POLYPHENOLS AND BIOLOGICAL ACTIVITIES OF *Feijoa sellowiana* LEAVES AND TWIGS

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ABSTRACT

A phytochemical analysis of the *Feijoa sellowiana* O. Berg (Myrtaceae) leaves and twigs led to the isolation of a new phenolic compound together with nine known metabolites. Based on chemical and spectroscopic analyses, including UV, 1D and 2D NMR spectroscopy and HRESI-MS, the structures were elucidated as 3`-*O*-methylellagic acid 3-sulphate **1**, nilocitin **2**, strictinin **3**, casuarin **4**, castalagin **5,** ellagic acid **6**, ellagic acid 4-*O*-methyl ether **7,** avicularin **8**, hyperin **9** and quercitrin **10**. It was found that 80 % aqueous methanol extract of *F. sellowiana* leaves and twigs (AME-80) is non toxic up to 5 g/kg b. wt. and it exhibited significant analgesic, anti-inflammatory, antiulcer, antioxidant and hepatoprotective activities. Histopathological study was done for the liver tissues, and revealed improvement of the liver tissue damage induced by oral paracetamol administration. This result supports the hepatoprotective activity of the tested extract.

Key words: *Feijoa sellowiana*; Myrtaceae; Tannins; Flavonoids; Pharmacological activities.

RESUMEN

Del análisis fitoquímico de las hojas y ramas de *Feijoa sellowiana* O. Berg (Myrtaceae) se aislaron nuevos compuestos fenolicos y nueve metabolitos ya conocidos. A través de análisis químicos y espectroscópicos (UV, NMR 1D y 2D, y HRESI-MS) se elucidaron los siguientes compuestos: ácido 3`-*O*-metilelagico 3-sulfato **1**, nilocitina **2**, estrictinina **3**, casuarina **4**, castalagina **5**, ácido elagico **6**, ácido elagico 4-*O*-metil eter **7**, avicularina **8**, hiperina **9** y quercitrina **10**.

El extracto metanolico al 80 % en agua (AME-80) de *F. sellowiana* no es tóxico hasta 5g/kg. En los estudios sobre su actividad biológica los resultados mostraron que tiene una significativa actividad analgésica, aintiinflamatoria, antiulcerosa, antioxidante y hepatoprotectiva. Se realizaron estudios histopatológicos de tejido hepático con daños inducidos por administración de paracetamol v.o., encontrando mejoras en el tejido, lo que comprueba la actividad hepatoprotectora del extracto AME-80 de *F. sellowiana.*

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INTRODUCTION

Feijoa sellowiana, O. Berg (*syn Acca sellowiana*) is belonging to family Myrtaceae. It is native to southern Brazil, northern Argentina, western Paraguay (Trease and Evans, 1978) being traditionally known as pineapple guava. The plant is used in many industrialized products particularly in the Australian area, in the form of jams, syrups, liquors and crystallized fruits (Di. Cesare *et al.*, 1998). *F. sellowiana* leaves, fruits and stems were reported to exhibit antimicrobial, antitumor, anti-inflammatory and antioxidant effects (Isobe *et al.*, 2003; Vuotto *et al.*, 1999). Few studies dealt with the chemistry of *F. sellowiana* as a flavone (Ruberto and Tringali, 2004), volatile components (Fernandez *et al.*, 2004; Di. Cesare *et al.*, 2000; Shaw *et al.*, 1990), lipids (Di. Cesare *et al.*, 1998) and tannins (Okuda *et al.*, 1982).

This study aims at the isolation and identification of the constitutive polyphenols in the aqueous methanol extract of the leaves and twigs of *F. sellowiana* in addition to evaluation of the analgesic, anti-inflammatory, antiulcer, antioxidant and hepatoprotective effects of the investigated extract.

MATERIALS AND METHODS

Equipments

The NMR spectra were recorded at 300, 400 and 500 (^{1}H) and 75, 100, 125 (^{13}C) MHz, on a Varian Mercury 300, Bruker APX-400 and JEOL GX-500 NMR spectrometers and δ-values are reported as ppm relative to TMS in the convenient solvent. HRESI-MS analyses were run on LTQ-FT-MS spectrometer (Thermo Electron, 400, Germany). UV analyses for pure samples were recorded as MeOH solutions and with different diagnostic UV shift reagents on a Shimadzu UV 240 (P/N240–58000) and Ebeckman DU7 spectrophotometer. For column chromatography, Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (E. Merck, Darmstadt, Germany) and polyamide 6 (Fluka Chemie AG, Switzerland) were used. For paper chromatography; Whatman No. 1 sheets (Whatman Ltd., Maidstone, Kent, England) were used.

Plant material

Leaves and twigs of *F. sellowiana* O. Berg were collected in February 2004 and April 2005 from Zohria Botanical Garden, Cairo, Egypt. The identification of the plant was performed by Dr. Amal Abdel-Aziz, Lecturer of Taxonomy, Institute of Horticulture, Zohria Botanical Garden. A voucher sample (No.: F-1) is kept in the Herbarium, Pharmacognosy Department, Faculty of Pharmacy, Helwan University.

Extraction and Isolation

Air-dried ground leaves and twigs (850 g) were extracted with hot 80 % aqueous methanol (5L, then 4 x 4L) under reflux (80 ˚C). After evaporation of solvent under reduced pressure, the residue (60 g) was defatted with petroleum ether (60-80 ˚C) under reflux (5 x 1.5 L, 60 ˚C). The residue (40 g), after evaporation of petroleum ether was re-dissolved in pure methanol to yield 29 g as dry methanol soluble portion. It was preliminary fractionated on a polyamide column (300 g, 110 x 7 cm) using a step gradient elution with H_2O-MeOH in ratio of 100:0 – 0:100 giving 28 fractions of 1L each, which were collected and monitored by Comp-PC (systems S_1 and S_2) and UV-light into three major collective fractions (I-III) with other non-phenolic fractions. Fraction I (3.5 g) was subjected to repeated column chromatography (CC) on microcrystalline cellulose using *n*-BuOH-2-propanol-H_2O BIW (4:1:5, organic layer as an eluent) followed by repeated and separately cellulose column for each major subfraction using MeOH/BIW (50 %) to give compounds **8** (13 mg), **9** (25 mg) and **10** (20 mg). Fraction II (1.7 g) was chromatographed on a Sephadex C and eluted with MeOH to give

pure sample of **3** (19 mg). Compound **5** (22 mg) was obtained by precipitation of fraction III (4.1 g) from conc. MeOH solution by excess EtOAc. Methanol insoluble portion (10 g) was re-dissolved in H_2O-MeOH (1:10), filtered and dried under vacuum to gives 8.5 g dry residue. Thereafter, it was fractionated on cellulose C (150 g, 70 x 5 cm) using aq. MeOH (10-70 %) to give three main fractions A, B and C. Fractionation of fraction A (250 mg) on Sephadex C (10 % aq. MeOH as eluent) led to isolate **2** (15 mg). Fraction B (2.8 g) was chromatographed on Sephadex C (10 % aq. MeOH as eluent) to give two major subfractions. The first main subfraction was then fractionated on Sephadex with BIW for elution to afford pure sample of **7** (11 mg), while the second one was separated on Sephadex C and eluted with 40 % aq. MeOH to give isolate **1** (9 mg). Fractionation of fraction C (95 mg) on Sephadex C using 10 % aq. MeOH followed by repeated and separately Sephadex C for each of the two major subfractions led to compounds **4** (9 mg) and **6** (20 mg). The homogeneity of the fractions was tested on 2D- and Comp-PC using Whatman No. 1 paper (systems S_1 and S_2); S_1: *n*-BuOH-HOAc-H_2O (4:1:5, top layer) and S_2: 15% aqueous HOAc. The compounds were visualized by spraying with Naturstoff reagent for detection of flavonoids and nitrous acid or KIO_3-reagents for detection of tannins.

3`-O-methyl-ellagic acid 3-sulphate (1)

Brown amorphous powder; R_f-values: 0.33 (S_1), 0.56 (S_2) on PC; mauve fluorescent spot by long and short UV-light turned to greenish-yellow with ammonia vapours, faint indigo-red colour with nitrous acid and faint blue with $FeCl_3$; UV λ_{max} (MeOH), nm: 240, 270 sh, 353 sh and 380; Negative HRESI-MS/MS: *m/z* 394.97169 $[M-H]^-$ (*calcd.*: 394.97145), (MS^2) 315.05426 $[M-H-SO_3]^-$, (MS^3) 300.06757 $[M-H-SO_3-CH_3]^-$ = [Ellagic acid-2H]$^-$. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 7.14 (2 H, br s, H-5/5`), 3.74 (3 H, s, $OCH_3$-3`).

Nilocitin (2)

Brown amorphous powder; R_f-values: 0.19 (S_1), 0.7 (S_2) on PC; it gives dark purple fluorescent spot by short UV-light, turned to indigo-red and deep blue colour by spraying with $NaNO_2$-glacial AcOH and $FeCl_3$, respectively. UV λ_{max} (MeOH), nm: strong absorption band at 245 with 283 sh. Negative ESI-MS/MS: *m/z* 481.25 $[M-H]^-$, (MS^2) 301.22 [Ellagic acid-H]$^-$, (MS^3) 257.07 [Ellagic acid-H-CO_2]$^-$, 229.10 [Ellagic acid-H-CO_2-CO]$^-$. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 6.43 (1 H in total, s, H-3``), 6.32 (1 H in total, s, H-3`), 5.23 (1/2 H, br s, H-1α), 5.10 (1/2 H, t-like, J = 9.6 Hz, H-3α), 4.85 (1/2 H, t-like, J = 9.9 Hz, H-3β), 4.81 (1/2 H, d, J = 8.1 Hz, H-1β), 4.69 (1 H, br d, J = 9.6 Hz, H-2α), 4.47 (1/2 H, t-like, J = 8.7 Hz, H-2β), 4.00 – 3.00 (m, hidden by H_2O-signal, remaining sugar protons). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 169.00 (C-7``α, β), 168.57, 168.34 (C-7`α, β), 144.46 (C-6`/6``α, β), 144.30 (C-4`/4``α, β), 134.91, 134.79 (C-5`/5``α, β), 125.49, 125.287 (C-2``α, β), 124.849 (C-2`α, β), 113.78, 113.70 (C-1``α, β), 113.67, 113.50 (C-1`α, β), 105.30, 104.92 (C-3`/3``α, β), 93.27 (C-1β), 89.76 (C-1α), 79.54 (C-3α), 77.12 (C-3β), 76.91, 76.68 (C-2α/2β), 74.24 (C-4β), 72.17 (C-5α), 67.51 (C-4α), 67.03 (C-5β), 60.46 (C-6α, β).

Strictinin (3)

Brown amorphous powder; R_f-values: 0.27 (S_1), 0.36 (S_2) on PC; it gives dark purple fluorescent spot by short UV-light, turned to indigo-red and deep blue colour by spraying with $NaNO_2$-glacial AcOH and $FeCl_3$ respectively; UV λ_{max} (MeOH), nm: 246, 288 sh. Negative HRESI-MS/MS: *m/z* 633.07410 $[M-H]^-$ (*calcd.* 633.07317), (MS^2) 481.04634 [M-H-galloyl]$^-$, 300.98930 [Ellagic acid-H]$^-$, (MS^3) 257.01764 [Ellagic-H-CO_2]$^-$, 229.02834 [Ellagic acid-H-CO_2-CO]$^-$, 213.01816 [Ellagic acid-H-OH-CO-CO_2]$^-$, (MS^4) 185.03564 [Ellagic acid-H-CO-2 CO_2]$^-$. ^{1}H NMR (500 MHz, DMSO-d_6): δ ppm 6.98 (2 H, s, H-2`/6` G), 6.48 (1 H, s, H-3```HHDP), 6.29 (1 H, s, H-3`` HHDP), 5.56

(1 H, d, J = 7.7 Hz, H-1), 4.94 (1 H, dd, J = 13, 6 Hz, H-6_a), 4.58 (1 H, t-like, J = 10 Hz, H-4), 4.03 (1 H, br dd, J = 9.2, 6.2 Hz, H-5), 3.67 (1 H, br d, J = 13 Hz, H-6_b), 3.55 (1 H, t-like, J = 9.2 Hz, H-3), 3.35 (1 H, t-like, J = 8.4 Hz, H-2). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 167.83, 167.09 (C-7```/7``HHDP), 164.62 (C-7` G), 145.62 (C-3`/5` G), 144.50, 144.41 (C-6``/6``` HHDP), 144.26. 144.23 (C-4``/4``` HHDP), 139.17 (C-4` G), 135.33, 134.96 (C-5``/5``` HHDP), 124.70, 124.42 (C-2``/2```HHDP), 118.42 (C-1` G), 115.55, 115.23 (1``/1```HHDP), 109.09 (C-2`/6` G), 106.29, 105.51 (C-3``/ 3``` HHDP), 94.73 (C-1), 73.82 (C-3), 73.33 (C-5), 71.61 (C-4/2), 62.74 (C-6); G = galloyl and HHDP = hexahydroxydiphenoyl moieties.

Casuarin (4)

Brown amorphous powder; R_f-values: 0.14 (S_1), 0.30 (S_2) on PC; it gives dark purple fluorescent spot by short UV-light and dull brown under long UV-light, turned to indigo-red and deep blue colour with $NaNO_2$-glacial AcOH and $FeCl_3$, respectively. UV λ_{max} (MeOH), nm: 246, 285 sh. ^{1}H NMR (500 MHz, DMSO-d_6): δ ppm 6.44, 6.24, 6.15 (1 H each, s, H-3````/3``/3``` HHDP), 5.38 (1 H, br s, H-1), 5.18 (1 H, br s, H-3), 4.8 (1 H, br s, H-4), 4.65 (1 H, br s, H-2), 4.34 (1 H, br d, J = 9.2 Hz, H-6_a), 3.91 (1 H, br s, H-5), 3.73 (1 H, br d, J = 10.7 Hz, H-6_b). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 169.27, 168.87, 167.57 (C-7``, C-7````, C-7```), 163.63 (C-7`), 145.79, 144.92, 144.64, 144.60 (C-6`/6``/6```/6```` HHDP), 144.42, 144.30, 144.11 (C-4``/4```/4```` HHDP), 142.62 (C-4` HHDP), 137.64 (C-5`), 135.68, 134.30, 133.74 (C-5``/5```/5```` HHDP), 126.06 (C-2``), 125.55 (C-2````), 123.56 (C-2```), 119.35 (C-2`), 115.89 (C-1`), 115.70, 115.41 (2C) (C-1``/1```/1```` HHDP), 114.32 (C-3` HHDP), 106.27 (C-3````), 104.67 (C-3``), 103.03 (C-3``` HHDP), 75.78 (C-2), 75.33 (C-4), 69.69 (C-3), 67.50 (C-1), 66.91 (C-5), 66.14 (C-6).

Castalagin (5)

Brown amorphous powder; R_f-values: 0.04 (S_1), 0.61 (S_2) on PC. It gives dark purple fluorescent spot by short UV-light and dull brown under long UV-light, turned to indigo-red and deep blue colour with $NaNO_2$-glacial AcOH and $FeCl_3$, respectively. UV λmax (MeOH), nm: 246, 288 sh. Negative HRESI-MS/MS: *m/z* 933.06324 $[M-H]^-$ (*calcd*. 933.06363), (MS^2) 914.92332 $[M-H-H_2O]^-$, 631.06571 [M-H-ellagic acid]$^-$, (MS^3) 896.95412 $[M-H-2H_2O]^-$, (MS^4) 878.95503 $[M-H-3H_2O]^-$, 301.01411 [Ellagic acid-H]$^-$; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 6.62 (1 H, s, H-3``` FLG), 6.52 (1 H, s, H-3```` HHDP), 6.38 (1 H, s, H-3````` HHDP), 5.47 (1 H, d, J = 4.5 Hz, H-1), 5.40 (1 H, br d, J = 7.8 Hz, H-5), 4.91 (1 H, t-like, J = 7.8 Hz, H-4), 4.89 (1 H, dd, J = 12.6, 3 Hz, H-6a), 4.82 (2 H, m, H-3/2), 3.96 (1H, br d, J = 12.6 Hz, H-6b). ^{13}C NMR (100 MHz, DMSO-d_6): δ ppm 168.14, 165.84 (C-7````/C-7````` HHDP), 165.80 (C-7``` FLG), 165.32 (C-7`` FLG), 162.87 (C-7` FLG), 146.40 (C-4``` FLG), 144.85, 144.73 (C-6````/6````` HHDP), 144.46, 144.12 (C-4````/4````` HHDP, 4`/4`` FLG), 143.67, 142.47, 142.35 (C-6`/6``/6``` FLG), 137.14 (C-5``` FLG), 135.75, 135.57 (C-5````/5````` HHDP), 135.11 (C-5`` FLG), 133.20 (C-5``` FLG), 125.91 (C-2`` FLG), 124.80, 123.52 (C-2````/2````` HHDP), 123.42 (C-2``` FLG), 121.10 (C-2` FLG), 115.25, 114.47, 114.25 (each 2C), (C-1````/1````` HHDP, C-1`/1``/1``` FLG, C-3` FLG), 111.70 (C-3`` FLG), 106.84 (each 2 C), 105.60 (C-3``` FLG, C-3````/3````` HHDP), 72.73 (C-2), 69.86 (C-5), 67.90 (C-4), 66.68 (C-1), 65.99 (C-3), 65.12 (C-6).

Ellagic acid (6)

Off-white amorphous powder; R_f-values: 0.39 (S_1), 0.19 (S_2) on PC; it gives buff fluorescent spot by long and short UV light, turned to dull yellow fluorescence on exposure to ammonia vapours, greenish yellow with Naturstoff, and faint blue colour with $FeCl_3$. UV λ_{max} (MeOH), nm: 215, 256, 362.

Negative HRESI-MS, *m/z* 300.99789 $[M-H]^-$ (*calcd.* 300.99896).

Ellagic acid 4-O-methyl ether (7)

Pale yellow amorphous powder; R_f-values: 0.29 (S_1), 0.30 (S_2) on PC; it gives faint mauve fluorescent spot under long and short UV light turned to greenish-yellow with ammonia vapours and faint blue with $FeCl_3$. UV λ_{max} (MeOH), nm: 245, 272 sh, 353 sh and 385. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 7.91 (1 H, s, H-5), 7.33 (1 H, s, H-5`), 3.86 (3 H, s, OCH_3-3).

Avicularin (8)

Yellow amorphous powder; R_f-values: 0.38 (S_1), 0.44 (S_2) on PC; it gave dark purple spot by long UV light turned to green with $FeCl_3$ and orange with Naturstoff reagent. UV λ_{max} (MeOH), nm: 296, 296 sh, 351; (+ NaOMe): 273, 327 sh, 403; (+ NaOAc): 270, 320 sh, 361; (+ NaOAc/ H_3BO_3): 270, 400; (+ $AlCl_3$): 273, 304 sh, 335 sh, 421; (+ $AlCl_3$/HCl): 268, 290 sh, 361 sh, 398. Negative HRESI/MS: *m/z* 433.07730 $[M-H]^-$ (*calcd.*: 433.07763), 867.16187 $[2M-H]^-$, 300.02756 [M-pentoside]$^-$ = [quercetin-H]$^-$. ^{1}H and ^{13}C NMR data are listed in **Table 1.**

Hyperin (9)

Yellow amorphous powder; R_f-values: 0.48 (S_1), 0.61 (S_2) on PC; it gave dark purple spot by long UV light turned to green with $FeCl_3$ and orange with Naturstoff reagent. UV λ_{max} (MeOH), nm: 261, 359; (+ NaOMe): 273, 411; (+ NaOAc): 265, 374; (+ NaOAc/ H_3BO_3): 262, 374; (+ $AlCl_3$): 252 sh, 260, 270, 408; (+ $AlCl_3$/HCl): 252 sh, 260, 270, 364. Negative HRESI/MS: *m/z* 463.08789 $[M-H]^-$ (*calcd.*: 463.08820), 927.18396 $[2M-H]^-$, 301.02255 [M-deoxyhexoside]$^-$ = [quercetin-H]$^-$, (MS^3) 178.89877 ($C_9H_7O_4$, cinnamoyl fragment), 150.89915 ($C_6H_3O_4$,

Table 1: ^{1}H and ^{13}C NMR spectral data of **8**-**10** (DMSO-d_6, 300 and 75 MHz)

Carbon	8		9		10	
No	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**
C-2	156.91		156.28		157.65	
C-3	133.37		133.49		134.58	
C-4	177.67		177.46		178.10	
C-5	161.18		161.17		161.66	
C-6	98.64	6.2 (d, 2.1)	98.68	6.2 (d,2.4)	99.05	6.2 (d,2.4)
C-7	164.19		164.06		164.58	
C-8	93.53	6.4 (d, 2.1)	93.52	6.4 (d,2.4)	93.98	6.39 (d,2.4)
C-9	156.32		156.25		156.80	
C-10	103.94		103.92		104.43	
C-1`	121.68		121.34		121.09	
C-2`	115.53	7.47 (d, 2.1)	115.19	7.58 (d,2.4)	115.82	7.23 (d,2.1)
C-3`	145.05		144.78		145.56	
C- 4`	148.43		148.43		148.80	
C-5`	115.53	6.85 (d, 8.1)	115.59	6.83 (d, 8.7)	116.01	6.86 (d,8.7)
C-6`	120.94	7.56 (dd, 8.1, 2.1)	121.10	7.66 (dd, 8.2, 2.4)	121.46	7.27 (dd, 8.7,2.1)
C-1``	107.86	5.58 (d, 1.2)	101.86	5.36 (d,7.8)	102.20	5.25 (d,1.5)
C-2``	82.09	4.15 (dd, 3.6, 1.2)	71.22	3.67-3.26 (m, H-2``, H-3``, H-4``, H-5``)	70.72	3.97dd (3.3,1.5)
C-3``	76.96	3.72 (m)	73.20		70.94	3.77-3.05 (m, H3``, H-4", H-5")
C-4``	85.86	3.57 (m)	67.34		71.55	
C-5``	60.66	3.33 (dd, 11.7, 2.75``a) 3.26 (dd, 11.7, 6.95``b)	75.81		70.41	
C-6``		-	60.15		17.85	0.80 (d, 5.7)

benzoyl fragment). ^{1}H and ^{13}C NMR data are listed in **Table 1.**

Quercitrin (10)

Yellow amorphous powder; R_f-values: 0.50 (S_1), 0.64 (S_2) same as (**9**) on PC; it gave dark purple spot by long UV light turned to green with $FeCl_3$ and orange with Naturstoff reagent. UV λ_{max} (MeOH) nm: 255, 299 sh, 353; (+ NaOMe): 268, 325 sh, 400; (+ NaOAc): 270, 320 sh, 370; (+ NaOAc/H_3BO_3): 260, 299 sh, 374; (+ $AlCl_3$): 273, 304 sh, 408; (+ $AlCl_3$/HCl): 268, 304 sh, 353 sh, 398. Negative HRESI/MS: *m/z* 447.09296 $[M-H]^-$ (*calcd.*: 447.09312), 301.03607 $[M\text{-deoxyhexoside}]^-$ = $[quercetin\text{-}H]^-$, (MS^3) 178.93030 ($C_9H_7O_4$, cinnamoyl fragment), 150.93245 ($C_6H_3O_4$, benzoyl fragment). ^{1}H and ^{13} C NMR data are listed in **Table 1.**

Pharmacological studies

Animals

Adult male pathogen-free Sprague-Dawley rats (120-130 g) and Swiss mice weighing (20-30 g) were purchased from the animal house of National Research Centre were used. The animals were housed in standard metal cages in an air-conditioned room at 22 ± 3°C, 55 ± 5% humidity, and 12 h light and provided with standard laboratory diet and water *ad libitum*. All experimental procedures were conducted in accordance with the guide for care and use of laboratory animals and in accordance with the Local Animal Care and Use Committee. The distilled water was used as a vehicle for extract and paracetamol and 5 % sodium bicarbonate for indomethacin.

Determination of median lethal dose (LD_{50})

The LD_{50} of the AME-80 was determined using rats. No percentage mortality was recorded after 24 hours up to dose of 5 g/kg and according to Semler (1992) who reported that if just one dose level at 5 g/kg is not lethal, regulatory agencies no longer require the determination of an LD_{50} value. So the experimental doses used were 1/20, 1/10 and 1/5 of 5/ g/kg of AME-80 (250, 500 and 1000 mg kg^{-1}).

Analgesic activity

This activity was determined by measuring the responses of animals to both thermal and chemical stimulus.

Thermal test

Hot-plate test was conducted using an electronically controlled hot-plate (Ugo Basile, Italy) adjusted at 52°C ± 0.1°C and the cut-off time was 30s (Eddy and Leimback, 1953). Five groups of mice each of six were used. The time elapsed until either paw licking or jumping is recorded 60 min before, and 1 and 2 h after oral administration of AME-80 (250, 500, 1000 mg kg^{-1}), saline and tramadol (20 mg kg^{-1} orally, a reference analgesic drug, October Pharma, 6th October, Egypt) (Sacerdote *et al.*, 1997).

Chemical test

Acetic acid-induced writhing in mice was performed according to the convenient published method (Collier *et al.*, 1968). The mice were divided into six groups each of six and received the same doses of AME-80, control as mentioned before, in thermal test. Indomethacin (25 mg kg^{-1}, Epico, Egypt) was used as reference drug. After 60 min interval, the mice received 0.6 % acetic acid *ip* (0.2 ml/mice). The number of writhes in 30 min period was counted and compared.

Anti-inflammatory activity

Anti-inflammatory activity in acute model was carried out according to the convenient reported method (Winter *et al.*, 1962). Rats were divided into five groups each of six and received orally saline as control, AME-80 (250, 500, 1000 mg kg^{-1}), and indomethacin (25 mg kg^{-1} orally) one hour before induction of oedema by subplanter injection of 100 µL of 1 % carrageenan injection (Sigma,

USA) in saline into the pad of right paw. The difference in hind foot pad thickness was measured immediately before and 1, 2, 3, 4 h after carrageenan injection with a micrometer caliber (Obukowicz *et al.*, 1998). The oedema was expressed as a percentage of change from the control group.

The percentage of oedema inhibition was calculated from the mean effect in the control and treated animals according to the following equation:

$$\% \text{ Oedema inhibition} = \frac{\% \text{ oedema formation of control group} - \% \text{ oedema formation of treated group}}{\% \text{ oedema formation of control group}} \times 100$$

Gastric ulcerogenic studies

Gastric lesions were induced in rats by ethanol (1 ml of 50 % orally). Animals fasted for 24 h and then divided into four groups, one group received ethanol and served as control group, and the remaining groups received AME-80 (250, 500 and 1000 mg kg^{-1}) one hour before the ethanol was given. Rats were killed 1 h following ethanol administration after being lightly anaesthetized with ether and the stomach was excised, opened along greater curvature, rinsed with saline, extended on plastic board and examined for mucosal lesions, using an ulcer score of 5 as described by Mozsik *et al.*, 1982. The ulcer scores were evaluated as follows: petechial lesions = 1, lesions less than 1 mm = 2, lesions between 1 and 2 mm = 3, lesions between 2 and 4 mm = 4, and lesions more than 4 mm = 5. A total lesion score for each animal is calculated as the total number of lesions multiplied by the respective severity score. Results are expressed as severity of lesions/rat.

Hepatoprotective study

The hepatic damage was induced in rats by oral administration of paracetamol 1 g/kg (Silva *et al.*, 2005). Fifty four rats were divided into nine groups of six animals each as following: group 1 (normal control group) received a daily oral dose of 1ml saline; groups 2, 3, 4 received a daily oral dose of AME-80 (250, 500 and 1000 mg/kg b. wt.) alone for successive 10 days; group 5 received a single oral dose of paracetamol (1 g kg^{-1} b. wt.); groups 6, 7, 8 received a daily oral dose of AME-80 (250, 500 and 1000 mg/kg b. wt.) for 10 successive days before paracetamol administration (1g/kg b. wt.); and group 9 received a daily oral dose of silymarin (25 mg/kg b. wt.) for successive 10 days before paracetamol (reference drug for hepatoprotective studies). At the end of the experimental period (24 h after paracetamol administration), the blood was obtained from all groups of rats after being lightly anaesthetized with ether by puncturing rato-orbital plexus (Sorg and Buckner, 1964). Finally, the following biochemical tests: Alanine aminotransferase (ALT) (Bergmeyer *et al.*,1986), aspartate aminotransferase (AST) (Klauke *et al.*, 1993) and serum alkaline phosphatase (ALP) (Tietz and Shuey, 1986) were performed.

Antioxidant activity

The activity of (1,1-Diphenyl,2-picryl hydrazyl) DPPH radical scavenging activity was investigated *in vitro* according to the method of Peiwu *et al.*, 1999. A methanolic solution of DPPH (2.95 ml) was added to 50 µl sample (AME-80) dissolved in methanol at different concentrations (10-100 mg/ml) in a disposable cuvette. The absorbance was measured at 517 nm at regular intervals of 15 seconds for 5 minutes. Ascorbic acid was used as a standard (0.1 M) as described by Govindarajan *et al.,* 2003.

$$\% \text{ inhibition (Reactive reaction rate)} = \frac{\text{Abs. (DPPH solution)-Abs. (sample)}}{\text{Abs (DPPH solution)}} \times 100$$

Statistical analysis

The results are expressed as mean ± S.E. and the statistical significance was evaluated by the student's *t*-test (Sendocor and Cechran, 1971) and one way ANOVA

(Dunnnett's multiple comparison test). The * P values < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Ten polyphenolic metabolites (**1**-**10**) were isolated from the aqueous methanol extract of *F. sellowiana* leaves and twigs using repeated column chromatographic separations with convenient adsorbents and solvent systems (see Materials and Methods section). Compounds **2** and **3** appeared as dark purple fluorescent spots under short UV-light changed into deep blue colour with $FeCl_3$ and indigo-red colour with HNO_2 spray reagent characteristic for hexahydroxydiphenoyl esters (ellagitannins), (Gupta *et al.*, 1982). The UV-spectrum showed intrinsic broad absorption band with a shoulder at about λ_{max} 283 nm characteristic for the ellagitannins. On complete acid hydrolysis **2** gave ellagic acid and glucose, whereas **3** yielded ellagic acid, gallic acid and glucose as well (Comp-PC). Negative ESI-MS of **2** exhibited a molecular ion peak at *m/z* 481.25 $[M-H]^-$ corresponding to a mono-hexahydroxydiphenoyl-glucose, while that of **3** showed a molecular ion peak at *m/z* 633.07410 with 152 amu more than that of **2** for extra galloyl moiety. As well as, at high fragmentation energy MS^2, MS^3 and MS^4 spectra exhibited the fragments, which were confirmative for the presence of a hexahydroxydiphenoyl group (HHDP) in **2** and HHDP with gallic in case of **3**. The positions of the attachment of galloyl or HHDP onto glucose core and its stereochemistry were concluded from NMR analysis. ^1H NMR of **2** exhibited two singlet signals, each one proton, at δ ppm 6.43 and 6.32 of one HHDP group (H-3``, 3`), while **3** exhibited two singlet signals, each of one proton (δ 6.48 and 6.29) with a singlet signal of two protons (δ 6.98) characteristic for a galloyl moiety. In the aliphatic region, **2** exhibited duplication of all ^1H-resonances which was indicative to a free anomeric-OH and the presence of **2** in the form of α/β-anomeric mixture (Okuda *et al.*, 1989). The downfield shift of H-3 and H-2 in both anomers at 5.10, 4.85 (H-3α/β) and 4.69, 4.47 (H-2α/β) indicated the attachment of the HHDP-group at C-2 and C-3 in case of **2**. In case of **3**, the downfield shift (Δ ≈ +1 ppm) of H-1, H-6a and H-4 at 5.56, 4.94 and 4.58 was intrinsic for the attachment of galloyl ester on OH-1 and bifunctional esterification of both OH-4 and OH-6 with HHDP moiety (Isaza *et al.*, 2004). The large J-values for all sugar signals were confirmative evidence for an *a/β*-$^4C_1$-pyranose structure of the glucose moiety in both compounds (Gupta *et al.*, 1982; Okuda *et al.*, 1983). The ^{13}C NMR spectrum of **2** showed also the duplication of all aliphatic and aromatic signals to confirm the *a/β*-configuration of the glucose, especially those of the anomeric carbon at 89.76 and 93.27 for C-1α and C-1β. In case of **3** the downfield location of C-1 (94.73, + 2.5 ppm) and upfield of C-2 (71.61, (≈ -2 ppm) gave an evidence of galloyl moiety at OH-1 (Okuda *et al.*, 1989). Similar effects were recorded due to esterification of OH-6 and OH-4 with HHDP as a downfield shift of C-4 and C-6 at δ 71.61 and 62.74 (Δ ≈ + 2.5 ppm) and upfield shift of C-3 and C-5 at 73.82 and 73.33, (Δ ≈ - 2 and 3 ppm). The remaining ^{13}C-signals were assigned by the comparison with published data of structural related compounds (Okuda *et al.*, 1983). Thus, **2** was confirmed as 2,3-(*S)*-hexahydroxydiphenoyl-(*a/β*)-D-glucopyranose (nilocitin) and **3** as 1-*O*-galloyl-4,6-(*S)*-hexahydroxydiphenoyl-*β*-D-glucopyranose (strictinin).

Like previous tannins **2** and **3**, isolates **4** and **5** exhibited characteristic chromatographic behavior and UV spectral data for ellagitannins. In addition the intensifying of UV-absorption and detection of these compounds as dull brown spot under long UV-light, referring to probability of more than one HHDP chromophore in the structure

of **4** and **5**. On complete acid hydrolysis, **4** and **5** gave ellagic acid together with an ellagitannin intermediate in the organic phase. The absence of the glucose in the aqueous hydrolysate and detection of the ellagitannin intermediate was strong evidence for *C*-glycosidic structure of **4** and **5**. ^{1}H NMR spectrum of **4** showed in its aromatic region three singlets at δ 6.44, 6.24 and 6.15 (each one proton) of two HHDP ester moieties with absence of one proton (H-3`) due to oxidative coupling and formation of extra C-C linkage with the anomeric carbon. Whereas, ^{1}H NMR of **5** exhibits three singlets, each of one proton, at δ 6.62, 6.52 and 6.38 ppm for a *C*-glycosidic flavogallonoyl attached to C-1, C-2, C-3 and C-5 and HHDP at C-4 and C-6 of an open chain glucose structure. The δ- and J- values of the glucose moiety, especially that of H-1 at ~ 5.4 as broad singlet in **4** and with J = 4.5 in **5**, were confirmative for full substituted open chain glucose with an anomeric axial hydroxyl group (Okuda *et al.*, 1983; Moharram *et al.*, 2003). Unlike **2** and **3**, the nature of glucose in **4** and **5** as open chain instead of hemiacetal $^{4}C_{1}$–pyranose was clearly proved due to the strong upfield location of C-1 at about 67 ppm in comparison to that of pyranose at about δ 90 – 95 ppm. As well as the *C*-glycosidic nature was further evidenced from the typical down field shift of C-3` at ~114 (≈ +10 ppm) and upfield location of C-7` at ~ 163 (≈ - 6 ppm) of HHDP in **4** and flavogallonoyl in **5** with respect to those of HHDP in case of **2** and **3** (Okuda *et al.*, 1983; Moharram *et al.*, 2003). All other ^{13}C resonances were assigned as it was mentioned above according to a comparison study with previously reported data of *C*-glycosidic tannins (Okuda *et al.*, 1983; Moharram *et al.*, 2003; Vivas *et al.*, 1995). Accordingly, **4** was identified as 2,3:4,6-bis-[(*S*)-hexahydroxydiphenoyl]-*ß*-D-glucopyranose (casuariin), and **5** as 2,3,5-(*S*)-flavogallonoyl-4,6-(*S*)-hexahydroxydiphenoyl-D-glucose (castalagin).

Isolates **1**, **6** and **7** displayed more or less the same chromatographic properties (R_f-values, colour under UV-light and fluorescence changes on exposure to ammonia vapours and with different spray reagents) of ellagic acid and ellagic acid derivatives (Hillis and Yazaki, 1973). Also, their UV spectral data are characteristic for ellagic acid and its derivatives (Tanaka *et al.*, 1986). Negative HRESI-MS revealed a molecular ion peak at *m/z* 300.99789 $[M-H]^-$ (MF $C_{14}H_5O_8$) and 394.97169 $[M-H]^-$ (MF $C_{15}H_7O_{11}S_1$) for **6** and **1**, consistent with ellagic acid and methoxy ellagic acid sulphate, respectively. ^{1}H NMR spectrum of **1** exhibited a relatively upfield broad singlet of two protons at δ 7.14 together with a singlet of three protons at δ 3.74, conforming the attachment of the methoxy and sulphate groups to C-3 and C-3`. In case of **7**, the downfield shift of H-5 at δ 7.91 together with other singlet for H-5 at δ 7.33 and singlet of three protons at δ 3.86 were informative for the methoxylation of 4-OH. Therefore, **6** was identified as ellagic acid, **7** as ellagic acid 4-*O*-methyl ether and **1** as ellagic acid 3`-*O*-methyl ether 3-sulphate.

Based on their chromatographic properties and UV spectral data, compounds **8-10** were expected to be quercetin 3-*O*-glycoside. UV-spectrum in MeOH showed the two characteristic absorption maxima at about λ_{max} 260 (band II) and 354 (band I) for a quercetin aglycone (Mabry *et al.*, 1970). The presence of a free 4`-OH group was deduced from the bathochromic shift in band I (+ ~50 nm) that was accompanied with hyperchromic effect (Mabry *et al.*, 1970). On addition of NaOAc, a bathochromic shift in band II was an evidence for a free 7-OH. Moreover, the remaining of the bathochromic shifts occurred in band I and II in both NaOAc and $AlCl_3$ spectra after the addition of H_3BO_3 and HCl revealed the *ortho* 3`,4`-dihydroxy-function in ring B and the free OH-5 in ring A. Complete acid hydrolysis produced quercetin in the organic phase of the three compounds (Comp-PC), while

arabinose, galactose or rhamnose were separately detected in the aqueous hydrolysate of **8**, **9**, and **10**, respectively. Negative HRESI-MS spectrum exhibits a molecular ion peak at *m/z* 433.07730, corresponding to a Mwt of 434.36 and MF $C_{20}H_{18}O_{11}$ for a quercetin pentoside in **8**, 463.08789 corresponding to the MF $C_{21}H_{20}O_{12}$ of a quercetin hexoside for **9** and 447.09296 corresponding to the MF $C_{21}H_{19}O_{11}$ in case of **10**. This evidence was further supported by the fragment ions at *m/z* 301.05, 179.00 and 151.00 as characteristic peaks for quercetin aglycone. The ^{1}H NMR spectra of **8-10** showed in the aromatic region the two characteristic spin coupling systems i.e. ABX (H-2`d, H-6` dd, H-5`d) and AM (H-8 d; H-6 d) for 3`,4`-dihydroxy ring B and 5,7-dihydroxy ring A protons. In the aliphatic region, a doublet at 5.58 with J_{12} = 1.2 Hz, was characteristic for an *α*-L-arabinofuranoside moiety (Servettaz *et al.*, 1984) in case of **8**. Also, a doublet at 5.36 (J_{12} = 7.8) gave an evidence for a *β*-galactopyranose structure of galactoside in **9**; as well as the sugar moiety in **10** was identified as α-rhamnopyranoside from the two doublet signals at 5.25 (J_{12} = 1.5) and 0.8 (J = 5.7) for H-1`` and $CH_3$-6``. ^{13}C NMR spectra of **8-10** showed 15 carbon resonances among which the key signals at about δ 177 (C-4), 148 (C-4`), 145 (C-3`) and 133 (C-3) for a 3-*O*-glycosylquercetin (Agrawal and Bansal, 1989). Concerning sugar moiety of **8**, the presence of five highly strained and downfield shifted ^{13}C resonances particularly those at 107.86, 85.86 and 82.09 assignable to C-1``, C-4`` and C-2`` gave an evidence for an arabinofuranoside moiety (Agrawal and Bansal, 1989). In case of **9** the presence of galactose moiety was confirmed from typical six signals, particularly those of C-5`` and C-3``, which have a difference more than 1 ppm (Agrawal and Bansal, 1989). Finally sugar moiety in **10** was identified as rhamnose from the presence of characteristic six carbon signals particularly that of $CH_3$-6`` at δ 17.85. Thus, **8** was identified as quercetin 3-*O*-α-L-arabinofuranoside (avicularin), **9** as quercetin 3-*O*-β-D-$^{4}C_1$-galactopyranoside (hyperin) and **10** as quercetin 3-*O*-α-L-$^{1}C_4$-rhamnopyranoside (quercitrin).

The determination of the LD_{50} in rats revealed that oral administration of AME-80 was non toxic up to 5 g/kg. In the thermal test, it produced a significant prolongation in the reaction time to the thermal stimulus in mice by 26.4, 34.9 and 40.1 (after 1 h) and 31.8, 39.2 and 44.6 % (after 2 h) for the three doses used as compared with control pre-drug value. Moreover, tramadol exhibited a significant prolongation in the reaction time by 52.8 and 87.2 % at 1 and 2 h, respectively **(Fig. 1a)**. On the other hand, in the chemical test the oral administration of AME-80 showed a significant decrease of the number of writhes in mice after acetic acid injection in dose-dependant manner (27.2, 45.8 and 67.5 %, at 250, 500 and 1000 mg kg^{-1}, respectively) as compared with saline control group. Indomethacin showed a significant decrease of number of writhes by 82.29 % **(Fig. 1b)**. In addition, AME-80 exhibited a significant anti-inflammatory effect at 250 and 500 mg kg^{-1} after 1 and 2 h post carrageenan injection, while the dose of 1000 mg kg^{-1} exhibited a significant inhibition of oedema after 1, 2, 3 and 4 hours as compared with saline control **(Fig. 2)**. The administration of AME-80, 1 h before the induction of gastric lesions by oral administration of ethanol, induced the reduction of the number and severity of gastric mucosal lesions at 500 and 1000 mg kg^{-1} by 31.8, 34.3 and 45.9, 42.5 %, respectively compared to ethanol control group **(Fig. 3)**.

Concerning the hepatoprotective effect, AME-80 given to rats at 250, 500 and 1000 mg kg^{-1} showed significant reduction in elevated serum levels of ALT and AST by 44.2, 51.1, 50.2 % and 20.7, 21.9, 30.4 %, respectively in dose dependant manner as compared with paracetamol treated group **(Fig. 4a & b)**. Also, a significant decrease in ALP serum level was recorded on treatment

with the same above mentioned doses by 20.1, 23.3 and 36.9 %, respectively. Additionally, silymarin (25 mg/kg) exhibited significant reduction in serum ALT, AST and ALP levels as compared with paracetamol treated group (Isaza *et al.*, 2004; Moharram *et al.*, 2003; Vivas *et al.,* 1995).

The hepatoprotective study of the AME-80 was supported by histopathological study of the liver tissues, which showed a significant gradual improvement in liver architecture. The high dose level (1000 mg kg^{-1}) led to a noticeable improvement in the portal area oedema, congestion and fibrosis. Blood sinusoids were returned to approximately normal size. Normal-shaped and sized hepatocytes were also **(Fig. 5a, b, c)**. Also normal shape hepatocytes are observed. Finally, the AME-80 exhibited an *in vitro* marked significant scavenging activity for DPPH radicals at different concentrations (10-100 mg/ml). The maximum reactive reaction rate after 5 minutes was 88.8, 87.1, 87.7, 86.6, 88.1, 84.9, 88.1, 85.8, 85.4 and 85.6 %, respectively as compared with 96.1 % solution of L-ascorbic acid (antioxidant reference drug) **(Fig. 6a & b)**.

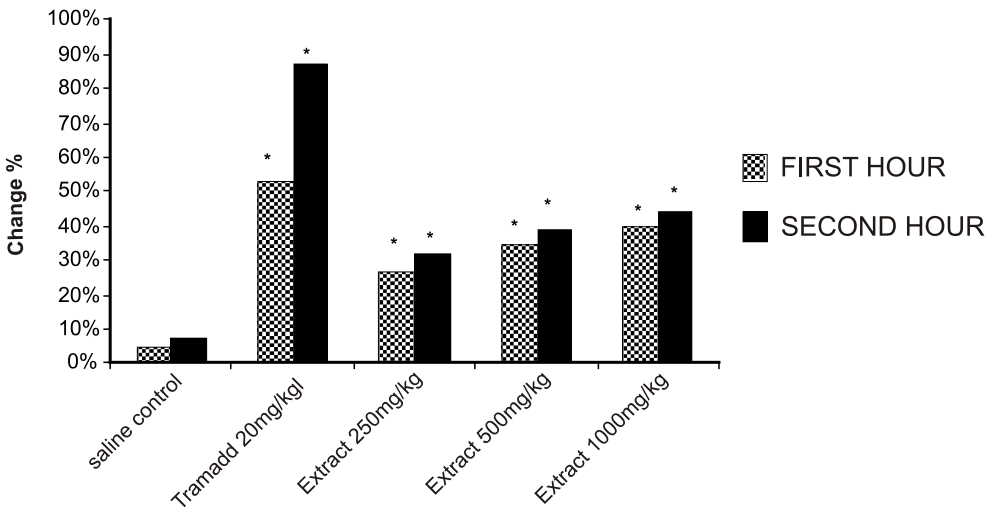

Figure 1a. Analgesic effect of oral administration of AME-80 (250, 500 and 1000 mg/kg) and tramadol (20 mg/kg) on thermal pain induced by using the hot plate test in mice. Data represent the percentage change from the basal (zero time), 1h and 2h values for each group (saline, tramadol and tested extract). Statistical comparisons between basal (pre-drug values) and post-drug values. Data were analyzed by using (Student´s t test). * = $P < 0.05$

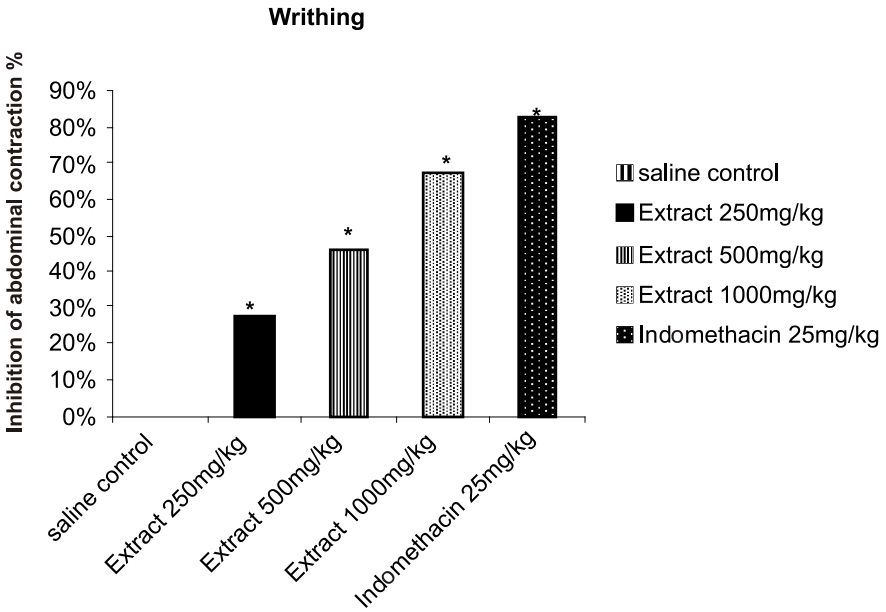

Figure 1b. Analgesic effect of oral administration of AME-80 (250, 500 and 1000 mg/kg) and indomethacin (25 mg/kg) on visceral pain [total number of abdominal stretches (contractions)] by using writhing test in mice. Data represent the percentage inhibition of number of writhes/30 min. Statistical comparison of the difference between saline control group and treated groups is done by (Student´s t test), * = $p < 0.001$

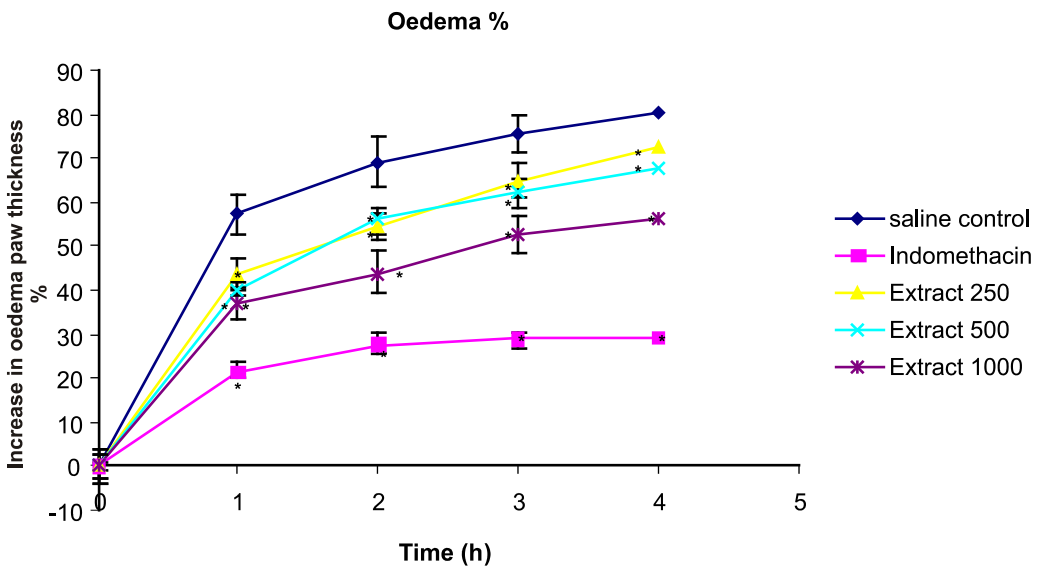

Figure 2. The anti-oedema effect of AME-80 and indomethacin in rats. Result are expressed as a percentage change from control (pre-extract) values, each point represents mean ± S.E of rats per group. Data were analyzed using one way ANOVA and Duncan's multiple comparison test * $P < 0.05$. Asterisks indicate significant change from control value at respective time points.

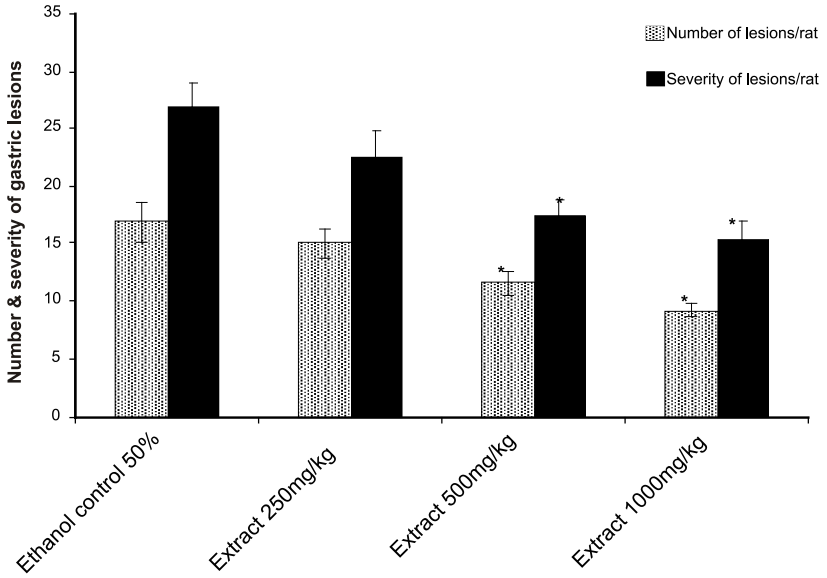

Figure 3. The effect of AME-80 (250, 500 and 1000 mg/kg) on the number and severity of gastric mucosal lesion in the ethanol treated rats (1ml of 50 % ethanol orally). Data represent the mean value ± SE of six rat per group. Asterisks indicate significant change from ethanol group. Data were analyzed using one way ANOVA and Duncan's multiple comparison test * $P < 0.05$.

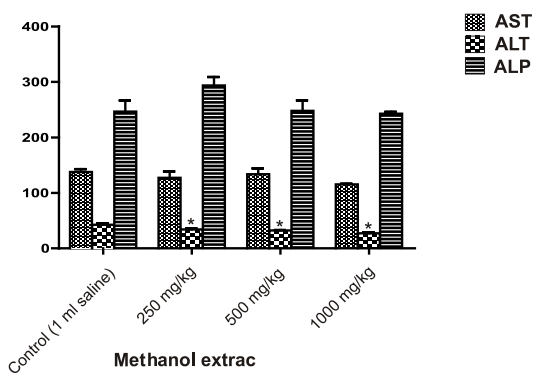

Figure 4a: The effect of rats´ oral administration of AME-80 on AST, ALT and ALP serum activity

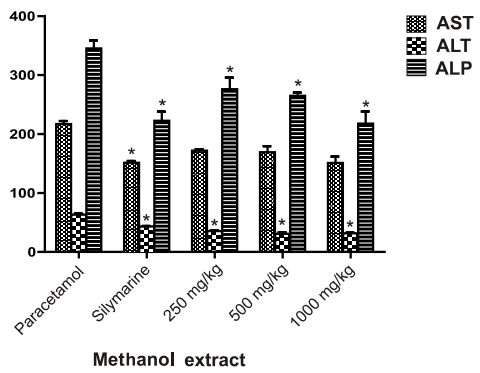

Figure 4b: Effect of oral rats´ oral administration of AME-80 on AST, ALT and ALP serum activity in paracetamol induced hepatotoxicity

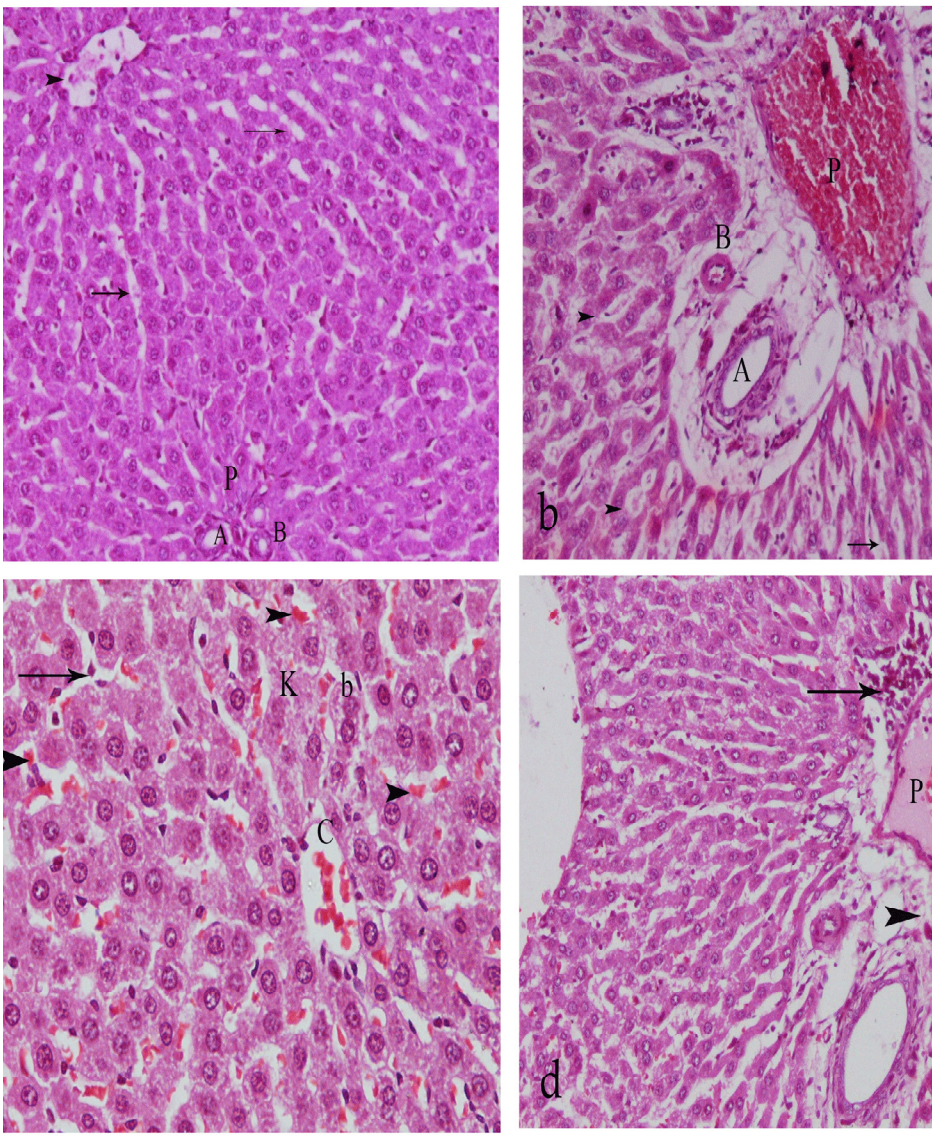

Figure 5a. Photomicrograph of a liver section of:
(a) a control rat
(b) a paracetamol treated rat
(c) a silymarin treated rat, 10 days before paracetamol administration
(d) a rat treated with AME-80 in a dose of 250 mg, 10 days before paracetamol administration
(Hx. & E. x 200)

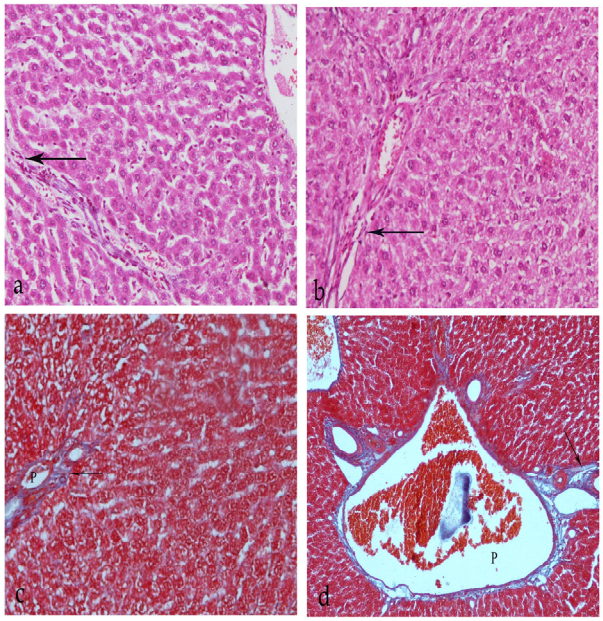

Figure 5b. Photomicrograph of a liver section of:
(a) a rat treated with AME-80 in a dose of 500 mg, 10 days before paracetamol administration
(b) a rat treated with AME-80 in a dose of 1000 mg, 10 days before paracetamol administration (Hx. & E. x 200)
(c) a control rat (M.T. x 100)
(d) a rat treated with paracetamol

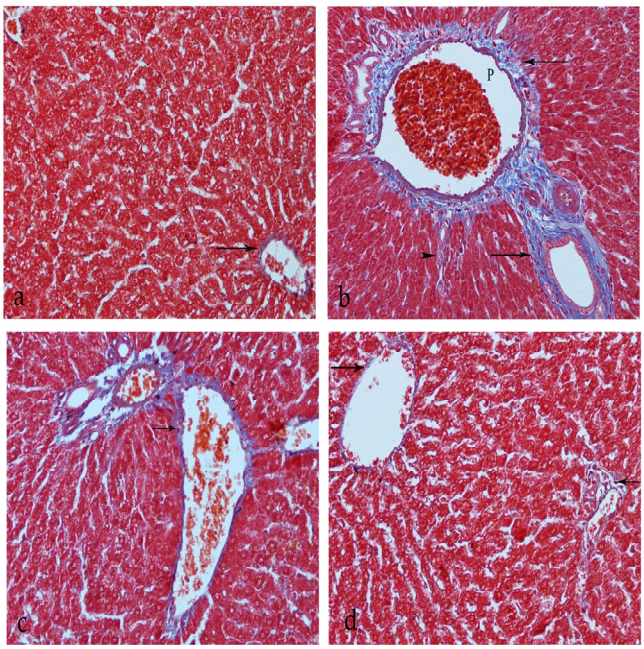

Figure 5c. Photomicrograph of a liver section of:
(a) a rat treated with silymarin, 10 days before paracetamol injection
(b) a rat treated with AME-80 in a dose of 250 mg, 10 days before paracetamol administration
(c) a rat treated with AME-80 in a dose of 500 mg, 10 days before paracetamol administration
(d) a rat treated with AME-80 in a dose of 1000 mg, 10 days before paracetamol administration
(M.T. x 200)

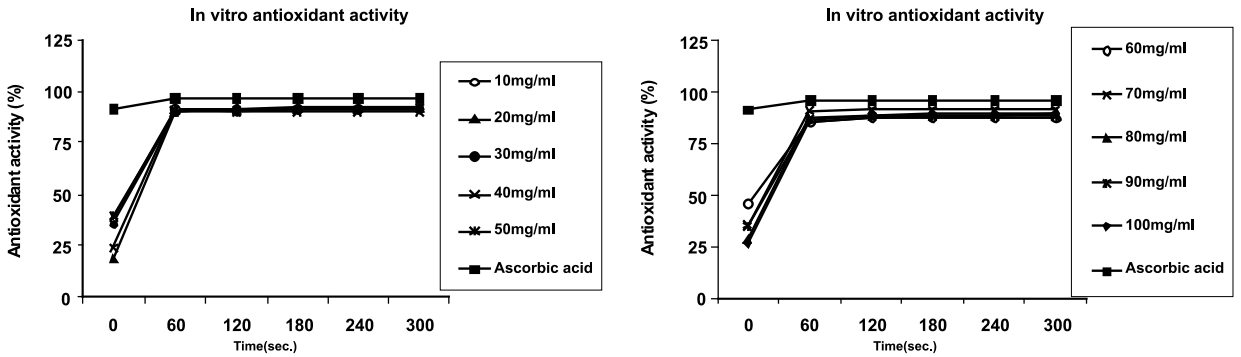

Figure 6a & b. Antioxidant activity of AME-80 and ascorbic acid *in vitro*, using DPPH radical scavenging activity method.

CONCLUSION

The 80 % aqueous methanol extract of *F. sellowiana* leaves and twigs (AME-80), that is rich in tannins and flavonoids, exhibited significant analgesic, anti-inflammatory, antiulcer, antioxidant and hepatoprotective activities. These effects appeared to be dose-dependant.

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