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Polyphenolic profile and biological activity of *Salvia splendens* leaves

Fatma Abd-elkader Moharram[a,d], Mohamed Soubhi Marzouk[b], Siham Mustafa El-Shenawy[f], Ahmed Hamed Gaara[c,e] and Wafaa Mostafa El Kady[g]

[a]Department of Pharmaceutical Science, College of Clinical Pharmacy, King Faisal University, Al – Hasa, [b]Department of Pharmacetical Chemistry, College of Pharmacy, King Saud University, Riyadh, [c]Department of Chemistry, College of Science, Jazan University, Jazan, Saudi Arabia, and [d]Faculty of Pharmacy, Department of Pharmacognosy, Helwan University, Helwan, Departments of [e]Natural Compound Chemistry and [f]Pharmacology, National Research Centre, Dokki, [g]Departments of Pharmacognosy, Faculty of Pharmacy, Future University, Cairo, Egypt

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Correspondence
Fatma Abd-elkader Moharram, College of Clinical Pharmacy, King Faisal University, Pharmaceutical Science, Al- Ahsa 400, Saudi Arabia.
E-mail: famoharram1@hotmail.com

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Abstract

Objectives The aim of this study was to investigate a new flavone triglycoside, together with eleven phenolic metabolites from 80% aqueous methanol extract of *S. splendens* leaves (**AME**) and assessment of its hypoglycemic and antiinflammatory activities along with *in vitro* antioxidant effect.

Methods The phenolic composition of *S. splendens* leaves was analyzed using UV, 1D and 2D NMR and negative ESI-MS spectroscopy. Hypoglycemic activity of **AME** was assessed by measuring blood glucose in streptozotocin induced-diabetic rats. Antiinflammatory activity was evaluated using the carrageenan-induced paw oedema test. Antioxidant activity was evaluated *in vitro* using DPPH test.

Key findings Twelve phenolic metabolites including three phenolic acids, namely caffeic acid **1**, rosmarinic acid **2** and methyl rosmarinate **3**; four flavone glycosides *viz* the new compound luteolin 7-*O*-(4″,6″-di-*O*-α-**L**-rhamnopyranosyl)-β-**D**-glucopyranoside **4**, apigenin 7-*O*-β-**D**-rutinoside **5**, cosmosiin **6** and cinaroside **7**, together with four flavones aglycone, luteolin **8**, apigenin **9**, pedalitin **10** and crisiliol **11** in addition to one coumarin, 6,7-dihydroxycoumarin **12** were isolated from the leaves of *S. splendens* Sellow ex Roem & Schult. The **AME** of *S. splendens* was non toxic to mice up to 5 g/kg b.wt. it exhibited a significant hypoglycemic activity at 250 and 500 mg/kg as compared with control pre-drug (zero time) for each group as well as the diabetic control. Moreover, **AME** exhibited a significant antiinflammatory activity only at 1000 mg/kg in comparison to indomethacin. Finally, **AME** exhibited a marked significant scavenging activity against DPPH; the maximum reactive reaction rate after 5 min was 62.9, 82.5, 83.7, 84.3 and 85.1% for the concentrations 10, 20, 30, 40 and 50 mg/ml, respectively in comparison to L-ascorbic acid (86.8%).

Conclusions This is the first study reporting the identification of a new flavone triglycoside, along with eleven known phenolic metabolites from **AME** of *S. splendens*. It showed significant hypoglycemic and antiinflammatory effects in dose dependant manner. Moreover it showed an *in vitro* antioxidant activity.

Introduction

Salvia splendens Sellow ex Roem & Schult (Lamiaceae), commonly has known as Scarlet sage.[1] It is a perennial herb native to Brazil and widely cultivated for its ornamental value. The genus *Salvia* is known to be a rich source of flavonoids and phenolic acids with an excess of 160 polyphenols been identified.[2] Flavone aglycones and glycosides based on apigenin and luteolin and their 6-hydroxylated and methyl ether derivatives are mainly present.[3,4] In addition to many phenolic acid based on caffeic acid building unit as caffeic, ferulic[5] and rosmarinic acids.[6,7] From the biological point of view, the importance of *Salvia* was derived from their broad antioxidant, antiplatelet, antiviral, hepatoprotective, antimutagenic, antitumor, antipyretic and antidiabetic activities.[2,8,9] Previous reports demonstrated that only anthocyanin

derivatives from the flowers,[10–13] as well as diterpenes[14–17] were isolated from *S. splendens* in addition to its toxicity, antidiabetic[18] antimicrobial[19] and anticoagulant[20] were done. This prompted us to investigate the bioactive constitutes of this plant. The main aim was the determination of hypoglycemic and antiinflammatory activities and in addition to the *in vitro* antioxidant effects of the **AME** of *S. splendens* leaves, and for the first time isolation and structure characterizations of its phenolic content supported by full ^{1}H and ^{13}CNMR and negative ESI-MS spectroscopy.

Materials and methods

Plant material

Salvia splendens Sellow ex Roem & Schult. leaves were collected from plants grown in Al-Zohria Botanical Garden, Giza, Egypt in June 2006 during the flowering stage, since the plant grow in warm-weather and high humidity regions. Authentication of the plant was performed by Dr. Mohamed El-Gibaly, Lecturer of Taxonomy and Consultant for Central Administration of Plantation and Environment, Cairo, Egypt. Voucher specimen (No. S.S. 1) has been deposited in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt.

General experimental procedures

For column chromatography, sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (E. Merck, Darmstadt, Germany) and polyamide S (Fluka Chemie AG, Switzerland) were used. For paper chromatography; Whatman no. 1 sheets (Whatman Ltd, Maidstone, Kent, England) were used. The pure compounds were visualized by spraying with Naturstoff reagent[21] [(a)1% diphenyl boryloxyethanolamine in ethanol, (b) 5% polyethylene glycol 400 in ethanol, heating the dry chromatogram at 120°C for 10 min. and visualizing under UV light (365 nm)] and $FeCl_3$ (1% in ethanol). Solvent systems S_1 (*n*-BuOH/HOAc/H_2O; 4 : 1 : 5 v/v/v top layer), S_2 (15% aqueous HOAc) and S_3 (*n*-BuOH/*iso*-propanol/H_2O; 4 : 1 : 5, v/v/v top layer) were used.

The NMR spectra were recorded at 300, 400 and 500 (^{1}H) and 75, 100 and 125 (^{13}C) MHz, on a Varian Mercury 300, a JEOL GX-400 and a 500 NMR spectrometers, respectively. The results were reported as δ ppm values relative to TMS in the convenient solvents.

ESI-MS analyses were run on LCQ (Finnigan MAT, Bremen, Germany) and LCQ-FT-MS mass spectrometers (Thermo Electron 400, Waltham, USA).

UV analyses for pure samples were recorded on MeOH solutions and with different diagnostic UV shift reagents on a JASCO (V-630) UV spectrophotometer.

Extraction and isolation

The powder of air-dried leaves (760 g) was exhaustively extracted with 80% aqueous methanol under reflux (4 L × 6, 70°C, 4 h). The residue left after evaporation of the solvent (220 g) was pre-purified with $CHCl_3$ under reflux (2 L × 2, 50°C). After evaporation of $CHCl_3$ under vacuum, dry chloroform extract (27 g) and 180 g residue were obtained. This residue was precipitated from H_2O with excess methanol (1 : 10) and the filtrate was evaporated under reduced pressure to give 150 g dry residue. It was fractionated on a polyamide S column (300 g, 110 × 7 cm) using a step-gradient of H_2O-MeOH, 100 : 0 – 0 : 100 to give 27 fractions of 1 L each, collected into 8 major collective fractions (I-VIII), monitored by comparative paper chromatography (comp-PC) and UV-light. Fraction I (3.55 g) was found to be polyphenolic-free ($FeCl_3$ spray reagent/PC). Fraction II (650 mg) was chromatographed on a microcrystalline cellulose column using H_2O/MeOH mixture (50 : 50) as an eluent, followed by repeated fractionation on sephadex columns to give pure sample of **1** (15 mg). Fraction III (430 mg) was purified on consecutive cellulose columns using 10% aqueous MeOH to give pure samples of **2** (25 mg) and **3** (18 mg). Purification of fraction IV (180 mg) was achieved by sephadex LH-20 column using MeOH/H_2O 40–60% to afford **4** (18 mg). Fraction V (100 mg) was purified by repeated chromatography on several sephadex columns using step gradient aqueous MeOH (10–60%) for elution to give pale yellow pure **5** (20 mg). Fraction VI (1.1 g) was applied on a sephadex LH-20 column using BIW [*n*-BuOH/*iso*–propanol/ H_2O (4 : 1 : 5, v/v/v upper layer)] for elution giving two main sub fractions. Each sub fraction was further purified on cellulose column using water as eluent and decreasing the polarity by MeOH to give pure sample of **6** (13 mg) and **7** (17 mg). Fraction VII (670 mg) was chromatographed on sephadex LH-20 columns using aqueous MeOH with decreasing polarity followed by repeated cellulose columns for each of the three major sub fractions using 50% methanol, which led to the isolation of pure compounds **8** (14 mg), **9** (11 mg) and **12** (20 mg). Fraction VIII (290 mg) was chromatographed on microcrystalline cellulose using H_2O/MeOH mixture (30 : 70) as an eluent, followed by repeated sephadex columns for each of the two major sub fractions to yield pure **10** (22 mg) and **11** (25 mg). The homogeneity of all fractions was tested by 2D- and comp-PC on Whatman no. 1 paper sheets with solvent systems S_1 and S_2.

Caffeic acid (1)

Off-white amorphous powder; R_f values 0.72 (S_1), 0.42 (S_2) on PC. It gave sky blue fluorescence under UV-light, changed to greenish blue fluorescence with Naturstoff (NA/PE) and blue color with $FeCl_3$ spray reagent. UV λ_{max} nm: (MeOH) 283, 313

Table 1 ^{1}H and ^{13}C NMR data of compounds 1–3

	1[a]	2[b]		3[b]	
C No.	δ_H	δ_H	δ_C	δ_H	δ_C
1			125.88		125.86
2	7.03 *br s*,	7.03 *br s*	115.35	7.04 *br s*	115.38
3			144.46		144.49
4			145.43		145.45
5	6.76 *d* (8.4)	6.73 *d* (8)	116.31	6.74 *d* (8)	116.33
6	6.95 *br d* (8.1)	6.96 *br d* (8.5)	122.10	6.97 *br d* (8.4)	122.11
7	7.40 *d* (15.9)	7.41 *d* (16.1)	146.28	7.42 *d* (16.1)	146.35
8	6.15 *d* (15.9)	6.20 *d* (16.1)	113.95	6.20 *d* (16.1)	113.88
9			166.52		166.52
10		4.96 *dd* (8.4, 3.8)	73.74	4.98 *dd* (8.4, 3.8)	73.64
11_a		2.99 *dd* (13.2, 3.8)	36.75	2.95 *dd* (13.2, 3.8)	36.72
11_b		2.90 *dd* (13.2, 8.4)		2.86 *dd* (13.2, 8.4)	
12			128.12		128.03
13		6.64 *br s*	117.19	6.66 *br s*	117.22
14			146.14		146.15
15			149.12		149.16
16		6.60 *d* (8.4)	115.90	6.61 *d* (8.2)	115.92
17		6.48 *br d* (8.4)	120.53	6.49 *b d* (7.7)	120.54
18			171.74		170.50
Me				3.60 *s*	52.54

J-values (Hz) were reported in parenthesis. [a](300 and 75 MHz, DMSO-d_6); [b](500 and 125 MHz, DMSO-d_6).

(+NaOH) 298, 350. ^{1}H NMR (300 MHz, DMSO-d_6) data are listed in Table 1.

Rosmarinic acid (2)

Creamy-white amorphous powder; R_f values 0.72 (S_1), 0.53 (S_2) on PC. It gave blue fluorescence under UV-light, changed to greenish blue fluorescence with ammonia vapors, shine bluish green fluorescence with NA/PE and blue color with $FeCl_3$. UV λ_{max} nm: (MeOH) 250 sh, 290, 329; (+NaOH) 256 sh, 302, 368. Negative ESI-MS: *m/z* 315.01 $[M\text{-}H_2\text{-}CO_2]^-$. ^{1}H and ^{13}C NMR (500 and 125 MHz, DMSO-d_6) data are listed in Table 1.

Methyl rosmarinate (3)

Gave the same physical and chromatographic behavior as **2**. R_f values 0.69 (S_1), 0.49 (S_2) on PC. UV λ_{max} nm: (MeOH) 250 sh, 289, 331; (+NaOH) 254, 303, 373. Negative ESI-MS: *m/z* 373.02 $[M\text{-}H]^-$, 315.14 $[M\text{-}CH_3\text{-}CO_2]^-$, 179.0 [M-dihydromethylcaffeate]$^-$ = [caffeate]$^-$. ^{1}H and ^{13}C NMR (500 and 125 MHz, DMSO-d_6) data are listed in Table 1.

Luteolin7-O-(4″, 6″-di-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside (4)

Pale yellow amorphous powder, R_f values 0.16 (S_1), 0.17 (S_2) on PC. It gave a green color on spraying with $FeCl_3$; orange fluorescence with Naturstoff reagent. UV λ_{max} nm: MeOH 258, 272, 347; (+NaOMe) 270, 300 sh, 394; (+NaOAc) 278, 389; (+NaOAc/H_3BO_3) 273, 360; (+$AlCl_3$) 272, 297 sh, 337, 410; (+$AlCl_3$/HCl) 272, 297 sh, 388. Negative ESI-MS: *m/z* 739.0 $[M\text{-}H]^-$, 591.0 [M-deoxyrhmnosyl-2H]$^-$, 447.0 [M-deoxydirhamnosyl]$^-$, 284.9 [deoxyrhmnosylrutinoside]$^-$ = [luteolin-H]$^-$. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 7.39 (1H, *br s*, H-2′), 7.32 (1H, *m*, H-6′), 6.86 (1H, *d*, J = 8.4 Hz, H-5′), 6.69 (1H, *s*, H-3), 6.64 (1H, *br s*, H-8), 6.41 (1H, *br s*, H-6), 5.02 (1H, *d*, J = 7 Hz, H-1″), 4.63 (1H, *br s*, H-1‴), 4.50 (1H, *br s*, H-1⁗), 4.83 (1H, *t-like*, J = 9.1 Hz, H-4″), 3.89 (1H *br d*, J = 11.4, Hz H-6_a″), 3.81(1H, *br s*, H-2‴), 3.76 (1H, *br s*, H-2⁗), 3.67 (1H *dd*, J = 12 &4.5 Hz, H-6_b″), 3.53- 3.10 (*m*, remaining sugar protons hidden by H_2O-signal), 1.07 (3H, *d*, J = 6.1 Hz, CH_3-6‴), 1.02 (3H, *d*, J = 6.1 Hz, CH_3-6⁗). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 181.90 (C-4), 163.39 (C-2), 162.40 (C-7), 159.61 (C-5), 157.53 (C-9), 149.99 (C-4′), 147.11 (C-3′), 121.20 (C-1′), 121.01 (C-6′), 116.71 (C-5′), 115.90 (C-2′), 103.50 (C-10), 101.30 (C-3), 101.18 (C-1⁗), 101.02 (C-1″/1‴), 100.38 (C-6), 94.5 (C-8), 78.29 (C-4″), 76.78 (C-3″), 76.04 (C-5″), 73.62 (C-2″), 72.56 (C-4⁗/4‴), 71.23 (C-2⁗/2‴), 70.79 (C-3⁗/3‴), 68.84 (C-5⁗/5‴), 66.53 (C-6″), 18.32 (C-6⁗/6‴).

Apigenin 7-O-β-D-rutinoside (5)

Pale yellow amorphous powder; R_f values 0.37 (S_1), 0.26 (S_2) on PC. It gives a green color when sprayed with $FeCl_3$ and greenish yellow fluorescence with Naturstoff reagent. UV λ_{max} nm: MeOH 265, 332; (+NaOMe) 268, 298 sh, 385; (+NaOAc) 267, 389; (+NaOAc/H_3BO_3) 267, 332; (+$AlCl_3$) 276, 340, 385

sh; (+$AlCl_3$/HCl) 276, 299 sh, 341, 384 sh. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 7.94 (2H, *d*, J = 7.5, H-2′/6′), 6.96 (2H, *d*, J = 7.5 Hz, H-3′/5′), 6.85 (1H, *s*, H-3), 6.77 (1H,*br s*, H-8), 6.45 (1H, *br s*, H-6), 5.05 (1H, *d*, J = 7.0 Hz, H-1″), 4.55 (1H, *br s*, H-1‴), 3.85 (1H, *br d*, J = 12 Hz, H-6_a″), 3.77 (1H, *br d*, J = 10.5 Hz, H-6_b″), 3.75 (1H, *br s*, H-2‴), 3.70–3.10 (7 H, *m*, remaining sugar protons hidden by H_2O signal), 1.05 (1H, *d*, J = 6 Hz, CH_3-6‴). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 180.96 (C-4), 164.37 (C-2), 162.82 (C-7), 161.10 (C-5/4′), 157.72 (C-9), 128.56 (C-2′/6′), 120.76 (C-1′), 116.06 (C-3′/5′), 108.87 (C-10), 103.05 (C-3, C-1‴), 100.50 (C-1″), 99.87 (C-6), 94.19 (C-8), 76.24 (C-5″/3″), 73.06 (C-2″/4‴), 70.71 (C-2‴), 70.28 (C-3‴), 69.57 (C-4″), 68.28 (C-5‴/6″), 17.74 (C-6‴).

Cosmosiin (6), Cinaroside (7), Luteolin (8) and Apigenin (9)

Yellow amorphous powder. Their chromatographic properties, UV date and ^{1}H NMR data listed in Tables 2 and 3.

Pedalitin (10)

Yellow amorphous powder; R_f values 0.36 (S_1), 0.06 (S_2) on PC, dark purple fluorescence under long UV light, changed into orange with Naturstoff and green color with $FeCl_3$ spray reagents. UV λ_{max} nm: (MeOH) 283, 339 sh, 344; (+NaOMe) 264, 370 sh, 385; (+NaOAc) 282 346; (+NaOAc/H_3BO_3) 285, 349; (+$AlCl_3$) 273, 300, 413; (+$AlCl_3$/HCl) 273, 300 sh, 413. Negative ESI-MS, ^{1}H and ^{13}C NMR are given in Table 4.

Crisiliol (11)

Yellow amorphous powder; R_f values 0.45 (S_1), 0.11 (S_2) on PC, dark purple fluorescence under long UV light, changed into orange with Naturstoff and green color with $FeCl_3$ spray reagents. UV λ_{max} nm: (MeOH) 276, 335; (+NaOMe) 269, 297 sh, 380; (+NaOAc) 271, 338 sh, 398; (+NaOAc/H_3BO_3) 289, 353; (+$AlCl_3$) 280, 344, 430 sh; (+$AlCl_3$/HCl) 282, 354, 425 sh, Negative ESI-MS, ^{1}H and ^{13}C NMR are given in Table 4.

6, 7-Dihydroxycoumarin (12)

Off-white amorphous powder; R_f values 0.67 (S_1), 0.48 (S_2) on PC. It gave sky blue fluorescence under UV-light, changed to greenish blue fluorescence with Naturstoff reagent. UV λ_{max}, nm: (MeOH) 260 sh, 290, 343; (+NaOH): 278 sh, 216 sh, 386. ^{1}H NMR (500 MHz, DMSO-d_6): δ ppm 7.82 (1H, *d*, J = 9.9 Hz, H-4), 6.94 (1H, *br s*, H-5), 6.70 (1H, *br s*, H-8), 6.12 (1H, *d*, J = 9.9 Hz, H-3). ^{13}C NMR (125 MHz, DMSO-d_6): δ ppm 161.39 (C-2), 151.09 (C-10), 149.05 (C-6), 145.00 (C-4), 143.44 (C-7), 112.74 (C-9), 111.91 (C-3), 111.18 (C-8), 103.13 (C-5).

Table 2 Chromatographic behavior qnd UV spectral data of compounds 6–9

	R_f values			Spray reagents response		UV data λ_{max} (nm)						
			Fluorescence		NA/PE				+NaOAc			+$AlCl_3$
	BAW	HOAc-15	in long UV	$FeCl_3$	UV	MeOH	+NaOMe	+NaOAc	+H_3BO_3	+$AlCl_3$	+$AlCl_3$	+HCl
6	0.51	0.12	d.pr	gr.	gr. y.	267, 336	271, 296 sh, 382	268, 269 sh, 347,382	268, 332	274, 300, 339, 384	275, 300 sh, 345, 385	
7	0.53	0.18	d.pr	gr.	or.	254, 268 (sh)	264 ,300 sh, 388	256,267 sh, 354, 405 sh	259, 376	274, 299 sh, 331, 431sh	275, 291 sh, 346, 390	
8	0.73	0.07	d.pr	gr.	or.	253, 265, 290 sh, 351	267 sh, 330 sh, 404	268, 325 sh, 387	260, 302 sh, 371, 429 sh	275, 300 (sh), 325, 425	273, 291, 355, 383	
9	0.80	0.20	d.pr	gr.	gr. y.	265, 296 (sh), 335	274, 326, 392	272, 300, 376	265, 302, 336	276, 299, 348, 380	275, 298, 338, 380	

d.pr, deep purple; gr., green; or., orange; sh, shoulder; y., yellow.

Table 3 ^{1}H NMR spectral data of compounds 6–9

H	6[a]	7[a]	8[b]	9[b]
H-3	6.83, *s*	6.70, *s*	6.53, *s*	6.59, *s*
H-6	6.42, *d* (2.0)	6.40, *d* (2.4)	6.20, *d* (2.1)	6.20, *d* (2.1)
H-8	6.76, *d* (2.4)	6.73, *d* (2.4)	6.43, *d* (2.1)	6.45, *d* (2.1)
H-5′	–	6.91, *d* (8.4)	6.89, *d* (8.7)	–
H-2′	–	7.44, *d* (2.4)	–	–
H-6′	–	7.40, *dd* (2.4, 8.4)	–	–
H-3′/5′	6.94, *d* (8.4)		–	6.92, *d* (8.4)
H-2′/6′	7.94, *d* (8.4)		7.37, *m*	7.91, *d* (8.4)
H-1″	5.05, *d* (7.6)	5.06, *d* (7.6)	–	–
Remaining sugar protons	3.8–3.0 (*m*)	3.8–3.0 (*m*)	–	–

J-values (Hz) were reported in parenthesis. [a](400 and 100 MHz, DMSO-d_6); [b](300 and 75 MHz $(CD_3)_2CO$ + CD_3OD).

Table 4 ^{1}H, ^{13}C NMR (300 and 75 MHz, DMSO-d_6) and negative ESI/MS spectral data of compounds 10 and 11

	10		11	
C No.	δ_C	δ_H	δ_C	δ_H
2	163.29		164.25	
3	102.46	6.86 *s*	102.65	6.89 *s*
4	182.00		182.06	
5	149.55		152.56	
6	129.88		131.80	
7	154.27		158.55	
8	90.99	6.68 *s*	91.45	6.72 *s*
9	146.21		152.04	
10	104.97		105.03	
1′	121.62		121.37	
2′	113.41	7.43 *d* (2.1)	113.55	7.45 *br s*
3′	145.76		145.81	
4′	149.67		149.99	
5′	115.95	6.89 *d* (9)	115.99	6.91 *d* (8.5)
6′	118.82	7.45 *dd* (9, 2.1)	118.97	7.45 *br s*
O-CH_3-7	56.24	3.92 *s*	56.42	3.92 *s*
O-CH_3-6			60.00	3.73 *s*
$[M-H]^-$	315.0		329.13	
$[M-CH_3]^-$			314.13	
$[M-H-CH_3]^-$	300.04			

J-values (Hz) were reported in parenthesis.

Animals

Adult male pathogen-free Sprague-Dawley rats (120–130 g) and Swiss mice (20–30 g), 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 ± 3ºC, 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. Sodium bicarbonate solution (5%) was used as vehicle for indomethacin.

Determination of LD_{50}

LD_{50} was determined by dissolving the **AME** in distilled water then given orally in graded doses to mice up to 5 g/kg while the control group received the same volume of the vehicle. The percentage mortality was recorded 24 h. later. No percentage mortality was recorded after 24 h up to a dose of 5 g/kg and according to Semler *et al*.,[22] 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 the **AME** (250, 500 and 1000 mg/kg, respectively).

Hypoglycemic activity

The hypoglycemic effect of **AME** was tested on Sprague Dawely rats of both sexes (150–175 g). The rats were divided into five groups, each of six. The first group received 1 ml saline (normal control). Induction of diabetes in the remaining four groups was carried out using a Streptozotocin (STZ) single dose (55 mg/kg, *i.p*) dissolved in 0.1 M of citrate buffer [pH = 4.5[23] followed by an overnight fasting. Diabetes was assessed by determining the blood glucose levels after 72 h. The first diabetic group received 1 ml saline (as diabetic control); the second and third diabetic groups received orally 250, 500 mg/kg of **AME** and the fourth diabetic group received rosiglitazone (0.5 mg/kg) as antidiabetic reference drug. The blood was obtained from all groups of rats by puncturing rato-orbital plexus of vein after being lightly anaesthetized with ether.[24] Blood glucose levels were measured at zero time and after 2 and 4 weeks of oral administration of the tested extract by using biodiagnostic kits of enzymatic colorimetric method.[25]

Antiinflammatory activity

Antiinflammatory activity in acute model was carried out according to the convenient reported method.[26] Rats were divided into five groups each of six; first group received saline

orally and served as control. The second, third and forth groups were given **AME** (250, 500, 1000 mg/kg) and the fifth was given indomethacin (25 mg/kg, orally) 1 h before induction of oedema by sub-planter injection of 100 µL of 1% carrageenan (Sigma, USA) in saline into the pad of right paw. The difference in hind footpad thickness was measured immediately before and 1, 2, 3, 4 h after carrageenan injection with a micrometer caliber.[27] The oedema was expressed as a percentage change from the control group.

DPPH antioxidant activity

This activity was investigated *in vitro* for **AME** using 1, 1-diphenyl, 2-picryl hydrazyl (DPPH).[28] Methanol solution of DPPH (2.95 ml) was added to 50 µl of **AME** dissolved in methanol at different concentrations (10–50 mg/ml) in a disposable cuvette. The absorbance was measured at 517 nm at regular intervals of 15 s for 5 min. Ascorbic acid was used as standard (0.1 M) as described by Govindarajan *et al*.[29]

Statistical analysis

The results were expressed as mean ± S.E and all the data were statistically evaluated using student's *t*-test[30] followed by one way ANOVA and Dunn's post-host multiple when the significance value was <0.05 using the same significant level. The criterion for statistical significance was generally taken as $P < 0.05$.

Results

Chemical results

The 2D-PC screening of the *S. splendens* leaves revealed the presence of a mixture of phenolic compounds (color properties under UV-light and responses to $FeCl_3$ and Naturstoff spray reagents). This mixture was fractionated on a polyamide column, followed by successive cellulose and sephadex LH-20 columns to obtain pure isolates **1–12.** All isolates were isolated from the *S. splendens* for the first time. The structures of these compounds, (Figure 1) were fully elucidated on the

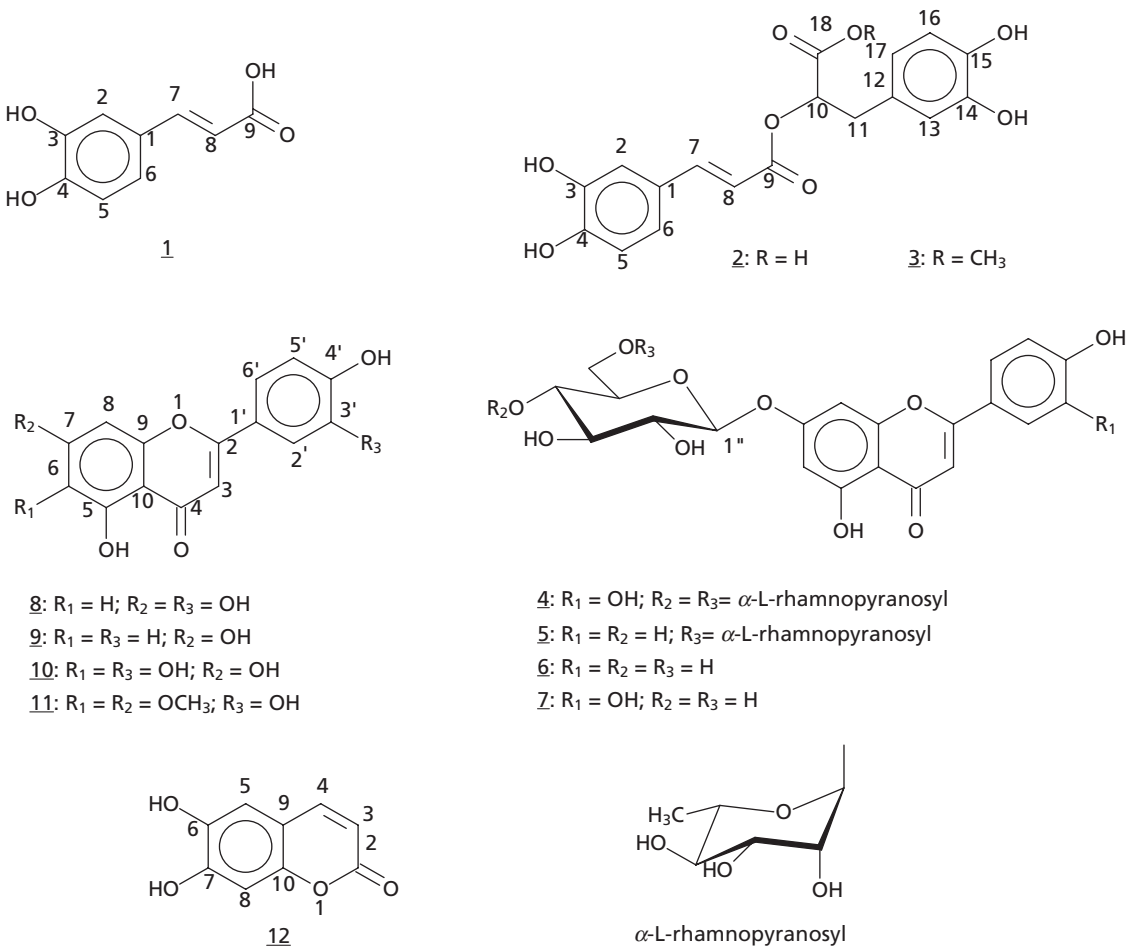

Figure 1 Structures of the identified compounds from **AME** of *S. splendens*.

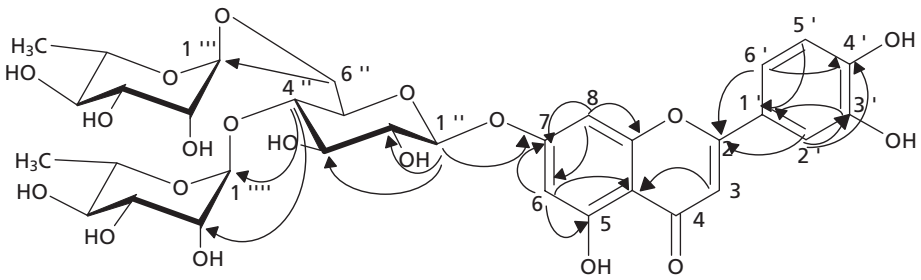

Figure 2 Key HMBC correlation of compound **4**.

basis of their physicochemical and spectral data (in particular UV, 1D and 2DNMR, ESI-MS) and by comparison with published data.[31–34]

On the bases of its chromatographic properties compound **4** was expected to be a luteolin –*O*- glycosides. On complete acid hydrolysis, it gave luteolin in the organic phase, while rhamnose and glucose were identified in the aqueous hydrolysate. Absence of bathochromic shift in band II upon addition of NaOAc gave a diagnostic document for an occupied 7-OH. As well as, the diagnostic batho- and hypsochromic shifts observed on the addition of the other shift reagents were indicative for free OH-5, OH-3′, OH-4′.[33] Negative ESI-MS revealed a molecular ion peak at *m/z* 739.0 $[M-H]^-$, corresponding to M.wt 740 of triglycosylluteolin with two rhamnosyl and one glucosyl moieties. This evidence was further supported by the three fragments at *m/z* 591.0, 447.0 and 284.9 due to the loss of one rhamnosyl, two rahmnosyl and then loss of the total triglycosyl moiety. ^{1}H NMR and COSY, spectra contained the resonances for B-ring protons of luteolin as expected. Downfield shift of AM-spin coupling system ($\Delta \sim +0.25$ ppm) of two *meta* coupled protons was assigned at δ6.64 and 6.41 for H-8 and H-6 was confirmative for luteolin 7-*O*-glycoside structure.[34] In the aliphatic region, the presence of three anomeric proton signals were assigned at δ 5.02 (*d*, J = 7 Hz, H-1″), 4.63 (*br s,* H-1‴) and 4.50 (*br s,* H- 1⁗) for one glucose and two rhamnose moieties. The anomeric configurations of **D**-glucose and **L**-rahmnose were β and α, respectively, according to the magnitudes of the $J_{1,2}$ coupling constants extracted from the ^{1}HNMR spectra, and all sugars were in the pyranose form. The site of glycosidation was confirmed from ^{1}HNMR spectrum through the downfield shift of both H-6″ (3.89) and H-4″ (4.83) of glucose moiety. Analysis of the ^{13}C NMR spectra indicated the presence of a conjugated ketone carbonyl signal at δ 181.90 characteristic for flavone nucleus. In addition the intrinsic downfield shift of C-4″ of the glucose moiety at δ 78.29 ($\Delta \sim +7$ ppm) and C-6″ at 66.53 ($\Delta \sim +6$ ppm) in the ^{13}C NMR spectra were diagnostic evidences for the two interglycosidic linkages as (1‴→4″) and (1⁗→6″).[34] Furthermore, the site of glycosidation was confirmed from the long rang correlation detected in HMBC experiments (Figure 2) between glucose H-1 and C-7 (162.40) of the luteolin aglycone. Similarly, the interglycosidic linkages of the branched trisaccharides were characterized by long range correlations detected between glu 6-CH_2 and 6^{glu}-*O*- Rha C-1 (101.12) and 6^{glu}-*O*- Rha H-1 and glu C-6 (66.53) likewise glu H-4 and 4^{glu}-*O*- Rha C-1(101.18) and 4^{glu}-*O*- Rha H-1 and glu C-4 (78.29). Compound **4** was therefore identified as luteolin 7-*O*-(4″, 6″-di-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside.

Biological activities

The result of LD_{50} study revealed that **AME** is non toxic up to a dose of 5 g/kg b.wt. which was the maximum soluble dose. Administration of **AME** exhibited a significant hypoglycemic effect in a dose dependant manner in streptozotocin induced-diabetic rats. The percentage of changes was 13.3, 18.7 for 250 and 500 mg/kg, respectively after 2 weeks. Furthermore, after four weeks both doses gave percent of change from the control pre-drug (zero time) more or less the same as the reference drug, rosiglitazone (35.5%), being 33.9 and 34.4 for 250 and 500 mg/kg, respectively (Table 5). Moreover, **AME** exhibited a significant antiinflammatory activity only at dose level 1000 mg/kg. with a potency being 0.39, 0.55, 0.50 and 0.51 at 1, 2, 3 and 4 h, respectively after carrageenan injection, in comparison to indomethacin (Table 6). Finally, **AME** exhibited a marked significant scavenging activity against DPPH *in vitro*. The kinetics of DPPH scavenging reaction revealed that the maximum reactive reaction rate after 5 min was 62.9, 82.5, 83.7, 84.3 and 85.1%, for the concentration 10, 20, 30, 40, 50 mg/ml, respectively (Figure 3), in comparison to L-ascorbic acid (86.8%).

Discussion

Salvia species are characterized by their richness in phenolic compounds mainly flavones based on apigenin and luteolin aglycone and phenolic acids based on caffeic acid building unit as caffeic, ferulic.[5] and rosmarinic acid.[6,7] *S. splendens* has been studied previously for its anthocyanin and diterpens

Table 5 Effect of the oral administration of 80% methanol extract of *S. splendens* leaves on blood glucose level of diabetic rats induced by streptozotocin injection ($n = 6$)

		Glucose mg/dl in diabetic rats				
		Before treatment	After treatment			
		Zero	15 days		30 days	
Groups	Dose	X ± S.E	X ± S.E	% of change	X ± S.E	% of change
Normal control	–	78.3 ± 3.2	75.4 ± 5.3	–	72.2 ± 2.8	–
Diabetic control	–	390.0 ± 12.3	385.8 ± 11.9	1.1	381.4 ± 13.3	2.2
80% aq. methanol extracts	250 mg/kg	369.7 ± 15.5	320.4 ± 10.6*•	13.3	244.4 ± 12.3***••	33.9
	500 mg/kg	401.8 ± 16.1	326.6 ± 16.5**•	18.7	263.5 ± 18.9***••	34.4
Rosiglitazone	0.5 mg/kg	416.5 ± 14.8	315.6 ± 12.2***•	24.2	270.5 ± 11.6***••	35.05

Significant difference from zero time (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Significantly difference from diabetic control (•$P < 0.01$, •• $P < 0.001$).

Table 6 Antiinflammatory activity of oral administration of 80% methanol extract of *S. splendens* leaves

Oedema (cm)								
	1 hr.		2 hr.		*3 hr.*		4 hr.	
Groups / doses	% increase	Potency	% increase	Potency	*% increase*	*Potency*	% increase	Potency
Control (0.2 saline)	122.34 ± 6.3	–	143.73 ± 10.3	–	148.85 ± 7.4	–	150.63 ± 5.7	–
Methanol extracts								
250 mg/kg	133.64 ± 3.5 (9.2)	0.16	136.94 ± 3.8 (4.7)	0.09	144.00 ± 4.3 (3.3)	0.07	145.57 ± 4.7 (–3.56)	0.05
500 mg/kg	108.91 ± 7.9 (10.98)	0.19	123.19 ± 8.9 (14.29)	0.28	132.50 ± 9.9 (10.98)	0.22	139.5 ± 9.4 (7.3)	0.15
1000 mg/kg	95.25 ± 4.3* (22.14)	0.39	103.78 ± 8.1* (27.79)	0.55	110.71 ± 7.9* (25.44)	0.5	112.70 ± 6.3* (25.18)	0.51
Indomethacine (25mg/kg)	54.31 ± 1.8* (55.62)	1	71.16 ± 5.5* (50.49)	1	73.16 ± 6.2* (50.73)	1	75.63 ± 4.9* (49.76)	1

Data represent the mean value ± SE. Data were analyzed using one way ANOVA and Dunnett's multiple comparison test *$P < 0.05$. Value between parentheses represents percent of inhibition as regard to control group. Potency was calculated as regard the percentage change of the indomethacin treated group.

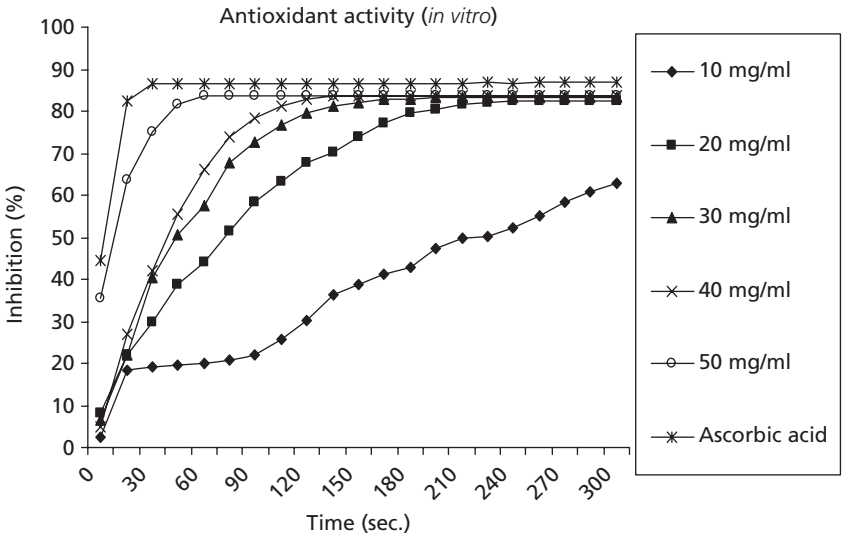

Figure 3 *In vitro* antioxidant activity of **AME** of *S. splendens* leaves and ascorbic acid, using DPPH radical scavenging activity method.

contents. In this investigation, for the first time by chemical and spectroscopic date we evidence that **AME** of *S. splendens* contains a broad structural diversity of phenolic constituents *viz* three phenolic acids (**1–3**), four flavones glycoside (**4–7**), together with three flavone aglycones (**8–11**) and one coumarin compound (**12**).

The **AME** of *S. splendens* contains rosmerinic acid, methyl rosmarinate as natural phenolic compounds contained in

many Lamiaceae herbs. The medicinal value of these substances has been will recognized, especially in regards to its antioxidant and antiinflammatory effects. Sabongi *et al*.,[35] observed that rosmarinic acid inhibited pathophysiological changes such as neutrophilic inflammation and oedema in lung of mice treated with rosmarinic acid. In *vivo,* study it is known to have inhibiting effect on cobra venum-factor-induced paw oedema. Several authors confirmed the high radical scavenging ability of rosmarinic acid and its relatively rapid kinetic behavior with DPPH radical.[36]

Many authors reported that caffeic acid, which is considered as a breakdown product of rosmerinic acid, as an antioxidant substance through its ability to scavenge a number of reactive species including DPPH.[37] Others studies reported also that caffeic also has antiinflammatory effect.[35,38]

Flavonoids have been shown to act as scavengers of various oxidizing species. Antioxidant properties of flavonoids are widely acknowledged.[39] The two classical antioxidant structural features of flavonoids are the presence of a B-ring catechol group and the presence of a C-2, C-3 double bond in conjugation with an oxo group at C-4; the first serves to donate hydrogen/electron to stabilize a radical species and the second serves to bind transition metal ions such as iron and copper.[39,40] In addition, the number and specific positions of phenolic hydroxyl groups and the nature of the substitutions determine whether flavonoids function as strong antioxidant[41][42–44] antinflammatory.[41,45] and antidiabetic effect.[40,46] The phenolic groups of flavonoids can dissociate to negatively charged phenolate ions under physiological conditions. Therefore, flavonoids can interact with several proteins by binding to them via hydrogen bridges and ion bonding. As a result the conformations of proteins are disturbed and in consequence their biological activity is altered.[47] These physicochemical properties can explain the wide range of activities of polyphenols. Because luteolin and apigenin and some of its glycosides fulfill these structural requirements, luteolin and apigenin-containing plants possess antioxidant properties.[40] The antioxidant activity of luteolin and apigenin and its glycosides has been associated with their capacity to scavenge reactive oxygen and nitrogen species[40] to chelate transition metals that may induce oxidative damage to inhibit pro-oxidant enzymes and to induce antioxidant enzymes. The antioxidant activity of them has not only been observed *in vitro* but also *in vivo*.[40]

Luteolin and apigenin and, its glycosides and plants containing them have been reported to exert antiinflammatory effects *in vitro* and *in vivo*.[40] They give their effect through several mechanisms.

In diabetes, the generation of reactive oxygen species (ROS) is greatly increased due to auto-oxidation and lipid peroxidation. Excessive ROS results in tissues damage and subsequent diabetic complications. As mentioned before, polyphenols modulate promoter activities of several antioxidant enzymes, so they will exerts its antidiabetic effect.

The presence of mixtures of these phenolic compounds in the **AME** of *S. splendens* may enhance its antioxidant, antiinflammatory and antidiabetic effect. Moreover this extract is non toxic to mice. Regarding these findings, it is encouraging to further investigate of the **AME** from phytochemical and biological points of view to confirm this possibility and for the development of new drugs.

Conclusion

Our results highlight that *S. splendens* leaves are characterized by a broad diversity of phenolic compounds in addition to being not toxic to mice. The **AME** exhibits significant hypoglycemic and antiinflammatory effects in dose dependant manner in addition to its antioxidant activity.

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