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Phytochemicals in leaves of *Cotoneaster mongolica*, their antioxidative, and acetylcholinesterase inhibitory activity

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ABSTRACT

The phytochemicals in the leaves of *Cotoneaster mongolica* Pojark, as well as their antioxidant and acetylcholinesterase (AChE) inhibitory activity, were studied. The methanol extract of the leaves showed acetylcholinesterase inhibitory activity (IC_{50}, 32.61 ± 0.51 μg/mL). The *n*-butanol fraction of this extract exhibited DPPH radical scavenging (IC_{50}, 55.70 ± 0.15 μg/mL) and AChE inhibitory activity (IC_{50}, 72.50 ± 0.60 μg/mL). From the *n*-butanol fraction quercetin (**1**), hyperoside (**2**), kaempferol-5-*O*-*β*-*D*-glucopyranoside (**3**), sissotrin (**4**), ursolic acid (**5**), corosolic acid (**6**), euscaphic acid (**7**), prunasin (**8**), (*2R*)-mandeloyl-*β*-*D*-glucopyranose (**9**), (*Z*)-3-hexenyl-*O*-*α*-*L*-rhamnopyranosyl-(1→6)-*β*-*D*-glucopyranoside (**10**), benzyl-*O*-*α*-*L*-rhamnopyranosyl-(1→6)-*β*-*D*-glucopyranoside (**11**) and arbutin (**12**) have been isolated and identified. Hyperoside, one of the major constituents among the isolated compounds, was active in both tested assays. Flavonol derivatives could provide the activity of this plant species.

Keywords: *Cotoneaster mongolica*, prunasin, hyperoside, DPPH scavenging, AChE inhibition

INTRODUCTION

In the Mongolian flora, the genus *Cotoneaster* (Rosaceae) contains 4 represented species: *C.mongolica*. Pojark., *C.megalocarpa* M.Pop., *C.melanocarpa* Lodd. and *C.uniflora* Bge. [1-3]. Fruits, shoots, twigs and leaves of *Cotoneaster* species as crude drugs are mainly used in the form of infusion, extract, tincture, tea and juice in traditional Mongolian medicine for the cure of intestinal inflammatory diseases, diarrhea, stomach indigestion, bowel disorders and abdominal cavity ascites, as well as rheumatoid arthritis. Fruits of *C.mongolica*, (rarely *C.melanocarpa*) under a Tibetan name "*dat-rig*" is the major component of the complex formulation *"dat-rig 9",* which supports digestion and "*dat-rig 7*", effective for the treatment of diarrhea [2, 3]. Moreover, *Cotoneaster* species occurring in Russia, Iran, Uzbekistan, Turkey and Caucasia are used in traditional medicine, especially for the treatment of neurasthenia, nervous prostration, nasal hemorrhage, excessive menstruation, neonatal jaundice and cough [4-6]. Besides medicinal utilization, some shrubs of the *Cotoneaster* genus are used as ornamental plants, for which some species were cultivated as a small *bonsai* with flowers and fruits [7, 8]. *C.mongolica* is a sub-indigenous deciduous shrub. Isoquercetin, quercitrin, rutin and isoorientin, quercetinglycosides, were identified in the leaf samples of *C.mongolica* Pojark with HPLC method [9]. However, no data were found on the biological activity of this plant species.In this study, phytochemicals in the leaves of *C.mongolica*, their antioxidant and acetylcholinesterase inhibitory activity were investigated.

EXPERIMENTAL

General experimental procedures: Thin layer chromatography (TLC) was performed on pre-coated silica gel 60 F_{254} plate (Merck, Darmstadt, Germany) and the spots were detected under UV radiation (365 nm) by spraying with 1 % methanolic diphenylboric acid-β-ethylamino ester (NP) and 5 % ethanolic polyethylene glycol (PEG); under visible light by spraying and heating at 100-105 °C with 5 % sulfuric acid; 1 % vanillin and 5 % sulfuric acid. Silica gel 60 (40-60 μm, Merck, Darmstadt, Germany) Sephadex LH-20 (25-100 mm, Pharmacia, Uppsala, Sweden), MCI gel-CHP-20P (75-150 μm, Mitsubishi Chemical Corporation, Japan) and

Sepra™ C18-E (50 µm, 65Å) were used for column chromatography (CC). NMR spectra were recorded on Bruker AM-300 Spectrometer in MeOH-d_4 ^{1}H (300 MHz) and ^{13}C (75 MHz). UV spectroscopic analysis was carried on a spectrophotometer UV-160 (Shimadzu, Japan). Mass spectra were carried out at 70 eV. All reagents and solvents used were of analytical grade.

Plant materials: Leaves and fruits of *Cotoneaster mongolica* Pojark were collected from shrubs grown alongside the dry, rocky creek, in the vicinity (Khailaast) of Ulaanbaatar, Mongolia (47° 57' N, 106' 53' E), in September 2005. Prof. Sanchir Ch., in Institute of Botany, Mongolian Academy of Sciences, authenticated the plant specimen. The voucher specimen (Cm 050911) was deposited in the Herbarium of the Natural Product Chemistry Laboratory of ICCT, MAS.

Extraction and fractionation: The air-dried and powdered leaves of *C.mongolica* (400 g) were macerated with pure MeOH (3 x 1200 mL, each 24 h) and 70 % MeOH twice (2 x 500 mL, each 24 h) at room temperature. The total extract was concentrated to dryness under reduced pressure. The concentrated solid (119 g) was reconstituted with 120 mL of dist. water, then partitioned successively with chloroform (800 mL), and *n*-butanol (800 mL) to yield 42.3 g $CHCl_3$ and 32 g *n*-BuOH fractions (fr), respectively. The aqueous soluble residue (Wat. res, 40 g), after fractionation, was kept for its free radical scavenging activity tests.

The air-dried and powdered fruits of *C.mongolica* (115 g) were macerated by MeOH (3 x 300 mL, each 24 h) at room temperature. Each time the MeOH extract was concentrated to dryness *in vacuo* and combined to yield 17 g of thick extract.

The quality of phytoconstituents of all extracts and fractions were analyzed by TLC method with $CHCl_3$: MeOH - 9 :1 and $CHCl_3$: MeOH : H_2O - 7 : 3 : 0.4 solvent systems by spraying with NP/PEG for flavonoids and phenolics, 5 % sulfuric acid and 1 % vanillin / 5 % sulfuric acid for terpenoids.

Separation and isolation: The *n*-BuOH fr. (32 g) was separated and divided into IX subfractions over silica gel 60 column (5 x 100 cm), eluted gradiently with $CHCl_3$: MeOH - 99 : 1, 98 : 2, 95 : 5, 90 : 10 and 85 : 15. Subfractions I (110 mg), II (115 mg) and V (1.17 g) were subjected to sephadex LH 20 CC eluted with CH_2Cl_2 : MeOH - 1 : 1. Subfractions II (313.2 mg), VI (2.19 g), VII (1.15 g), VIII (1.19 g) and IX (3.88 g) were subjected to MCI gel CHP 20P CC eluted with 10 %, 20 %, 40 %, 60 %, 70 %, and 80 % MeOH in water, respectively. Subfraction III (74.2 mg) was separated over silica gel 60 column with the solvent CH_2Cl_2 : MeOH - 98 : 2. The final purification and isolation of all **1-12** compounds were carried out by CC using sephadex LH 20 or C 18-E eluting with 10 %, 20 %, 30 %, 40 %, 50 % MeOH in water.

Compound **1** (10 mg) was isolated from the subfractions VII and VIII, **2** (559.7 mg), **3** (9.0 mg), **11** (185.7 mg) and **12** (36.1 mg) from the IX, **4** (17.1 mg) from the V and VI, **5** (59.4 mg) from the I and II, **6** (11.7 mg) from the II and IV, **7** (37.4 mg) from the II, **8** (2.38 g) from the II – VIII, **9** (131.4 mg) and **10** (43 mg) from the VII and IX.

Acid hydrolysis: Each compound (**2**, **3** or **8**, 2-5 mg) in vials was added 2 mL 2 N HCl in MeOH and the vial was capped tightly. The vial was heated for 3 hours at 100 °C. After being allowed to cool, the solution was diluted with dist. water (5 mL). 10 mL of ethylacetate (EA) was added, and the solution was shaken vigorously for 2 minutes. The two phases were allowed to separate, then the organic (top) layer was pipetted off and collected in another vial. This procedure was repeated 2 more times with EA, collecting a total of EA extract into the vial and reducing the volume of the extract. Aglycones were identified by comparing them with corresponding authentic quercetin and kaempferol in TLC developed with $CHCl_3$: MeOH - 9 : 1, and TLC was sprayed with NP / PEG and visualized under UV 365 nm. The water residue after hydrolysis was evaporated and dissolved in a small amount of MeOH followed by the identification of sugars by TLC in comparison to standard ones. The TLC was developed with EA : H_2O : MeOH : CH_3COOH - 13 : 3 : 3 : 4 and sprayed with 5 % sulfuric acid followed by heating at 100 °C for 10 min. [10].

DPPH scavenging activity: DPPH radical (2,2-diphenyl-2-picrylhydrazyl hydrate, TCI, Co., Ltd) scavenging activity of the crude extracts, fractions and pure compounds was determined according to the known spectrophotometric assay [11]. The absorbance was measured at 517 nm and the anti-oxidative activity (AA) was expressed in percentage:

$$AA\%=100-\{[(Abs_{sample}-Abs_{blank})\times100]/Abs_{control}\};$$

Methanol (1.5 mL) added to the plant extract solution (1.5 mL) was used as a blank. DPPH solution (1.5 mL, $6x10^{-5}$ M) plus methanol (1.5 mL) was used as a control and rutin was used as a positive control.

Acetylcholinesterase inhibiting activity: The acetylcholinesterase inhibiting activity of crude extracts, fractions and isolated pure substances was determined using a previously reported Ellman spectrophotometric method with DTNB (5,5'-dithiobis(2-nitrobenzoisc acid), TCI, Co., Ltd) color reagent [12]. The absorbance was measured at 412 nm and the AChE inhibiting activity (I) was expressed in percentage:

$$I\ (\%) = 100 \times (Abs\ 10_{control} - \Delta\ Abs_{sample}) / Abs\ 10_{control}$$

$$\text{Where; } \Delta\ A_{sample} = Abs\ 10\ min_{sample} - Abs\ 00\ min_{sample}$$

Blank solution was prepared from 0.1 mL sample (1 mg/mL) with DTNB prepared in Tris-HCl, while the positive control was prepared from DMSO (dimethylsulfoxide) with the same amount of the sample. Eserin (physostigmine, TCI, Co., Ltd) was used as a positive control.

Statistical analysis: The results were expressed as mean values and standard deviation (mean ± SD). Student's *t*-test at a level of $p<0.05$ was considered as statistically significant. The IC_{50} value, defined as the amount of the sample that could reduce the initial concentration of DPPH and AChE by 50 %, was calculated from the linear regression plots of test samples concentration against the mean inhibition in percentage. IC_{50} values were calculated using concentration of tested plant extracts, fractions and isolated substances and average percent of the antioxidant activity from three separate tests, and AChE inhibiting activities 6-8 separate tests.

RESULTS AND DISCUSSION

The methanol extract of leaves of *C.mongolica* and its derived chloroform and *n*-butanol fractions, including water residue, as well as the methanol extract of fruits were evaluated for their anti-oxidative and AChE inhibitory activities (Table 1 and 2).

Table 1. Results of the DPPH radical scavenging activity, IC_{50} ± SD (μg/mL)

Extracts and fractions	Leaves	Fruits
Methanol extract	> 200	108.50 ± 0.71
Chloroform fraction	> 200	-
n-Butanol fraction	55.70 ± 0.15	> 200
Water residue	> 200	-
Standard - Rutin	22.66 ± 0.15	

Notice: - - not screened;

Table 2. Results of the AChE inhibition activity, IC_{50} ± SD (μg)

Extracts and fractions	Leaves	Fruits
Methanol extract	32.60 ± 0.51	45.0 ± 0.50
Chloroform fraction	405.60 ± 0.92	-
n-Butanol fraction	72.50 ± 0.60	-
Water residue	442.35 ± 0.64	-
Standard - Physostigmine	0.228	

Notice: - - not screened;

Among all the DPPH assayed crude extracts and fractions, only *n*-butanol fraction of leaves exhibited higher activity (IC_{50}, 55.70 ± 0.15 μg/mL); however, the activity was half that of standard rutin. The methanol extracts of both leaves and fruits and the *n*-butanol fraction of leaves showed AChE inhibitory activity (IC_{50}, 32.61 ± 0.51 μg, 44.97 ± 0.50 μg, and 72.50 ± 0.60 μg). Over the past years, the anti-oxidative activity of several species of *Cotoneaster* has been assayed by the hydrogen donating DPPH radical scavenging method. The anti-oxidative activity of 70 % methanol extracts of leaves from 12 species of *Cotoneaster* Medik. varied between EC_{50}, 18.5-34.5 μg/mL [13]. The leaf methanol extract of *C.melanocarpus* showed activity with IC_{50}, 106.41 μg/mL [4]. The twig ethanol extract of *C.horizontalis* possessed high scavenging activity of IC_{50},19.3 μg/mL [14], compared to the same crude drug methanol extracts of *C.nummularia* (IC_{50}, 104.0 mg/mL) [15] and *C.integerrimus* (IC_{50}, 1.06 mg/mL) [16]. The variability in results of different species of *Cotoneaster* depends on many reasons, in particular on various natures of phytochemicals and their contents.

Previously, the AChE inhibitory activity of the twig and fruit methanol extracts of *C.integerrimus* was determined with results IC_{50}, 1.07 mg/mL and 1.72 mg/mL, while the twig methanol extract of *C.nummularia* gave 4.77 mg GALAEs/g (galanthamine equivalent) extract, respectively [15,16]. In our experiments, methanol extracts of *C.mongolica* leaves and fruits at 1mg/mL concentration exhibited comparable activities against AChE enzyme with over 60 % inhibition, which is the same level as ethanol extract, ethylacetate and *n*-butanol fractions of *Dasiphora fruticosa* leaves, as well as the ethylacetate fraction of *Myricaria alopecuroides* branches [17]. The experiments were carried out in the same conditions. In contrast to methanol extract, it was observed that the *n*-butanol fraction showed lower AChE inhibitory activity. This phenomen could be explained by the presence of both various classes of nonpolar and polar compounds with greater enzyme inhibitory activity in the methanol extract than the polar compounds, which are predominantly extracted in the *n*-butanol fraction. In particular, it is likely that the nonpolar aglycones extracted in the methanol fraction are responsible for much of enzyme inhibitory activity present in the leaves. Thus, the biological activity of extracts and fractions depends on the complex nature of phytochemicals and their synergistic and antagonistic effects [15].

In this study, 12 compounds have been isolated from the *n*-butanol fraction, including quercetin (**1**) [18, 19], hyperoside (**2**) [20], kaempferol-5-*O*-*β*-*D*-glucopyranoside (**3**) [21], sissotrin (**4**) [22], ursolic acid (**5**) [23, 24], corosolic acid (**6**) [24], euscaphic acid (**7**) [24], prunasin (**8**) [25], (2*R*)-mandeloyl-*β*-*D*-gluco pyranose (**9**) [26], (*Z*)-3-hexenyl-*O*-*α*-*L*-rhamnopyranosyl-(1→6)-*β*-*D*-glucopyranoside (**10**) [27], benzyl-*O*-*α*-*L*-rhamnopyranosyl-(1→6)-*β*-*D*-gluco pyranoside (**11**) [28] and arbutin (**12**) [29], respectively (Figure 1). They were identified on the basis of their physicochemical properties and spectral data. The sugars in the glycosides (**2, 3** and **8**) were characterized by acidic hydrolysis.

The phytochemical investigation of leaf, twig and fruit samples of other *Cotoneaster* species indicated that flavonoids, cyanogenic glycosides and triterpenoids are the main secondary metabolites in these plants. In this study, 2.38 g prunasin (**8**), a cyanogenic glycoside, was isolated. The presence of prunasin and amygdalin in the leaves and fruits of *Cotoneaster*-Arten was confirmed by gas-liquid chromatography [30].

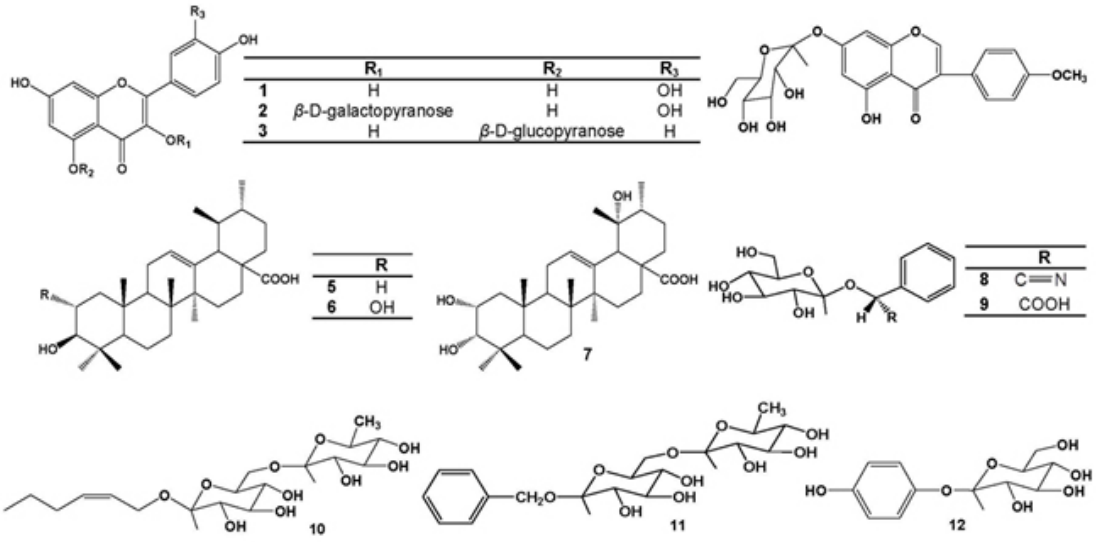

Fig. 1. Compounds 1 - 12 isolated from leaves of *C.mongolica* Pojark.

Hyperoside (**2**), a second rich flavonol glycoside, identified in leaf samples of 12 *Cotoneaster* Medik. species and *C.melanocarpus* by HPLC analysis, was isolated [13, 4]. In addition, quercetin (**1**) was identified in the twig of *C.integerrimus* [16], and ursolic acid (**5**), corosolic acid (**6**), euscaphic acid (**7**) and arbutin (**12**), respectively were identified in the leaves of *C.simonsii* by HPLC analysis [31]. Compounds **3, 4, 9, 10, 11** were isolated, and identified for the first time from *C.mongolica*.

All isolated compounds were tested for their antioxidant effect and AChE inhibitory activity by the previously described methods. Results are given in Table 3.

Table 3. Anti-oxidative and AChE inhibitory activities of isolated compounds

Compounds	DPPH scavenging activity, IC_{50}, μM	AChE inhibiting activity, IC_{50}, mM
1	41.36 ± 0.89	**0.110** ± 0.001
2	**18.93** ± **0.72**	**0.021 ± 0.002**
3	156.72 ± 1.10	**0.053 ± 0.23**
4	na	1.530 ± 0.11
5	na	0.306 ± 0.003
6	na	ns
7	na	1.567 ± 0.09
8	na	2.072 ± 0.23
9	na	2.519 ± 0.09
10	na	2.340 ± 0.10
11	na	1.960 ± 0.06
12	ns	ns
Rutin	**38.7**	
Physostigmine		**0.000083**

Notice: na – not active; - - not screened;

Biological activity analysis indicated that flavonoid derivatives were distinguished by high activities in both examined assays over other classes of isolated compounds. In particular, compound **2** was most active as an antioxidant and as an AChE enzyme inhibitor. Quercetin (**1**) was the second most active compound, which exhibits excellent DPPH scavenging activity with a wide range of values IC_{50}, 95 nM - 226 μM [32]. Quercetin was also well studied for its AChE inhibitory activity. Researchers of different groups suggested that quercetin exhibited remarkable inhibitory activity with the IC_{50}, 19.8 μM [33], the inhibitory activity 76.2 % [34] and the inhibition zone 0.6 cm [35], respectively. Compound **3** demonstrated a low activity for both DPPH scavenging and AChE enzyme inhibition. All other tested components showed no activities. Consequently, it has been shown that the presence of hyperoside in considerable quantity causes anti-oxidative and AChE inhibitory activities in the leaf sample of *C.mongolica*. Hyperoside widely occurs in the plant kingdom and it demonstrates definite free radical scavenging and oxidative stress protective activity, as well as anti-inflammatory, anti-thrombotic, anti-diabetic, anti-viral, anti-fungal and hepatoprotective effects [36-38]. In a study by Zhao Y. *et al.,* hyperoside exhibited DPPH scavenging and AChE inhibition activities by the IC_{50}, 11.19 μM and 94.61 μM (or 0.0946 mM), respectively, which to a certain degree is comparable to our results.

Flavonoids are well-known antioxidants. According to the literature survey, both anti-oxidative and AChE inhibitory activities of various flavonoid aglycones and glycosides are related to their structural features. The presence of multiple OH groups in A and B rings, and unsaturation of the C ring produces a positive effect on the biological activity [32, 39]. In this study, anti-oxidative and AChE inhibitory activities of the main compound prunasin were assayed for the first time. However, it did not exhibit any AChE inhibition or anti-oxidative activity.

CONCLUSIONS

The study of *Cotoneaster mongolica* leaves indicated that the methanol extract exhibited the highest acetylcholinesterase inhibitory, while the *n*-butanol fraction exhibited the highest DPPH radical scavenging activities. The latter fraction also showed activity against acetylcholinesterase enzyme. Flavonol derivatives, isoflavoneglycoside, phenol derivatives, triterpenoid acids, cyanogenic glycoside and olefinylglycoside were isolated from the *n*-butanol fraction. Hyperoside and its aglycone quercetin reflected the antioxidative and AChE inhibitory activities of the leaf sample. These results may provide a scientific basis to explain the use of *C.mongolica* in traditional and complementary medicine.

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