancer Cells

	IC_{50} (μM) for cell line		
compound	HL-60	HepG2	COLO 205
1	>100	>100	>100
2	>100	>100	>100
3	>100	>100	>100
4	>100	>100	>100
5	>100	>100	>100
6	>100	>100	>100
7	82.0 ± 3.6[b]	>100	>100
8	39.6 ± 0.7	>100	100 ± 5.8
9	>100	>100	>100
10	>100	>100	>100
11	>100	>100	>100
12	>100	>100	>100
13	>100	>100	>100
14	1.7 ± 2.3	>100	32.8 ± 3.4
15	5.5 ± 4.6	>100	29.9 ± 6.1
doxorubicin[a]	5.4 ± 0.9	15.2 ± 0.9	7.7 ± 0.7

[a] Positive control. [b] Each experiment was independently performed three times and expressed as mean ± SE.

potency of the inhibitory effects on nitrite production showed the sequence as **15** > **14** > **8** > **5** > **2** (**Table 2**). Among them, compounds **14** and **15** were the strongest inhibitors of nitrite production in macrophages in the agreement with the literature (*2*). The inhibitory effect of luteolin (**8**) has been reported with an IC_{50} value 27 μM (*20*), whereas our result showed a very weak activity for **8**. Future study is needed. The cytotoxicity of compounds **1**−**15** was evaluated in vitro against HL-60, HepG2, and COLO 205 cell lines. These cell lines were treated with different concentrations (5−100 μM) of selected compounds for 24 h, and the viability of the cells was determined by MTT assay. As shown in **Table 3**, **14** and **15** were more sensitive as antiproliferative agents to HL-60 cells with IC_{50} values of 1.7 and 5.5 μM, respectively, followed by **8** and **7** with IC_{50} values of 39.6 and 82.0 μM, respectively. In addition, **14** and **15** also showed potent antiproliferative effects on COLO 205 cells with IC_{50} values of 32.8 and 29.9 μM, respectively. However, there was no cytotoxicity observed for any compounds in HepG2 cells. Most flavonoids were inactive, except **7** and **8** showed weak activities in these bioassays. Therefore, the current study confirms that the major components responsible for the activities of anti-inflammatory and cytotoxicities were carnosic acid and carnosol as the literature reported.

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Supporting Information Available: ^{1}H, ^{13}C NMR, COSY, HMQC, HMBC, and ROESY spectra of **1**, **2**, and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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