SUPPLEMENTARY MATERIAL

Comparative secondary metabolites profiling and biological activities of aerial, stem and root parts of *Salvadora oleoides* Decne (Salvadoraceae)

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Abstract

In this study, different parts (aerial, stem and root) of *Salvadora oleoides* Decne were investigated in order to explore their phytochemical composition and biological potential. The bioactive contents were evaluated by conventional spectrophotometric methods. Additionally, the secondary metabolite compounds were identified by UHPLC-MS analysis. Biological potential was evaluated by determining antioxidant (DPPH, FRAP and Phosphomolybdenum) and enzyme inhibitory (butrylcholinesterase and lipoxygenase) effects. Higher total bioactive contents were found in methanolic extracts which tend to correlate with higher radicalscavenging and reducing potential of these extracts. LC/MS spectrum revealed the presence of 16 different secondary metabolites belonging to terpene, glucoside and sesquiterpenoid derivatives. Glucocleomin and emotin A were the main compounds present in all three parts. The strongest butrylcholinesterase and lipoxygenase inhibitory activity was observed for root and stem DCM extracts. Demonstrated biological potential of *S. oleoides* plant can trace a new road map for developing newly designed bioactive pharmaceuticals.

**Keywords:** *Salvadora oleoides*, antioxidant, enzyme inhibition, phytochemical, LC-MS
Experimental

1. **Plant collection and extraction**

Aerial, stem and root parts of *S. oleoides* were collected in July from Bahawalpur, Pakistan and identified by Dr. H. Waris, Taxonomist at Cholistan Institute of Desert Studies, The Islamia University of Bahawalpur, Pakistan. In addition a voucher specimen number (SO-WP-01-15-116) were also deposited in the herbarium of Department of Pharmacy and Alternative Medicines, The Islamia University of Bahawalpur. The shade-dried parts were subjected for extraction by maceration (72 hrs) successively with dichloromethane and methanol at room temperature with occasionally shaking for 24 hrs. The resultant extracts were concentrated by Rotavapor-R20 at 35 °C.

2. **Total phenolic and flavonoid contents**

Total phenolic content assay was done by utilizing well-established Folin–Ciocalteu reagent method (Kahkonen et al. 1999). The total phenolic content was expressed as mg gallic acid equivalent per gram of fresh sample (mg GAE/g). Total flavonoid contents was determined with the aluminium chloride colorimetric method as described by Chew et al. (Chew et al. 2009). The flavonoid concentration was expressed as mg quercetin equivalent per gram sample (mg QE/100).

3. **LC-MS analysis**

UHPLC Accurate-Mass Q-TOF (Agilent 1290 Infinity LC system coupled to Agilent 6520) mass spectrometer with dual ESI source was used. Column specifications were as: XDB-C18 Agilent Zorbax Eclipse, narrow-bore 2.1 x 150 mm, 3.5 micron (P/N: 930990-902). The temperature of column was maintained at 25 °C, while auto-sampler temperature was 4 °C. The following two mobile phases used were: A (0.1% formic acid in water), B (0.1% formic acid in acetonitrile) at flow rate of 0.5 mL/min. Injection volume was 1.0 µL. Run time was 25 min and post-run time was 5 min. MS analysis full scan was carried out over a range of m/z 100-1000 employing electrospray ion source in the negative ionization mode. Flow rate for nitrogen as nebulizing and drying gas was 25 and 600 L/hour, respectively with drying gas temperature of 350 °C. The fragmentation voltage was optimized to 125. Capillary voltage for analysis was 3500 V.
4. Antioxidant assays

4.1. DPPH assay

In this method, 1 mL of plant extract of different concentrations (1000-15.625 µg/mL) was added to 2 mL of DPPH solution (0.059 mg/mL methanol). Absorbance was measured at 517 nm after 30 min incubation, (Miliauskas et al. 2004). Data was expressed as:

\[
\text{RSC} \, (\%) = 100 - \frac{(\text{abs}_c - \text{abs}_s)}{\text{abs}_c}
\]

\(\text{Abs}_s\) = absorbance of sample, \(\text{Abs}_c\) = absorbance of control.

4.2. FRAP assay

Plant sample (1000 µg/mL) was added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1% w/v), incubated for 20 min at 50°C. After 20 min, trichloroacetic acid (2.5 mL, 10% w/v) was added. The contents were divided into two halves; equal volume of water was added in one half of 2.5ml and then 0.5 mL of FeCl₃ solution (0.1% w/v) was added. The contents were incubated for 30 min at 25 ºC and the absorbance was measured at 700 nm (Chan et al. 2010). The results were expressed as mg GAE/g.

4.3. Phosphomolybdenum assay

Total antioxidant capacity (TAC) of was determined by phosphomolybdenum method (Prieto et al. 1999). Briefly, Plant extract solution (0.3 mL, 1 mg/mL) was mixed with 3 ml of molybdate reagent solution, incubated at 95 ºC for 90 min and the absorbance of the solution was measured at 695 nm against blank. TAC was expressed as equivalent of gallic acid (mg GAE/g) (Prieto et al. 1999).

5. Enzyme inhibition studies

5.1. Butrylcholinesterase assay

The BChE inhibition activity was performed according to the method (Ellman et al. 1961) with slight modifications. Total volume of the reaction mixture was 100 µl. It contained 60 µl Na₂HPO₄ buffer with concentration of 50 mM and pH 7.7. 10 µl test compound (0.5 mM well⁻¹) was added, followed by the addition of 10 µl (0.005 unit/ well) enzyme. The contents were mixed and pre-read at 405 nm. Then, contents were pre-incubated for 10 min at 37 ºC. The
reaction was initiated by the addition of 10 µl of 0.5 mM/well substrate (butyrylthiocholine chloride), followed by the addition of 10 µl DTNB (0.5 mM/well). After 30 min of incubation at 37 ºC absorbance was measured at 405 nm using 96-well plate reader (Synergy HT, Biotek, USA). All experiments were carried out with their respective controls in triplicate. Eserine (0.5 mM/well) was used as a positive control. The percent inhibition was calculated with the help of following equation 1.

\[
\text{Inhibition (\%)} = \left( \frac{\text{Control} - \text{Test}}{\text{Control}} \right) \times 100 \quad \ldots \ldots \ldots \text{Eq. 1}
\]

Where,

\[
\text{Control} = \text{Total enzyme activity without inhibitor.}
\]

\[
\text{Test} = \text{Activity in the presence of test compound.}
\]

IC\textsubscript{50} values were calculated using EZ–Fit Enzyme kinetics software (Perrella Scientific Inc. Amherst, USA).

5.2. Lipoxygenase assay

Lipoxygenase (LOX) activity was performed according to the method (Baylac and Racine 2003) with slight modifications. A total volume of 200 µl lipoxygenase assay mixture contained 140 µl sodium phosphate buffer (100 mM, pH 8.0), 20 µl test compound and 15 µl purified lipoxygenase enzyme (600 units/well, Sigma Inc.). The contents were mixed and pre-read at 234 nm and pre-incubated for 10 minutes at 25 ºC. The reaction was initiated by addition of 25 µl substrate solution. The change in absorbance was observed after 6 min at 234 nm using 96-well plate reader. All reactions were performed in triplicates. The positive and negative controls were included in the assay. Baicalin (0.5 mM/well) was used as a positive control. The percentage inhibition (\%) was calculated by Eq. 1.

6. Statistical analysis

All the experiments were carried out in triplicates to calculate the mean values which are expressed as the mean ± standard deviation (SD). The results were analysed employing one way analysis of variance (ANOVA). Tukey’s test was used for the post hoc treatment using SPSS (Statistical Package for Social Science) 24.0 for windows.
References

Baylac S, Racine P. 2003. Inhibition of 5-lipoxygenase by essential oils and other natural fragrant extracts. Int. J. Aromather. 13: 138-142.

Chan E, Lim Y, Chong K, Tan J, Wong S. 2010. Antioxidant properties of tropical and temperate herbal teas. J Food Compost Anal. 23: 185-189.

Chew Y-L, Goh J-K, Lim Y-Y. 2009. Assessment of in vitro antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia. Food Chem. 116: 13-18.

Ellman GL, Courtney KD, Andres V, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 7: 91-95.

Kahkonen MP, Hopia AI, Vuorela HJ, Rauha J-P, Pihlaja K, Kujala TS, Heinonen M. 1999. Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food chem. 47: 3954-62.

Miliauskas G, Venskutonis P, Van Beek T. 2004. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chem. 85: 231-237.

Prieto P, Pineda M, Aguilar M. 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem. 269: 337-341.
Figure captions

Figure S1. LC-MS total ion chromatograms (TICs) of aerial methanol extract of *S. oleoides*.
Figure S2. LC-MS total ion chromatograms (TICs) of stem methanol extract of *S. oleoides*.
Figure S3. LC-MS total ion chromatograms (TICs) of root methanol extract of *S. oleoides*.
Figure S4. *In vitro* enzyme inhibition (%) of different parts of *S. oleoides*.
Tables and Figures

Table S1. Extraction yield and total bioactive contents of *S. oleoides* different parts

| Plant part | Solvents | Abbr. | Yield (%) | Total phenolic content (mg GAE/g) | Total Flavonoid content (mg QE/g) |
|------------|----------|-------|-----------|-----------------------------------|----------------------------------|
| Aerial     | Methanol | SA-M  | 21%       | 0.4±0.78                          | 167.55±0.21                      |
|            | DCM      | SA-D  | 16%       | 0.34±0.61                         | 158.51±3.51                      |
| Stem       | Methanol | SS-M  | 19%       | 0.46±1.05                         | 88.06±1.48                       |
|            | DCM      | SS-D  | 14%       | 0.13±0.45                         | 42.87±1.70                       |
| Root       | Methanol | SR-M  | 18%       | 0.82±1.21                         | 154.76±2.21                      |
|            | DCM      | SR-D  | 11%       | 0.01±1.21                         | 45.21±0.64                       |

SA-M: *S. oleoides* aerial methanol extract; SA-D: *S. oleoides* aerial DCM extract; SS-M: *S. oleoides* stem methanol extract; SA-D: *S. oleoides* stem DCM extract; SR-M: *S. oleoides* root methanol extract; SR-D: *S. oleoides* root DCM extract. Data from three repetitions, with mean ± standard deviation; GAE: gallic acid equivalent; QE: quercetin equivalent;
Table S2: Secondary metabolites identified in different parts of *S. oleoides*

| S.no | RT (min) | B. peak m/z | Compound Identified | Compound class | Mol. formula | Mol. Mass |
|------|----------|-------------|---------------------|----------------|--------------|-----------|
|      |          |             | **S. oleoides** aerial methanol extract (negative ionization mode) | | | |
| 1    | 0.791    | 317.06      | Prekinamycin         | Napthalene     | C18 H10 N2 O4 | 318.06    |
| 2    | 7.411    | 404.07      | Glucocleomin         | Glucoside      | C12 H23 N O10 S2 | 405.07    |
| 3    | 7.636    | 315.11      | Hydroxytyrocol 1-O-glucoside | Oleuropein | C14 H20 O8 | 316.11    |
| 4    | 14.005   | 675.36      | (S)-Nerolidol 3-O-[a-L-Rhamnopyranosyl-(1->4)-a-L-rhamnopyranosyl-(1->2)-b-D-glucopyranoside] | Terpene | C33 H56 O14 | 676.36    |
| 5    | 15.781   | 595.29      | Salannin             | Limonoid       | C34 H44 O9 | 596.29    |
| 6    | 16.826   | 571.29      | Ganoderic acid H     | Triterpenoid   | C32 H44 O9 | 572.29    |
|      |          |             | **S. oleoides** aerial methanol extract (positive ionization mode) | | | |
| 7    | 15.233   | 331.27      | 1-Monopalmitin       | Fatty acid     | C19 H38 O4 | 330.27    |
| 8    | 17.173   | 278.15      | Emmotin A            | Sesquiterpenoid| C16 H22 O4 | 278.15    |
| 9    | 18.65    | 279.22      | 9Z,12Z,15E-octadecatrienoic acid | Fatty acid | C18 H30 O2 | 278.22    |
| 10   | 19.543   | 625.26      | Kanokoside D         | Terpene        | C27 H44 O16 | 624.26    |
|      |          |             | **S. oleoides** stem methanol extract (negative ionization mode) | | | |
| 11   | 8.961    | 404.07      | Glucocleomin         | Glucoside      | C12 H23 N O10 S2 | 405.07    |
|      |          |             | **S. oleoides** stem methanol extract (positive ionization mode) | | | |
| 12   | 10.502   | 331.13      | Gambirtannine        | Alkaloid       | C21 H18 N2 O2 | 330.13    |
| 13   | 17.155   | 279.15      | Emmotin A            | Sesquiterpenoid| C16 H22 O4 | 278.15    |
|      |          |             | **S. oleoides** root methanol extract (negative ionization mode) | | | |
| 14   | 7.371    | 404.07      | Glucocleomin         | Glucoside      | C12 H23 N O10 S2 | 405.07    |
|      |          |             | **S. oleoides** root methanol extract (positive ionization mode) | | | |
| 15   | 0.714    | 317.20      | Cyrneine A           | Terpene        | C20 H28 O3 | 316.20    |
| 16   | 17.161   | 279.15      | Emmotin A            | Sesquiterpenoid| C16 H22 O4 | 278.15    |

RT: retention time; B. peak: base peak
Table S3. Antioxidant activities of *S. oleoides* different parts.

| Plant code | Radical scavenging activity | Reducing antioxidant power | Total antioxidant capacity |
|------------|------------------------------|----------------------------|---------------------------|
|            | DPPH (%) inhibition          | FRAP (mg GAE/g)            | Phosphomolybdenum (mg GAE/g) |
| SA-M       | 51.66±0.41                   | 37.08±1.24                 | 11.59±0.13                |
| SA-D       | 11.20±0.45                   | 9.42±0.96                  | 30.12±1.43                |
| SS-M       | 37.18±0.70                   | 26.42±1.59                 | 10.24±3.04                |
| SS –D      | 29.90±1.05                   | 16.75±0.45                 | 18.32±0.25                |
| SR-M       | 41.07±0.93                   | 22.33±1.45                 | 2.02±0.14                 |
| SR-D       | 31.72±0.38                   | 17.92±0.12                 | 16.64±0.35                |
| Quercetin  | 93.21±0.97                   | nd                         | nd                        |

* Values are expressed as means ± S.D. of three replicates; nd: not determined, FRAP: ferric reducing anti-oxidant power; GAE: gallic acid equivalent
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