SUPPLEMENTARY MATERIAL

Anticholinesterase, Antioxidant activity and phytochemical investigation of aqueous extracts from five species of *Agrimonia* genus

Renata Kubínová, Emil Švajdlenka and Dagmar Jankovská*

*Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and
Pharmaceutical Sciences Brno, Palackého 1/3, Brno 61242, the Czech Republic*

Aqueous extracts of aerial flowering parts of five *Agrimonia* species (Rosaceae): *Agrimonia coreana* Nakai, *Agrimonia japonica* (Miq.) Koidz, *Agrimonia procera* Wallr., *Agrimonia eupatoria* L., *Agrimonia leucantha* Kunze were investigated on their antioxidant activity, measured using five different methods, the best was the extract from *A. procera* with IC₅₀ values from 6 to 29 µg/mL. All the extracts displayed inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) at the tested concentration of 100 µg/mL. The highest inhibition of cholinesterase was found in extract of *A. japonica* with inhibition 70.4 % for AChE and 79.8 % for BuChE. These findings are statistically significant in comparison with other extracts (p<0.001). The phytochemical analyses showed that the antioxidant activity of *Agrimonia* extracts can be affected especially by hexahydroxydiphenoyl (HHDP)-glucose and quercetin glycosides, inhibition of cholinesterases by apigenin, luteolin and quercetin glycosides.

Keywords: *Agrimonia* species; flavonoids; polyphenols; antioxidant activity; cholinesterase inhibition

*Correspondence to: Dagmar Jankovska. E-mail: djankovska@seznam.cz

1. Experimental

1.1. Plant material

Agrimonia species were collected in Medicinal Herbs Centre of Masaryk University Brno (the Czech Republic) in July 2008. *Agrimonia coreana* Nakai, *Agrimonia japonica* (Miq.) Koidz, *Agrimonia procera* Wallr., *Agrimonia eupatoria* L., *Agrimonia leucantha* Kunze were identified by chief scientific researcher of Medicinal Herbs Centre Ing. Pavel Musil and confirmed by prof. Václav Suchý, Department of Natural Drugs, Faculty of Pharmacy, Brno. Voucher specimens are deposited in the Herbarium of Department of Natural Drugs, Pharmaceutical Faculty, Brno (the Czech Republic) under numbers AE2009, AP2009, AL2009, AC2009, AJ2009.

1.2. Chemicals

All chemicals, including standards for HPLC, were purchased from Sigma-Aldrich, Prague, the Czech Republic.

1.3. General experimental procedures

HPLC-DAD-MS analyses were performed on amide-type column (Supelcosil ABZ+Plus column, length 150 x 4.6 mm, particle size 3 µm) at a temperature of 40 °C using an Agilent 1100 instrument (Agilent Technologies, Böblingen, Germany with SW HPChem). The injection volume was 5 µl and the UV detector was set to 254, 280 and 350 nm. The mobile phase for the gradient elution was a mixture of acetonitrile and 40 mM formic acid and the flow rate was 0.3 mL/min. For LC-MS detection, a Hewlett Packard Agilent 1100 Series LC(Agilent Technologies) equipped with DAD and Mass Spectroscopy Detector trap with electrospray ion source was used. The nebulising and drying gas was high-purity nitrogen (t=350°C) flowing at a rate of 10 L/min; the nebuliser pressure was 40psi and the needle voltage was 3.5 kV. The full mass scan covered the range from m/z 50 to 1000. Full scan mass spectra were collected in the negative mode. For determination of total polyphenols, flavonoids and biological activities was used a Microplate reader (Synergy HT BioTech, Highland Park, IL, USA).

1.4. Extracts

The aqueous extracts from aerial parts of *Agrimonia* sp. were prepared as decoction with water at 100°C: 120 mL water was added to 1g of dried aerial parts and was decocted 10 min. After filtration, 100 mL the extracts were lyophilised. The percentage yields (w/w) of the

lyophilised extracts were 14.6 % for *A. coreana*, 15.5 % for *A. leucantha*, 7.7 % for *A. eupatoria*, 8.5 % for *A. procera* and 7.5 % for *A. japonica*. The lyophilised extracts were kept in a dessicator, protected from light.

1.5. Determination of total polyphenols

Total polyphenols content was measured using the Folin-Ciocalteu reagent (Singleton & Rossi 1965). The reaction mixture contained: 10 μ L of extract (1 mg/mL), 10 μ L of Folin-Ciocalteu reagent and 310 μ L of sodium carbonate (7.5%). The absorbance was measured at 760 nm after 5 min. The amount of total polyphenols was calculated as a gallic acid equivalent (GAE) from the calibration curve of gallic acid standard solutions and expressed as mgs of gallic acid per gram of lyophilised extract (mg GAE/g LE).

1.6. Determination of flavonoids

Total flavonoid content was measured using aluminium chloride (Correia et al. 2006). The reaction mixture contained: 100 μ L of extract (1 mg/mL), 900 μ L of methanol and 20 μ L of aluminium chloride (10%). The absorbance was measured at 425 nm after 30 min. The amount of total flavonoids was calculated as a quercetin equivalent (QE) from the calibration curve of quercetin standard solutions and expressed as mgs of quercetin per gram of lyophilised extract (mg QE/g LE).

1.7. 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined routinely using 60 μ M DPPH (Blois 1958). The reaction mixture contained: 10 μ L of extract and 320 μ L of DPPH solution. The absorbance (A) was measured at 517 nm after 5 min. Scavenging effect (%) was calculated according to the formula: $(1 - (A_{\text{sample}} / A_{\text{control}})) * 100$

1.8. Scavenging of dihydrogen dioxide

The dihydrogen dioxide (H₂O₂) scavenging activity was measured by monitoring the H₂O₂ induced oxidation of luminol, accordingly to a described procedure (Guo et al. 2007). The reaction mixture contained: 100 μ L of 1% H₂O₂, 120 μ L of 0.1 M phosphate buffer (pH 7.0) and 10 μ L of extract. The reaction was started by the injection of 1 mM luminol in 0.1 M sodium hydroxyde (100 μ L). The resulting chemiluminescence was monitored for 15 minutes. Area under curve (AUC) was recorded in each experiment and scavenging effect (%) was calculated according to the formula:

$$(1 - (\text{AUC}_{\text{sample}} / \text{AUC}_{\text{control}})) * 100$$

Area under curve (AUC)

1.9. Scavenging of hydroxyl radical

The hydroxyl radical scavenging activity was measured by monitoring the hydroxyl radical induced oxidation of luminol, accordingly to a described procedure with modifications (Pajero et al. 2000). Hydroxyl radical was generated by a Fenton system. The reaction mixture contained: 140 μL of 0.1 M phosphate buffer (pH 7.0), 100 μL of 1 mM luminol in 0.1 M sodium hydroxyde and 10 μL of extract. Initiation of reaction was achieved by adding 30 μL of 25 μM FeSO_4 and 50 μL of 0.5 mM H_2O_2 into the mixture. The resulting chemiluminescence was monitored for 30 minutes. Scavenging effect (%) was calculated according to the formula: $(1 - (\text{AUC}_{\text{sample}} / \text{AUC}_{\text{control}})) * 100$

1.10. Scavenging of peroxy radical

The luminescence of luminol was induced by thermolysis of 2,2'-azo-bis(2-amidinopropane) (AAPH), which produce the alkylperoxyl radicals (Ritov et al. 1995). The reaction mixture contained: 120 μL of 0.1 M phosphate buffer (pH 7.0), 100 μL of 0.1 M AAPH and 10 μL of extract. Initiation of reaction was achieved by adding 1 mM luminol (100 μL) in 0.1 M NaOH into the mixture after thermal decomposition of AAPH to carbon-centred radical at 37 °C. The dynamic curves were recorded after the initiation for 30 minutes. Scavenging effect (%) was calculated according to the formula: $(1 - (\text{AUC}_{\text{sample}} / \text{AUC}_{\text{control}})) * 100$

1.11. Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)

AChE inhibitory activity was measured by the spectrophotometric method from the literature (Fan et al. 2010) which was initially developed by Ellman with slightly modification. The reaction mixture contained: 120 μL of 0.1 M phosphate buffer (pH 7.0), 20 μL of 1 U/mL AChE/BuChE solution in buffer, 20 μL of extract and 20 μL of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). After incubation at 37 °C (15 min), 20 μL of 1.5 mM acetylthiocholine iodide (ATCI)/ butyrylthiocholine iodide (BTCI), was added to initiate the reaction. The hydrolysis was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the enzyme-catalyzed reaction of DTNB with thiocholines at a wavelength of 405 nm. One set of mixtures prepared with an equivalent volume of water, instead of tested samples was used as a control. Another set of mixtures prepared with an equivalent volume of phosphate buffer instead of enzyme was used as a blank. The inhibitory

rates (%) were calculated according to the formula: $(1 - (A_{\text{sample}} - A_{\text{blank}}) / A_{\text{control}}) * 100$. Galanthamine was used as standard.

1.12. Statistical analysis

By one-way ANOVA followed by Tukey's post *hoc* test were used for statistical evaluation. Values of $p < 0.05$ were considered to be statistically significant. GraphPad Prism 5.01 was used for the analysis.

References

- Blois MS. 1958. Antioxidant determination by the use of a stable free radical. *Nature*. 81:1199-1200.
- Correia H, Gonzalez-Paramas A, Amaral MT, Santos-Buelga C, Batista MT. 2006. Polyphenolic profile characterization of *Agrimonia eupatoria* L. by HPLC with different detection device. *Biomed Chromatogr*. 20:88-94.
- Fan P, Terriera L, Hay AE, Marston A, Hostettmann K. 2010. Antioxidant and enzyme inhibition activities and chemical profiles of *Polygonum sachalinensis* F.Schmidt ex Maxim (Polygonaceae). *Fitoterapia*. 81:124-131.
- Guo S, Deng Q, Xiao J, Xie B, Sun Z. 2007. Evaluation of antioxidant activity and preventing DNA damage effect of pomegranate extracts by chemiluminescence method. *J Agric Food Chem*. 55:3134-3140.
- Pajero I, Petrakis CH, Kefalas PJ. 2000. A transition metal enhanced luminol chemiluminescence in the presence of a chelator. *J Pharmacol Toxicol Methods*. 43:183-190.
- Ritov BR, Goldman R, Stoyanovsky AD, Menshikova EV, Kagan EV. 1995. Antioxidant paradoxes of phenolic compounds: peroxyl radical scavenger and lipid antioxidant, etoposide (VP-16), inhibits sarcoplasmic reticulum Ca^{2+} -ATPase via thiol oxidation by its phenoxyl radical. *Arch Biochem Biophys*. 321:140-152.
- Singleton V, Rossi J. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic*. 16:144-158.

Table S1. HPLC retention times, UV absorption maxima and ESI-MS data for identified compounds of *Agrimonia* species aqueous extracts.

	t_R (min)	UV; λ_{max} (nm)	$[M-H]^-$ (m/z) ^a	$[M-H]^-$ fragments (m/z)	Identification
1	1.6	Slope	481	301	Hexahydroxydiphenoyl-glucose
2	5.2	280	577	451, 425, 407, 289	Procyanidin dimer
3	5.7	280	289		Catechin ^b
4	6.5	280	865	739, 695, 577, 207	Procyanidin trimer
5	8.1	267, 340	431	311	Vitexin ^b
6	8.7	260, 338	431	413, 341, 311	Isovitexin ^b
7	8.8	256, 355	609	301	Rutin ^b
8	9.1	255, 267sh, 350	447	285	Luteolin 7- <i>O</i> -glucoside ^b
9	9.4	260, 266sh, 354	463	301	Quercetin 3- <i>O</i> -galactoside ^b
10	9.6	255, 265sh, 354	463	301	Quercetin 3- <i>O</i> -glucoside ^b
11	9.8	265, 295sh, 350	593	285	Keampferol 3- <i>O</i> -rutinoside ^b
12	10.6	256, 345	431	269	Apigenin 7- <i>O</i> -glucoside ^b
13	10.8	265, 348	447	285	Kaempferol 3- <i>O</i> -glucoside ^b
14	11.7	255, 267sh, 350	461	285, 175	Luteolin <i>O</i> -glucuronid ^c
15	12.7	265, 342	445	269, 175	Apigenin 7- <i>O</i> -glucuronid ^b
16	14.5	265, 340	593	285	Kaempferol <i>O</i> -(coumaroyl)-hexoside ^c

^a ESI-MS (negative mode) data for the protonated molecular ion.

^b Identification was confirmed by comparison with standard.

^c Position of sugar moiety could not be determined by LC/MS

Table S2. Antioxidant activity (IC₅₀), polyphenol and flavonoid content of aqueous extracts

Plant	DPPH ($\mu\text{g/mL}$)	OH ($\mu\text{g/mL}$)	Peroxy ($\mu\text{g/mL}$)	H ₂ O ₂ ($\mu\text{g/mL}$)	Polyphenols (mg GAE/g LE)	Flavonoids (mg QE/g LE)
<i>A. coreana</i>	13.0 $\pm$ 1.6 ^a	30.0 $\pm$ 3.0 ^a	30.5 $\pm$ 0.2 ^a	177.7 $\pm$ 3.3 ^a	338.0 $\pm$ 5.1 ^d	14.9 $\pm$ 0.2 ^b
<i>A. japonica</i>	10.3 $\pm$ 1.7 ^b	16.0 $\pm$ 0.7 ^d	21.0 $\pm$ 0.3 ^c	41.3 $\pm$ 1.0 ^c	449.7 $\pm$ 0.5 ^b	26.3 $\pm$ 0.8 ^a
<i>A. procera</i>	6.0 $\pm$ 0.3 ^c	19.3 $\pm$ 1.0 ^c	16.1 $\pm$ 1.1 ^d	28.7 $\pm$ 0.3 ^b	526.6 $\pm$ 23.7 ^a	19.3 $\pm$ 1.0 ^c
<i>A. eupatoria</i>	13.3 $\pm$ 0.3 ^a	30.3 $\pm$ 4.3 ^a	30.7 $\pm$ 0.7 ^a	29.0 $\pm$ 0.3 ^b	402.5 $\pm$ 15.7 ^c	25.8 $\pm$ 0.9 ^a
<i>A. leucantha</i>	9.5 $\pm$ 0.5 ^b	26.7 $\pm$ 1.0 ^b	43.2 $\pm$ 0.7 ^b	49.5 $\pm$ 3.8 ^d	484.0 $\pm$ 6.2 ^a	11.9 $\pm$ 0.1 ^d
Quercetin	4.3 $\pm$ 0.1	1.6 $\pm$ 0.4	0.7 $\pm$ 0.4	15.7 $\pm$ 3.1		

Results in this table are the average of five replications $\pm$ mean standard deviation. Same letters on the same column show no significant difference ($p > 0.05$)

Figure S1. HPLC polyphenolic profile of the aqueous extract of *A. eupatoria* recorded at 254 nm. The identification of the peaks is shown in Table S1.