Zagazig J. Pharm. Sci., June, 2011
Vol. 20, No. 1, pp, 22-29

ISSN 1110-5089

Phenolic constituents from the leaves of *Tylophora indica* and their antioxidant activities

Mohammed H. Hussein, Ehab M. Mostafa, El-Sayed M. El-Ghaly and Ehab A. Ragab
Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

ABSTRACT

Four flavonoids ; kaempferol-3-*O*-galactoside (trifolin) [1], quercetin-3-*O*-galactoside (hyperoside) [2], quercetin-3-*O*-glucoside (isoquercitrin) [3] and kaempferol-3-*O*-rutinoside [4] together with chlorogenic acid [5] and chlorogenic acid methyl ester [6] have been isolated and purified for the first time from the leaves of *Tylophora indica* by HPLC. The structures were elucidated by the application of MS, IR, UV, 1D- and 2D-NMR spectroscopic analyses and by comparison with literature data. All compounds [1-6] were found to exhibit antioxidant effects, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical.

INTRODUCTION

Tylophora indica, (Burm.f.) Merr. previously known as *T. asthmatica*, Wight et Arn. (Family Asclepiadaceae) is a perennial climbing plant native to the plains, forests and hills of southern and eastern India and other tropical regions [1]. The leaves and roots are used in folk medicine as antiasthmatic [2], anti-inflammatory and anti-arthritic [3], anti-snakebite [4], anti viral [5], antitumor [6], antibacterial [7], free radical scavenging [8], insecticide [9], hepatoprotective [10] and to suppress cellular immune responses [11].

Previous phytochemical studies with *Tylophora indica* have led to the isolation of several indolizidine alkaloids [12-14], fatty acids, sterols and triterpenes [15] and flavonoids [16]. The present paper describes an efficient method for the preparative isolation and purification of four flavonoids and two quinic acid derivatives from *Tylophora indica* by high-performance liquid chromatography (HPLC) followed by the identification of these components using electrospray ionization mass spectrometry (ESI-MS), ultraviolet (UV), infrared (IR) and nuclear magnetic resonance (NMR). These phenolics were evaluated for their antioxidant activities using 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) assay system.

EXPERIMENTAL

General Experimental Procedures

IR spectra were carried out on a Nicolet 205 FT IR spectrometer connected to a Hewlett-Packard Color Pro. Plotter. The ^{1}H- and ^{13}C-NMR measurements were obtained with a Bruker ARX-500 NMR spectrometer operating at 500 MHz (for ^{1}H) and 125 MHz (for ^{13}C) in DMSO-d_6 or CD_3OD solution, and chemical shifts were expressed in δ (ppm) with reference to TMS, and coupling constant (*J*) in Hertz. ^{13}C multiplicities were determined by the DEPT pulse sequence (135°). COSY, HMBC, and HMQC NMR experiments were carried out using a Bruker ARX-500 NMR spectrometers. All 1D and 2D spectra were obtained using the standard Bruker software. LCESI mass spectra were obtained on a Thermo finnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector. Analytical HPLC was performed on a Eurospher-100 C_{18} (5 μm) column (125 x 2 mm, i.d., Knauer, Berlin, Germany) attached with a photodiode-array detector UVD 340S (Dionex, Munich, Germany) and Dionex P580A LPG pump with flow rate 1 mL/min with Chromeleon (V. 6.3) HPLC Program. Routine detection was at 254 nm in aqueous MeOH. Semipreparative HPLC was performed on Merck-Hitachi Eurospher-100- 10 C_{18} column (300 x 4 mm, i.d., Knauer, Berlin, Germany), L-7100 pump, and L-7400 UV detector with flow rate 5 mL/min. Preparative HPLC was performed on Varian Dynamax (250 × 4.6 mm, ID and 250 × 21.4 mm, ID) column, pre-packed with Microsorb 60-8 C_{18} connected to Varian, PrepStar 218 pump and Varian, ProStar 320 UV-Vis detector with flow rate 20 mL/min, Varian Star (V. 6) HPLC Program. Detection was achieved with a diode array detector, and chromatograms were recorded at 235, 254, 280 and 340 nm. Column chromatography was carried out using Sephadex LH 20, 0.25-0.1 mm mesh size (Merck). VLC was carried out using Silica Gel 60, 0.04-0.063 mm mesh size (Merck).

Plant material

Tylophora indica leaves were collected in July, 2007 from New Delhi, India and identified in Plant Biotechnology Institute, New Delhi, India under supervision of Dr. Nutan Kaushik.

Extraction and isolation

Air-dried powdered leaves of *T. indica* (850 g) were subjected to exhaustive extraction with methanol (4 x 5L), (pH 11 with NH_4OH). The combined methanolic extract was concentrated under *vacuum* at 40°C to dryness (88.5 g). The concentrated methanolic extract was then

suspended in distilled water (500 ml), (pH 2 with 0.5 N H_2SO_4) and filtered through filter paper. The water-soluble portion (67 g) was subjected to extraction with ethyl acetate (4 x 1L) to remove non-alkaloidal compounds (37 g), [EtOAc phase I]. The pH of aqueous extract was elevated to 11 with NH_4OH then partitioned several times with EtOAc (4 x 1L) to extract the total alkaloidal bases (10 g) [EtOAc phase II]. The EtOAc phase I fraction was subjected to vacuum liquid chromatography (VLC) using silica gel 60, 0.04 – 0.063 mm mesh size (Merck). Gradient fractionation with 10%, 20%, 30% up to 70% dichloromethane in *n*-hexane then with dichloromethane 100% yielded eight fractions (EtOAc1,1-EtOAc1,8). The fractionation was continued with 5% and 10% methanol in dichloromethane to give two fractions (EtOAc1,9 and EtOAc1,10). Fraction EtOAc1,9 (320 mg) derived from VLC was further subjected to Sephadex LH 20 eluted with methanol to give seven fractions (EtOAc1,9,seph1- EtOAc1,9,seph7). Fraction EtOAc1, 9, seph6 furnished compound **1** (13 mg) as a pale yellow precipitate upon standing. This precipitate was dissolved in methanol (HPLC-grade) and filtered through 0.45 µ (illipore nylon membrane, Merck) filter and injected (20 µL) into the reverse phase analytical HPLC using a linear gradient, 2-90% methanol in nanopure water with a run time of over 35 min, flow rate 1.0 ml/min, detection at 278 nm for purity determination of compound **1** (t_R= 22.29). Fraction EtOAc1,9,seph7 was injected (20 µL) into the reverse phase preparative HPLC with the solvent system water and methanol without acid, starting with 5% methanol and installing a gradient to obtain 40% methanol at 10 min, 50% methanol at 15 min, 70% methanol at 20 min, 100% methanol at 22 min, returning after that in 25 minutes to the initial condition, to give compounds **3** (8 mg, t_R= 22.45) and **2** (17 mg, t_R= 21.36), respectively. Fraction EtOAc1, 10 (1.1 g) derived from VLC was subjected to Sephadex LH 20 eluted with methanol to give eleven fractions (EtOAc1,10, seph1- EtOAc1,10,seph11). Fractions EtOAc1,10,seph6, 8 and 11 were injected (40 µL) into the reverse phase semi-preparative HPLC with the solvent system water and methanol without acid, using the same solvent program described above to give compounds **5** (25 mg, t_R= 14.87), **6** (8.5 mg, t_R= 17.52) and **4** (28 mg, t_R= 22.59), respectively.

Compound (1): Yellow powder (MeOH); UV (MeOH) λ_{max} 265, 344-360 nm; IR (KBr) ν_{max} 3440, 1640, 1615, 1580; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.06 (2H, d, *J*=8.5 Hz, H-2', 6'), 6.85 (2H, d, *J*=8.5 Hz, H-3', 5'), 6.42 (1H, d, *J*=1.9 Hz, H-8), 6.19 (1H, d, *J*=1.9 Hz, H-6), 5.39 (1H, d, *J*=7.5 Hz, H-1''), 3.65 (1H, d, *J*=2.8 Hz, H-4''), 3.53 (1H, t, *J*=8.5 Hz, H-2''), 3.45 (1H, dd, *J*=10.4, 5.6 Hz, H-6''a), 3.37 (1H, dd, *J*=9.4, 3.1 Hz, H-3''), 3.33 (1H, t, *J*=6.0 Hz, H-5''), 3.29 (1H, dd, *J*=10.1, 6.0 Hz, H-6''b), OH signals: 12.60, 10.26, 5.18, 4.86, 4.50, 4.45; ^{13}C NMR (DMSO-d_6, 125 MHz) δ 177.55 (C, C-4), 164.23 (C, C-7), 161.23 (C, C-5), 159.99 (C, C-4'), 156.41 (C, C-9), 156.38 (C, C-2), 133.25 (C, C-3), 131.02 (CH, C-2', 6'), 120.89 (C, C-1'), 115.09 (CH, C-3', 5'), 103.96 (C, C-10), 101.68 (CH, C-1''), 98.74 (CH, C-6), 93.71 (CH, C-8), 75.80 (CH, C-5''), 73.10 (CH, C-3''), 71.23 (CH, C-2''), 67.91 (CH, C-4''), 60.23 (CH_2, C-6''); positive ESIMS *m/z* 448 $[M]^+$, 449 $[M+H]^+$, 471 $[M+Na]^+$, 286 $[aglycon]^+$, 287 $[aglycon+H]^+$; negative ESIMS *m/z* 447 $[M-H]^-$, 285 $[aglycon-H]^-$.

Compound (2): Yellow powder (MeOH); UV (MeOH) λ_{max} 265, 345-360 nm; IR (KBr) ν_{max} 3420, 1635, 1610, 1580; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.65 (1H, dd, *J*=8.5, 2.2 Hz, H-6'), 7.51 (1H, d, *J*=2.2 Hz, H-2'), 6.80 (1H, d, *J*=8.5 Hz, H-5'), 6.39 (1H, d, *J*=2.2 Hz, H-8), 6.18 (1H, d, *J*=2.2 Hz, H-6), 5.36 (1H, d, *J*=7.5 Hz, H-1''), 3.63 (1H, d, *J*=2.8 Hz, H-4''), 3.53 (1H, dd, *J*=9.4, 7.5 Hz, H-2''), 3.44 (1H, dd, *J*=10.1, 5.6 Hz, H-6''a), 3.36 (1H, dd, *J*=9.4, 3.1 Hz, H-3''), 3.31 (1H, t, *J*=6.0 Hz, H-5''), 3.29 (1H, dd, *J*=10.1, 6.0 Hz, H-6''b), OH signals: 12.62, brs, 5.12, 4.84, 4.42; ^{13}C NMR (DMSO-d_6, 125 MHz) δ 177.47 (C, C-4), 164.15 (C, C-7), 161.22 (C, C-5), 156.29 (C, C-9), 156.21 (C, C-2), 148.46 (C, C-4'), 144.82 (C, C-3'), 133.46 (C, C-3), 122.0 (CH, C-6'), 121.08 (C, C-1'), 115.91 (C, C-2'), 115.17 (CH, C-5'), 103.89 (C, C-10), 101.76 (CH, C-1''), 98.66 (CH, C-6), 93.49 (CH, C-8), 75.84 (CH, C-5''), 73.16 (CH, C-3''), 71.19 (CH, C-2''), 67.91 (CH, C-4''), 60.13 (CH_2, C-6''); positive ESIMS *m/z* 464 $[M]^+$, 465 $[M+H]^+$, 302 $[aglycon]^+$, 303 $[aglycon+H]^+$; negative ESIMS *m/z* 463 $[M-H]^-$, 301 $[aglycon-H]^-$.

Compound (3): Yellow powder (MeOH); UV (MeOH) λ_{max} 256, 357 nm; IR (KBr) ν_{max} 3420, 1640, 1620, 1585; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.58 (1H, dd, *J*=8.8, 2.2 Hz, H-6'), 7.56 (1H, d, *J*=2.2 Hz, H-2'), 6.83 (1H, d, *J*=9.1 Hz, H-5'), 6.39 (1H, d, *J*=1.9 Hz, H-8), 6.19 (1H, d, *J*=1.9 Hz, H-6), 5.45 (1H, d, *J*=7.2 Hz, H-1''), 3.58 (1H, d, *J*=10.0 Hz, H-6''a), 3.32 (1H, dd, *J*=11.6, 5.7 Hz, H-6''b), 3.24 (1H, t, *J*=9.1 Hz,

ISSN 1110-5089

H-3``), 3.22 (1H, dd, *J*=7.2, 9.1 Hz, H-2``), 3.07 (2H, m, H-4``, 5``), OH signals: 12.63, 5.28, 5.06, 4.94, 4.25; ^{13}C NMR (DMSO-d_6, 125 MHz) δ 177.51 (C, C-4), 164.12 (C, C-7), 161.25 (C, C-5), 156.32 (C, C-9), 156.19 (C, C-2), 148.48 (C, C-4`), 144.83 (C, C-3`), 133.32 (C, C-3), 121.62 (CH, C-6`), 121.19 (C, C-1`), 116.22 (C, C-2`), 115.20 (CH, C-5`), 104.00 (C, C-10), 100.85 (CH, C-1``), 98.69 (CH, C-6), 93.52 (CH, C-8), 77.60 (CH, C-5``), 76.51 (CH, C-3``), 74.10 (CH, C-2``), 69.94 (CH, C-4``), 60.99 ($CH_2$, C-6``); positive ESIMS *m/z* 464 $[M]^+$, 465 $[M+H]^+$, 302 $[aglycon]^+$, 303 $[aglycon+H]^+$; negative ESIMS *m/z* 463 $[M-H]^-$, 301 $[aglycon-H]^-$.

Compound (4): Yellow powder (MeOH); UV (MeOH) λ_{max} 265, 347-370 nm; IR (KBr) ν_{max} 3440, 1640, 1610, 1590; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.97 (2H, d, *J*=8.8 Hz, H-2`, 6`), 6.86 (2H, d, *J*=8.8 Hz, H-3`, 5'), 6.39 (1H, d, *J*=2.2 Hz, H-8), 6.19 (1H, d, *J*=1.9 Hz, H-6), 5.30 (1H, d, *J*=7.5 Hz, H-1``), 4.36 (1H, br s, H-1```), 3.68 (1H, dd, *J*=10.1, 5.6 Hz, H-6``a), 3.40 (1H, t, *J*=9.4 Hz, H-4``), 3.34 (1H, m, H-5``), 3.26 (1H, dd, *J*=10.1, 6.0 Hz, H-6``b), 3.25 (1H, m, H-5```), 3.24 (1H, d, *J*=4.4 Hz, H-2```), 3.17 (1H, dd, *J*=7.5, 9.4 Hz, H-2``), 3.15 (1H, t, *J*=9.4 Hz, H-3``), 3.08 (1H, t, *J*=9.1 Hz, H-4```), 3.05 (1H, t, *J*=9.4 Hz, H-3```), 0.97 (3H, d, *J*=6.3 Hz, H-6```), OH signals: 12.55, 5.34, 5.08, 5.06, 4.53, 4.41; ^{13}C NMR (DMSO-d_6, 125 MHz) δ 177.36 (C, C-4), 164.15 (C, C-7), 161.16 (C, C-5), 159.87 (C, C-4`), 156.82 (C, C-9), 156.47 (C, C-2), 133.20 (C, C-3), 131.18 (CH, C-2`, 6`), 120.87 (C, C-1`), 115.33 (CH, C-3`, 5`), 103.94 (C, C-10), 101.18 (CH, C-1``), 100.71 (CH, C-1```), 98.49 (CH, C-6), 93.95 (CH, C-8), 76.27 (CH, C-3``), 75.65 (CH, C-5``), 74.07 (CH, C-2``), 71.85 (CH, C-4```), 70.52 (CH, C-3```), 70.37 (CH, C-2```), 69.97 (CH, C-4``), 68.16 (CH, C-5```), 66.89 (CH_2, C-6``), 17.51 (CH_3, C-6```); positive ESIMS *m/z* 594 $[M]^+$, 595 $[M+H]^+$, 617 $[M+Na]^+$, 448 $[M-rhamnose]^+$, 286 $[aglycon]^+$, 287 $[aglycon+H]^+$; negative ESIMS *m/z* 593 $[M-H]^-$, 285 $[aglycon-H]^-$.

Compound (5): Colorless needles (MeOH), UV (MeOH) λ_{max} 245, 316, 327, 340 nm; IR (KBr) ν_{max} 3400, 3200-2500, 1705, 1640, 1610, 1520; ^{1}H NMR (*CD$_3$OD*, 500 MHz) δ 7.54 (1H, d, *J*=16.1 Hz, H-7`), 7.03 (1H, d, *J*=1.9 Hz, H-2`), 6.94 (1H, dd, *J*=8.2, 1.9 Hz, H-6`), 6.76 (1H, d, *J*=8.2 Hz, H-5`), 6.25 (1H, d, *J*=15.7 Hz, H-8`), 5.32 (1H, ddd, *J*=7.9, 7.6, 4.1 Hz, H-5), 4.16 (1H, m, H-3), 3.72 (1H, dd, *J*=7.6, 3.1 Hz, H-4), 2.22 (1H, dd, *J*=13.8, 3.5 Hz, H-6_{eq}), 2.18 (1H, dd, *J*=13.8, 4.1 Hz, H-2_{ax}), 2.12 (1H, dd, *J*=13.8, 7.9 Hz, H-6_{ax}), 2.07 (1H, dd, *J*=13.8, 6.9 Hz, H-2_{eq}); ^{13}C NMR (*CD$_3$OD*, 125 MHz) δ 177.47 (C, C-7), 168.65 (C, C-9`), 149.52 (C, C-4`), 147.05 (CH, C-7`), 146.74 (C, C-3`), 127.75 (C, C-1`), 122.97 (CH, C-6`), 116.45 (CH, C-5`), 115.22 (CH, C-2`), 115.16 (CH, C-8`), 76.58 (C, C-1), 73.44 (CH, C-4), 71.99 (CH, C-5), 71.31 (CH, C-3), 38.76 (CH_2, C-6), 38.12 (CH_2, C-2); positive ESIMS *m/z* 354 $[M]^+$, 355 $[M+H]^+$, 377 $[M+Na]^+$, 731 $[2M+Na]^+$; negative ESIMS *m/z* 353 $[M-H]^-$, 191 [M – caffeoyl]$^-$, 163 [caffeoyl]$^-$.

Compound (6): Colorless needles (MeOH), UV (MeOH) λ_{max} 221, 244, 327 nm; IR (KBr) ν_{max} 3410, 1735, 1680, 1630, 1600, 1520; ^{1}H NMR (*CD$_3$OD*, 500 MHz) δ 7.51 (1H, d, *J*=15.7 Hz, H-7`), 7.03 (1H, d, *J*=1.9 Hz, H-2`), 6.93 (1H, dd, *J*=8.2, 1.9 Hz, H-6`), 6.77 (1H, d, *J*=8.2 Hz, H-5`), 6.20 (1H, d, *J*=15.7 Hz, H-8`), 5.26 (1H, ddd, *J*=7.6, 7.5, 4.4 Hz, H-5), 4.12 (1H, ddd, *J*=3.1, 3.5, 6.6 Hz, H-3), 3.72 (1H, dd, *J*=7.6, 3.1 Hz, H-4), 3.68 (3H, s, OCH_3), 2.20 (1H, dd, *J*=13.5, 3.4 Hz, H-6_{eq}), 2.17 (1H, dd, *J*=13.5, 4.4 Hz, H-2_{ax}), 2.12 (1H, dd, *J*=13.5, 7.9 Hz, H-6_{ax}), 2.00 (1H, dd, *J*=13.5, 6.9 Hz, H-2_{eq}); ^{13}C NMR (*CD$_3$OD*, 125 MHz) δ 175.41 (C, C-7), 168.25 (C, C-9`), 149.67 (C, C-4`), 147.19 (CH, C-7`), 146.84 (C, C-3`), 127.62 (C, C-1`), 122.97 (CH, C-6`), 116.52 (CH, C-5`), 115.10 (CH, C-2`), 115.03 (CH, C-8`), 75.80 (C, C-1), 72.52 (CH, C-4), 72.10 (CH, C-5), 70.27 (CH, C-3), 38.01 (CH_2, C-6), 37.74 (CH_2, C-2); positive ESIMS *m/z* 368 $[M]^+$, 391 $[M+Na]^+$, 736 $[2M]^+$, 759 $[2M+Na]^+$; negative ESIMS *m/z* 367 $[M-H]^-$, 205 [M-caffeoyl]$^-$, 163 [caffeoyl]$^-$, 135 [caffeoyl-C=O]$^-$.

Determination of the effects of 1–6 on DPPH radical

The test sample in solution was added to 1 ml of a methanolic solution of DPPH radical (Sigma-Aldrich, St. Louis, USA.) (final DPPH concentration was 300 μM). The mixture was shaken vigorously and left to stand for 30 min. The absorbance of the resulting solution was measured at 517 nm. The percent inhibition of each sample was calculated [17, 18] according to the equation:

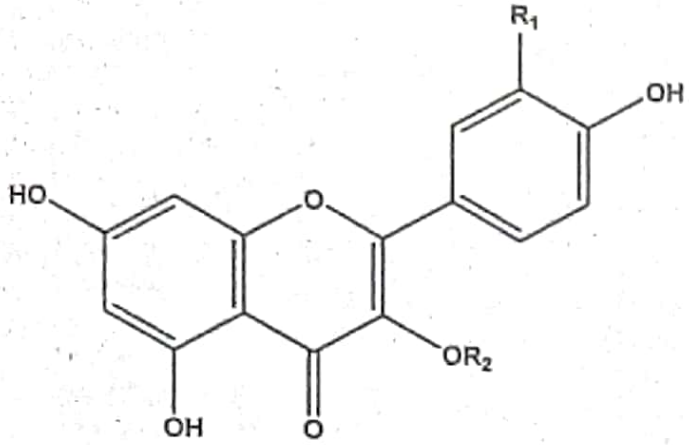
$$Inhibition\% = (1 - Sample_{515} / blank_{515}) \times 100.$$

Percentage inhibition was plotted versus compound concentration (mg/ml). From the equation of the slope, the test sample concentration required to reduce the absorbance at 515 nm by 50 % (IC_{50}) was calculated. All tests and analyses were run in triplicate and averaged. The value of 50% inhibition (IC_{50})

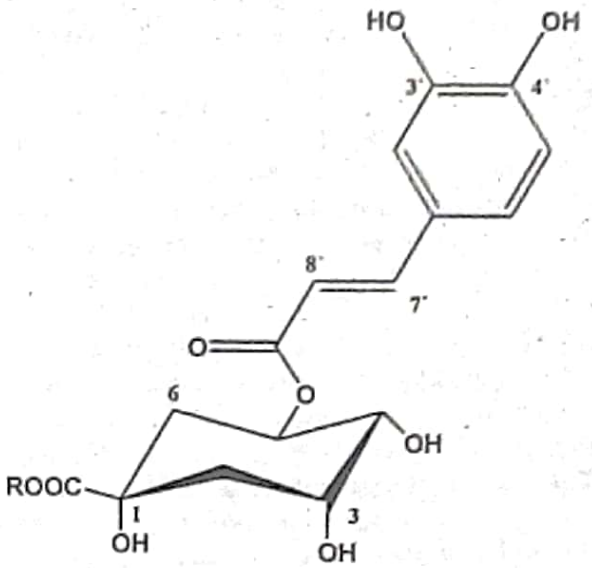
denotes the concentration of sample required to scavenge 50% of the DPPH free radicals.

RESULTS and DISCUSSION

Fractions derived from VLC of the non-alkaloidal EtOAc extract of *T. indica* leaves were subjected to preparative and semi-preparative HPLC separation using the program described under the experimental section. Four flavonoids [1-4] and two quinic acid derivatives [5 and 6] were isolated and identified through LC-ESIMS, LC-UV, IR and NMR data analysis. Analysis of the COSY, HMQC and HMBC spectra allowed the complete assignments for the ^{1}H and ^{13}C NMR of all compounds.



	R_1	R_2
Compound 1	**H**	**Galactose**
Compound 2	**OH**	**Galactose**
Compound 3	**OH**	**Glucose**
Compound 4	**H**	**Glucose(6-1)Rhamnose**



	R
Compound 5	**H**
Compound 6	**CH_3**

Compounds **1-4** were obtained as pale yellow amorphous powder. IR spectra of compounds **1-4** showed the same pattern, suggesting the presence of hydroxyl (3420-3433 cm-1) and carbonyl (1730-1735 cm-1) groups. In addition, their UV absorption patterns (244-245sh, 296-300, 328-329 nm) were nearly identical to each other and similar to those of flavonoids [19]. In the ESIMS, peaks corresponding to $[M]^+$, $[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$ were observed at *m/z* 448, 449, 471 and 447 showing the molecular formula of **1** to be $C_{21}H_{20}O_{11}$. Another fragment ion peaks at *m/z* 286, 287 and 285 corresponding to $[aglycone]^+$, $[aglycone+H]^+$ and $[aglycone-H]^-$, respectively, was indicative of the kaempferol aglycon of **1**. The ESIMS of **2** and **3** showed the same molecular ions $[M]^+$ at *m/z* 464, $[M+H]^+$ at *m/z* 465, and $[M-H]^-$ ions at *m/z* 463, which were in accordance with quercetin galactoside or quercetin glucoside and appropriate for the molecular formula $C_{21}H_{20}O_{12}$. In addition, fragment ion peaks at *m/z* 302 $[aglycone]^+$, 303 $[aglycone+H]^+$ and 301 $[aglycone-H]^-$ confirmed the quercetin aglycone. Compound **4** exhibited an $[M]^+$ peak at *m/z* 594, an $[M+H]^+$ peak at *m/z* 595, an $[M+Na]^+$ peak at *m/z* 617 and an $[M-H]^-$ peak at *m/z* 593, which were indicative of the molecular formula 11 $C_{27}H_{30}O_{15}$. Fragment ions similar to those of **1** were observed at *m/z* 286, 287 and 285, suggesting that, kaempferol is the aglycone of **4**. Furthermore, the fragment ion peak at *m/z* 448 $[M-146]^+$, confirmed that **4** has a rhamnose unit.

The ^{1}H NMR spectrum of **1** showed the expected kaempferol-3-*O*-galactoside proton pattern of signals. Two doublets at δ6.19 and 6.42 with a coupling constant of 1.9 Hz, typical of two *meta*-coupled protons, were assigned to H-6 and H-8, respectively. The disubstituted aromatic B ring appeared as AA'BB' spin system at δ6.85 (H-3', H-5') and δ8.06 (H-2', H-6'), each 2H, J_{AB}= 8.5 Hz. ^{13}C NMR spectral analysis further confirmed the structure of **1**. Thus, most of the chemical shift values were the

ISSN 1110-5089

Zagazig J. Pharm. Sci., June, 2011
Vol. 20, No. 1, pp, 22-29

same as for kaempferol-3-*O*-galactoside. It displayed 19 signals but it was obvious that two signals at δ131.02 and 115.09 had intensities suggesting that they represent two carbons each. The ^{1}H and ^{13}C NMR of **1** exhibited one sugar anomeric proton at δH 5.39 (1H, *d*, *J*= 7.5 Hz,) and carbon at δC 101.68 of galactose. The ^{13}C-NMR chemical shifts of galactose and $^{3}J_{H-H}$ analysis of H-3 and H-4 (9.4, 3.1 Hz) confirmed the D-galactose moiety. The galactose was established to be in the β-configuration from the anomeric proton chemical shift and $^{3}J_{H1,H2}$ coupling constant [20]. Glucosidation at the 3-hydroxyl of kaempferol was deduced from the recognized upfield shift of the C-3 (δ133.25) carbon resonance and the downfield shift of the C-2 (δ156.38) carbon resonance. Furthermore, the HMBC correlations clearly revealed the galactose unit attached to C-3 in the kaempferol moiety. Thus, compound **1** was identified as kaempferol-3-*O*-galactoside (trifolin) in good agreement with the reported literature [21].

The ^{1}H and ^{13}C NMR spectral data indicated that **2** is the quercetin analogue of **1**. The ^{1}H and ^{13}C NMR spectra were similar to those of compound **1** and also showed a galactose proton and carbon signals. The only difference between compounds **1** and **2** in ^{1}H NMR was the presence of an ABX system instead of AA`BB` system corresponding to the B ring protons at δ7.65 (1H, dd, *J*=8.5, 2.2 Hz, H-6`), 7.51 (1H, d, *J*=2.2 Hz, H-2`), 6.80 (1H, d, J=8.5 Hz, H-5`). The ^{13}C NMR spectrum displayed 21 signals consistent with quercetin galactoside. HMBC analysis established the site of galactosidation at C-3 as in **1**. Therefore, assigned compound **2** was as quercetin-3-*O*-galactoside (hyperoside) ,being in close agreement with hyperoside by literature comparison [19,22].

Similarly, compound **3** showed ^{1}H and ^{13}C NMR resonances comprising typical quercetin signals, almost identical to those of compound **2**. The difference between **2** and **3** is that, the galactose moiety was replaced by glucose. ^{1}H and ^{13}C NMR chemical shifts and coupling constants were in good agreement with β-D-glucose [20]. By direct comparison with corresponding spectroscopic data published previously [19, 22-24], the structure of compound **3** was identified as quercetin-3-*O*-glucoside (isoquercitrin).

The ^{1}H NMR of compound **4** was similar to that of **1**, except the galactose moiety being replaced by rutinose as indicated by the signals of two anomeric protons, one at δ5.30 (1H, d, *J*= 7.5 Hz, H-1`` of glucose) and the second at δ4.36 (1H, br s, H-1``` of rhamnose). The ^{13}C NMR spectrum of **4** showed the characteristic 13 distinct carbon resonances of a kaempferol moiety. In addition, two anomeric carbons at δ101.18 and 100.71, confirmed the disaccharide moiety of **4**. The appearance of the strong sharp signal at δ0.97 (3H, *d*, *J*= 6.3 Hz) and δ17.51 in the ^{1}H- and ^{13}C-NMR spectra confirmed the rhamnose unit. The configuration at C-1 of the glucose was determined to be β; while configuration at C-1 of the rhamnose was determined to be *α*, according to their anomeric protons chemical shifts and $^{3}J_{H1,H2}$ coupling constants [20,22]. The signal of the glycosidic carbon, C-6`` was downfield- shifted as compared to the unsubstituted C-6`` of the glucose moiety. This is the shift expected for a 1→6 linked rhamnoglucoside (rutinoside) and the ^{1}H NMR signal of the rhamnose H-1 was consistent with a rutinose linkage [22, 25]. This was confirmed by HMBC cross-peaks between the anomeric proton of rhamnose and and C-6`` of glucose, and between H2-6`` of glucose and the anomeric carbon of rhamnose. The attachment of the rutinose moiety to C-3 of the kaempferol moiety followed from the upfield shift of this carbon resonance and the accompanying downfield shift of the resonance of C-2 (all in comparison with the chemical shifts of the corresponding carbon resonances in the ^{13}C NMR spectrum of kaempferol itself [22]. Furthermore, the HMBC spectrum of **4** showed correlations between the anomeric proton of the glucose and C-3 of the kaempferol skeleton, confirming the attachment of the disaccharide moiety to this position. The structure of **4** is, therefore, that of kaempferol-3-*O*-rutinoside and in good agreement with the reported literature [19, 23, 24].

Compound **5** was obtained as colourless needles, and exhibited strong absorption bands due to hydroxyl, carbonyl and an aromatic ring in the IR spectrum. In addition, the UV absorption pattern (245, 316, 327, 340 nm) was similar to those of caffeic, chlorogenic, and ferulic acids, indicating the presence of a –CH=CH-CO2H group linked to the phenyl ring [26]. Peaks corresponding to $[M]^{+}$, $[M+H]^{+}$, $[M+Na]^{+}$, were observed at *m/z* 354, 355, 377 in the positive ESIMS mode and a peak corresponding to $[M-H]^{-}$, was observed at *m/z* 353, in the negative ESIMS mode showing the molecular formula of **5** to be $C_{16}H_{18}O_{9}$. Analysis of the HMQC and HMBC spectra allowed the complete assignments for the ^{1}H and ^{13}C NMR

of compound 5. The ^{1}H NMR spectrum of 5 displayed an AMX spin system consisting of δ7.03 (1H, d, *J*=1.9 Hz, H-2`), 6.76 (1H, d, *J*=8.2 Hz, H-5`), and 6.94 (1H, dd, *J*=8.2, 1.9 Hz, H-6`); indicating a 1`,3`,4`-trisubstituted benzene ring [27]. The presence of a *trans* double bond was noted in the ^{1}H NMR spectrum of 5; δ6.25 (1H, d, *J*=15.7 Hz, H-8`) and 7.54 (1H, d, *J*=16.1 Hz, H-7`), suggesting the presence of a *trans* caffeoyl moiety in 5. The signals of H-3, H-4 and H-5 of the quinic acid moiety were assigned according to their multiplicity and their spin-spin coupling constants [28]. The ^{1}H NMR spectrum of 5 displayed H-4 as a dd at δ3.72 with *J* values of 3.1 and 7.6 Hz, indicating axial-axial (*J*=7.6 Hz) and axial-equatorial (*J*=3.1 Hz) couplings. The large coupling of H-4 and H-5 (*J*=7.6 Hz) demonstrates axial-axial coupling, and the small coupling of H-3 and H-4 (*J*=3.1 Hz) shows axial-equatorial coupling. These results confirm that 5 has the same stereochemistry in its quinic acid moiety as chlorogenic acid [29]. In the ^{1}H NMR spectrum, the downfield shift of the resonance of the axial proton signal of H-5 (δ5.32, ddd, *J*=7.9, 7.6, 4.1 Hz) indicated that the hydroxyl group on C-5 was acylated with a caffeic acid. The ^{13}C NMR data revealed the presence of a quinic acid moiety characterized with two methylenes (δ38.12 and 38.76), three oxymethines (δ71.31, 71.99, and 73.44), one quaternary carbon (δ76.58), and one carboxyl group (δ177.47), as well as a caffeoyl moiety. Furthermore, the location of the caffeoyl substitution on the quinic acid moiety was determined by the HMBC spectrum. The signal at δ5.32 due to H-5 of the quinic acid moiety showed a correlation with the carboxyl carbon signal at δ168.65. These results established the location of the caffeoyl substitution as being at the C-5 position of the quinic acid moiety. The ^{1}H and ^{13}C NMR data of 5 were similar to those of chlorogenic acid [29].

Compound 6 was obtained as colourless needles. The IR and UV absorption patterns were nearly identical to those of 5. The ESIMS of 6 showed molecular ions at *m/z* 368 $[M]^+$, 391 $[M+Na]^+$, 367 $[M-H]^-$, which were in accordance with chlorogenic acid containing a methoxyl group. Compound 6 exhibit 1D and 2D NMR data similar to those of 5, except that 6 displays chemical shifts for an *O*-methyl group. Therefore, 6 was identified as chlorogenic acid methyl ester. The ^{1}HNMR spectrum exhibited signals belonging to a caffeic moiety, a quinic acid moiety and a methoxyl group. Analysis of ^{1}H NMR data was used to determine the stereochemistry of 6. The results showed that 6 has the same stereochemistry in the quinic acid moiety as chlorogenic acid (Pauli *et al*, 1999). The ^{13}C NMR spectrum exhibited 17 signals comprising two ester carbonyls (δ175.41 and 168.25), one methoxyl carbon (δ52.96), two methylene carbons (δ37.74 and 38.01), three oxymethine carbons (δ70.27, 72.10 and 72.52), one quaternary carbon (δ 75.80), two olefinic carbons (δ147.19 and 115.03) and two hydroxylated aromatic carbons (δ149.67 and 146.84). The position of caffeoyl substitution and the location of the methoxyl group on the quinic acid moiety were confirmed as follows: The HMBC spectrum showed a correlation between the signal of H-5 (δ5.26) and a carboxyl carbon signal (δ168.25). The carboxyl carbon signal was also found to be correlated with two *trans* olefinic proton signals, while a methoxy proton signal (δ3.68) showed correlation with C-7 (δ175.41). These spectral data confirmed the location of the caffeoyl substitution at the C-5 position of the quinic acid methyl ester moiety. The ^{1}H and ^{13}C NMR data of 6 are consistent with previously published data for chlorogenic acid methyl ester [30]. The structure of 6 is, therefore, that of chlorogenic acid methyl ester.

Antioxidant activities of 1-6

In this paper, the scavenging activities of compounds 1–6 toward the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical were determined (Table 1). The DPPH free radical is a stable free radical, which has been widely used as a tool to estimate the free radical scavenging activities of antioxidants [31]. To evaluate the six phenolics 1-6 for antioxidant potential, their DPPH free radical scavenging activities were compared with those of selected standard antioxidants including quercetin and α-tocopherol (Table 1). In terms of DPPH scavenging activity, quercetin was found to be the most potent antioxidant, whereas compounds 6 showed the lowest activity. The values obtained for compounds 1 and 4, are comparable to those of α-tocopherol, while compounds 2 and 3 were stronger than α-tocopherol and their DPPH scavenging activities were slightly higher than those of α-tocopherol; Among the six compounds, compounds 5 and 6 were the weakest in antioxidant activity. In comparison with the values of quercetin, all compounds 1-6, showed weak antioxidant activity.

ISSN 1110-5089

Zagazig J. Pharm. Sci., June, 2011
Vol. 20, No. 1, pp, 22-29

(Table 1): Scavenging effect of 1-6 on DPPH radicals.

Compound	Antioxidant activity/(IC_{50} µM)
1	84.22 ± 2.44
2	61.53 ± 1.33
3	58.21 ± 1.22
4	86.12 ± 2.44
5	165.67 ± 3.11
6	182.44 ± 3.28
α-tocopherol	82.23 ± 1.23
Quercetin	37.08 ± 1.62

α-tocopherol and quercetin were used as positive controls.
*The data are presented as a mean of at least three independent measurements ± standard deviation.

REFERENCES

1. Sharma, O. P., **Plant taxonomy**, New Delhi, Tata Mc Graw-Hill, 248-251, 327-330 (1993).
2. Gupta, S., George, P., Gupta, V., Tandon, V. R., Sundaram, K. R., **Ind. J. Med. Res.**, 69, 981 (1979).
3. Biren, S. N., Nayak, B. S., Seth, A. K., Jalalpure, S. S., Patel, K. N., Patel, M. A. Mishra, A. D., **Pharmacogn. Mag.**, 2 (6), 77 (2006).
4. Samy, R. P., Thwin, M. M., Gopalakrishnakone, P. Ignacimuthu, S., **J Ethnopharmacol.**, 115 (2), 302 (2008).
5. Balasubramanian, G., Sarathi, M., Rajesh, K. S. and Sahul, H. A. S., **Aquaculture**, 263, 15 (2007).
6. Gao, W., Chen, A. B. C., Leung, C. H., Gullen, E. A., Fürstner, A., **Bioorg. Med. Chem. Lett.**, 18, 704 (2008).
7. Parekh, J. and Chanda, S. V., **Turk. J. Biol.**, 32, 63 (2008).
8. Chandra, J. G. Shrinath, B. M., **J. med. Food.**, 7(3), 343 (2004).
9. Kathuria, V. Kaushik, N., **Insect Sci.**, 12, 249-254 (2005).
10. Gujrati, V., Patel, N., Rao, N. V., Nandakumar, K., Gouda, T. S. , Shalam, M. d., Shanta Kumar, S. M., **Indian J Pharmacol.**, 39 (1), 43 (2007).
11. Ganguly, T. and Sainis, K. B., **Phytomedicine**, 8, 348 (2001).
12. Gellert, E., **J. Nat. Prod.**, 45 (1), 50 (1982).
13. Ali, M., Bhutani, K. K., **Phytochemistry**, 28 (12), 3513 (1989).
14. Ali, M., Ansari, S. H, Qadry, J. S., **J. N. P.**, 54 (5), 1271 (1991).
15. Jain, D. C.; Agrawal, P. K. **Fitoterapia**, 62 (1), 93 (1991).
16. Chandrashekar, V.; Seshadri, T. R., **Curr. Sci.**, 37(15), 432 (1968).
17. Mossman, B. T., **Free Rad. Biol. Med**, 28, 1315 (2000).
18. Janssen, Y. M. W, Houten, B., Borm, P. J. A. Mossman, B. T., **Lab. Invest.**, 69, 261 (1993).
19. Markham, K. R., Ternai, B., Stanley, R., Geiger, H. Mabry, T. J., **Tetrahedron**, 34, 1389 (1978).
20. Gorin, P. A. J. Mazurek, M., **Can. J. Chem.**, 53, 1212 (1975).
21. D´ıaz, J. G., Carmona, A. J., Torres, F., Quintana, J., Est´evez, F. Herz, W., **Planta. Med.** 74,171 (2008).
22. Harborne, J. B. **The Flavonoids, Advances in research since 1986**, 116-238, 446-471.Chapman and Hall, London, New York (1994).
23. Moco, S., Tseng, L. H., Spraul, M., Chen, Z. and Vervoort, J., **Chromatographia** , 64, 503 (2006).
24. Lee, J. H., Ku, C. H., Baek, N. I., Kim, S. H., Park, H. W. Kim, D. K., **Arch. Pharm. Res.** 27 (1), 40 (2004).
25. Mabry, T. J., Markham, K. R. Thomas, M. B. **The Systematic Identification of Flavonoids.**, Springer, New York (1970).
26. Perry, G., Raina, A. K. L, Nonomura, A., Wataya, T., Sayre, L. M., Smith, M. A., **Free Rad. Biol. Med.**, 28, 831 (2000).
27. Kweon, M. H., Hwang, H. J., Sung, H. C., **J. Agric. Food Chem.** , 49, 4646 (2001).
28. Karplus, M. J., **Chem. Phys.**, 30, 11 (1959).
29. Pauli, G. F., Kuczkowiaky, U. Nahrstedt, A. **Magn. Reson. Chem.** 37, 827 (1999).
30. Ida, Y., Satoh, Y., Ohtwka, M., Nagasao, M., Shoji, J. Phytochem., 35(1), 209 (1994).
31. Jin, Z. Q., Chen, X., **J. Pharmacol. Toxicol. Methods.**, 39, 63 (1998).

Received: 27/03/2011
Accepted: 10/06/2011

مركبات فينولية من أوراق نبات تيلوفورا اندكا و فاعليتها المضادة للأكسدة

محمد حسنى و إيهاب محمد مصطفى و السيد محمد الغالي و إيهاب عبد الوهاب رجب

قسم العقاقير – كلية الصيدلة – بنين بالقاهرة – جامعة الأزهر – مصر

تم فصل و تنقية اربع مركبات من مشتقات الفلافونيدات و هي كالاتى، كامفيرول-٣-جالاكتوزايد (ترايفولين) و كوارستين-٣-جالاكتوزايد (هيبروزايد) و كوارستين-٣-جلوكوزايد (ايزوكوارسترين) و كامفيرول-٣-روتينوزايد مع مركب حمض الكلوروجينيك و مركب أسترات ميثيل حمض الكلوروجينيك لأول مرة من أوراق نبات تيلوفورا اندكا، وذلك باستخدام جهاز الفصل الكروماتوجرافى للسائل ذو الكفاءة العالية. وقد تم التعرف على هذه المركبات باستخدام الطرق الطيفية الحديثة المختلفة والممثلة بالأشعة دون الحمراء والأشعة فوق البنفسجية والرنين النووي المغناطيسي لذرات الهيدروجين والكربون-١٣ أحادى وثنائي البعد بالإضافة إلى الدبت وكذلك طيف الكتلة وبالمقارنة مع الابحاث العلمية المنشورة. كما تم دراسة الفاعلية المضاد للأكسدة لهذه المركبات باستخدام الراديكل الحر (دى بى بى اتش) ووجد أن لجميع هذه المركبات فاعلية مضادة للأكسدة.