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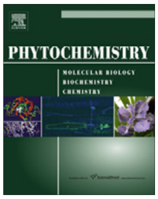

Antioxidant constituents of *Nymphaea caerulea* flowers

Vijai K. Agnihotri [a], Hala N. ElSohly [a], Shabana I. Khan [a], Troy J. Smillie [a], Ikhlas A. Khan [a,b,*], Larry A. Walker [a]

[a] *National Center for Natural Products Research, School of Pharmacy, University of Mississippi, MS 38677, USA*
[b] *Department of Pharmacognosy, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, MS 38677, USA*

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ABSTRACT

As part of an ongoing search for antioxidants from medicinal plants, 20 constituents were isolated from the *Nymphaea caerulea* flowers, including two 2*S*,3*S*,4*S*-trihydroxypentanoic acid (**1**), and myricetin 3-*O*-(3″-*O*-acetyl)-α-L-rhamnoside (**2**), along with the known myricetin 3-*O*-α-L-rhamnoside (**3**), myricetin 3-*O*-β-D-glucoside (**4**), quercetin 3-*O*-(3″-*O*-acetyl)-α-L-rhamnoside (**5**), quercetin 3-*O*-α-L-rhamnoside (**6**), quercetin 3-*O*-β-D-glucoside (**7**), kaempferol 3-*O*-(3″-*O*-acetyl)-α-L-rhamnoside (**8**), kaempferol 3-*O*-β-D-glucoside (**9**), naringenin (**10**), (*S*)-naringenin 5-*O*-β-D-glucoside (**11**), isosalipurposide (**12**), β-sitosterol (**13**), β-sitosterol palmitate (**14**), 24-methylenecholesterol palmitate (**15**), 4α-methyl-5α-ergosta-7,24(28)-diene-3β,4β-diol (**16**), ethyl gallate (**17**), gallic acid (**18**), *p*-coumaric acid (**19**), and 4-methoxybenzoic acid (**20**). The structures were determined by spectroscopic means. Compounds were tested for antioxidant activity and nine compounds **2**–**7**, **11**, **12** and **18** were considered active with IC_{50} of 1.16, 4.1, 0.75, 1.7, 1.0, 0.34, 11.0, 1.7 and 0.95 μg/ml, respectively, while **1** was marginally active ($IC_{50} > 31.25$ μg/ml). The most promising activity was found in the EtOAc fraction (IC_{50} 0.2 μg/ml). This can be attributed to the synergistic effect of the compounds present in it.

1. Introduction

Nymphaea caerulea Savigny (Nymphaeaceae), is an aquatic perennial plant growing on the shores of lakes and rivers and known by the common names blue lotus, blue water lily and Egyptian lotus. In folk medicine, the plant is reported to be soothing with tranquilizing effects and is reputedly a detoxicant and aphrodisiac along with astringent, diuretic properties. It is used in Ayurvedic medicine for dyspepsia, enteritis, diarrhea, urinary problems, fevers and heart palpitations (Encyclopedia of Herbs and their uses, 1995). Previous work on the flowers of this species resulted in the isolation of seven flavonol glycosides (Fossen et al., 1999) and three acylated anthocyanins (Fossen and Andersen, 1999). This work is a continuation of phytochemical investigations of unexploited flora for new sources of potential antioxidants. A preliminary screening showed that an EtOH extract of this plant and subsequent $CHCl_3$, EtOAc fractions displayed significant antioxidant activity without any associated cytotoxicity. It is well known that having a diet rich in antioxidants, such as herbs, vegetables, fruits, and grains can prevent various diseases caused by reactive oxygen species (ROS) (Halliwell, 1994). Hence, the aim of this study was to investigate the phytochemical constituents of this plant and to evaluate their antioxidant activities. This paper describes the isolation, structure elucidation and evaluation of the antioxidant activity of two new compounds, 2*S*,3*S*,4*S*-trihydroxypentanoic acid (5-deoxyribonic acid) (**1**), and myricetin 3-*O*-(3″-*O*-acetyl)-α-L-rhamnoside (**2**) along with several known compounds that were first reported from this plant (**4**, **7–20**) in addition to previously reported flavonol glycosides (**3–6**) (Fossen et al., 1999) from the flowers of *N. caerulea*.

2. Results and discussion

Compound **1** was isolated as colorless, optically active, needles. Its molecular formula of $C_5H_{10}O_5$ was determined by HRESIMS and indicated one degree of unsaturation. ^{13}C NMR and DEPT-135 spectra showed the presence of five carbon signals, including one methyl (δ_C 18.3), three oxymethine groups (δ_C 75.7, 78.8 and 80.9) and one carboxyl (δ_C 176.6). The presence of a carboxyl group was further supported by a peak in the IR spectrum at 1757 cm^{-1}, indicative of a monomeric saturated aliphatic acid (Silverstein and Webster, 1998). The ^1H NMR spectrum gave rise to a methyl signal at δ 1.42 (3H, *d*, $J = 6.4$ Hz, CH_3-5), three oxymethines at δ 4.34 (1H, *d*, $J = 8.8$ Hz, *C*H-2), 3.75 (1H, *dd*, $J = 8.8$, 8.4 Hz, *C*H-3) and δ 4.16 (1H, *m*, *C*H-4). The above data suggested that **1** is a polyhydroxy aliphatic acid, identified as 2,3,4-trihydroxypentanoic acid, and these inferences were confirmed by HMQC and HMBC correlations (Fig 1). Because of the presence of three asymmetric centers in **1**, eight stereoisomers are possible. However the specific rotation of **1** was found to be $[\alpha]_D^{25}$ −33.2 (MeOH, *c* 1.0). This rotation value

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[*] Corresponding author. Address: National Center for Natural Products Research, School of Pharmacy, University of Mississippi, MS 38677, USA. Tel.: +1 662 915 7821; fax: +1 662 915 7989.
E-mail address: ikhan@olemiss.edu (I.A. Khan).

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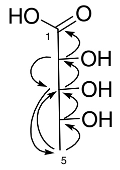

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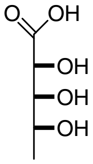
Fig. 1. Key HMBC correlations for compound **1**.

favors isomers with *S* configuration at position four, as determined by Holy (1982) who synthesized eight stereoisomers of 5-(adenyl-9-yl)-2,3,4-trihydroxy pentanoic acid derivatives. In addition, the rotation of the molecule suggests that the compound is an oxidation product of 5-deoxy-D-ribose (Kissman and Baker, 1957) and thus the absolute stereochemistry at all the three stereo centers was determined as *S*. On the basis of the above evidence, the molecule was characterized as 2*S*,3*S*,4*S*-trihydroxy pentanoic acid (5-deoxy-D-ribonic acid).

Compound **2** was obtained as a yellow amorphous powder. Its molecular formula of $C_{23}H_{22}O_{13}$ was determined by HRESIMS and indicated 13 degrees of unsaturation. The ^{13}C NMR and DEPT-135 spectra of **2** displayed 23 signals for two methyls, nine methines, and 12 quaternaries. The aromatic region of the ^{1}H NMR spectrum of **2** was in accordance with a myricetin derivative (Fossen et al., 1999). The cross-peaks at δ 6.94/159.2 (H-2′ and H-6′/C-2), δ 6.25/158.2 (H-8/C-9) and δ 6.1/162.8 (H-6/C-5) in the HMBC spectrum were particularly useful for assignment of the quaternary carbons of the aglycone (Fig 2). The sugar was identified as 3″-*O*-acetyl-α-L-rhamnoside with the help of COSY, HMBC and comparison with the literature values for 3″-*O*-acetyl-α-L-rhamnoside (Fossen et al., 1999). A HMBC correlation between H-1″ (δ 5.27)



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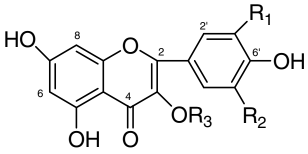
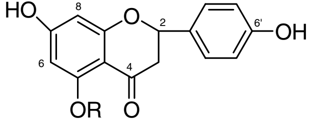
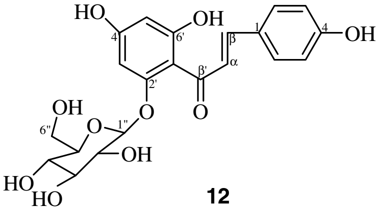


2 $R_1 = R_2 =$ OH, $R_3 =$ rha-(3"-O-Ac)
3 $R_1 = R_2 =$ OH, $R_3 =$ rha
4 $R_1 = R_2 =$ OH, $R_3 =$ glc
5 $R_1 =$ OH, $R_2 =$ H, $R_3 =$ rha-(3"-O-Ac)
6 $R_1 =$ OH, $R_2 =$ H, $R_3 =$ rha
7 $R_1 =$ OH, $R_2 =$ H, $R_3 =$ glc
8 $R_1 = R_2 =$ H, $R_3 =$ rha-(3"-O-Ac)
9 $R_1 = R_2 =$ H, $R_3 =$ glc



10 = R= H
11 = R= glc



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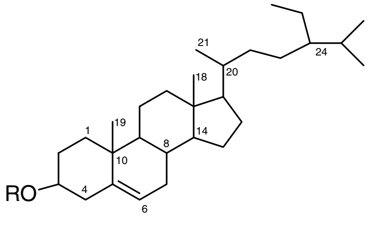
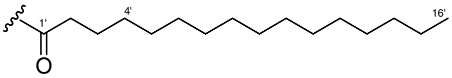
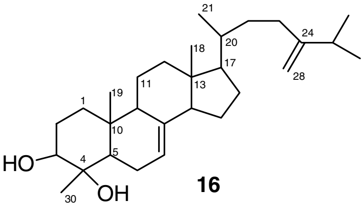


13 R=H

14 R=





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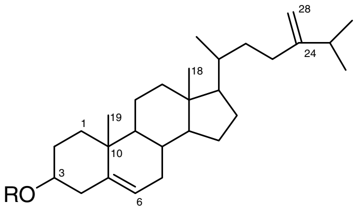
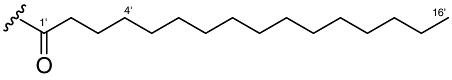
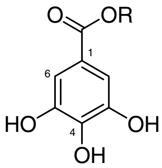
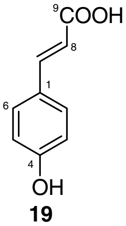


15 R=





17 R= C_2H_5
18 R= H



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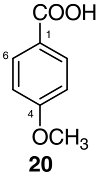

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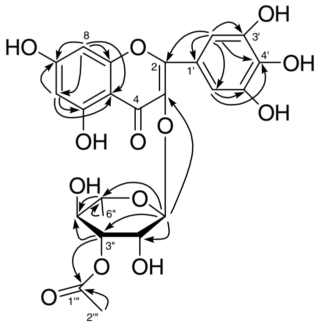

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Fig. 2. Key HMBC correlations for compound **2**.

of rhamnose and C-3 (*δ* 136.2) of aglycone established that the 3″-*O*-acetyl-α-L-rhamnose was attached to the C-3 position. Thus, the structure of compound **2** was determined to be a novel flavonol myricetin 3-*O*-(3″-*O*-acetyl)-α-L-rhamnoside.

The known compounds were identified as myricetin 3-*O*-α-L-rhamnoside (**3**) (Fossen et al., 1999; Markham et al., 1978), myricetin 3-*O*-β-D-glucoside (**4**) (Agrawal and Bansal, 1989), quercetin 3-*O*-(3″-*O*-acetyl)-α-L-rhamnoside (**5**) (Fossen et al., 1999), quercetin 3-*O*-α-L-rhamnoside (**6**) (Fossen et al., 1999; Markham et al., 1978), quercetin 3-*O*-β-D-glucoside (**7**) (Markham et al., 1978), kaempferol 3-*O*-(3″-*O*-acetyl)-α-L-rhamnoside (**8**) (Masuda et al., 1991), kaempferol 3-*O*-β-D-glucoside (**9**) (Markham et al., 1978) naringenin (**10**) (Zhang et al, 2003), (*S*)-naringenin 5-*O*-β-D-glucoside (**11**) (Zhang et al, 2003; Gaffield, 1970), 2′,4′,6′,4-tetrahydroxychalcone 2′-*O*-β-D-glucoside (isosalipurposide) (**12**) (Zhang et al, 2003), β-sitosterol (**13**) (Goad, 1991), β-sitosterol palmitate (**14**) (Nielsen and Kofod, 1963), 24-methylenecholesterol palmitate (**15**) (Rashkes et al., 1990), 4α-methyl-5α-ergosta-7,24(28)-diene-3β,4β-diol (**16**) (Greca et al., 1991), ethyl gallate (**17**) (Hussain et al., 1979), gallic acid (**18**) (Hussain et al., 1979), *p*-coumaric acid (**19**) (Ji et al., 2005), and 4-methoxybenzoic acid (**20**) (Gadgoli and Mishra, 1999) by comparison of their physical and spectroscopic data with literature values (see Supporting Information).

For compound **5**, some ^{1}H and ^{13}C NMR spectroscopic data were not previously determined (Fossen et al., 1999). In this paper, we have included the complete ^{1}H and ^{13}C NMR data for **5**, along with HRESIMS, IR, UV, mp and specific rotation. Compound **15** was previously reported from the leaves of the cotton plant and identified on the basis of mass fragmentation (Rashkes et al., 1990). In this study, we have included additional spectroscopic data for compound **15**.

The ethanolic extract, $CHCl_3$ and EtOAc fractions together with compounds **1–12**, **16**, and **18–20** were evaluated for antioxidant activity by determining the inhibition of ROS generation in HL-60 cells. The EtOH extract and the subsequent $CHCl_3$ and EtOAc fractions showed antioxidant effect with IC_{50} values of 2.8, 4.4 and 0.2 μg/ml, respectively. Compounds **2–7**, **11**, **12** and **18** were considered active with IC_{50} values of 1.16, 4.1, 0.75, 1.7, 1.0, 0.34, 11, 1.7 and 0.95 μg/ml, respectively. Vitamin C, used as an internal standard, displayed an IC_{50} of 1.95 μg/ml. The samples cytotoxicity in HL-60 cells was also determined to rule out the possibility that antioxidant effect was not observed due to their toxicity. Most of the compounds did not show cytotoxicity in HL-60 cells up to the highest concentration tested (Table 1) except compound **16** which exhibited cytotoxicity with an IC_{50} of 5.1 μg/ml but this compound did not show any antioxidant effect (Table 1).

Structure activity relationship for myricetin and quercetin derivatives showed that when the rhamnose of myricetin becomes acetylated at position 3″ (**2**) antioxidant activity increased, however in the case of quercetin acetylation at position 3″ (**5**) decreased the activity. Both glucosides (**4**, **7**) displayed the greatest activity among the tested compounds. None of the kaempferol derivatives were active (**8**, **9**) however when we tested naringenin (**10**) and its glucoside (**11**), only the glucoside was active. The promising activity of the EtOAc fraction (0.2 μg/ml) may be accounted for by the possible synergistic effect of all the compounds present in this fraction, as none of the isolated compounds from this fraction exhibited a strong antioxidant effect.

Table 1
Antioxidant and cytotoxic activities of extract, fractions and compounds **1–12**, **16**, **18–20**

Sample	Antioxidant activity IC_{50} (μg/ml)	Cytotoxicity (HL-60 cells) IC_{50} (μg/ml)
2*S*,3*S*,4*S*-trihydroxypentanoic acid (**1**)	>31.25	NC
Myricetin 3-*O*-(3″-*O*-acetyl)-α-L-rhamnoside (**2**)	1.16 (2.3 μM)	NC
Myricetin 3-*O*-α-L-rhamnoside (**3**)	4.1 (8.8 μM)	NC
Myricetin 3-*O*-β-D-glucoside (**4**)	0.75 (1.6 μM)	>15.6
Quercetin 3-*O*-(3”-*O*-acetyl)-α-L-rhamnoside (**5**)	1.7 (3.5 μM)	NC
Quercetin 3-*O*-α-L-rhamnoside (**6**)	1.0 (2.2 μM)	NC*
Quercetin 3-*O*-β-D-glucoside (**7**)	0.34 (0.7 μM)	>15.6
Kaempferol 3-*O*-(3”-*O*-acetyl)-α-L-rhamnoside (**8**)	NA	NC
Kaempferol 3-*O*-β-D-glucoside (**9**)	NA	NC*
Naringenin (**10**)	NA	>31.25
Helichrysin B (**11**)	11	>31.25
Isosalipurposide (**12**)	1.7 (3.9 μM)	NC
4α-Methyl-5α-ergosta-7,24(28)-diene-3β, 4β-diol (**16**)	NA	5.1
Gallic acid (**18**)	0.95	NC
p-Coumaric acid (**19**)	NA	NC
4-Methoxybenzoic acid (**20**)	NA	15.0
95% Ethanolic extract	2.8	NC**
Chloroform fraction	4.4	NC**
EtOAc fraction	0.2	NC**
Vitamin C	1.95	

NA = No activity up to 31.25 μg/ml, NC = No cytotoxicity up to 31.25 μg/ml, NC* = No cytotoxicity up to 15.6 μg/ml, NC** = No cytotoxicity up to 125 μg/ml.

3. Concluding remarks

This study discloses that the EtOAc fraction of *N. caerulea* flowers was found to be rich with the derivatives of food derived flavonoids such as flavonols, quercetin, kaempferol and myricetin that have reported antimutagenic and anticarcinogenic effects in vitro and in vivo (Hertog et al., 1992). According to Kuhnau (1976) 1 g of mixed flavonoids are necessary to provide pharmacologically significant concentration in body fluids and tissue (Kuhnau, 1976) and the lack of flavonoids in quantity in the western diet is increasing the demand for natural flavonoid supplements. The EtOAc fraction of *N. caerulea* flowers can be used as a new source of flavonoids and because of its promising display of antioxidant activity shown here, this fraction can also be considered for further detailed pharmacological studies related to develop a new natural product for the treatment of oxidative stress-related diseases (Halliwell, 1994).

4. Experimental

4.1. General methods

Optical rotations were determined with a AUTOPOL IV polarimeter. UV spectra were obtained on a VARIAN 50 BIO UV–Visible spectrophotometer and IR spectra were recorded on a JASCO 302-A spectrometer. The 1D and 2D NMR spectra were run on a Varian 400 Mercury plus NMR spectrometer. Chemical shifts are expressed in *δ* values relative to the deuterated solvent. Multiplicity determination (DEPT) and 2D NMR spectra (COSY, HMQC, HMBC) were performed with standard pulse programs. GC/MS analysis was conducted using a Hewlett Packard 5890 Series II plus/5972 MSD system, using a DB-5 capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). Column temperature, 90 °C (5 min) to 240 °C at 7 °C/min, injector temperature 240 °C, detector temperature 260 °C; acquisition mass range 700–10 amu, helium was used as carrier gas (39.9 cm/s). The mass spectra were obtained using an Agilent Series 1100 SL HPLC connected to a time of flight mass detector (model G1969A, Agilent technologies) equipped with an ESI interface. Acquisitions were performed under either positive or negative ionization mode with a capillary voltage of +4000 V (or 3500 for negative mode). Nitrogen was used as the nebulizer gas (30 psig) as well as the drying gas at 13 l/min at 325 °C. Data acquisition and processing were done with the Analyst QS software. Column chromatography was run using silica gel (J.T. Baker), 5% $AgNO_3$–silica gel and RP-18 (Bakerbond, J.T. Baker). TLC was performed on silica gel G_{254} aluminum-backed plates or RP-18 (E. Merck). The compounds were visualized by spraying with 0.5% vanillin in 5% ethanolic sulfuric acid.

4.2. Plant material

The flowers of *N. caerulea* were purchased in 2004 from Bouncing Bear Botanicals, and authenticated in-house by Dr. Vaishali C. Joshi. A voucher specimen is deposited at the medicinal plant repository, at The National Center for Natural Products Research, University of Mississippi (Voucher # NYCEN 05-2769).

4.3. Extraction and isolation

The dried powdered whole flowers (1400 g) were percolated with EtOH–H_2O (95:5, v/v) (8×, 1 × 6 l followed by 7 × 3 l). The ethanolic extracts were combined and evaporated to dryness under vacuum at 35 °C to yield a semi-solid residue (218.6 g, 15.6%). Part of this extract (128 g) was suspended in deionized water and extracted with $CHCl_3$ (500 ml × 3, 26.2 g), and with EtOAc (500 ml × 5, 22.7 g).

Part of the $CHCl_3$ fraction (24 g) was applied to a dry silica gel column (270 g, 4 × 50 cm) using a step gradient consisting of pet. ether/EtOAc (99:1 to 83:17 each 1.0 l) to afford eight successive fractions: A (1.2 g, 2.5 l), B (4.5 g, 0.4 l), C (4.4 g, 6.5 l), D (1.0 g, 1.6 l), E (0.6 g, 2.2 l), F (0.15 g, 0.6 l), G (0.15 g, 1.0 l), H (0.22 g, 1.4 l) then followed by a MeOH wash (3.7 g, 1 l). Fraction B was crystallized from acetone (100 mg, fraction B_1). Part of B_1 (92 mg) was subjected to dry silica gel CC (35 g, 1 × 65 cm) using an isocratic solvent system of pet. ether/$CHCl_3$ (95:5) to afford **14** (20 mg, 325 ml) and fraction B_3 (7 mg, 340 ml). Fraction B_3 (7 mg) was applied to column 5% $AgNO_3$–silica gel (8 g, 1 × 29 cm) column using a $CHCl_3$/pet. ether (5:95) solvent system to afford **15** (1.4 mg, 56 ml). Fraction D (1.0 g) was crystallized with acetone to afford **13** (600 mg). Fraction F was crystallized with acetone to afford **16** (8 mg). Fraction H (0.22 g) was crystallized with EtOAc and washed with $CHCl_3$ to afford **20** (14 mg).

Part of the EtOAc fraction (19.6 g) was dissolved in MeOH (25 ml) and the soluble portion was separated out (19.3 g). Part of the MeOH soluble fraction (18.8 g) was applied to a silica gel column (150 g, 3 × 43 cm) using an isocratic solvent system consisting of EtOAc/$CHCl_3$/MeOH/H_2O (15:8:2:0.5) to afford seventeen successive fractions: I (74 mg, 100 ml), J (28 mg, 25 ml), K (43 mg, 45 ml), L (420 mg, 110 ml), M (170 mg, 45 ml), N (185 mg, 25 ml), O (910 mg, 50 ml), P (2.18 g, 100 ml), Q (1.12 g, 150 ml), R (650 mg, 250 ml), S (843 mg, 300 ml), T (2.0 g, 650 ml), U (1.5 g, 650 ml), V (450 mg, 230 ml), W (557 mg, 370 ml), X (900 mg, 720 ml), and Y (1 g, 1.4 l), followed by washing with MeOH (2.8 g, 1 l).

Fraction J was crystallized from MeOH to afford **10** (24 mg). Fraction L was subjected to silica gel CC (35 g, 1.25 × 60 cm) using EtOAc/$CHCl_3$/MeOH/H_2O (15:8:1.5:0.25) as the eluting solvent to afford the following four successive fractions L_1 (5 mg, 30 ml), L_2 (180 mg, 20 ml), L_3 (90 mg, 16 ml) and L_4 (16 mg, 16 ml). Fraction L_3 was crystallized with $CHCl_3$/acetone (1:1) to afford a crystalline material (8 mg), which was applied to a silica gel column (10 g, 1 × 28 cm) eluting with EtOAc/$CHCl_3$/MeOH (5:5:0.1) to afford **17** (1.6 mg, 4 ml). Fraction N was crystallized with MeOH to afford **18** (50 mg). Fraction P was subjected to a silica gel CC (65 g, 2 × 56 cm) eluting with a solvent system of the following $CHCl_3$/MeOH mixtures {99:1 (0.5 l), 98.5:1.5 (1.0 l), 98:2, 97.5:2.5 (each 2.0 l), 97:3, 96.5:3.5, 96:4 (each 1.0 l)} to afford successive seven fractions: P_1 (58 mg, 2.5 l), P_2 (23 mg, 180 ml), P_3 (210 mg, 2.72 l), P_4 (380 mg, 0.6 l), P_5 (560 mg, 0.84 l), P_6 (100 mg, 160 ml), and P_7 (260 mg, 1.5 l). Fraction P_2 was crystallized from MeOH to afford **1** (16 mg). Fraction P_4 was applied to a RP-18 column (60 g, 2 × 36 cm) using a MeOH/H_2O (6:4) solvent system to afford **8** (150 mg, 90 ml). Fraction P_7 was also subjected to RP-18 CC using a MeOH/H_2O (5.5:4.5) solvent system to afford compound **5** (20 mg, 60 ml). Refractionation of fraction Q using a RP-18 column and a MeOH/H_2O (5.5:4.5) solvent system afforded **2** (150 mg, 30 ml). Fraction S was applied to a RP-18 column using a MeOH/H_2O (1:1) solvent system to afford six successive fractions: S_1 (270 mg, 110 ml), **3** (30 mg, 22 ml), S_3 (25 mg, 22 ml), S_4 (150 mg, 86 ml), S_5 (25 mg, 110 ml) and S_6 (15 mg, 70 ml). Fraction S_4 was further purified over a RP-18 column using MeOH/H_2O (1:1) to yield **12** (23 mg, 16 ml) and **6** (10 mg, 80 ml). Fraction S_6 was crystallized from MeOH/H_2O (1:1) to afford compound **19** (12 mg). Fraction U (1.4 g) was dissolved in MeOH/H_2O (4.5:5.5) and loaded onto a RP-18 column (60 g, 2 × 36 cm) and eluted with the same solvent system to yield **9** (140 mg, 320 ml). Fraction V was crystallized from MeOH/H_2O (1:1) to yield **11** (140 mg). Fraction W was loaded onto a RP-18 column (60 g, 2 × 36 cm) and eluted with MeOH/H_2O (6:4) to yield an additional quantity of **11** (40 mg, 72 ml) and **7** (13 mg, 468 ml). Fraction X was re-purified using a RP-18 column (60 g, 2 × 36 cm) that was eluted with MeOH/H_2O (6.5:3.5) to yield **4** (18 mg, 144 ml).

4.4. 2S,3S,4S-trihydroxypentanoic acid (**1**)

Colorless needles; m.p. 104 °C; $[\alpha]_D^{25}$ −33.2 (MeOH, *c* 1.0); IR (neat) ν_{max} cm^{-1} 3388, 1757; ^1H NMR (400 MHz, CD_3OD) δ 4.34 (1H, *d*, 8.8, H-2), 3.75 (1H, *dd*, 8.8, 8.4, H-3), 4.16 (1H, *m*, H-4), and 1.42 (3H, *d*, 6.4, CH_3-5); ^{13}C NMR (100 MHz, CD_3OD) δ 176.6 (C), 75.8 (CH, C-2), 78.8 (CH, C-4), 80.9 (CH, C-3) and 18.3 (CH_3, C-5); HRESIMS *m/z* 167.0468 $[M+OH]^-$ (calcd. for $C_5H_{11}O_6$ 167.0556).

4.5. Myricetin 3-O-(3″-O-acetyl)-α-L-rhamnoside (**2**)

Yellow-colored powder; m.p. 178–182 °C, $[\alpha]_D^{25}$ −124 (MeOH, *c* 1.0); UV (CH_3OH) λ_{max} nm (logε): 261 (4.28), 300 (sh), 352 (4.22); IR (neat) ν_{max} cm^{-1} 3348, 2982, 2937, 1718, 1655, 1607 and 1200 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 6.10 (1H, *d*, 1.6, H-6), 6.25 (1H, *d*, 1.6, H-8), 6.94 (2H, *brs*, H-2′ and 6′), 5.27 (1H, *d*, 1.6, H-1″), 4.42 (1H, *dd*, 1.6, 3.2, H-2″), 5.05 (1H, *dd*, 3.2, 9.2, H-3″), 3.58 (1H, *t*, 9.2, H-4″), 3.64 (1H, *dd*, 6.4, 9.2, H-5″), 0.96 (3H, *d*, 6.4, H-6″) and 2.10 (3H, *s*, H-2‴); ^{13}C NMR (100 MHz, CD_3OD) δ 159.2 (C-2), 136.2 (C-3), 179.3 (C-4), 162.8 (C-5), 99.8 (C-6), 165.5 (C-7), 94.8 (C-8), 158.2 (C-9), 105.8 (C-10), 121.9 (C-1′), 109.7 (C-2′ and C-6′), 146.6 (C-3′ and C-5′), 137.7 (C-4′), 103.2 (C-1″), 69.7 (C-2″), 75.3 (C-3″), 70.6 (C-4″), 72.0 (C-5″), 17.6 (C-6″), 173.1 (C-1‴), and 21.2 (C-2‴); HRESIMS *m/z* 507.1094 $[M+H]^+$ (calcd. for $C_{23}H_{23}O_{13}$ 507.1138).

4.6. *Quercetin 3-O-(3″-O-acetyl)-α-L-rhamnoside (**5**)*

Yellow-colored powder; m.p. 192–194 °C, $[\alpha]_D^{26}$ −152 (MeOH, *c* 0.7); UV (CH_3OH) λ_{max} nm (logε) 256 (4.34), 350 (4.22); IR (neat) ν_{max} cm^{-1} 3386, 2981, 1719, 1655, 1606, 1266 and 1200; ^1H NMR (400 MHz, CD_3OD) δ 6.15 (1H, *brs*, H-6), 6.30 (1H, *brs*, 1.6, H-8), 7.33 (1H, *brs*, H-2′), 6.91 (1H, *d*, 7.6, H-5′), 7.33 (1H, *d*, 7.6, H-6′), 5.38 (1H, *d*, 1.6, H-1″), 4.41 (1H, *dd*, 1.6, 3.2, H-2″), 5.05 (1H, *dd*, 3.2, 9.2, H-3″), 3.57 (2H, *m*, H-4″ and H-5″), 0.98 (3H, *d*, 6.4, H-6″) and 2.14 (3H, *s*, H-2‴); ^{13}C NMR (100 MHz, CD_3OD) δ 159.2 (C-2), 136.1 (C-3), 179.4 (C-4), 163.0 (C-5), 99.9 (C-6), 165.7 (C-7), 94.8 (C-8), 158.4 (C-9), 105.9 (C-10), 123.0 (C-1′), 116.9 (C-2′), 149.7 (C-3′), 146.3 (C-4′), 116.5 (C-5′), 123.2 (C-6′), 103.1 (C-1″), 69.8 (C-2″), 75.3 (C-3″), 70.6 (C-4″), 72.1 (C-5″), 17.7 (C-6″), 173.0 (C-1‴), and 21.2 (C-2‴); HRESIMS *m/z* 491.1258 $[M+H]^+$ (calcd. for $C_{23}H_{23}O_{12}$ 491.1189).

4.7. *24-Methylenecholesterol palmitate (**15**)*

White-colored amorphous solid; $[\alpha]_D^{25}$ −25 ($CHCl_3$, *c* 0.05); IR (neat) ν_{max} cm^{-1} 2915, 2849, 1740 and 885; ^1H NMR (400 MHz, $CDCl_3$) δ 5.34 (1H, *m*, H-6), 4.63 (1H, *s*, 1H-28), 4.68 (1H, *s*, 1H-28), 4.58 (1H, *m*, H-3), 2.27 (2H, *m*, 2H-4), 2.25 (2H, *m*, 2H-2′), 2.21 (1H, *m*, H-25), 2.09 (1H, *m*, 1H-23), 1.98 (1H, *m*, 1H-12), 1.97 (1H, *m*, H-7), 1.90 (1H, *m*, 1H-23), 1.88 (1H, *m*, 1H-2), 1.84 (2H, *m*, 1H-1, 1H-16) 1.58 (1H, *m*, 1H-15), 1.55 (2H, *m*, H-3′), 1.53 (1H, *m*, 1H-22), 1.48 (3H, *m*, 1H-2, 1H-7, 1H-8), 1.45 (2H, *m*, 2H-11), 1.38 (1H, *m*, 1H-20), 1.25 (1H, *m*, 1H-16), 1.22 (25H, *m*, 1H-22, 24H-4′-15′), 1.12 (1H, *m*, H-12), 1.09 (1H, *m*, H-1), 1.03 (1H, *m*, H-17), 1.00 (6H, *d*, 6.4, 3H-26, 3H-27), 0.99 (3H, *s*, 3H-19), 0.97 (2H, *m*, 1H-14, 1H-15) 0.91 (1H, *m*, H-9), 0.92 (3H, *d*, 6.4, 3H-21), 0.85 (3H, *t*, 7.2, 3H-16′), 0.65 (3H, *s*, 3H-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.6 (C-1′), 157.1 (C-24), 139.9 (C-5), 122.8 (C-6), 106.1 (C-28), 73.9 (C-3), 56.9 (C-14), 56.2 (C-17), 50.2 (C-9), 42.6 (C-13), 39.9 (C-12), 38.4 (C-4), 37.2 (C-1), 36.8 (C-10), 36.0 (C-20) 34.9 (C-22, 2′), 34.0 (C-25), 32.1 (C-2, 7, 8), 32.2 (C-14′), 31.2 (C-23), 29.9 (C-5′-12′), 29.3 (C-4′, C-13′), 28.4 (C-16), 25.3 (C-3′), 24.5 (C-15), 22.9 (C-15′), 22.2 (C-27), 22.1 (C-26), 21.3 (C-11), 19.6 (C-19), 18.9 (C-21), 14.3 (C-16′), and 12.1 (C-18); ESIMS *m/z* 551 $[M-84-H]^-$ (calcd. for $C_{44}H_{75}O_2-C_6H_{12}$, 551).

4.8. *Hydrolysis of fraction B_1*

Fraction B_1, enriched with compounds **14** and **15** (8 mg), was heated under reflux with 5% methanolic KOH (2 ml) for 3 h (Mustafa et al., 2000). The reaction product was diluted with H_2O (10 ml) and extracted with $CHCl_3$ (10 ml × 2). The $CHCl_3$ solutions were dried (anhydr Na_2SO_4), and the residue following solvent removed was subjected to silica gel CC (4 g, 1 × 14 cm) using hexane/EtOAc (98:2, 100 ml). The hexane/EtOAc (98:2) fraction afforded, after evaporation, methyl palmitate which was identified by comparison with an authentic sample of methyl palmitate (Sigma–Aldrich Inc.), (GC, R_t = 22.72 min, GC/MS *m/z* = 270 $[M]^+$, 241, 239, 227, 199, 185, 171, 157, 143, 129, 87, 74, and 59).

4.9. *Assay for antioxidant activity*

Antioxidant activity of the extracts as well as the purified compounds was determined by the DCFH-DA (2′,7′-dichlorofluorescin diacetate) method (Rosenkranz et al., 1992). Myelomonocytic HL-60 cells (1×10^6 cells/mL, ATCC, Manassas, VA, USA) were suspended in RPMI-1640 medium with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 μg/ml). The cell suspension (125 μl) was added to the wells of a 96-well plate. After treatment with different concentrations of the extract / test compounds for 30 min, cells were stimulated with 100 ng/ml phorbol 12-myristate-13-acetate (PMA, Sigma) for 30 min. DCFH-DA (Molecular Probes, Invitrogen Corp. Carlsbad, CA, USA, 5 μg/ml) was added and cells were further incubated for 15 min. Levels of fluorescent DCF (produced by ROS catalyzed oxidation of DCFH) were measured on a PolarStar with excitation wavelength at 485 nm and emission at 530 nm. Antioxidant activity of test samples was determined in terms of % decrease in DCF production compared to the vehicle control. Vitamin C (Sigma) was used as the positive control in each assay.

IC_{50} values were obtained from dose curves generated by plotting % DCF production versus test concentrations.

The cytotoxicity was also determined in HL-60 cells after incubating the cells (2×10^4 cells/well in 225 μl) with test samples for 48 h by XTT method (Scudiero et al., 1988). Briefly, 25 μl of XTT-PMS solution (1 mg/ml XTT solution supplemented by 25 μM of PMS) was added to each well. After incubating for 4 h at 37 °C, absorbance was measured on a plate reader (EL312e; Bio-Tek instruments, Winooski, VT) at a dual wavelength of 450–630 nm.

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