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FLAVONOID PROFILES, POLYPHENOLIC CONTENT AND ANTIRADICAL PROPERTIES OF CULTIVATED PLANTS OF *Arnica montana* L.

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Abstract

Total flavonoid and phenolic content, antiradical properties and external flavonoid profiles on extracts of six samples of flower heads of cultivated Arnica montana was assessed. The studied samples were compared in relation to location of cultivation, origin of the seeds, time of harvesting, method of propagation: in vitro and in vivo. Four flavonoid aglycones were detected of examined exudates by co-chromatography with known compounds - scutellarein-6-methyl ether (hispidulin), scutellarein 6,4'-dimethyl ether (pectolinarigenin), 6-hydroxyluteolin 6-methyl ether and kaempferol 6-methyl ether. Quality differences in flavonoid composition were not observed between the studied samples while quantitative variations of total flavonoid and phenolic content were detected but in the most cases they a statistically not significant. The highest antioxidant properties displayed the samples from in vivo grown plants. As a result of the present preliminary study it was established that the best values of studied parameters showed the sample of in vivo grown plants, from seeds with Ukrainian origin, cultivated in Rhodope Mountain, collected at full flowering stage.

Key words*: DPPH, surface flavonoids, TLC.*

INTRODUCTION

Arnica montana L. (Asteraceae) is a rare species under strict protection in several European countries and at the same time is medicinal plant with high commercial value (Lange, 1998). The alcohol extract of flowering heads are traditionally used for treatment of skin wounds, bruises and contusions (Smallfield and Douglas, 2008). The herb is rich in sesquiterpene lactones (e.g. helenalin), phenolic acids (caffeic acid derivatives), flavonoids and essential oils, that are determining its pharmacological properties – antiseptic, anti-inflammatory, antibacterial and antioxidant (Woerdenbag et al., 1994; Lyss et al., 1997; Smallfield and Douglas, 2008)

In Bulgaria, *Arnica montana* L. has been reported to grow only in Rila mountain (Assov and Petrova, 2006), but its distribution has not been confirmed. So, breeding of the plant is becoming increasingly necessary. An *in vitro* propagation protocol for *A. montana* has been reported by several authors including our team (Petrova et al., 2005; Surmacz-Magdziak and Sugier, 2012; Petrova et al., 2012). Although in many countries such as Italy, Germany, Finland and others, studies on the cultivation of *A. montana* was carried out for years (Aiello et al., 2012) in Bulgaria such kind of researches began only in recent years.

Phenolic compounds and flavonoids are among the main components of *A. montana* but they are also the main components determining antioxidant properties, therefore we selected them as indicators of the quality of the material. The total polyphenol content and antioxidant activity of *A. montana* extracts have been reported in several publications (Hărmănescu et al., 2008; Fraisse et al., 2011; Moldovan et al., 2011). It has been shown that phenolic profiles of *A. montana* depends on environmental conditions (Albert et al., 2009; Spitaler et al., 2008). Therefore important point in the cultivation of the plant is to define the growing conditions and time of flower harvest for obtaining material with the best quality.

In the present preliminary study the effect of some factors (location of cultivation, harvesting stage, origin of seed, method of propagation: *in vitro* and *in vivo*) on qualitative and quantitative parameters (external flavonoids, total phenolic and flavonoids, antiradical properties) of cultivated plants of *A. montana* was studied.

MATERIALS AND METHODS

Plant material

The used plant material was derived from seeds of both origins - natural habitat in the Carpathians, Ukraine (UO) and Botanical garden, Germany, cultivar "Arbo" (GO). The plants were grown in the experimental stations "Golden Bridge" of the Vitosha Mountain (VM) - 1404 m a.s.l. and "Beglika" of Rhodope Mountain (RM) - 1500 m a.s.l. The plant material was collected at full flowering (FFS) and end of flowering (EFS) stages. One of the samples represents *in vitro* adapted plant.

Preparation of the extracts

Acetone exudates. Air-dried (but not ground) plant material (1g) was briefly (2-3 min) rinsed with acetone to dissolve the lipophilic components accumulated on the surface. After evaporation of the acetone the dry extracts were dissolved in 250 μL methanol and subjected on TLC.

Methanol extracts. Dry, ground plant material (1 g) was extracted with 80% (3 x 30 mL) methanol by classical maceration for 24 h. After evaporation of the solvent the crude extract was subject to subsequent analysis.

Thin layer chromatographic analysis of flavonoid aglycones

The acetone exudates were screened for surface flavonoids by TLC analysis. Three TLC sorbents and three mobile phases were used for the analysis of the flavonoid exudates. Toluene-dioxan-acetic acid (95:25:4, v/v/v) was applied for the development of the aglycones mixture on silica gel plates Kiselgel 60 F_{254} (10x20 cm, 0.2 mm layer).Toluene-methylethylketone-methanol (60:25:15, v/v/v) was used for DC-Alufolien Polyamid 11 F_{254} plates (10 x 20 cm, 0.15 mm layer). Acetic acid–water (30:70, v/v) was used for cellulose plates DC-Alufolien Cellulose 5552 (10 x 20 cm, 0.1 mm layer). Chromatograms were viewed under UV light before and after spraying with ''Natural product reagent A", 1% solution of diphenylboric acid 2-aminoethyl ester complex in methanol. The identification of the compounds was achieved by co-chromatography with authentic markers obtained from Prof. Eckhard Wollenweber.

Determination of total phenolic content

Total phenolic content of the methanol extracts was determined by employing the methods given in the literature involving Folin–Ciocalteu reagent and gallic acid as standard (Giorgi et al., 2009; Nićiforović et al., 2010).

Determination of total flavonoid content

Total flavonoid content was determined using method described by Miliauskasa et al. (2004), using rutin as a reference compound.

DPPH radical scavenging activity

The effect of methanolic extracts on DPPH radical was estimated according to Stanojević et al., (2009). The IC_{50} values were calculated by Software Prizm 3.00.

Statistical analysis

Statistical analysis was carried out using excel. All experiments were performed in triplicate. Results were presented as a value ± standard deviation (SD). Differences at $P \leq 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSIONS

Six samples of cultivated plants of *Arnica montana* were analyzed for their external flavonoid profiles, total content of phenolics and flavonoids as well as for their antiradical properties. The plants were grown in different localities (Vitosha Mountain and Rhodope Mountain), were collected in different flowering stages (full and end of flowering stages) also they were derived from the seeds of various origin (German and Ukrainian), in one case it is a *in vitro* adapted plants.

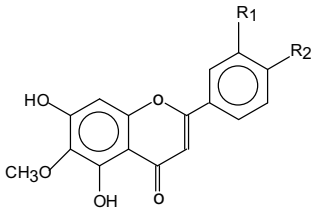
External flavonoid aglycones

Four surface flavonoid aglycones were detected in the acetone exudates of the studied samples: scutellarein 6-methyl ether (hispidulin) (**1**), scutellarein 6,4'-dimethyl ether (pectolinarigenin) (**2**), 6-hydroxyluteolin 6-methyl ether (**3**) and kempferol 6-methyl ether

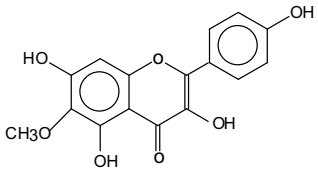
(**4**). Tree of detected flavonoid structures belong to flavone class of flavonoids and one to flavonol class (Figure 1). Quality differences in flavonoid composition were not observed between the exudates of flower heads of studied samples.

Total phenolic and flavonoid content

The total flavonoid content was the highest in the samples collected at full flowering stage but the differences were not statistically significant (Table 1). With regard to total content of phenolics two samples showed statistically significant differences. The both samples with lower level of phenolics were collected at end of flowering stage, in terms of the other factors the meanings are multifaceted.



(**1-3**)



(**4**)

Figure 1. Structures of the identified flavonoid aglycones (1-4)

(**1**) R_1=H; R_2=OH scutellarein 6-methyl ether (hispidulin);
(**2**) R_1=H; R_2=OCH_3 scutellarein 6.4'-dimethyl ether (pectolinarigenin);
(**3**) R_1=OH; R_2=OH6-OH luteolin 6-methyl ether
(**4**) kempferol-6-methyl ether

Antiradical properties

Methanol extracts of studied samples were examined for their antiradical properties using a DPPH assay and expressed as IC_{50} value - extract concentration providing 50% inhibition of the DPPH solution. The highest antiradical properties were determined for the samples obtained from *in vivo* grown plants. Flower heads collected at full flowering stage, developed with seed with Ukrainian origin showed the strong antioxidant activity.

From the testing factors (localition of cultivation, time of harvesting, origin of seed, method of propagation: *in vitro* and *in vivo*) the biggest impact on examined parameters (flavonoid composition, total phenolic and flavonoids, antiradical properties) have the time of harvesting and method of propagation of plant material: *in vitro* and *in vivo*. The best values of examined parameters are obtained for materials that were collected at full flowering stage. Method of propagation shows effect mainly on the antioxidant properties, less on phenolic and flavonoid content.

The plant materials of *in vitro* adapted plants exhibited the lowest values of examined parameters - antiradical properties, total phenolic and flavonoids. Other authors have been reported higher antioxidant activity and poliphenolic content of *in vivo* developed plants compare to *in vitro* developing individuals too (Joshi et al., 2009; Taware et al., 2010). It will be interesting to study after a few growing seasons, whether *in vitro* and *in vivo* grown plants will show equal values of studied parameters.

Regarding to the origin of the seed comparing the couples samples (EFS-UO-RM and EFS-GO-RM; FFS-UO-RM and FFS-GO-RM) with better values for the studied parameters are those originating from Ukraine, but the differences were not statistically significant (Table 1). With regard to the locality of cultivation comparing couple samples EFS-UO-RM and EFS-UO-VM no clearly outlined trend.

CONCLUSIONS

As a result of present preliminary study it was found that materials of *A. montana* with the best quality in terms of total flavonoids and phenolics, antiradical properties and external flavonoid profiles were obtained when it were collected at full flowering stage, *in vivo* developed from seed with Ukrainian origin and grown in Rhodope Mountain.

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Table 1. Total content of flavonoids, phenolics and antiradical properties of methanol extracts of cultivated plants of *Arnica montana*

Samples (time of harvesting, origin of seed, locality of cultivation, method of propagation)	**Total phenols* mg GAE/g extract**	**Total flavonoids* (mg RE/g extract)**	**DPPH scavenging activity IC50 (µg/mL)**
FFS-UO-RM: full flowering stage, Ukrainian origin, Rhodope Mountain, *in vivo*	33.34±2.32565[a]	4.9965±1.75150[a]	85.64
FFS-GO-RM: full flowering stage, German origin, Rhodope Mountain, *in vivo*	34.24±1.60724[a]	4.9695±1.82489[a]	105.7
EFS-UO-RM: end of flowering stage, Ukrainian origin, Rhodope Mountain, *in vivo*	31.97±1.13137[a]	4.77±1.24364[a]	130
EFS-GO-RM: end of flowering stage, German origin, Rhodope Mountain, *in vivo*	29.17±0.90423[b]	4.566±0.89304[a]	200
EFS-UO-VM: end of flowering stage, Ukrainian origin, Vitosha Mountain, *in vivo*	34.69±1.30615[a]	3.822±1.43244[a]	106
EFS-UO-VM: end of flowering stage, Ukrainian origin, Vitosha Mountain *in vitro*	28.47±1.38713[b]	4.12±1.32617[a]	274

Legend: *Data are the means of three replications. Values within column followed by the same letter are not significantly different at $P > 0.05$

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