SUPPLEMENTARY MATERIAL

Evaluation of antioxidant activity, total phenolics, total flavonoids and LC–MS/MS characterisation of phenolic constituents in *Stachys lavandulifolia*

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Abstract: The aim of this study was to evaluate phenolics profile and antioxidant activity of *Stachys lavandulifolia*. Total phenolics (TP), total flavonoids (TF), DPPH[•] assay (IC₅₀), ferric ion reducing antioxidant power (FRAP), and total antioxidant activity (TAC) of the methanolic extract were measured. The content of TP, TF, IC₅₀, FRAP and TAC, were obtained 16.59 gallic acid equiv./g dry matter (DM), 4.48 mg quercetin equiv. /g DM, 2.07 (μg/ml), 0.014 (absorbance /mg phenolic), and 14.61 (mg BHT equiv./g DM), respectively. The results showed that *Stachys lavandulifolia* compared to other species of *Stachys* had moderate TP content with desirable antioxidant activity. Subsequently, 59 various phenolic compounds were identified and confirmed in the methanolic extract of *Stachys lavandulifolia* using high mass accuracy by MS² experiments. The compounds consisted of 6 hydroxybenzoic acids and hydroxybenzoic aldehydes, 9 hydroxycinnamic acids, 1 coumarin, 32 flavonoids, 3 lignans, 2 stilbenes, 3 tannins and 3 other phenolics.

Keywords: *Stachys lavandulifolia*; phenolic compounds; antioxidant activity; LC-ESI-MS/MS

3. Experimental

3.1. Materials

Stachys lavandulifolia was gathered from Zagross Mountains in Isfahan province, Iran and identified by Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran, using voucher specimens which were deposited in the cited institute (no. MPH-211). The samples were dried under darkness at room temperature and then ground. All chemicals were of analytical grade, obtained from Merck (Darmstadt, Germany), Sigma-Aldrich and used without any further purification.

3.2. Extraction method

The extraction was performed using ultrasonic probe (Top sonic model, Iran) with frequency of 20 KHz. 2 g of the sample was mixed with 80 ml methanol/water (60/40) and the mixture was treated for 14 min.

3.3. Determination of total phenolic (TP) and total flavonoid (TF)

The TP of extracts was quantified by colorimetric analysis using Folin–Ciocalteu reagent described by Waterhouse (2002), and the TF was measured by a colorimetric method as explained by Hatamnia et al. (2014).

3.4. Antioxidant activity

Antioxidant activity of extract was determined by three methods of DPPH[•] assay, ferric ion reducing antioxidant power (FRAP) assay and total antioxidant capacity (TAC) assay. DPPH[•] assay was determined by the method described by Hatamnia et al. (2014). FRAP assay is based on the reduction of Fe⁺³ to Fe⁺² with blue colored. The FRAP assay was performed as described by Lim & Murtijaya (2007). TAC assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. Results were expressed as mg BHT equivalents (mg BHT /g DM) (Kubola & Siriamornpun 2008).

3.5. LC-ESI-MS/MS analyses

Qualitative LC-ESI-MS/MS was carried out using LC-API3200 (made in Canada) with Xterra RP18 column (150mm×2.1 mm., 3.5µmd). Linear gradient elution with a mobile phase comprising acetonitrile (solvent A) and ammonium acetate (10mM, pH=4) (solvent B) commenced at 20:80 (A:B) and was kept for 2 min, then it was gradually changed from 20:80 (A:B) to 90:10 (A:B) in 20 min, after that was kept at 90:10 (A:B) for 5 min, finally it was gradually changed from 90:10 to 20:80 in 5 min. The flow rate of 300µl.min⁻¹ from the chromatograph was injected directly into the ESI source, and temperature was kept at 500°C.

The mass spectrometer was equipped with an ESI interface in the negative mode with the following settings: ion spray voltage -5500 V; nebulizer gas (N2), curtain gas (N2), collision gas (N2), declustering voltage 61 V; entrance potential, 10 V; drying gas (N2). Full scan acquisition was done scanning from *m/z* 150 to 1000 u. In the product ion scan, MS/MS product ions were created by collision-activated dissociation of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer (Q2), and mass analyzed by the second analyzer of the instrument (Q3). In the precursor ion scan, Q1 scanned over all possible precursors of the selected ion in Q3 of the triple

quadrupole. The MS and MS/MS data were processed by Analyst 1.6.1 software (AB Sciex, Foster City, CA, USA). The tentative identification was based on the precise mass measurements, isotope pattern, previous literatures, and available databases, because in main cases no commercial standards were available.

References

- Dou J, Lee VS, Tzen JT, Lee MR. 2007. Identification and comparison of phenolic compounds in the preparation of oolong tea manufactured by semifermentation and drying processes. *J. Agric. Food chem.* 55:7462-7468.
- Flamini R. 2013. Recent applications of mass spectrometry in the study of grape and wine polyphenols. *ISRN spectroscopy.* 2013:45
- Hatamnia AA, Abbaspour N, Darvishzadeh R. 2014. Antioxidant activity and phenolic profile of different parts of Bene (*Pistacia atlantica subsp. kurdica*) fruits. *Food chem.* 145:306-311.
- Jaiswal R, Müller H, Müller A, Karar MGE, Kuhnert N. 2014. Identification and characterization of chlorogenic acids, chlorogenic acid glycosides and flavonoids from *Lonicera henryi* L. (Caprifoliaceae) leaves by LC–MSⁿ. *Phytochem.* 108:252-263.
- Khoo HE, Azlan A, Ismail A, Abas F. 2012. Antioxidative properties of defatted dabai pulp and peel prepared by solid phase extraction. *Molecules.* 17:9754-9773.
- Kubola J, Siriamornpun S. 2008. Phenolic contents and antioxidant activities of bitter gourd (*Momordica charantia* L.) leaf, stem and fruit fraction extracts in vitro. *Food Chem.* 110:881-890.
- Lim YY, Murtijaya J. 2007. Antioxidant properties of *Phyllanthus amarus* extracts as affected by different drying methods. *LWT-Food Sci. Technol.* 40:1664-1669.
- Lin LZ, Harnly JM. 2010. Identification of the phenolic components of chrysanthemum flower (*Chrysanthemum morifolium* Ramat). *Food Chem.* 120:319-326.
- Sapozhnikova Y. 2014. Development of liquid chromatography–tandem mass spectrometry method for analysis of polyphenolic compounds in liquid samples of grape juice, green tea and coffee. *Food Chem.* 150:87-93.
- Simirgiotis MJ, Bórquez J, Schmeda-Hirschmann G. 2013. Antioxidant capacity, polyphenolic content and tandem HPLC–DAD–ESI/MS profiling of phenolic

compounds from the South American berries *Luma apiculata* and *L. chequén*. Food Chem. 139:289-299.

Suárez M, Macià A, Romero MP, Motilva MJ. 2008. Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil. J. Chromatogr A. 1214:90-99.

Torras-Claveria L, Jauregui O, Bastida J, Codina C, Viladomat F. 2007. Antioxidant Activity and Phenolic Composition of Lavandin (*Lavandula x intermedia* Emeric ex Loiseleur) Waste. J. Agric. Food Chem. 55:8436-8443.

Waterhouse AL. 2002. Determination of total phenolics. Current protocols in food analytical chemistry.

Wojdyło A, Teleszko M, Oszmiański J. 2014. Antioxidant property and storage stability of quince juice phenolic compounds. Food Chem. 152:261-270.

Table S1. Retention time and mass spectrometric data of 59 phenolic compounds in *Stachys lavandulifolia* determined by LC/MS/MS

	Tentative identification	Retention time (min)	[M-H] ⁻	MS-MS ion	References
1	Coumestrol	0.719	267	210.9,238.8	(Sapozhnikova 2014)
2	Eriodictyol	0.862	286.8	150.7,134.9	(Sapozhnikova 2014)
3	Naringenin	0.933	270.8	150.8,119	(Sapozhnikova 2014)
4	Epigallocatechin gallat	1.005	457	331,169	(Simirgiotis et al. 2013)
5	Gallic acid	1.148	168.7	124.9,106.8	(Sapozhnikova 2014)
6	Pelargonidin	1.362	268.8	240.9,200.7	(Sapozhnikova 2014)
7	Biochanin	1.505	282.9	267.8,238.8	(Sapozhnikova 2014)
8	Quercetin-3- <i>O</i> -ribose	1.674	433	179	(Simirgiotis et al. 2013)
9	3,5- <i>O</i> -dicafeoyl quinic acid	1.719	515	353	(Jaiswal et al. 2014)
10	Rutin	2.005	609	299.8,270.9	(Sapozhnikova 2014)

11	Hesperetin	2.219	300.8	163.9,150.9	(Sapozhnikova 2014)
12	Genistin	3.650	268.6	159,132.9	(Sapozhnikova 2014)
13	Cyaniding-3- <i>O</i> -arabinoside	3.939	417	285	(Khoo et al. 2012)
14	Cyanidin	4.004	284.9	240.8,174.9	(Sapozhnikova 2014)
15	Oleuropein derivate	4.147	365	229,185	(Suárez et al. 2008)
16	Tyrosol	4.504	137	119,106	(Suárez et al. 2008)
17	Myricetin	5.361	316	178.9,150.8	(Sapozhnikova 2014)
18	5-Sinapoylquinic acid	5.861	387	223,191	(Lin and Harnly 2010)
19	Delphinidin	6.432	300.9	256.7,190.8	(Sapozhnikova 2014)
20	Epicatechin	7.004	288.8	245,108.9	(Sapozhnikova 2014)
21	Luteolin-7- <i>O</i> -glucoside	7.147	447	285	(Lin and Harnly 2010)
22	Quercetin-3- <i>O</i> - α -arabinoside	7.575	433	301,271	(Jaiswal et al. 2014)
23	Entrodiol	8.218	301	252.9,106	(Sapozhnikova 2014)
24	Rosmarinic acidmethylester	8.361	373	179,135	(Torras-Claveria et al. 2007)
25	Methyl gallat	8.575	183	169,125	(Flamini 2013)
26	Luteolin-7- <i>O</i> -(6- <i>O</i> -glucosyl-glucoside)	8.718	609	285	(Jaiswal et al. 2014)
27	Caffeic acid-4-glucoside	8.861	341	179,161	(Lin and Harnly 2010)
28	Petrosilbene	9.004	254.9	239.9,196.9	(Sapozhnikova 2014)
29	<i>p</i> -coumaroyl quinic acid derivatives	9.432	337	136	(Wojdyło et al. 2014)
30	5- <i>O</i> -Feruloyl quinic acid	9.861	367	191	(Jaiswal et al. 2014)
31	Equol	9.932	240.8	120.9,119.1	(Sapozhnikova 2014)
32	Furosinin	10.075	815	481,301	(Simirgiotis et al. 2013)

33	Pinoresinol	10.289	357	151,136	(Suárez et al. 2008)
34	Quercetin glucoside acylated by <i>p</i> -coumaric acid	11.146	609	463	(Wojdyło et al. 2014)
35	<i>Cis</i> -resveratrol-3- <i>O</i> -glucoside	11.289	389	227	(Flamini 2013)
36	Apigenin-7- <i>O</i> -6- <i>O</i> -pentosyl hexoside	11.717	563	225	(Jaiswal et al. 2014)
37	Procyanidin B1	12.432	577	425,289	(Flamini 2013)
38	Enterodiol	13.220	301	252.9,106	(Sapozhnikova 2014)
39	Formonetin	13.646	266.8	251.7,222.8	(Sapozhnikova 2014)
40	Hydroxytyrosol	15.64	153	123,95	(Suárez et al. 2008)
41	Eriodictyol- <i>O</i> -hexoside	15.717	449	151,135	(Torras-Claveria et al. 2007)
42	3- <i>O</i> -hydroxydihydrocaffeoylquinic acid	17.431	371	353,233	(Jaiswal et al. 2014)
43	Myricitrin 3- <i>O</i> -rhamnose	18.216	463	317	(Simirgiotis, 2013)
44	Protocatechuic acid	19.716	153	109	(Torras-Claveria et al. 2007)
45	Kaempferol	20.430	285	151,92.8	(Sapozhnikova 2014)
46	Prodelphinidin B-4	21.144	609	441	(Dou et al. 2007)
47	Trihydroxycinnamic acid	22.501	357	177	(Torras-Claveria et al. 2007)
48	5-Galloylquinic acid	23.715	343	191,169	(Dou et al. 2007)
49	Matairesinol	24.501	356.9	137,82.9	(Sapozhnikova 2014)
50	Luteolin	25.644	285	150.9,132.9	(Sapozhnikova 2014)
51	Caffeic acid	25.715	178.8	134.9,107.2	(Sapozhnikova 2014)
52	Vanillin	26.286	151	136,92	(Suárez et al. 2008)
53	Apigenin 4- <i>O</i> -hexoside	27.530	431	225	(Jaiswal et al. 2014)

54	Galocatechin	28.070	305	261	(Dou et al. 2007)
55	Vanillic acid	28.270	166.6	123.1,107.8	(Sapozhnikova 2014)
56	Luteolin- <i>O</i> -hexoside	28.480	447	447	(Torras-Claveria et al. 2007)
57	Apigenin	28.570	268.8	116.9,150.8	(Sapozhnikova 2014)
58	Apigenin 7- <i>O</i> -(6- <i>O</i> - rhamnosylhexoside)	28.715	577	269	(Jaiswal et al. 2014)
59	Cyanidin 3- <i>O</i> -galactoside	29.300	447	285	(Khoo et al. 2012)

Figure S1. Chromatogram of *Stachys lavandulifolia* extract. Peaks assignment listed in Table S1.