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Acetylcholinesterase inhibitory guided fractionation of *Melissa officinalis* L.

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ABSTRACT

The plant *Melissa officinalis* L. has been used traditionally in the treatment of cognitive dysfunction. Based on its traditional medicinal use, it was assessed for its clinical efficacy in mild to moderate Alzheimer's patients. The plant was effective in the management of the disease. Therefore, based on this result, a similar plant extract was prepared in order to be screened for bioactivities which are relevant in Alzheimer's disease therapy. The extract was recently screened for antioxidant activity and it showed a wide range of antioxidant properties. Another important bioactivity is acetylcholinesterase inhibition, which the extract was screened for in the current investigation. The extract was capable of inhibiting the enzyme in a time and dose-dependent manner. Activity of the extract at 10 min was estimated as 1.72 ± 0.16 μg equivalents of physostigmine/mg of the extract. Acetylcholinesterase inhibitory guided fractionation of the extract was then carried out. Most of the fractions showed inhibitory activity and were more potent than the extract. The contents of the most potent fraction were identified as *cis*- and *trans*-rosmarinic acid isomers and a rosmarinic acid derivative using LC-DAD-ESI-MS and NMR methods.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that primarily affects the elderly population and is considered to be responsible for the majority of dementia cases in people aged 65 or older. This disease is characterised by numerous symptoms such as memory and language impairment, cognitive dysfunction and behavioural disturbances (i.e., depression, agitation and psychosis), which become progressively more severe. Due to its debilitating nature, an enormous social and economic burden is placed on society. The significance of AD is further compounded as the number of identified cases is estimated to quadruple by 2050.[1] Currently there is no cure for the disorder, thus there is a real need for novel molecular templates for AD therapy. Plants have been used since antiquity in the treatment of cognitive dysfunction. It has been shown in a review carried out by Dastmalchi et al., that ethnopharmacological screening of plants may provide useful leads in the discovery of new drugs for AD therapy.[2] Based on its traditional medicinal use, *Melissa officinalis* L. was assessed for its therapeutic efficacy in AD in a clinical study carried out by Akhondzadeh et al.[3] The plant was found to be effective in the treatment of mild to moderate AD patients. Therefore, a similar plant extract using the same solvent and plant material was prepared and screened for bioactivities considered of relevance in the treatment of AD. The extract was recently screened for antioxidant activity in a battery of in vitro assays by Dastmalchi et al., and it showed a wide range of antioxidant properties.[4]

Another bioactivity of great relevance to AD therapy is acetylcholinesterase inhibition, which was used for screening and activity guided fractionation of the extract in the current investigation.

2. Results and discussion

2.1. Extraction yield

The extract yield was 308.7 mg/g (dry weight) plant material.

2.2. Screening for acetylcholinesterase inhibition

The acetylcholinesterase inhibitory effects of the extract and the reference standard physostigmine increased in a linear concentration and time-dependent manner (data not shown). Therefore, the extract and physostigmine inhibited acetylcholinesterase in a similar fashion throughout the assay period. Since the inhibitory

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activities of physostigmine and *M. officinalis* extracts were highest at the end of the assay, acetylcholinesterase inhibition at 10 min was used as the basis of screening fractions later on (Section 2.2). Using the linear regression parameters (LRPs) calculated for physostigmine from the standard response curve, the potency of the extract at 1 min intervals from $t = 2$ min (10 min in toto) is presented as physostigmine equivalents (PhE) expressed as micrograms physostigmine per milligrams of dry weight of the extract (μg PHY/mg) in Table 1.

2.3. Bioactivity-guided fractionation

The crude *M. officinalis* extract was fractionated into 12 fractions (F1–F12) on a time based scheme as it can be seen from the chromatogram presented in Figure 1. All fractions, with the exception of fractions F10–F12, inhibited acetylcholinesterase activity (Fig. 2). The PhE values at 10 min were used for estimating the potency of the fractions. The majority of the active fractions were more potent than the crude extract (1.72 ± 0.16 μg PHY/mg), which is indicative of the complex nature of possible interactions amongst the compounds within the extract. Antagonistic and synergistic effects between various constituents within plant extracts are responsible for the extracts showing lower or higher activity compared to their fractions.

Table 1
Acetylcholinesterase inhibition by *M. officinalis* L.

Time (min)	PhE[a]
2	1.73 ± 0.41
3	1.46 ± 0.28
4	1.43 ± 0.24
5	1.45 ± 0.23
6	1.46 ± 0.00
7	1.44 ± 0.18
8	1.50 ± 0.18
9	1.61 ± 0.16
10	1.72 ± 0.16

Data are presented as mean values ± 95% confidence intervals ($n = 16$).
[a] Physostigmine equivalents, expressed as micrograms physostigmine per milligram (dry weight) extract.

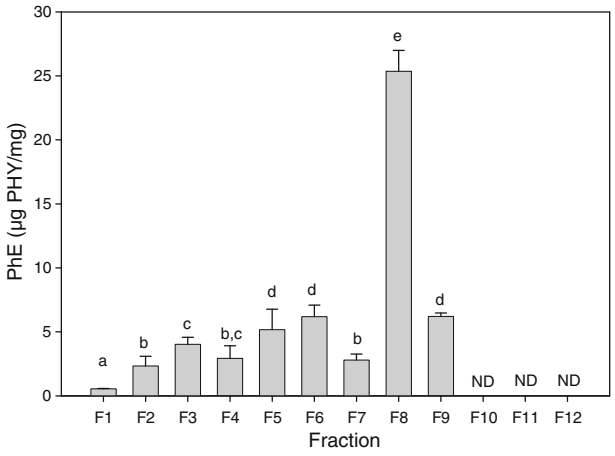

Figure 2. Acetylcholinesterase inhibitory activity of the extract fractions. Values are presented as means ± 95% confidence intervals ($n = 8$). Bars with the same lower case letters (a–e) are not statistically ($P > 0.05$) different. ND, not determined.

Fraction F8 was identified as the most potent fraction (25.36 ± 1.63 μg PHY/mg), whilst the least potent fraction was identified as fraction F1 (0.55 ± 0.03 μg PHY/mg). Interesting, the least potent fraction (F1) had the highest inhibitory activity (44.35 ± 1.31%). This shows the importance of using PhE as opposed to percentage inhibition for potency estimation. In calculating the former, yield of the sample being screened is taken into account, however, that is not the case when estimating the latter.

2.4. Compositional analysis of the most potent fraction

2.4.1. LC-DAD-ESI-MS analysis

The retention time of three components present in fraction F8 and their mass data are mentioned in Table 2.

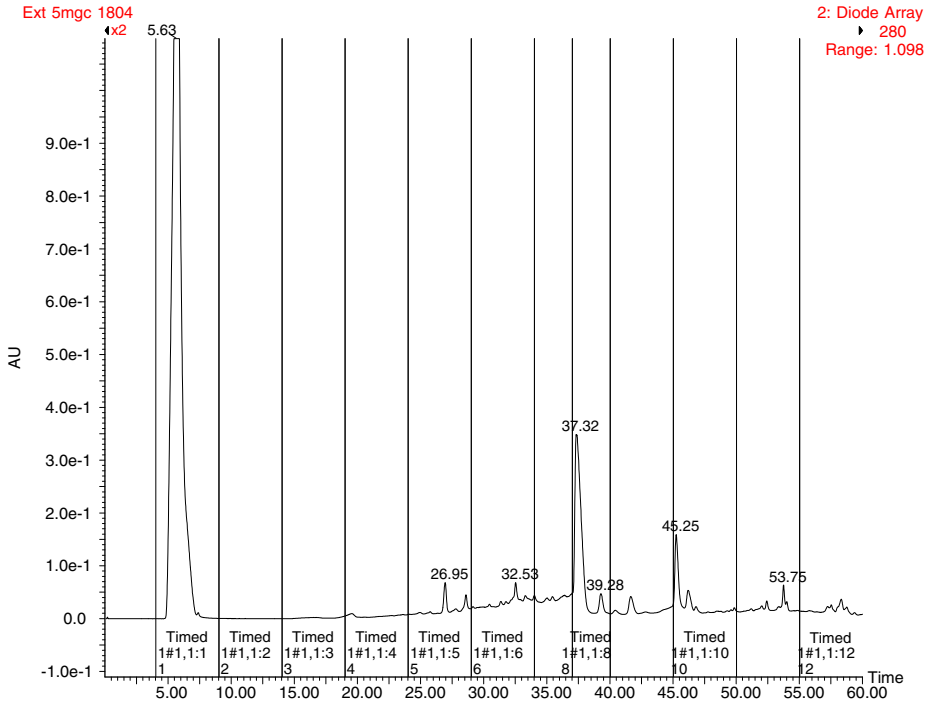

Figure 1. Semi-preparative HPLC based fractionation of the *M. officinalis* extract.

Table 2
LC-DAD-ESI-MS analysis of fraction F8

Compound	Retention time (min)	UV λ_{max} (nm)	Negative mode	
			$[M-H]^-$ (m/z)	Other fragments (m/z)
I	38.1	288, 324	359 (100)	333 (2.5), 193 (2.4), 161 (4.5)
II	38.9	290, 330	359 (100)	199 (2.9), 163 (6.1)
III	39.5	298, 334	359 (100)	249 (27.3), 171 (12.8), 155 (13.6)

Compound **II** eluting at 38.9 min was identified as rosmarinc acid, based on its retention characteristics, mass and UV spectral data (Figs. 3 and 4). The presence of rosmarinic acid in *M. officinalis* has been reported previously.[4,5] Compounds **II** and **III** have been tentatively identified as rosmarinic acid derivatives based on their similar mass and UV spectra.

2.4.2. NMR studies

Coupling constants are reported in Hertz (Hz). Spectral splitting patterns are designated as follows: s, singlet; d, doublet; dd, doublet of doublets. Deuterated solvents were purchased from Aldrich.

Fraction 8 major peaks: ^{1}H NMR δ_{ppm} 7.52 (d, 1H, J = 16.2 Hz), 7.11 (d, 1H, J = 1.8 Hz), 7.03 (dd, 1H, J = 1.8 and 8.1 Hz), 6.87 (d, 1H, J = 8.1 Hz), 6.82 (d, 1H, J = 1.8 Hz), 6.78 (d, 1H, J = 8.1 Hz), 6.70 (dd, 1H, J = 2.1 and 8.1 Hz), 6.30 (d, 1H, J = 15.6 Hz), 5.06 (dd, 1H, J = 3.6 and 9.6 Hz), 3.11 (dd, 1H, J = 3.6 Hz and 14.1 Hz), 2.95 (dd, 1H, J = 9.6 and 14.1 Hz). ^{13}C NMR δ_{ppm} 176.7, 169.4, 148.6, 147.2, 145.8, 145.2, 144.0, 130.8, 127.8, 123.4, 122.2, 117.8, 116.8, 116.6, 115.6, 115.0, 76.9, 38.0. In addition to these peaks, other minor peaks could be detected in both the ^{1}H and ^{13}C spectrum that were then investigated in more detail with 600 MHz NMR spectrometer equipped with cold probe.

Fraction 8 minor peaks: ^{1}H NMR δ_{ppm} 7.40 (d, 1H, J = 1.8 Hz), 7.05 (dd, 1H, J = 1.8 and 8.1 Hz), 6.83 (d, 1H, J = 12.3 Hz), 6.70 (d, 1H, J = 1.8 Hz), 6.69 (d, 1H, J = 8.1 Hz), 6.60 (d, 1H, J = 8.1 Hz), 6.53 (dd, 1H, J = 1.8 Hz and 8.1 Hz), 5.77 (d, 1H, J = 12.3 Hz), 5.12 (dd, 1H, J = 3.6 and 9.6 Hz), 3.04 (dd, 1H, J = 3.6 and 14.1 Hz), 2.94 (dd, 1H, J = 9.6 and 14.1 Hz). ^{13}C NMR δ_{ppm} 176.3, 168.8, 149.6, 148.2, 146.8, 145.2, 144.0, 130.8, 129.8, 123.4, 122.2, 117.8, 117.5, 116.6, 115.6, 115.0, 79.5, 39.0.

In the ^{1}H spectrum the main peaks correspond to *trans*-rosmarinic acid.[6] The minor peaks seem to correspond to *cis*-rosmarinic acid as can be seen by doublets at 5.83 and 5.77 ppm with lower J-values than for the *trans*-isomer (12.3 Hz for the *cis*-isomer vs 15.9 Hz for the *trans*-isomer). Structures were confirmed using TOCSY (*total correlation spectroscopy*), HSQC (*heteronuclear single quantum coherence*) and HMBC (*heteronuclear multiple bond correlation*) NMR experiments, which confirmed identical HMBC correlation signal patterns between both two major compounds and previously reported HMBC signal data from rosmarinic acid.[7] Small traces of a third aromatic compound could also be seen from ^{1}H NMR spectra, in too low concentration to do 2D NMR experiments for structural confirmation, but signal intensity integrals from ^{1}H spectrum support that all those signals are most likely coming from a derivative of rosmarinic acid that has a methyl ester or an aromatic methoxy group (singlet at 3.17 ppm).

3. Experimental

3.1. General procedures

Chromatographic based fractionation was carried out using a HPLC system (Waters 2545) consisting of a binary gradient module and active flow splitter coupled to a PDA detector (Waters 2996), a mass selective detector (Waters), a make up pump (Waters 515) and a sample manager (Waters 2767) interfaced to a PC running MassLynx 4.1 software (Waters). Separations were performed using Supelco C18 Discovery column (250 × 10 mm, 5 μm) along with a Supelguard Discovery C18 precolumn (10 × 10 mm, 5 μm).

3.2. Plant material

The air-dried leaves of *M. officinalis* (Specimen No. GeLM175) were obtained from the Institute of Medicinal Plants, Iranian Academic Centre for Education, Culture and Research (Halejrad, Iran). The taxonomic identity of the plant was established by a botanist from the Department of Cultivation and Development of the Institute of Medicinal Plants, Tehran, Iran.

3.3. Extraction

The air-dried leaves of the plant were extracted with 45% (v/v) aq. ethanol using medium pressure solid liquid extraction. The column used was manufactured by Büch (Büch Laboratorium Tecknic Ag, Switzerland). The material/extractant ratio was 1:5.4 (w/v). The incubation period and the time between the runs was 3 h and each run took 45 min at a flow rate of 3 mL/min. The plant material was extracted in two batches of 200 g and 250 g and the number of runs was 8 and 10, respectively. Finally, the extracts from all the runs were pooled and concentrated in vacuo at 45 °C, lyophilized and stored at 4 °C.

3.4. Acetylcholinesterase inhibitory assay in 96-well plate

Acetylcholinesterase inhibitory activity was determined using a 96-well plate format as previously described by Oinonen et al. (2006).[8] Twenty five microlitres of plant extract dissolved in 50 mM Tris buffer (pH 8) was added to the wells in a 96-well plate, followed by 25 μL of 15 mM acetylthiocholine iodide in H_2O, 125 μL of 3 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 50 mM Tris buffer containing 0.1 M NaCl and 0.02 M $MgCl_2$ (pH 8) and 50 μL of 50 mM Tris buffer containing 0.1% (w/v) bovine serum albumin (BSA). The absorbance at 405 nm was measured 10 times during 10 min (spontaneous hydrolysis) using a Victor2 multilable counter (Perkin-Elmer Life and Analytical Sciences/Wallac Oy, Turku, Finland). Then, 25 μL of 0.226 U/mL acetylcholinesterase in 50 mM Tris buffer containing 0.1% (w/v) BSA was added to start the reaction and the measurement was repeated. The absorbance readings were corrected by subtracting from them the spontaneous hydrolysis values. Acetylcholinesterase inhibitory effect of the sample was estimated in terms of percentage change in average absorbance compared to the blank, which is referred to as percentage inhibition (n = 16). Physostigmine was used as the reference standard. Concentration–response curves for the sample and physostigmine were plotted using percentage inhibition values vs. concentration. The potency of the extract was calculated from the standard response curve of physostigmine using linear regression analysis. The potency value is expressed in PhE.

3.5. Fractionation

Fractionation of the crude *M. officinalis* extract was carried out by semi-preparative RP-HPLC using a binary non-linear gradient of 0.02% aq. trifluoroacetic acid (Solvent A) and methanol (Solvent B). The composition of the mobile phase was changed as described in Table 3. The injection volume of the extract was 3 mL at a concentration of 5 mg/mL.

UV monitoring was carried out at 280 nm and the fractionation was time based as shown in Figure 1. The fractions were collected in vials, freeze dried and stored at 4 °C.

3.6. Fraction screening and compositional analysis

Each fraction was screened for acetylcholinesterase inhibitory activity using the assay mentioned above. The potency values of the fractions were estimated in terms of PhE. Once the most potent fraction was identified it was further analysed by LC-DAD-ESI-MS.

Analysis was carried out using HPLC system (Agilent 1100 series) consisting of an automatic sampler, binary pump an online degasser, heated column compartment, coupled to a DAD detector (Agilent 1100 series) interfaced to a PC running HP ChemStation Plus A.07.01. software (Agilent Technologies, Palo Alto, CA). Separation was performed using Luna C18(2) (150 × 1 mm, 5 μm, Phenomenex Ltd). Elution was carried using binary non-linear gradient of 0.02% aq. trifluoroacetic acid (Solvent A) and 0.02% methanolic trifluoroacetic acid (Solvent B). The mobile phase composition changed according to Table 4. The column was maintained at a constant temperature of 30 °C. The injection volume of the fraction was 3 μL. UV monitoring was done at 280 nm and 360 nm channels as shown in Figure 3.

Mass spectrometric analysis was carried out using an Esquire-LC quadruple ion trap mass spectrometer equipped with an electrospray interface (Bruker Daltonics, Berman, Germany) controlled using Esquire-LC NT 3.1 (Bruker Daltonics) software. Electrospray ionization was performed in negative mode. When using negative mode scan range of 100–700 *m*/*z*, capillary exit voltage of 85 V and trap drive value of 36 were used. Helium was used as the collision gas.

NMR spectra were acquired on a Varian Mercury 300 Plus spectrometer in CD_3OD. Chemical shifts for ^1H NMR (300 MHz) spectra are reported relative to internal standards CH_3OH in CD_3OD (3.31 ppm). In ^{13}C NMR spectra, peaks are referenced to the central line of the signal arising from the solvent, CD_3OD (49.00 ppm).

4. Conclusion

The plant extract demonstrated acetylcholinesterase inhibitory activity, which is a bioactivity of great relevance to AD therapy. Based on this result activity guided fractionation of the extract was carried out. Most of the fractions (F2–F9) were significantly ($P < 0.05$) more potent than the extract which can be explained in terms of antagonism between phytochemical constituents within the plant extract.

The most potent fraction F8 was found to contain rosmarinic acid and two its derivatives based on their retention characteristics, mass and UV spectral characteristics. Based on 1D and 2D NMR data *cis*- and *trans*-rosmarinic acid isomers were tentatively identified. The data also point to the existence of a derivative of rosmarinic acid that possibly has a methyl ester or a methoxy group. Rosmarinic acid possesses neurological properties relevant to AD.[9] Therefore, there is a need to investigate constituents of fraction F8 in an in vivo model of AD.

It is also recommended that the compositional analysis of remaining potent fractions should be carried out in order to determine the identity of their chemical constituents. Also there is a need to investigate the possibility of a correlation existing between antioxidant and AChE inhibitory activities.

Table 3
Mobile phase composition during preparative HPLC run

Time (min)	Mobile phase composition (A:B)[a]	Flow rate (mL/min)
0	90:10	3.5
5	90:10	3.5
15	70:30	3.5
25	57:43	3.5
37	57:43	3.5
55	20:80	3.5
57	20:80	3.5
60	90:10	3.5
72	90:10	3.5

[a] Post-column run was 30 min during which the initial run conditions were maintained for the column to be equilibrated before the next run.

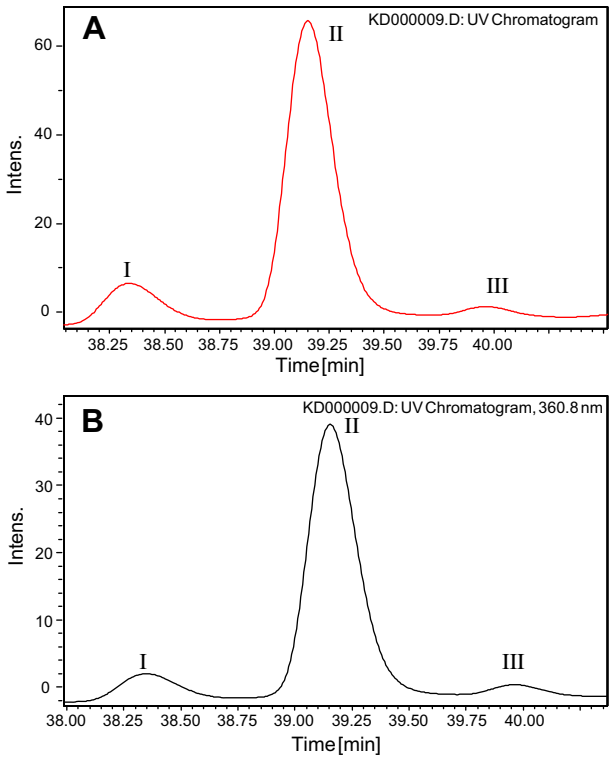

Figure 3. Chromatographic separation of components in fraction F8 at 280 nm (A) and 360 nm (B).

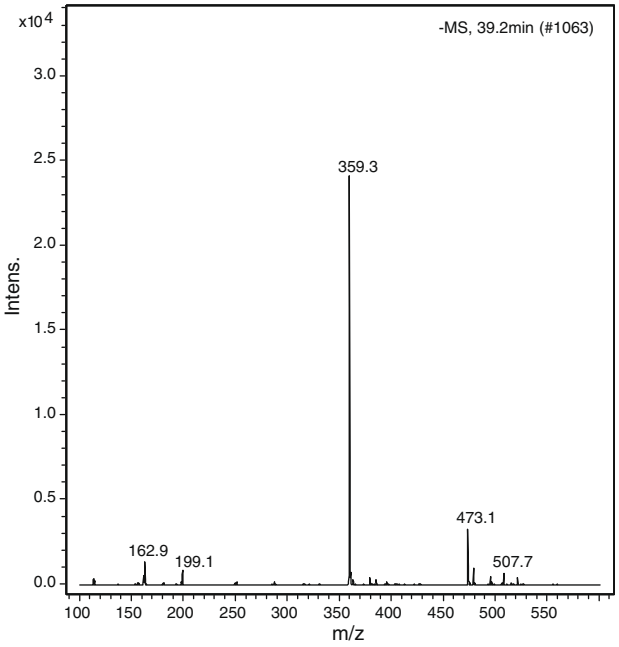

Figure 4. MS spectrum of component **II**.

Table 4
Mobile phase composition during LC-DAD-ESI-MS

Time (min)	Mobile phase composition (A:B)[a]	Flow rate (μL/min)
0	95:5	75
5	95:5	75
15	70:30	75
25	55:45	75
30	55:45	75
65	5:95	75
70	5:95	75
75	95:5	75

[a] Post-column run was 30 min during which the initial run conditions were maintained for the column to be equilibrated before the next run.

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