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Flavonoid glycosides and free radical scavenging activity of Bulgarian endemic *Alchemilla jumrukczalica* Pawl.

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Dedicated to Acad. Bogdan Kurtev on the occasion of his 100th birth anniversary

The aim of this study was to identify the flavonoid constituents of the Bulgarian endemic *Alchemilla jumrukczalica* Pawl. and evaluate the antiradical scavenging activity of the total extract, fractions and individual compounds. The total MeOH extract exhibited a significant DPPH activity (IC_{50} 10.7±0.4 μg/ml), while EtOAc fraction obtained after partition of the total extract was found to be the most active radical scavenger (IC_{50} 5.1±0.1 μg/ml). Catechin and seven flavonoid glycosides (guajaverin, hyperoside, isoquercitin, quercitrin, miquelianin, tiliroside and trifolin) were isolated from EtOAc fraction.Their structures were elucidated on the basis of spectral data. Quercetin glycosides (guajaverin, hyperoside and miquelianin) were found to be better DPPH radical scavengers than kaempferol-3-O-galactoside and catechin.

Key words: *Alchemilla jumrukczalica*; Rosaceae; flavonoid glycosides; DPPH assay

INTRODUCTION

Species of the genus *Alchemilla* L. are valuable medicinal plants referred to the collective name *Alchemilla vulgaris* complex (Lady's mantle). These plants are used in phytotherapy as Herba Alchemillae. The drug possesses astringent, diuretic and antispasmodic properties, and is commonly used in traditional medicine as a cure for excessive menstruation and wounds [1, 2]. Different studies showed that the phenolic compounds (tannins, flavonoids, *etc.*) presented in the plant are responsible for the pharmacological activity of Lady's mantle [3–9].

The genus *Alchemilla* (Rosaceae) is represented in Bulgarian Flora by 35 species, four of them are Bulgarian and seven - Balkan endemics [10]. The endemic *A. jumrukczalica* Pawl. occurs only in the National Park "Central Balkan" (Stara Planina Mt). The clon-populations can be found along the mountain streams at an altitudinal range between 1600–1800 m a.s.l. and gullies in the subalpine mountain belt [11]. The species was protected as rare and critically endangered one according IUSN criteria and is included in the Red List of Bulgarian vascular plants [12] and Red Data Book of R. Bulgaria [13]. Because of its very limited occurrence, *ex situ* conservation of *A. jumrukczalica* started a few years ago [14]. It was found that the species could be easily cultivated at places with mountain climate close to that of its natural distribution. The plants grown *ex situ* were larger and more robust than those from natural clone-populations and cultivation did not cause significant changes in the total content of flavonoids and tannins in the aerial parts [14]. All these preliminary results prompted us to continue our investigations on flavonoid constituents of *A. jumrukczalica* and to assess its antioxidant capacity.

EXPERIMENTAL

Plant material

A. jumrukczalica plants with origin of native Bulgarian population (Central Stara planina Mt., 1600 m a.s.l.) cultivated in the experimental field of the Institute of Biodiversity and Ecosystem Research (Vithosha Mt., 1400 m a.s.l.) were used in the experiments.

The aerial parts were collected within phenophase full blossoming, air-dried and kept in dark place.

The voucher specimen (SOM 165678) was deposited in the Herbarium of Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia.

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Extraction and isolation

Powdered plant material (70 g) was extracted with CH_3OH (2 x 1 L) at room temperature in an ultrasonic bath for 30 min each and once with 1 L of CH_3OH at room temperature for 24 hrs. After filtration, the solvent from combined extracts was evaporated under vacuum to give total methanolic extract (CH_3OH, 12.71 g). The latter was further dissolved in distilled water (200 ml) and partitioned with petroleum ether (PE, 4 x 80 ml), chloroform ($CHCl_3$, 4 x 80 ml) and ethyl acetate (EtOAc, 4 x 80 ml) to yield corresponding PE (0.50 g), $CHCl_3$ (0.50 g) and EtOAc (1.33 g) fractions. The remaining aqueous phase was evaporated to dryness (H_2O residue, 10.28 g).

The total methanolic extract of wild growing *A. jumrukczalica* and the corresponding fractions were obtained from 5 g of dry plant material using the same procedure. TLC comparison was performed on Silica gel (EtOAc/CH_3OH/H_2O, 5:0.8:0.6 and EtOAc/HCOOH/CH_3COOH/H_2O, 100:11:11:26), spraying with NP/PEG reagent and UV visualization at 366 nm [15].

EtOAc extract was dissolved in CH_3OH (15 ml) and filtered through celite in order to remove insoluble parts. Clear methanolic solution was concentrated up to 5 ml and applied to a Sephadex LH-20 column (equilibrated with CH_3OH) to give 2 main fractions A (0.92 g) and B (0.33 g). Flavonoid containing fraction (B) was further applied to MPLC on LiChroprep RP-18 and eluted with increasing concentrations of CH_3OH in H_2O (20 to70%). Repeated MPLC (LiChroprep RP-18, CH_3OH/H_2O, 50:50) of selected fractions yielded **1** (16 mg), **2** (11 mg), **3** (4 mg), **4** (4 mg), **5** (4 mg), **6** (10 mg), **7** (1 mg) and **8** (6 mg).

Free Radical Scavenging Activity on DPPH radical

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method was used for determination of antioxidant capacity of the extracts and individual compounds [16]. Different concentrations of the extracts (2.5–100 μg/ml) and individual compounds (2–20 μM) in CH_3OH were added at an equal volume (2.0 ml) to CH_3OH solution of $DPPH^{\bullet}$ (0.1 mM, 2 ml). After 30 min at room temperature and darkness, the absorption values were spectrophotometrically measured at 517 nm and converted into the percentage antioxidant activity using the following equation:

$$DPPH^{\bullet}\ (\%) = [1-(A_{sample} - A_{blank})/ A_{control}]x100.$$

CH_3OH (2.0 ml) plus plant extract solution (2.0 ml) was used as a blank, while $DPPH^{\bullet}$ solution plus CH_3OH was used as a control. The measurements were performed in triplicate. The results are presented as a mean ± SD. The IC_{50} values were defined as the concentration of antioxidant necessary to decrease the absorbance of DPPH solution by 50%.

RESULTS AND DISCUSSION

The DPPH scavenging assay is widely used for preliminary evaluation of the antioxidant potential of extracts and individual compounds [15, 16]. In this work, the methanol extract of *A. jumrukczalica* and its fractions obtained after re-extraction with light petroleum (PE), chloroform ($CHCl_3$), and ethyl acetate (EtOAc), as well as the remaining H_2O residue were studied for their potential to scavenge the stable DPPH radical (Table 1). The total extract exhibited a significant dose dependent inhibition of DPPH activity with a 50% inhibition (IC_{50}) at a concentration of 10.7±0.4 μg/ml. As shown in Table 1, the scavenging activities of the fractions on DPPH increased in the order of PE < $CHCl_3$ < H_2O < EtOAc. Although the DPPH free radical scavenging ability of the EtOAc fraction (IC_{50} 5.1±0.2 μg/ml) was less than that of quercetin and ascorbic acid (IC_{50} 2.4±0.2 and 3.8±0.2 μg/ml, respectively) it was evident that this fraction could serve as free radical inhibitor or scavenger.

Table 1. DPPH radical scavenging activity of *A. jumrukczalica*.

Sample	IC_{50} (μg/ml)
total CH_3OH extract	10.7±0.4
PE fraction	>200
$CHCl_3$ fraction	57.5±0.5
EtOAc fraction	5.1±0.2
H_2O residue	7.9±0.3
Quercetin (Reference)	2.4±0.1
Ascorbic acid (Reference)	3.8±0.1

The presence of flavonoids in the most active EtOAc fraction was initially determined by TLC and visualization of the spots with NP/PEG reagent [14]. Two main types of flavonoid glycosides were detected: quercetin (orange coloured spots) and kaempferol (yellow-green coloured spots). The isolation of the individual compounds from EtOAc fraction was achieved by SephadexLH-20 column chromatography and further purification by column chromatography and preparative TLC. Catechin (**1**) [18], guajaverin (**2**) [19], hyperoside (**3**) [20], isoquercitin (**4**) [20], quercitrin (**5**) [21],

miquelianin (**6**) [20], tiliroside (**7**) [22] and trifolin (**8**) [23] (Fig. 1) were identified based on their spectral data (UV, ^{1}H NMR and MS) compared with those published in the literature.

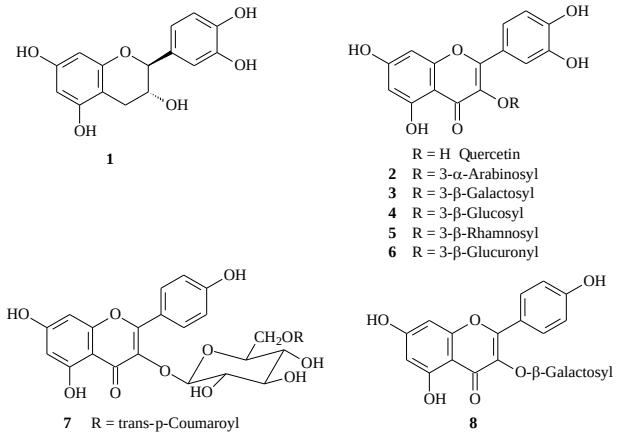

Fig. 1. Structures of the isolated flavonoid glycosides

The obtained in this study results are in accordance with those found previously for the content of quercetin (**2**-**6**) and kaempferol (**7** and **8**) glycosides in the species of the genus *Alchemilla* [24–27]. In addition, catechin (**1**) has been descried as a component of *A. mollis* [26] only.

Selected pure compounds were also studied for their ability to quench the DPPH radical (Table 2) and quercetin was used as reference compound. As can be expected, the radical scavenging activity of the flavonoids depends on the molecular structure and the substitution pattern of hydroxyl groups [17, 28]. Guajaverin (**2**), hyperoside (**3**) and miquelianin (**6**) are quercetin derivatives with free hydroxyl groups at C-3' and C-4' and glycosylated at C-3.The nature of sugar moiety did not affect the activity. They had similar IC_{50}, but lower activity than that of quercetin (7.95±0.10 μM). Trifolin (**8**), a kaempferol derivative contained only one free hydroxyl group at C-4'in B ring and therefore possessed the lowest activity (31.03±0.49 μM) [17, 28]. Catechin (**1**) was a weaker antioxidant compared to quercetin and its derivatives because of the absence of C-2/C-3 double bond and carbonyl group at C-4 in its structure [17, 28].

Table 2. DPPH radical scavenging activity of isolated compounds.

Compound	IC_{50} (μM)
Catechin (**1**)	14.14±0.34
Guajaverin (**2**)	10.83±0.23
Hyperoside (**3**)	11.51±0.21
Miquelianin (**6**)	12.14±0.36
Trifolin (**8**)	31.03±0.49
Quercetin (Reference)	7.95±0.10

CONCLUSION

TLC comparison of the total extracts and corresponding EtOAc fractions obtained from wild growing and cultivated *A. jumrukczalica* in the presence of isolated compounds did not show any significant qualitative and quantitative differences. The obtained results revealed the possibility to use *ex situ* cultivated *A. jumrukczalica* as a source of secondary metabolites with potential antioxidant activity.

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ФЛАВОНОИДНИ ГЛИКОЗИДИ И АНТИРАДИКАЛОВА АКТИВНОСТ НА БЪЛГАРСКИЯ ЕНДЕМИЧЕН ВИД *ALCHEMILLA JUMRUKCZALICA* PAWL.

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(Резюме)

Целта на настоящето изследване е да се идентифицират флавоноидните съединения в българския ендемичен вид *Alchemilla jumrukczalica* Pawl. и да се определи антирадикаловата активност на тоталния екстракт, фракции и индивидуални съединения. Тоталният метанолен екстракт проявява значителна DPPH активност (IC_{50} 10.7±0.4 μg/ml), но за етилацетатната фракция (EtOAc) получена след ре-екстракция от тоталния екстракт бе установено, че е най-активният уловител на DPPH радикали (IC_{50} 5.1±0.1 μg/ml). Катехин и седем флавоноидни гликозиди (гуайаверин, хиперозид, изокверцетин, кверцитрин, микуелианин, тилирозид и трифолин) бяха изолирани от етилацетатната фракция. Структурите на тези съединения е установена с помощта на спектрални данни. Установено бе, че кверцетиновите гликозиди (гуайаверин, хиперозид и микуелианин) са по-добри уловители на DPPH радикали от трифолин (кемпферол-3-глюкозид) и катехин.