SUPPLEMENTARY MATERIAL

Chemical composition, in vitro antioxidant activity and α-glucosidase inhibitory effects of the essential oil and methanolic extract of Elsholtzia densa Benth.

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Abstract

Elsholtzia densa Benth. is a traditional aromatic herb used in the pharmaceutical and flavoring industries. To analyze and compare the chemical composition, the oils and nonvolatile compounds in E. densa and Mosla chinensis Maxim. were extracted via hydrodistillation, solvent extraction or ultrasound-assisted extraction. 73 volatile compounds in the volatile oil (0.35±0.06%) obtained by E.densa via hydrodistillation were investigated by Gas Chromatography-Mass Spectrometer and compared based on different parameters. Also, the antioxidant activity and α-glucosidase inhibitory effects of the five sub-fractions of the methanolic extract were studied and the ethyl acetate sub-fraction (EC50=7.9μg/mL) and petroleum ether sub-fraction (EC50=0.0955mg/mL) showed the strongest activity respectively. This study has provided a scientific basis for scientific collection, effective development, use of Elsholtzia densa Benth., and suggested that it can be used as a potential source of antioxidants in food and a potential candidate for the management of type 2 diabetes mellitus.

Keywords: Elsholtzia densa Benth.; comparisons of the chemical composition; antioxidant activity; α-glucosidase inhibitory effects

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Experimental

1. Plant materials

Samples of *E. densa* were collected from the Qinling mountains (Shaanxi, China) in June 2014 and were dried at 30 °C. Samples of dried *M. chinensis* were purchased from the Shaanxi Guangji Pharmacy. Species identification was confirmed by Professor Guo Zengjun of the School of Pharmacy, Xi’an Jiaotong University. The voucher specimen was kept at the Pharmacy Department of the Medical School of Xi’an Jiaotong University for future reference.

2. Preparation of the methanol extracts

Methanol extract of dried plant (50g) was refluxed at a temperature of 70°C. The resulting extracts were filtered and concentrated in vacuum at 40°C using a rotary evaporator. The residues obtained was suspended in water using an ultrasonic bath and successively partitioned with petroleum ether, chloroform, ethyl acetate and butanol to obtain five sub-fractions. Then the solvents were removed by a rotary evaporator, and the dried residues were kept in a dark place at -4°C until the test.

3. Extraction of the essential oil

*Hydrodistillation*: The dried *E. densa* samples were subjected to HD with a Clevenger-type apparatus for 4 h, and the essential oil was collected. The aerial parts and roots of the dried *E. densa*, as well as the dried *M. chinensis* samples, were similarly treated. The obtained essential oils were dried with anhydrous sodium sulfate and stored at 4 °C until further use. The nonvolatile components were dissolved in water and fractionated by ethyl acetate; the samples were stored at 4 °C until further use.

*Solvent Extraction*: The dried plant samples were weighed and quantitatively transferred into a conical flask, before the chemical components were extracted with ethyl acetate for 48 h at room temperature. After extraction, the samples were stored at 4 °C until further use.

*Ultrasound-assistant Extraction*: The dried plant samples were weighed and quantitatively transferred into a conical flask before these samples were treated with a fixed ultrasound power of 150 W for 2 h at room temperature. After extraction, the samples were stored at 4 °C until further use.
4. Analysis of the essential oil

GC/MS was performed on a Shimadzu GCMS-QP2010 Plus detector coupled to a Shimadzu GC-2010 apparatus with a Rtx-5MS capillary column (5% diphenyl, 95% dimethyl polysiloxane; length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm). The injector, GC–MS interface, and ion source were maintained at 240, 280, and 200 °C, respectively. The following temperature program was employed: 60 °C (3 min), 60–260 °C (10 °C min), and holding time at 260 °C (30 min). All the compounds were identified by comparing their EI-mass spectra and their retention times with the National Institute of Standards and Technologies Mass Spectra Library.

5. Antioxidant capacity assays

DPPH radical scavenging activity was measured according to the method of Blois (1958) with minor modifications. Briefly, methanol-diluted sample was mixed with same volume of 0.1mmol/L DPPH solution dissolved in methanol. Following 30min incubation at 37°C in the dark, absorbance was measured at 517 nm and DPPH radical scavenging activity was calculated using the following formula: Inhibition% = [(Acontrol − A_sample)/Acontrol] × 100. The five sub-fractions of the methanol extract were successively determined and vitamin C was used as the standard.

6. α-glucosidase inhibition assay

Inhibitory activity against α-glucosidase was determined as described by Tibbot and Skadsen (1996). Briefly, the reaction mixture containing 20μL of diluted extract sample, 10μL 10U/mL α-glucosidase solution (25mM PBS buffer; pH 6.86) and 1.5mL 25mM PBS buffer (pH 6.86) was pre-incubated at 37°C for 10 min. Then, 20μL of 2.5mmol/L pNPG (25mM PBS buffer; pH 6.86) was added to the mixture, incubated in a water bath at 37°C for 20 min, and the reaction was stopped by 0.5mL 1mol/L Na₂CO₃ solution. The absorbance was measured at 405 nm. AGI activity was calculated as: Inhibition% = [(Acontrol − A_sample)/Acontrol] × 100. The five sub-fractions of the methanol extract were successively determined and acarbose was used as the standard.
| Constituents                                      | RI    | Whole Plants | Aerial Parts | Roots |
|--------------------------------------------------|-------|--------------|--------------|-------|
| Toluene                                          | 669.6 | 0.10         |              | 3.47  |
| **Hexanal**                                      | 687.5 | 0.43         | 0.66         | 4.06  |
| Camphene                                         | 786.4 | 0.18         | 0.40         |       |
| 1-Octen-3-ol                                     | 803.9 | 2.90         |              |       |
| 5-Hepten-2-one, 6-methyl-                         | 807.9 | 6.50         | tr           |       |
| 3-Octanone                                       | 813.2 | 1.68         |              | **8.68** |
| 3-Octanol                                        | 821.4 | tr           | 5.52         |       |
| **Eucalyptol**                                   | 839.3 | 1.38         | 1.69         | 2.22  |
| 1,3,6-Octatriene, 3,7-dimethyl-, (Z)-             | 848.2 | 5.06         | 6.20         |       |
| **Acetophenone**                                 | 864.1 | 6.22         | 9.31         | 1.89  |
| Octen-1-ol, acetate                              | 889.3 | 1.99         |              | 2.26  |
| Cyclobutanecarboxylic acid, octyl ester          | 896.1 | 1.46         | 1.66         |       |
| 2,6-Octadienal, 3,7-dimethyl-, (Z)-              | 975.5 | 7.62         | 3.63         |       |
| 2,6-Octadienal, 3,7-dimethyl-, (E)-              | 992.8 | 7.78         | 4.93         |       |
| 2,6-Octadienal-1-ol, 3,7-dimethyl-, acetate, (Z)-| 1004.5| 1.28         | 2.00         |       |
| Di-epi-α.-cedrene                                 | 1055.2| tr           | 3.91         |       |
| Benzene, 2-(1,1-dimethyleryl)-1,4-dimethoxy-     | 1070.2| tr           | 2.46         |       |
| **Caryophyllene**                                | 1075.2| 7.85         | 8.54         | 0.68  |
| Octen-1-ol, acetate                              | 1077.3| tr           | 0.74         | 6.21  |
| 1,6-Cyclohexadiene,1-methyl-5-methylene-8-(1-met | 1079.0| 2.44         | 4.22         |       |
| hylethyl)-, [s-(E,E)]-                           |       |              |              |       |
| **2-Methyl-2-octene**                            | 1084.6| 2.20         | 2.29         | 2.39  |
| α-Caryophyllene                                  | 1093.4| 14.34        | 9.16         |       |
| Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-    | 1100.7| 3.68         | 6.60         |       |
| α-Farnesene                                      | 1105.6| 0.92         | 1.60         |       |
| 1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, (E)- | 1137.5| 1.68         | 2.88         |       |
| 1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3  | 1166.7| 2.43         | 3.50         | 2.77  |
| 7-diene                                          |       |              |              |       |
| τau.-Muurolool                                   | 1183.9| 2.08         | 2.81         |       |
| α-Cadinol                                        | 1186.0| 2.21         | 2.66         |       |
| 1-Heptatriacotanol                                | 1194.4| tr           | 3.16         |       |
| 1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-  | 1223.6| 0.21         | 2.59         |       |
| 2-Pentadecanone, 6,10,14-trimethyl-               | 1249.8| 0.79         | 3.01         |       |
| Geranyl benzoate                                  | 1280.2| tr           | **14.75**    |       |
| Pregnenolone                                     | 1283.4| 1.65         | 1.04         | 3.15  |
| (E,E,E)-3,7,11,15-Tetramethylhexadeca-1,3,6,10,14-pentaene | 1298.9 | tr | 3.4 |       |
| Bicyclo[4.1.0]heptan-2-one,3,4,4-trimethyl-3-(3-methyl-1,3-butadienyl)- | 1546.9 | tr | 7.09 |   |
Table S2  Nonvolatile compounds obtained from the whole plants, aerial parts, and roots of *E. densa*.

| Constituents                        | RI   | MS%   | Whole plants | Aerial Parts | Roots |
|-------------------------------------|------|-------|--------------|--------------|-------|
| **Toluene**                         | 668.6| 36.25 | 34.34        | 55.01        |       |
| Oxirane-2-carboxylic acid, ethyl ester| 684.6| tr    |              |              | 2.44  |
| **Acetic acid, butyl ester**        | 692.9| 7.16  | 8.77         | 6.06         |       |
| **1-Butanol, 3-methyl-, acetate**   | 731.1| 5.62  | 5.71         | 5.02         |       |
| Octane, 3,3-dimethyl-               | 828.9| tr    |              |              | 3.88  |
| Decane, 4-methyl-                   | 830.0| 4.38  | 6.3          |              |       |
| Nonane, 4,5-dimethyl-               | 852.5| tr    |              |              | 5.45  |
| Undecane, 5,7-dimethyl-             | 853.2| 6.2   | 6.72         |              |       |
| Allyl n-octyl ether                 | 881.4| 9.31  | 10.1         |              |       |
| Decane, 2,6,7-trimethyl-            | 952.8| 4.22  | 4.81         |              |       |
| Undecane, 2,4-dimethyl-             | 982.3| 5.68  |              | 3.45         |       |
| Dodecane, 2,6,10-trimethyl-         | 991.7| 6.97  | 5.29         |              |       |
| **Hexadecane**                      | 1102.1| 4.18 | 13.48        | 8.02         |       |
| Heptadecane                         |      |       |              |              |       |
| Eicosane                            | 1195.8| 4.27 | 4.49         |              |       |

Table S3  Chemical composition of extracts from *E. densa* obtained via hydrodistillation, solvent extraction, and ultrasound-assisted extraction.

| Constituents                        | RI   | MS%   | HD  | SE  | UAE |
|-------------------------------------|------|-------|-----|-----|-----|
| **Toluene**                         | 668.6| 7.61  |     |     |     |
| Acetic acid, butyl ester            | 691.8| 2.83  | 2.52|     |     |
| 1-Octen-3-ol                       | 803.9| 2.90  |     |     |     |
| 5-Hepten-2-one, 6-methyl-           | 807.9| 6.50  |     |     |     |
| Allyl n-octyl ether                 | 832.9| -     | 1.08| 0.48|     |
| Eucalyptol                          | 839.8| 1.38  |     |     | 1.02|
| 1,3,6-Octatriene, 3,7-dimethyl-, (Z)-| 848.2| 5.06  |     |     |     |
| Acetophenone                        | 867.5| 6.22  |     |     |     |
| Octen-1-ol, acetate                 | 889.2| 4.99  |     | 3.53|     |
| 2,6-Octadienal, 3,7-dimethyl-, (Z)-| 975.5| 7.62  |     |     |     |
| 2,6-Octadienal, 3,7-dimethyl-, (E)-| 992.8| 7.78  |     |     |     |
| Caryophyllene                       | 1075.2| 7.85 |     |     |     |
| Cedrene                             | 1079.4| 2.44 | 2.24| 4.86|     |
| **α-Caryophyllene**                 | 1093.4| **14.34**| 9.03| 10.01|     |
| Benzene, 1-((1,5-dimethyl-4-hexenyl)-4-methyl-| 1104.9| 3.68 | 2.41|     |     |
| α-Farnesene                         | 1109.1| 2.92  |     |     |     |
| Naphthalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-| 1122.1| 2.68  |     |     |     |
| Compounds                                                                 | RI    | MS% | E. densa | M. chinensis |
|--------------------------------------------------------------------------|-------|-----|---------|--------------|
| Hexanal                                                                  | 684.3 | 0.43| 0.13    |              |
| Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene | 765.4 | 0.25| 0.96    |              |
| Camphene                                                                 | 771.1 | 0.18| 1.31    |              |
| Benzo[1,2-b:4,5-b']dioxol, 1-methyl-5-(1-methylethyl)-                    | 782.1 | 0.18| 0.17    |              |
| Hydrate                                                                  |       |     |         |              |
| Octadecane, 3-ethyl-5-(2-ethylbutyl)-                                    | 1590.0 | 8.19| 1.8     |              |
| Nonacosane                                                               | 1601.8 | 9.92| 2.47    |              |
| Stearic acid, 3-(octadecyloxy)propyl ester                               | 1649.6 | 3.63| 0.97    |              |
| 6,10-Dodecadien-3-ol, 3,7,11-trimethyl-                                 | 1663.5 | 3.19| 2.63    |              |
| Octadecane, 1,1'-[1,3-propanediylbis(oxy)]bis-[Hexatriacontane]          | 1684.0 | 1.5 | 0.97    |              |
| Hexatriacontane                                                          | 1924.5 | 12.91|        |              |
| Pentatriacontane                                                         | 2256.7 | 8.63|         |              |

HD: hydrodistillation; SE: solvent extraction; UAE: ultrasound-assistant extraction

Table S4  Chemical composition of oils extracted from E. densa and M. chinensis.
| Compounds | RI     | MS%  |
|-----------|--------|------|
| Thymol    | 1010.5 | 7.72 |
| Phenol, 2-methyl-5-(1-methylethyl)- | 1016.4 | 7.69 |
| Caryophyllene | 1075.2 | 7.85 |
| 1,6-Cyclodecadiene | 1079.0 | 2.44 |
| 1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (E)- | 1086.7 | 7.33 |
| α-Caryophyllene | 1093.3 | **14.34** |
| Asarone | 1096.9 | 2.54 |
| Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl- | 1104.9 | 3.68 |
| α-Farnesene | 1109.2 | 2.92 |
| Naphthalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylthethyl)-, (1S-cis)- | 1122.1 | 2.68 |
| 1,3-Benzodioxole, 4-methoxy-6-(2-propenyl)- | 1124.9 | 6.43 |
| Caryophyllene oxide | 1155.4 | 3.15 |
| 1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene | 1166.7 | 2.43 |
| Apiol | 1169.8 | 8.32 |
| 1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl- | 1208.1 | 0.21 |

**Table S5  Nonvolatile compounds in E. densa and M. chinensis.**

| Compounds | RI    | E.densa | M.chinensis |
|-----------|-------|---------|-------------|
| Toluene   | 668.6 | 6.25    | **32.24**   |
| Acetic acid, butyl ester | 692.9 | 7.16    | 3.10        |
| Heptane, 2,4-dimethyl- | 695.0 | 3.92    | 10.2        |
| 1-Butanol, 3-methyl-, acetate | 730.0 | 4.38    | 2.37        |
| Decane, 4-methyl- | 828.6 | 10.2    | 5.68        |
| Undecane, 5,7-dimethyl- | 852.1 | 6.97    | 3.04        |
| Nonane, 4,5-dimethyl- | 855.7 | 5.54    | 6.12        |
| Allyl n-octyl ether | 881.4 | 13.31   | **30.43**   |
| Bicyclo[3.1.0]hexan-3-one,4-methyl-1-(1-methylethyl)- | 893.9 | 5.54    | 6.12        |
| Decane, 2,6,7-trimethyl- | 952.1 | 4.22    | 3.04        |
| Undecane, 2,4-dimethyl- | 982.3 | 6.97    | 3.97        |
| Dodecane, 2,6,10-trimethyl- | 991.7 | 5.54    | 3.04        |
| Benzene, 1,2-dimethoxy-4-(2-propenyl)- | 1070.3 | 13.38  | **30.43**   |
| 1,6,10-Dodecatriene,7,11-dimethyl-3-methylene- | 1090.2 | 3.97    | 6.12        |
| Heptadecane | 1102.1 | 4.18    | 3.04        |
| Hexadecane | 1121.1 | 11.42   | 3.27        |
| Eicosane | 1195.8 | 10.27   | 6.12        |
| 1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)- | 1203.2 | 6.1     | 3.04        |
| Benzene, (1-ethyloctadecyl)- | 1243.5 | 3.96    | 3.04        |
| 9-Octadecenamide, (Z)- | 1450.7 | 30.43   | **30.43**   |
| 8-Methyl-6-nonanamide | 1657.8 | 5.68    | 6.12        |
Figure S1 Free radical scavenging properties of the methanolic sub-fractions of *E.densa*

Figure S2 α-glucosidase inhibitory effects of the methanolic sub-fractions of *E.densa*