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, FTIR, 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-obesity activity using pancreatic lipase inhibitory assay, anti-diabetic activity using α-amylase inhibition assay and anti-cancer activities using cell viability assay. **Results:** Six compounds were isolated including 1,2,4,5 tetrahydroxy benzene (1), leucantho flavone (2), rhamnetin (3), apigenin-7-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 IC₅₀ 13.11 compared to ascorbic acid, anti-obesity activity with IC₅₀ 59.3 compared to orlistate, anti-diabetic activity with IC₅₀ 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.

**INTRODUCTION**

Family *Lamiaceae* consists of about 250 genera and 7000 species worldwide.1 The genus *Salvia* has about 1000 species.2 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 which have the most appropriate antioxidant activity4,5 and showed anti-obesity, anti-diabetic, anti-oxidant and anti-microbial activities.6-13 On the other hand, the study of *S. hispanica* L. aerial parts indicate the presence of neoclerodane diterpenoids14,15 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.1H- and 13C-NMR spectral analyses were carried out using Bruker (Switzerland) at 400 and 100 MHz, respectively. Rotatory evaporator (Büchi, Germany). HPLC analysis were recorded on Shimadzu LC-10AT with a Shimadzu UV-10A detector. Infrared spectral analysis were recorded in a Pye-Unicam SP 3000 FTIR spectrophotometer of Alpha (100523), Jasko, Germany. Infrared spectral analysis were recorded in a Pye-Unicam SP 3000 FTIR spectrophotometer of Alpha (100523), Jasko, Merck, Germany. INFRA-IR analysis were recorded in a Pye-Unicam SP 3000 FTIR spectrophotometer of Alpha (100523), Jasko, 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.5-10) 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 (9x 500 ml) then...
dichloromethane (7x 500 ml) and finally ethyl acetate (5x 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 and the polarity was increased gradually using methanol to yield 55 fractions. Fractions (15-20) eluted by 4% MeOH/CH₂Cl₂ 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/CH₂Cl₂ were combined, concentrated then subjected to TLC examination, revealed the presence of two major yellow spots using ammonia vapour and crystallized to afford yellow powder of compound 2. Fractions (27-33) eluted by 8% MeOH/CH₂Cl₂ were combined, concentrated then subjected to TLC examination, revealed the presence of two major yellow spots using ammonia vapour and crystallized to afford yellow powder of compound 3. Fractions (34-40) eluted by 25% MeOH/CH₂Cl₂ 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 2. Fractions (41-53) eluted by 30% MeOH/CH₂Cl₂ 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 yellow powder with R f 0.48 (CH₂Cl₂ : CH₃OH; 9:1). FT-IR(KBr. 3163, 1685, 1582, 1444. EI-MS: m/z 414[(M + H) - Glu] +. 1 H-NMR(CD3OD, 100 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,dd,J=2,8Hz,H-3'), 6.69(1H,dd,J=8Hz,H-5'), 6.65(1H,dd,J=8Hz,H-5''), 2.97(1H,dd,J=8,12Hz,H-7a'), 3.12(1H,dd,J=12Hz,H-7b') and 5.11(1H,dd,J=8Hz,H-8'). C-NMR (CDOD, 100 MHz); δ(ppm) 128.95(C-5), 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-10), 112.30(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 170.7(C-9).

Kampferol-7-O-β-D-glucoside(5): yellow powder with m.p 247-250°C and Rf 0.43(ETHOAc:OCH₃:H₂O; 6:1:0.3). UV(λmax, MeOH): 268, 338 nm, (+NaOAc): 274, 394 nm, (+AlCl₃):273, 383 nm, (+AlCl₃ + HCl): 274, 389 nm, (+NaOAc+H₂BO₂): 269,338 nm.EI-MS: m/z = 361 [M+H] +. 1 H-NMR(CD3OD, 400 MHz); δ(ppm) 6.07(1H,s,H-5), 6.84(1H,t,J=8Hz,H-6'), 6.89(1H,d,J=8Hz,H-6''), 6.92(1H,d,J=8Hz,H-5''), 6.98(1H,d,J=8Hz,H-7'), 7.00(1H,d,J=8Hz,H-7''), 7.08(1H,d,J=8Hz,H-8'), 7.22(1H,d,J=8Hz,H-8''). C-NMR (CDOD, 100 MHz); δ(ppm) 115.27(C-2), 114.49(C-3), 137.76(C-4), 137.84(C-5), 124.91(C-6), 129.00(C-7), 129.50(C-8), 141.28(C-9), 141.55(C-10), 147.72(C-11), 147.94(C-12), 166.52(C-13), 166.55(C-14), 166.70(C-15), 167.80(C-16), 167.92(C-17).

**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 C18 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 H₂O 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. Briefly, ethyl acetate fraction was determined at different concentration 2.5, 5, 10, 20, 40, 80, 160, 320, 640 and 1280 μg/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. 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 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 (acarbose) were estimated.

**Anti-diabetic activity**

The anti-diabetic activity was determined by a -amylose inhibition method. Briefly, 1ml of the fraction of various 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 (acarbose) were estimated.

**Cytotoxic activity**

The anti-cancer activity using cell viability assay. 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 (orlistat) were estimated.

**RESULTS**

**Chemical investigations**

Six compounds were isolated and identified using physical investigations in addition to spectral analyses that 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 [galloloy moiety] and \( m/z \) 107 [-CO, (44Da)]\(^{26}\). The identification of compounds 2 and 4 were confirmed by the product ions at \( m/z \) 135\(^+\) and 29,\(^+\) 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 3 revealed to loss of arbutin moiety.\(^{33}\) 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\(_2\)O and CO\(_2\)as in case of compounds 16, 22 and 25.\(^{31}\) Compounds 17 and 27 gave the molecular ion peak [M-H] at \( m/z \) 359 and 373, respectively. The MS\(^+\) spectrum showed the product ions at

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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   | Caffeoyl 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 |
| 7   | or Orientin          | 7.99      | 448 | 447   |       | 357,327 | 28  |
| 8   | Iso-orientin         | 8.25      | 448 | 447   |       | 339,327 | 28  |
| 9   | Vitexin              | 8.52      | 432 | 431   |       | 341,311,283 | 30 |
| 10  | Iso-vitexin          | 8.54      | 432 | 433   |       | 413,313 | 30  |
| 11  | Naringenin-O-hexoside| 8.66      | 434 | 433   |       | 272   | 31  |
| 12  | Kaempferol-O-glucoside| 8.72     | 448 | 449   |       | 287   | 32  |
| 13  | Apigenin-O-glucoside | 8.95      | 432 | 431   |       | 269,153 | 33  |
| 14  | Scutellarein-O-hexoside| 9.04    | 448 | 449   |       | 287   | 34  |
| 15  | Rutin or Hesperidin  | 9.25      | 610 | 609   |       | 360   | 35  |
| 16  | Quercetin-O-glucoside| 9.27      | 464 | 463   |       | 360,171,131 | 36 |
| 17  | Syringic acid glucoside| 9.79     | 360 | 359   |       | 197,179,161,135 | 8 |
| 18  | Rosmarinic acid      | 10.20     | 360 | 359   |       | 197,179,161,135,73 | 8 |
| 19  | Methyl-O-ellagic acid| 10.65     | 316 | 315   |       | 300   | 37  |
| 20  | Medioresinol-O-glucoronide| 11.03  | 564 | 563   |       | 387,207,193 | 38 |
| 21  | Caftaric acid        | 11.17     | 312 | 313   |       | 181   | 39  |
| 22  | Quercetin-3-methyl ether| 11.23    | 316 | 317   |       | 302,153 | 40  |
| 23  | Ferulic acid hexoside| 11.30     | 356 | 357   |       | 179,177 | 41  |
| 24  | Rhamnetin or isorhamnetin| 11.31    | 316 | 315   |       | 360,165 | 40  |
| 25  | Myricetin            | 11.32     | 318 | 317   |       | 151   | 42  |
| 26  | Danshenu glucuronide | 11.34     | 374 | 373   |       | 197,179,175,135,123 | 8 |
| 27  | Embellifterone       | 11.35     | 162 | 163   |       | 135   | 43  |
| 28  | Methyl rosmarinic acid| 11.77     | 374 | 373   |       | 179,161,135 | 8 |
| 29  | Luteolin or kaempferol| 12.19     | 286 | 285   |       | 217,199,151,133 | 32 |
| 30  | Leucantholavine or eupatolin| 12.48   | 346 | 345   |       | 330,315 | 33, 44 |
| 31  | Syringetin           | 12.48     | 346 | 345   |       | 330,315 | 45  |
| 32  | VISIDULIN III        | 12.50     | 346 | 345   |       | 330,287,243 | 46 |
| 33  | 1,2,4,5-tetrahydroxy benzene| 12.65   | 142 | 143   |       | 110,78 | 8   |
| 34  | Medioresinol        | 12.84     | 388 | 387   |       | 207,179 | 47  |
| 35  | Jaceosidin or Tricin | 13.39     | 330 | 329   |       | 314,299 | 33, 48 |
| 36  | Carnosol            | 17.07     | 330 | 329   |       | 285   | 8   |
| 37  | Salvimarind B       | 28.90     | 390 | 391   |       | 491   | 49  |
| 38  | Triterpenoids d.v.s | 30.13     | 663 | 664   |       | 551,495,439 | 32 |

Underlined numbers represent the base peak.

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.
Flavonoid-Aglycones

Methodology

Data analysis was performed using the following steps:

• Identification of compound 29 showed the molecular ion peak \([\text{M-H}^{-}]\) at \(m/z\) 357.

• Fragmentation at \(m/z\) 110 and 78 of compound 32 formed due to loss of successive hydroxyl groups.

Lignans

Compounds 25 and 30 that were tentatively identified as medioresinol-O-glucoronide and medioresinol according to precursor ions \([\text{M-H}^{-}]\) at \(m/z\) 563 and 387, respectively. The MS\(^2\) spectrum of compound 25 showed the fragment ions at 387\([\text{M-H-Glu}]^{-}\) and 207\([\text{M-H-Glu-180}]^{-}\).

Coumarins

Compounds 26 (umbelliferone) showed the fragment ion at 135 \([-\text{CO(28 Da)}]\).

Biological activities

Anti-obesity 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. The DPPH scavenging percentage of ethyl acetate fraction of \(S. \text{hispanica}\) 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 fraction has anti-obesity activity with IC\(_{50}\) 114.9 compared to orlistat that showed IC\(_{50}\) 12.50 μg/ml.

Anti-diabetic activity

The inhibition activity of ethyl acetate fraction of \(S. \text{hispanica}\) 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 fraction significantly inhibited the α-amylase enzyme with IC\(_{50}\) 95.2 compared to acarbose with IC\(_{50}\) 34.71 µg/ml. \(S. \text{hispanica}\) is rich in omega-3 fatty acids which have positive effect on insulin resistance.

Cytotoxic activity

Cytotoxic activity of ethyl acetate fraction of \(S. \text{hispanica}\) 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: IC\(_{50}\) ≤ 20 μg/ml = highly active, IC\(_{50}\) 21 - 200 μg/ml = moderately active, IC\(_{50}\) 201 - 500 μg/ml = weakly active and IC\(_{50}\) > 501 μg/ml = inactive. The presence of flavonoids, phenolics, tannins and glycosides are responsible for cytotoxic activities. The results revealed...
that the ethyl acetate fraction showed a highly cytotoxic activity against A-549 and HCT-116 cell lines with IC₅₀ 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 IC₅₀ 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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