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Research Article

Phytochemical Investigation and Biological Screening of Ethyl Acetate Fraction of *Salvia hispanica* L. Aerial Parts

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ABSTRACT

Introduction: *Salvia hispanica* L. is an annual herbaceous plant commonly known as "Chia", native of southern Mexico and northern Guatemala. The aim of this study is isolation, identification of secondary metabolites and evaluation of biological activities of ethyl acetate fraction of *Salvia hispanica* L. aerial parts. **Methods:** Air dried powdered of *Salvia hispanica* L. aerial parts was extracted by maceration and fractionated using light petroleum, dichloromethane and ethyl acetate solvents. Ethyl acetate fraction was subjected to column and thin layer chromatography for isolation of secondary metabolites that are characterized by UV-Vis, FT-IR, EI-MS, 1D and 2D NMR spectral analyses. UPLC-ESI-MS/MS technique was used on the same fraction. *In-vitro* biological evaluation of the fraction carried out for anti-oxidant activity using DPPH assay, anti-obesityactivity using pancreatic lipase inhibitory assay,anti-diabetic activity usingα -amylase inhibition assay andanti-cancer activities usingcell viability assay. **Results:** Six compounds were isolated including 1,2,4,5 tetrahydroxy benzene (1), leucantho flavone (2), rhamnetin (3), apigenin-7-O-β-D-glucoside(4), rosmarinic acid (5) and kaempferol-7-O-β-D-glucoside (6). The identification of thirty seven compounds byUPLC-ESI-MS/MS analysis. A strong DPPH scavenging activity with IC_{50} 13.11compared to ascorbic acid, anti-obesity activity with IC_{50} 59.3 compared to orlistate, anti-diabetic activity with IC_{50} 95.2 compared to acarbose. High cytotoxic activity against lung carcinoma, colon carcinoma and moderately cytotoxic activity against prostate carcinoma cell lines. **Conclusions:** *Salvia hispanica* L. is a strong anti-oxidant and anti-carcinogenic against lung and colon cancer.

Key words: Anti-oxidant, Anti-obesity, Leucantho flavone, *Salvia hispanica*, 1,2,4,5-tetrahydroxy benzene, UPLC-ESI-MS/MS.

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INTRODUCTION

Family *Lamiaceae* consists of about 250 genera and 7000 species worldwide.[1] The genus *Salvia* has about 1000 species.[2] Mexican chia seeds protein content ranged from 18.5 to 22.3%, fat content ranged between 21.5 and 32.7%with their high-quality fatty acids[3]. The survey involving chia seeds indicate the presence of phenolic acids and flavonoids have the most appropriate antioxidant activity[4-9] and showed anti-obesity, anti-diabetic, anti-oxidant and anti-microbial activities.[10-13] On the other hand, the study of *S. hispanica* L. aerial parts indicate the presence of neoclerodane diterpenoids,[14, 15] also tentative identification of phenolics.[16] There is no phytochemical investigation about *S. hispanica* cultivated in Egypt so this work focuses on it, resulted in the isolation of main bioactive phytochemical constituents that including 1,2,4,5 tetrahydroxy benzene (first report to be isolated from nature), leucantho flavone and rhamnetin (first report to be isolated from *Salvia*), UPLC-ESI-MS/MS analysis for the first time on *S. hispanica* L. aerial parts cultivated in Egypt and evaluation of biological activities of ethyl acetate fraction.

MATERIALS AND METHODS

General

UV spectra were recorded on Shimadzu U.V.-1700 spectrophotometer (Japan). EI/MS spectra on Shimadzu GC-MS-QP5050A.^{1}H- and ^{13}C-NMR spectral analyses were carried out using Bruker (Switzerland) at 400 and 100 MHz, respectively. Rotatory evaporator (Büchi,Germany). Heidolph U.V lamp U.V.P.,GL-58 (λ_{max}254 and 365nm), ammonia and anisaldhyde sulfuric acid as visualizing agents for TLC. Circulating hot air oven (W.T-binder 7200, Germany).Silica gel column (5 x 120 cm), Sephadex LH_{20} (Fluka AG, Buchs SG).Pre-coated TLC plates (silica gel 60, GF_{254}, 60-250 mesh), Merck, Germany. Infrared spectral analysis were recorded in potassium bromide disks on a PyeUnicam SP 3000 and IR spectrophotometer of Alpha (100523), Jasko, FT/IR-460 plus, Japan. Melting point apparatus, Digital, electro-thermal LTD (England).

Plant material

The aerial parts were collected in the flowering stage from mushtohor farm, Tokh, Egypt in March 2018. The plant was identified and verified by Dr. Hussein Abdelbaset, (Professor of Plant Taxonomy, Faculty of Science, Zagazig University). A voucher specimen (Lam.S-10) was deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

Extraction and fractionation

The air dried powdered aerial parts of *Salvia hispanica* L. (3 kg) was extracted by cold maceration (5 times x 7 L) using 70% aqueous ethanol. The total extract was evaporated under reduced pressure at 50 °C yielded 540 gm of dark green viscous residue. The residue (400 gm) was dissolved in methanol: water mixture (1:9) then subjected to fractionation using light petroleum 60-80 °C (9× 500 ml) then

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dichloromethane (7× 500 ml) and finally ethyl acetate (5× 500 ml). The fractions were washed with distilled water and dried over anhydrous sodium sulphate then the solvent of each fraction was distilled off under reduced pressure at 50 °C to yield 68 gm of light petroleum fraction, 4 gm of dichloromethane fraction and 14 gm of ethyl acetate fraction.

Isolation

About 11gm of ethyl acetate fraction was dissolved in a least amount of methanol and adsorbed on 250 gm silica gel for column and the solvent was evaporated completely. The dry zone was applied on the top of silica gel column (5 x 120 cm, 200 g) packed by wet method using dichloromethane, the development was started with dichloromethane and the polarity was increased gradually using methanol to yield 55 fractions. Fractions (15-20) eluted by 4% MeOH/CH_2Cl_2 were combined, concentrated then subjected to TLC examination , revealed the presence of one major orange spot using anisaldehyde-sulphuric acid and crystallized from dichloromethane-methanol mixture to afford pale yellow needle shaped crystals of compound 1. Fractions (21-26) eluted by 6% MeOH/CH_2Cl_2were combined, concentrated then subjected to TLC examination, revealed the presence one major yellow spot using ammonia vapour and crystallized to afford yellow powder of compound 2. Fractions (27-33) eluted by 8% MeOH/CH_2Cl_2 were combined, concentrated then subjected to TLC examination, revealed the presence of two major yellow spots using ammonia vapour, the fractions were crystallized by dichloromethane to afford yellow powder of compound 3, the mother liquor was concentrated and recrystallized from dichloromethane-methanol mixture to afford compound 4. Fractions(44-47) eluted by 16% MeOH/CH_2Cl_2 were combined, concentrated then subjected to TLC examination, revealed the presence of one major pink spot using anisaldehyde-sulphuric acid, the fractions were crystallized from dichloromethane-methanol mixture to afford yellowish brown crystals of compound 5. Fractions (51-53) eluted by 30% MeOH/CH_2Cl_2 were combined, concentrated then subjected to TLC examination, revealed the presence of two yellow spots, fractions were pooled, concentrated and subjected to rechromatographic separation using Sephadex column LH-20 (2 × 50 cm, 3gm), eluted with 100% methanol. Compound 6 was isolated as a result of Sephadex column whose crystals was isolated after crystallized from dichloromethane-methanol mixture.

1,2,4,5-tetrahydroxybenzene(1): pale yellowcrystals with m.p 208-210°C andR_f0.48 (CH_2Cl_2:CH_3OH; 9:1). FT-IR(KBr. ν_{max}, cm-1) 3133, 3030, 1511, 1460, 1360. EI-MS: *m/z*= 142amu $[M]^+$.^{1}H-NMR (CD_3OD, 400 MHz); δ(ppm) 6.64 (2H, *s*, H-3, H-6) and 4.90 (OH).^{13}C-NMR-APT (CD_3OD, 100 MHz); 115.42 (C-3,C-6) and 149.85(C-1, C-2,C-4,C-5).

Leucanthoflavone(2): yellow powder with R_f0.39 (CH_2Cl_2:CH_3OH; 9:1). UV(λ_{max}, MeOH):287,346 nm, (+NaOMe):277,394 nm, (+$AlCl_3$):287,318(sh.),377,448(sh.) nm, (+$AlCl_3$+HCl): 292,317(sh.), 362 nm, (+NaOAc):280, 405 nm, (+NaOAc+H_3BO_3):279, 402 nm. FT-IR(KBr. ν_{max}, cm-1) 3500 -2500, 1655, 1599, 1576, 1486. EI-MS: *m/z*= 346amu $[M]^+$and 316(M-2CH_3).^{1}H-NMR (CD_3OD, 400 MHz); δ(ppm)7.51(1H,overlapped,H-2'), 7.49(1H,d,J=8Hz,H-6'), 6.92(1H,d,J=8Hz,H-5'), 6.59(1H,s,H-3), 4.04(3H,s,H-7) and 3.90(3H,s,H-6). ^{13}C-NMR (CD_3OD, 100 MHz); δ(ppm) 165.46(C-2), 101.98(C-3), 183.39(C-4), 147.73(C-5), 136.36(C-6), 60.58(OCH_3-6), 149.86(C-7), 59.93(OCH_3-7), 130.86(C-8), 145.68(C-9), 106.54(C-10), 122.19(C-1'), 112.89(C-2'), 144.87(C-3'), 141.48(C-4'), 115.35(C-5'), 119.28(C-6').^{1}H-^{1}HCOSY: δ 6.92 (d,*J* =8 Hz) is coupled withδ 7.48 (d,*J* =8 Hz) for H-5' and H-6', respectively. HSQC:δ 3.93(3H,s,H-6) and δ 4.04(3H,s,H-7) ppm correlated on at δ 60.58(OCH_3-6) and δ 59.93 ppm(OCH_3-7), respectively. HMBC showed the locations of two methoxy groups.

Rhamnetin(3):yellow powder with m.p 293-295°C and R_f0.32 (CH_2Cl_2:CH_3OH; 9:1). UV (λ_{max}, MeOH): 285, 344 nm, (+ NaOMe): 307, 398 nm, (+$AlCl_3$): 299,385 nm, (+$AlCl_3$+HCl): 295, 369 nm,(+NaOAc): 290, 391 nm, (+NaOAc+H_3BO_3): 292, 392 nm. ESI-MS:*m/z*= 316$[M]^+$ which is compatible with the molecular formula $C_{16}H_{12}O_7$ and 302 $[M+H-CH_3]^+$.^{1}H-NMR (CD_3OD, 400 MHz); δ(ppm)7.42(1H,d,J=3.2Hz,H-2'),7.40(1H,dd,J=3.2 Hz,7.6,H-6'),6.90(1H,d,J=8Hz,H-5'), 6.83(1H,s,H-8), 6.50 (1H,s,H-6) and 4.02(3H,s,H-7).

Apigenin-7-O-β-D-glucoside(4): yellow crystals with m.p 233-235°C and R_f0.72 (EtOAc:CH_3OH:H_2O; 6:1:0.3).UV(λ_{max}, MeOH): 269, 334 nm, (+NaOMe): 279,395 nm, (+$AlCl_3$):275,348,388 nm, (+$AlCl_3$+HCl):275,340,379nm,(+NaOAc):271,341nm,(+NaOAc+H_3BO_3): 270,337 nm. FT-IR(KBr. ν_{max}, cm-1) 3210, 1650, 1613, 1566,1505. ESI-MS: *m/z*= 271$[M+H-Glu]^+$.^{1}H-NMR (CD_3OD, 400 MHz); δ(ppm) 8.01(2H,d,J=8Hz,H-2',6'),6.96(2H,d,J=8Hz,H-3',5'),6.92(1H,s,H-3), 6.63(1H,s,H-8), 6.30(1H,s,H-6), 5.01(1H,d,J=8Hz,glucosyl H-1``) and 3.50-4.15(m,H-2", 3", 4", 5" and 6"). ^{13}C-NMR (CD_3OD, 100 MHz); δ(ppm) 163.3(C-2), 104.85(C-3), 182.72(C-4), 158.69(C-5), 100.86(C-6), 165.69(C-7), 96.79(C-8), 160.04(C-9), 106.15(C-10), 121.19(C-1'), 129.25(C-2',6'), 117.83(C-3',5'), 161.32(C-4'), 99.13(C-1"), 71.42(C-2"), 78.80 (C-3"), 66.38(C-4"), 76.09(C-5") and 62.68(C-6").

Rosmarinic acid (5): yellowish-brown crystals with m.p 173-175°C and R_f0.63(EtOAc:CH_3OH:H_2O; 6:1:0.3). UV(λ_{max}, MeOH): 288, 328 nm. FT-IR(KBr. ν_{max}, cm-1) 3185, 1685, 1582, 1520, 1444. ESI-MS: *m/z*= 361 $[M+H]^+$, 342$[M^+-H_2O]^+$.^{1}H-NMR(CD_3OD, 400 MHz); δ(ppm) 7.05(1H,s,H-2), 6.70(1H,s,H-5), 6.94(1H,d,J=8Hz,H-6), 7.53(1H,d,J=16 Hz,H-7), 6.30(1H,d,J=16 Hz,H-8), 6.79(1H,s,H-2'), 6.69(1H,d,J=8Hz,H-5'), 6.65(1H,d,J=8Hz,H-6'), 2.97(1H,dd,J=8,12Hz,H-7'a), 3.12(1H,d,J=12Hz,H-7'b) and 5.11(1H,d,J=8Hz,H-8').^{13} C-NMR (CD_3OD, 100 MHz); δ(ppm) 129.85(C-1), 114.29(C-2), 143.38(C-3), 144.54(C-4), 115.10(C-5), 124.58(C-6), 147.96(C-7), 114.83(C-8), 167.7(C-9), 129.90(C-1'), 120.38(C-2'), 145.24(C-3'), 145.32(C-4'), 116.13 (C-5'), 121.51 (C-6'), 37.41(C-7'), 76.47(C-8') and 171.70(C-9').

Kaempferol-7-O-β-D-glucoside(6): yellow powder with m.p 247-250°C and R_f0.43(EtOAc:CH_3OH:H_2O; 6:1:0.3). UV(λ_{max},MeOH): 268, 338 nm, (+ NaOMe): 274, 394 nm, (+ $AlCl_3$):273, 383 nm, (+$AlCl_3$ + HCl): 274, 389 nm, (+ NaOAc):271, 356 nm, (+NaOAc + H_3BO_3):269,338 nm.EI-MS: *m/z*= 414[(M^+-H_2O)] and268(aglycone-H_2O).^{1}H-NMR(CD_3OD, 400 MHz); δ(ppm)8.09(2H,d,J=6.8Hz,H2',6'),6.92(2H,d,J=8Hz,H-3',5'),6.44(1H,s,H-8),6.24(1H,s,H-6), 5.13(1H,d,J=9.6Hz,glucosyl H-1``) and 3.22-3.91(m,H-2", 3", 4", 5" and 6"). ^{13}C-NMR (CD_3OD, 100 MHz); δ(ppm) 158.31(C-2), 130.89(C-3), 179.70(C-4), 162.37(C-5), 98.82(C-6), 164.03(C-7), 95.14(C-8), 157.33(C-9), 108.16(C-10), 122.94(C-1'), 130.23(C-2',6'), 116.89(C-3',5'), 161.02(C-4'), 103.19(C-1"), 72.77(C-2"), 77.74 (C-3"), 68.43(C-4"), 78.12(C-5") and 67.73(C-6").

LC/MS instrument and separation technique

The sample (100µg/mL) solution was prepared using HPLC analytical grade solvent of MeOH, filtered using a membrane disc filter (0.2µm) then subjected to LC-ESI-MS analysis. Samples injection volumes (10µL) were injected into the UPLC instrument equipped with reverse phase C-18 column (ACQUITY UPLC - BEH C18 1.7 µm particle size - 2.1 × 50 mm Column). Sample mobile phase was prepared by filtering using 0.2 µm filter membrane disc and degassed by sonication before injection. Mobile phase elution was made with the flow rate of 0.2 mL/min using gradient mobile phase comprising two eluents: eluent A is H_2O acidified with 0.1% formic acid and eluent B is MeOH acidified with 0.1% formic acid. Elution was performed using the above gradient. The parameters for analysis were carried out using negative ion mode as follows: source temperature 150 °C, cone voltage 30 eV, capillary voltage 3 kV, desolvation temperature 440 °C, cone gas flow 50 L/h, and desolvation gas flow 900 L/h. Mass spectra were detected in

the ESI between *m/z* 100–1000. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its retention time and mass spectrum with reported data.

Biological activities

The biological activities of ethyl acetate fraction of *S. hispanica* L. aerial parts were carried out at Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University, Cairo, Egypt.

Antioxidant activity

The antioxidant activity using DPPH method ac.[17] Briefly, ethyl acetate fraction was determined at different concentration 2.5, 5, 10, 20, 40, 80, 160, 320, 640 and 1280µg/ml that were added respectively to 3 ml DPPH solution, the decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). The 50%inhibitory concentration (IC_{50}) of ethyl acetate fraction and the standard (ascorbic acid) were estimated

Anti-obesity activity

The anti-obesity activity was determined by pancreatic lipase inhibitory assay.[18] Briefly, ethyl acetate fraction with different concentrations (1000 to 7.81 µg/mL) were pre-incubated with 100 µg/mL of lipase for 10 min at 37°C. The reaction was then started by adding 0.1 mL p-nitrophenyl butyrate substrate, after incubation at 37°C for 15. The amount of p-nitrophenol released in the reaction was measured using Multiplate Reader. IC_{50} value of ethyl acetate fraction and the standard (orlistat) were estimated.

Anti-diabetic activity

The anti-diabetic activity was determined by α -amylase inhibition method[19]. Briefly, 1ml of the fraction of various concentrations (1000 to 7.81 µg/ml) and 1ml of enzyme solution were mixed together and incubated at 25°C for 10 min. After incubation, 1ml of starch (0.5%) solution was added to the mixture and incubated at 25°C for 10 min. The reaction was then stopped by adding 2ml of dinitro salicylic acid, heating the mixture in a boiling water bath (5min). After cooling, the absorbance was measured colorimetrically at 565 nm, the IC_{50} value of ethyl acetate fraction and the standard (acarbose) were estimated.

Cytotoxic activity

The anti-cancer activity using cell viability assay.[20,21] Briefly, the cell lines used were human Lung cancer cell line (A-549), human prostate carcinoma cells (PC-3) and colon carcinoma cells (HCT-116), ethyl acetate fraction used in various concentrations (500 to 0 µg/ml), the IC_{50} value of ethyl acetate fraction and the standard (vinblastine sulfate) were estimated.

RESULTS

Chemical investigations

Six compounds were isolated and identified using physical investigations in addition to spectral analyses that compared with reported data. 1,2,4,5 tetrahydroxy bezene(1) was isolated for first time from nature, leucanthoflavone (2), rhamnetin (3) and were isolated for first time from genus *Salvia*.Apigenin-7-O-β-D glucoside (4) and rosmarinic acid (5) kaempferol-7-O-β-D glucoside (6) were isolated for first time from *S. hispanica*L.aerial parts.

Compound 1

IR spectrum showed broad absorption band at 3100 cm^{-1} for OH groups, 3030, 1511, 1460 and 1360 cm^{-1} indicated aromaticity. EI-MS exhibited a molecular ion peak at *m/z* 142(M^+) for molecular formula $C_6H_6O_4$, the fragmentation pattern showed loss of hydroxyl groups by formation of peaks at *m/z* 110 (relative abundance 100%). ^{1}H-NMR spectrum showed a singlet signal at δ 6.56 ppm for two aromatic protons and singlet signal at δ 4.90 ppm for four protons of OH groups. ^{13}C-NMR (APT) spectrum showed only two types of carbons: at δ 115.42 ppm (C-H) and at δ 149.85ppm(C-O). These data confirmed the structure to be 1,2,4,5 tetrahydroxybenzene which was reported synthetically but it is the first time to be isolated from nature.

Compounds 2 and 3

UV showed a flavonol skeleton at 287, 346 nm and 285, 344 nm for compounds 2 and 3, respectively. Bathochromic shift with NaOAc/H_3BO_3 indicate the presence of hydroxyl groups at C-3' and C-4' of these compounds. EI-MS of compound 2 showed the loss of two methoxy groups by a fragment ion at *m/z* 316 from a molecular ion at *m/z* 346 but ESI-MS in positive mode of compound 3 showed the loss of one methoxy group by a fragment ion *m/z* 302 from a molecular ion at *m/z* 317 which were confirmed by ^{1}H-NMR at δ 3.90 and δ 4.04 in compound 2 and at δ 4.02 in compound 3. HSQC and HMBC of compound 2 showed the correlation of 3.93 (H-6) with 60.58 (OCH3-6) and136.36 (C-6), 4.04 (H-7) with 59.93 (OCH3-7) and 149.86 (C-7). So compound 2 was identified as leucanthoflavone andcompound 3 was identified as rhamnetin.[22]

Compounds 4 and 6

Compounds 4 (flavone) and 6 (flavonol) were identified as flavonoidal glucosides. UV showed bathochromic shift with NaOMe in band I indicate the presence of hydroxyl group at C-4' but no bathochromic shift with NaOAc/H_3BO_3 indicated the absence of hydroxyl group at C-3' and occupied hydroxyl group at C-7. The presence of singlet proton at δ 6.92 ppm (H-3) confirmed the flavone nature of compound 4, at the same time the absence of this signal confirmed the flavonol nature of compound 6. Signals at δ 5.01,δ 5.13 ppm (H-1") and δ 3.5-4.15,δ 3.22-3.91 ppm (H-2", 3", 4", 5" and 6") confirmed the presence of β-D glucose at C-7 for both compounds 4 and 6. ^{13}C-NMR showed anomeric carbons at 99.13and 103.19 (C-1") for compounds 4 and 6, respectively.

These compounds were confirmed to be apigenin-7-O-β-D glucoside and kaempferol-7-O-β-D glucoside.[23, 24]

Structural identification of constituents by UPLC-ESI-MS/MS

UPLC-ESI-MS/MS in both negative and positive ion modes were used to analyze ethyl acetate fraction of *S. hispanica* L. aerial parts. The tentative detection of **37** compounds based on the fragmentation patterns that were compared with the available literature data as seen in Table 1. The compounds were arranged according to retention time (R_t) and divided according to different classes to phenolic derivatives (11), flavonoid aglycones (8), flavonoid-O-glycosides (7), flavonoid-C-glycosides (4), tannins (1), diterpenoids (2), lignin (2), coumarin (1), and triterpenoids(1).

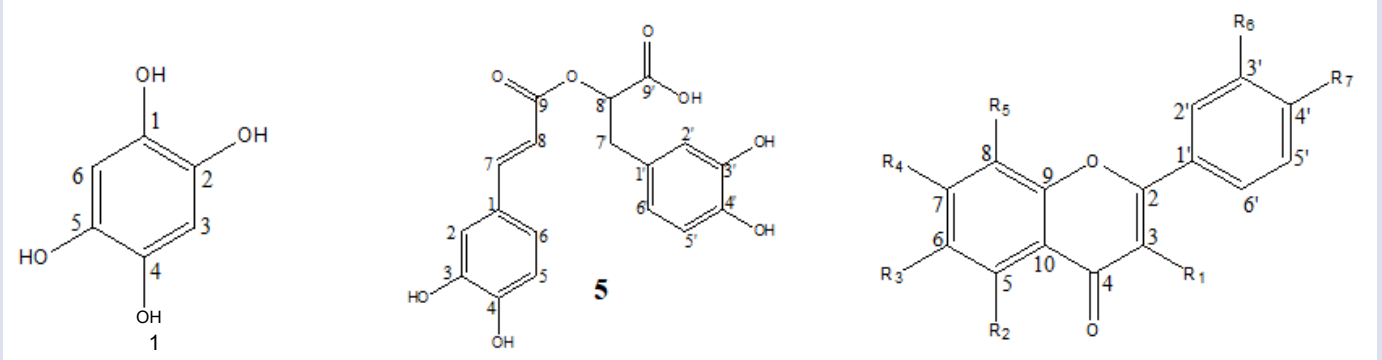
Phenol derivatives

Compound **1**with$[M-H]^-$ at *m/z* 317 showed the product ion at *m/z*151 [galloyl moiety]and *m/z* 107 [-CO_2 (44Da)][25]. The identification of compounds 2 and 4 were confirmed by the product ions at *m/z* 135[8] and 29,[27] respectively that formed by the neutral loss of CO_2(44Da). The product ion at *m/z* 179 $[(caffeic\ acid - H)]^-$ from the parent ion at *m/z* 433 of compound 3revelead to loss of arbutin moiety.[26] Phenolic acid glycosides were tentatively identified due to cleavage of the glycosidic bond resulting in the *m/z* of the phenolic acid, and then neutral losses of H_2O and CO_2as in case of compounds16, 22 and 25.[8, 41] Compounds 17and 27 gave the molecular ion peak $[M-H]^-$at *m/z* 359 and 373, respectively. The MS^2 spectrum showed the product ions at

Table 1: Tentatively identified Compounds from ethyl acetate fraction of *S. hispanica* L. aerial parts using UPLC-ESI-MS/M

No.	Tentative assignment	R_t (min)	MWT	[M-H]	[M+H]	MS/MS	Ref
1	Galloyl dihydrocoumaric acid	1.04	318	317		151,107	25
2	Caffeic acid	5.76	180	179		135	8
3	Caffeoyl arbutin	7.29	434	433		179,161,133	26
4	Caffoeyl shikmic acid	7.59	336	335		291	27
5	Herbacetin rhamnoside	7.97	448	447		357,327,297	28
6	Kaempferol-8-C-glucoside	7.99	448	447		357,327,299,297	29
	or Orientin	7.99	448	447		357,327	28
7	Iso-orientin	8.25	448	447		339,327	28
8	Vitexin	8.52	432	431		341,311,283	30
9	Iso-vitexin	8.54	432		433	415,313	30
10	Naringenin-O-hexoside	8.66	434	433		272	31
11	Kaempferol-O-glucoside	8.72	448		449	287	32
12	Apigenin-O-glucoside	8.95	432	431		269,153	33
13	Scutallarein-O-hexoside	9.04	448		449	287	34
14	Rutin or Hesperidin	9.25	610	609		300	35
15	Quercetin-O-glucoside	9.27	464	463		301,271,151	36
16	Syringic acid glucoside	9.79	360	359		197,179,161,135	8
17	Rosmarinic acid	10.20	360	359		197,179,161,135,73	8
18	Methyl-O-ellagic acid	10.65	316	315		300	37
19	Medioresinol-O-glucoronide	11.03	564	563		387,207,193	38
20	Caftaric acid	11.17	312		313	181	39
21	Quercetin-3-methyl ether	11.23	316		317	302,153	40
22	Ferulic acid hexoside	11.30	356		357	179,177	41
23	Rhamnetin or isorhamnetin	11.31	316	315		300,165	40
24	Myricetin	11.32	318	317		151	42
25	Danshenu glucuronide	11.34	374	373		197,179,175,135,123	8
26	Umbelliferone	11.35	162		163	135	43
27	Methyl rosmarinic acid	11.77	374	373		179,161,135	8
28	Luteolin or kaempferol	12.19	286	285		217,199,151,133	32
29	Leucanthoflavone or eupatolitin	12.48	346	345		330,315	33, 44
30	Syringetin	12.48	346	345		330,315	45
31	Visidulin III	12.50	346	345		330,287,243	46
32	1,2,4,5-tetrahydroxy benzene	12.65	142		143	110,78	
33	Medioresinol	12.84	388	387		207,179	47
34	Jaceosidin or Tricin	13.39	330	329		314,299	33, 48
35	Carnosol	17.07	330	329		285	8
36	Salvinorin B	28.90	390		391		49
37	Triterpenoids d.v.s	30.13	663		664	551,495,439	32

Underlined numbers represent the base peak.



2,3,4,6

2: R_1: H; R_2, R_5, R_6 and R_7:OH; R_3 and R_4:OCH_3

3: R_3 and R_5: H; R_1,R_2, R_6 and R_7:OH; R_4:OCH_3

4: R_1, R_3, R_5 and R_6: H; R_2 and R_7: OH; R_4: O-glucose

6: R_3, R_5 and R_6: H;R_1, R_2 and R_7: OH; R_4: O-glucose

Figure 1: Chemical structures of the isolated compounds.

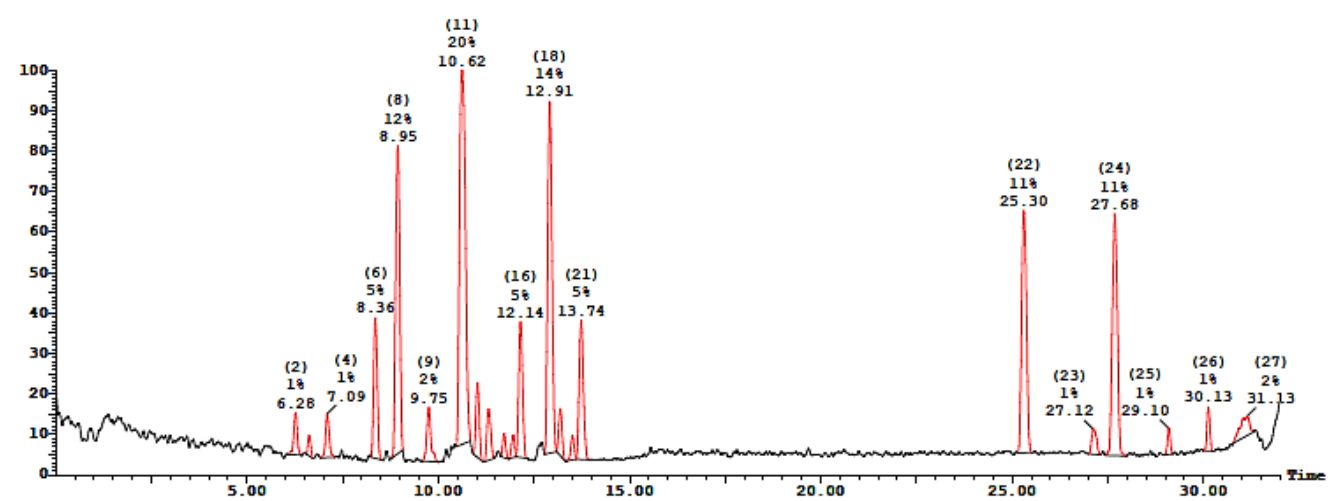


Figure 2: Negative mode UPLC-ESI-MS/MS chromatogram of ethyl acetate fraction of *S. hispanica* L. aerial parts.

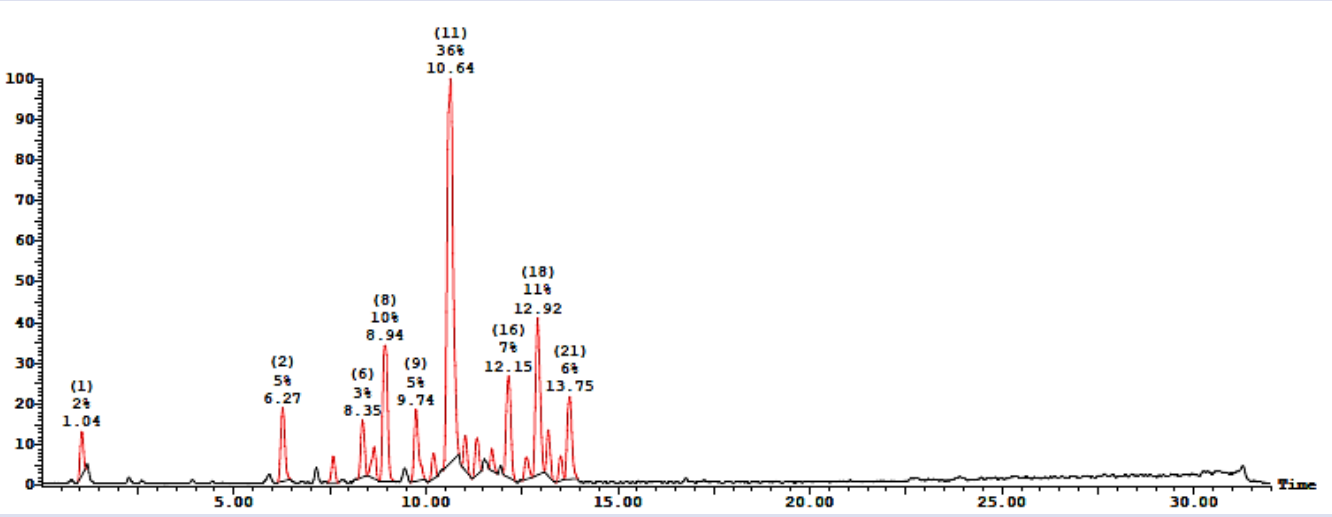


Figure 3: Positive mode UPLC-ESI-MS/MS chromatogram of ethyl acetate fraction of *S. hispanica* L. aerial parts.

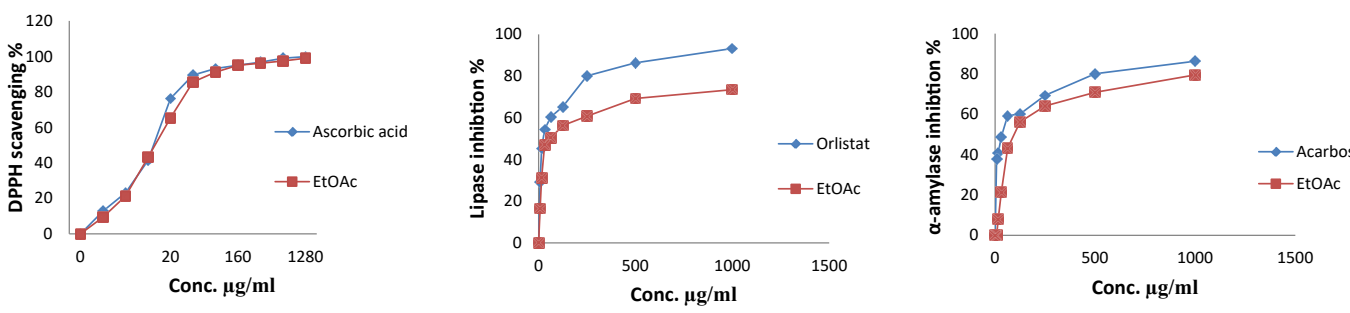


Figure 4: (**A**): DPPH scavenging capacity of *S. hispanica* L. ethyl acetate fraction and ascorbic acid. (**B**): Anti-obesity activity of *S. hispanica* L. ethyl acetate fraction and orlistat. (**C**): Anti-diabetic activity *S. hispanica* L. ethyl acetate fraction and acarbose.

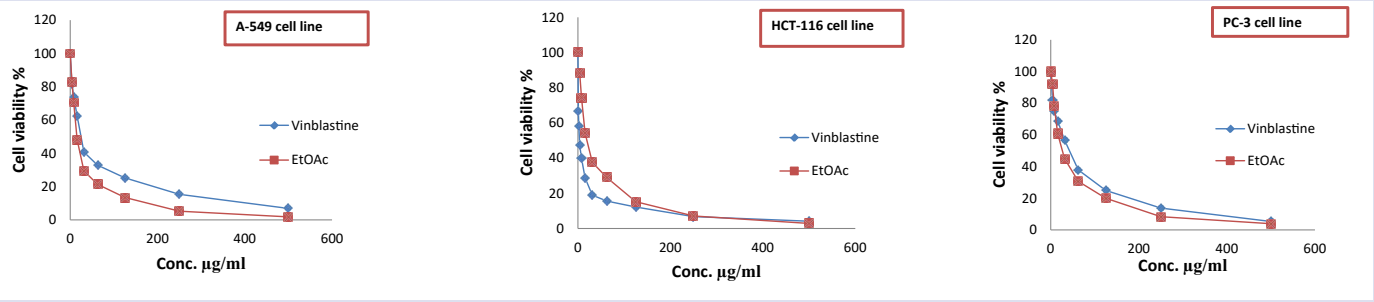


Figure 5: (**A**): Cytotoxic activity of *S. hispanica* L. ethyl acetate fraction against A-549 cell. (**B**): cytotoxic activity *S. hispanica* L. ethyl acetate fraction against HCT-116 cell. (**C**): cytotoxic activity *S. hispanica* L. ethyl acetate against PC-3 cell.

*m/z*179 due to breaking of ester linkage and fragment ion at *m/z*135[-CO_2 (44Da)].[8] Compound 20showed ion at *m/z* 181 corresponding to [M+H−tartaric].[+39] The fragment ions at *m/z* 110 and 78 of compound 32 formed due to loss of successive hydroxyl groups.

Flavonoid-Aglycones

Methoxylated flavonoids were identified, the fragmentation started by lossing of methyl groups (15 Da) then cleavage of flavonoid nucleus as in case of compounds 21 and 23. In positive ionization mode of compound 21, it showed product ions at *m/z* 302 $[M+H-CH_3]^+$and at*m/z* 153 but in negative ionization mode of compound 23, the product ions were formed at *m/z* 300 $[M-H-CH_3]^-$ and *m/z* 165.[40] Compound 29 showed the molecular ion peak $[M-H]^-$ at*m/z* 345,the first MS/MS fragmentation pattern showed fragment ions at *m/z* 330 and *m/z* 315.[33,44] In case of compound 31 the second fragmentation pattern showed characteristic fragment ions at *m/z* 330, 315, *m/z* 287 [-CO(28 Da)]and 243 [-CO_2 (44 Da)].[46]

Flavonoid-O-Glycosides

Fragmentation of flavonoid-O-glycosides depends on lossing of sugar moeity (162 Da) that resulting in formation of product ion coressponds to aglycone. According to this rule, compounds 10,11,12, 13,14 and 15 were identified according to,[31-36] respectively. Compound 5 showed the fragmentation ion at *m/z* 357 $[M-H -90]^-$ suggesting loss of [H_2O+3CH_2O] and 299 [M-H -90- 2CO].[28]

Flavonoid-C-Glycosides

The MS^2 spectrum of compound 6 showed the fragment ions at *m/z* 357$[(M-H-90)]^-$, 327$[(M-H-120)]^-$ and 297$[(M-H-150)]^-$ this fragmentation pattern was characteristic for Kaempferol-8-C-glucoside,[29] another fragmentation showed the fragment ions at *m/z* 357$[(M-H-90)]^-$and327$[(M-H-120)]^-$may be also corresponding to orientin but presecnce of a fragment at *m/z* 339$[(M-H-90-18)]^-$that related to 6-C-glycoside, so compound 7 was identified as iso-orientin.[28] The MS^2 spectrum of Compound 8 gave a fragment ions at m/z 341$[(M-H-90)]^-$, 311$[(M-H-120)]^-$ and 283 [(M-H-120-CO), this fragmentation pattern is corresponding to vitexin,the positive ionization of this peak showed a precursor ion at *m/z* 433$[M+H]^+$, the fragmentation showed neutral loss of H_2O (18 Da) at *m/z* 415 that related to 6-C-glycoside and the 313[(M+H -120)]$^+$socompound 9 was tentatively identified as iso-vitexin.[30]

Tannins

Compound 18 were identified as methyl-O-ellagic acid.[37]

Diterpenoids

Compound 35 (carnosol) fragmentation showed the ion at *m/z* 285 [-CO_2 (44Da)][8] while compound 36(salvinoron B) showed a fragment ion at *m/z* 373 [-H_2O (18Da)].[49]

Lignans

Compounds 25 and 30 that were tentatively identified as medioresinol-O-glucoronide and medioresinol according to precursor ions $[M-H]^-$at *m/z* 563 and 387, respectively. The MS^2 spectrum of compound 25 showed the fragment ions at 387$[M-H-Glu]^-$ and 207[M-H-Glu-180][38], compound 30 showed the same fragment at *m/z*207.[50]

Coumarins

Compound 26 (*umbelliferone*) showed the fragment ion at 135 [-CO(28Da)].[43]

Biological activities

Anti-oxidant activity

This promising result is due to presence of flavonoids and phenolic content as the presence of hydroxyl groups in the phenolic compounds are responsible of anti-oxidation effect as the hydroxyl group consider necessary component as a radical scavenger.[51] The DPPH scavenging percentage of ethyl acetate fraction of *S. hispanica* L aerial parts (IC_{50} = 13.11 µg/ml) is shown in (Figure 4A) in comparison with ascorbic acid (IC_{50} = 12.50 µg/ml).

Anti-obesity activity

There are many reports about *S. hispanica* L. seeds antiobesity action[10,52-56] but no reports about aerial parts activity. The anti-obesity activity is due to presence of poly phenolics, flavonoids and triterpenoides.[57] The results showed that ethyl acetate fraction has anti-obesity activity with IC_{50} 114.9 compared to orlistat that showed IC_{50} 23.8 (Figure 4B).

Anti-diabetic activity

The inhibition activity of ethyl acetate fraction of *S. hispanica* L. aerial parts was investigated on the α amylase enzyme using acarbose as standard then IC_{50} value was calculated (Figure 4C). The results showed that ethyl acetate farction significantly inhibited the α-amylase enzyme with IC_{50} 95.2 compared to acarbose with IC_{50} 34.71. *S. hispanica* is rich in omega-3 fatty acids which have positive effect on insulin resistance.[58]

Cytotoxic activity

Cytotoxic activity of ethyl acetate fraction of *S. hispanica* L. aerial part was evaluated against human Lung cancer cell line (A-549), human prostate carcinoma (PC-3) and colon carcinoma (HCT-116) using viability assay with vinblastine as standard. The criteria used to categorize the activity against cancer cell lines based on IC_{50} values as follows: IC50 ≤ 20 µg/ml = highly active, IC50 21 - 200 µg/ml = moderately active, IC50: 201 - 500 µg/ml = weakly active and IC50 > 501 µg/ml = inactive.[59] The presence of flavonoids, phenolics,tannin and glycosides are responsible for cytotoxic activities.[60] The results revealed

that the ethyl acetate fraction showed a highly cytotoxic activity against A-549 and HCT-116 cell lines with IC_{50} of 15 ± 0.8 µg/ml and 19.5 ± 0.6 µg/ml µg/ml respectively but showed a moderately cytotoxic activity against PC-3 with IC_{50} of 26.3 ± 1.1 µg/ml (Figures 5A, 5B and 5C).

CONCLUSION

The biological study of *S. hispanica* L. aerial parts indicates that the ethyl acetate fraction has powerful anti-oxidant, cytotoxic, anti-obesity and anti-diabetic activities. Phytochemical study indicated the presence of phenolic acids, flavonoids, tannins, diterpenoids, lignans and triterpenoids. Further studies are required to detect the exact mechanism of action and to characterize more chemical compounds responsible for the pharmacological activities of *S. hispanica* L.

DECLARATION OF COMPETING INTEREST

There are no conflicts to declare.

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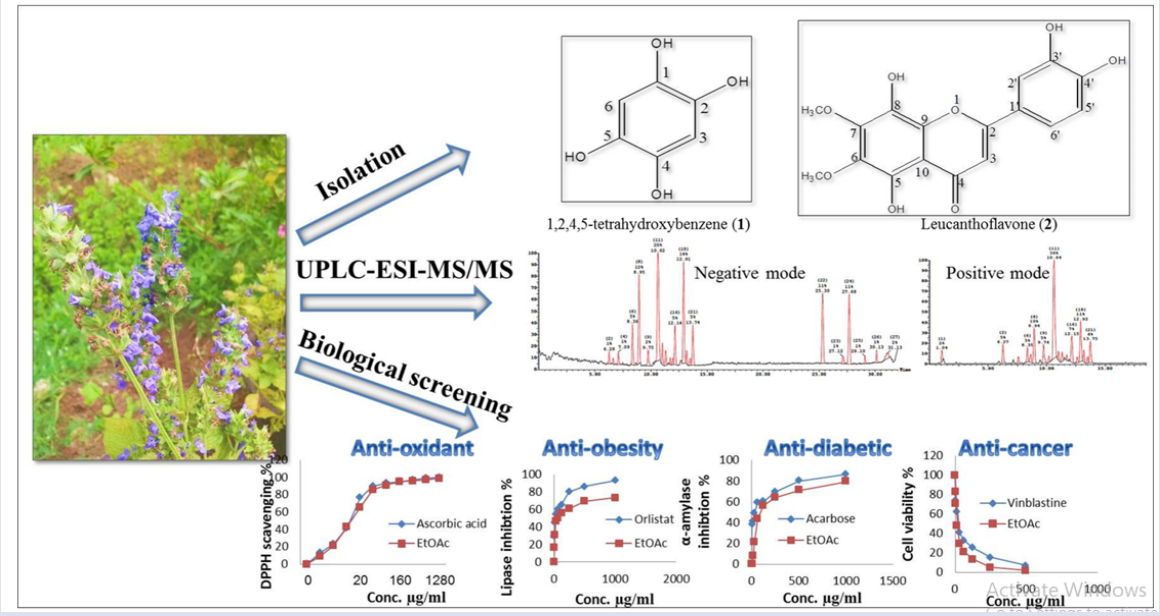
GRAPHICAL ABSTRACT



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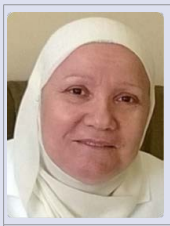	**Prof. Dr. Ehsan Mahmoud AbouZeid** graduated in pharmaceutical sciences in 1974, Cairo University and subsequently completed her MSc (1981) at University of Zagazig and PhD (1986). Assistant PhD (1986) through a channel system mission with institute of pharmaceutical chemistry, Storrs Univeristy, America. Professional in 2003 as Prof. of pharmacognosy at Faculty of Pharmacy, Zagazig University, Egypt.
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