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Comparative study of *in vitro, ex vitro* and *in vivo* grown plants of *Arnica montana* – polyphenols and free radical scavenging activity

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Abstract – *Arnica montana* L. is an endangered species rich in sesquiterpene lactones, phenolic acids and flavonoids with high pharmaceutical value. The polyphenolic content and free radical scavenging activity of plants that had passed all stages of cultivation: micropropagation and rooting (in vitro), adaptation in greenhouse (ex vitro) and mountain conditions (in vivo) were evaluated. Four surface flavonoid aglycones [scutellarein 6-methyl ether (hispidulin), scutellarein 6,4'-dimethyl ether (pectolinarigenin), 6-OH luteolin 6-methyl ether and kempferol-6-methyl ether] were detected in the acetone exudates of the studied samples by means of thin layer chromatography. No differences in the accumulation of surface flavonoids were found among the tested leaf extracts of in vitro, ex vitro and in vivo samples. However, the extracts from the flowers were richer in surface flavonoids than extracts from the leaves. The methanol extracts of the samples from ex vitro and in vivo grown *A. montana* plants had significantly higher radical scavenging activity and polyphenolic content than the extracts of in vitro samples. The observed differences in the contents of these biologically active compounds were related to different growth conditions and stages of plant development. The biotechnological method of *A. montana* established holds promise for the future production of antioxidants.

Keywords: antioxidant, flavonoid, phenol, *Arnica montana*

Introduction

Arnica montana L. (Asteraceae) is a valuable perennial herb. The species contains sesquiterpene lactones (e.g. helenalin), phenolic acids (caffeic acid derivatives) and flavonoids (quercetin 3-*O*-glucuronic acid) with significant antiseptic, anti-inflammatory, antibacterial and antioxidant effects (WOERDENBAG et al. 1994, LYSS et al. 1997, IAUK et al.

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2003, SANTOS et al. 2006). The plant has been used mainly as anti-inflammation drug and is applied topically for skin, bruises, rheumatic and muscle pains. The species is used as ingredient in many homeopathic remedies. *Arnica montana* is a rarely found in Bulgaria and has been reported to grow only on Rila mountain (ASSYOV and PETROVA 2006), however so far its distribution has not been confirmed. Therefore the development of a biotechnological method for in vitro propagation of the species and subsequently ex vitro and in vivo acclimatization is very important for its cultivation and sustainable use.

Phenolic compounds and flavonoids possess multiple biological effects including antioxidant, free radical scavenging abilities, inhibition of hydrolytic and oxidative enzymes, anti-inflammatory, anticarcinogenic, antibacterial, hypolipidemic, antimutagenic and other activities (BURDA and OLESZEK 2001, CAI et al. 2004, ROCHA-GUZMAN et al. 2007). They are also associated with the prevention and treatment of cardiovascular and cerebrovascular diseases (GAN et al. 2010). *Arnica montana* flavonoids are supposed to act synergistically with sesquiterpene lactones (WILLUHN 1991). They also serve as chemosystematic markers and are used to assess identity and purity of *A. montana* according to the European Pharmacopoeia (HÄNSEL and STICHER et al. 2007). The total polyphenol content and antioxidant activity of *A. montana* extracts have been reported in several publications (HAĂRMĂNESCU et al. 2008, FRAISSE et al. 2011, GAWLIK-DZIKI et al. 2011, MOLDOVAN et al. 2011). The literature survey showed that there is no information about the antioxidant activity of in vitro cultured *A. montana* plant material.

The purpose of this study was to evaluate the polyphenol content and antioxidant capacity of plants derived from in vitro culture and then adapted in greenhouse (ex vitro) and mountain conditions (in vivo).

Material and methods

Plant material

The origin of *Arnica montana* plants was Botanical Garden, Germany (AG). Six samples were analyzed: two in vitro samples: AGM (multiple plants), AGR (rooted plants); two ex vitro samples AG1: AG1_L (leaves), AG1_F (flower heads) of greenhouse plants from Sofia region 553 m a.s.l. and two in vivo sample AG2: AG2_L (leaves), AG2_F (flower heads) of cultivated plants from the Rhodope Mountains, Beglika.

Preparation of extracts

Acetone exudates. Air-dried (but not ground) 1g plant material was briefly (2–3 min) rinsed with acetone at room temperature to dissolve the lipophilic components accumulated on the surface. The obtained acetone filtrate was then dried using a rotary-evaporator to give a crude extract which was suspended in MeOH and then subjected on TLC.

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

Biotechnological tools: The *in vitro* propagation protocol of *A. montana* plants consisted of four stages: 1) an initial stage – obtaining seedlings on basal MS medium (MURASHIGE and SKOOG 1962) with 40 mg L^{-1} gibberellic acid; 2) a propagation stage –

shoot formation on MS medium supplemented with 1 mg L^{-1} 6-benzylaminopurine and 0.1 mg L^{-1} indole-3-acetic acid; 3) rooting stage – shoot rooting on half strength MS medium containing 0.5 mg L^{-1} indole-3-butyric acid; 4) *ex vitro* acclimatization of plants was carried out on a mix of peat, perlite and coconut fiber (2:1:1 v/v/v) for 4 weeks. Then the plants were adapted in the experimental field in the Rhodopes Mountains at an altitude of 1500 m a.s.l (*in vivo* plants).

Cultural conditions

In vitro cultures were maintained in a growth room at temperature of 22 ± 2 °C under a 16 h photoperiod with light intensity of 40 μM m^{-2} s^{-1} provided by cool white fluorescent tubes. The micropropagated plants were cultivated in *ex vitro* conditions ata temperature of 24 ± 2 °C under a 16 h photoperiod with a light intensity of 50 μM m^{-2} s^{-1} with subsequent transfer to greenhouse. Then the plants were adapted in the experimental field in the Rhodopes Mountains at an altitude of 1500 m a.s.l (*in vivo* plants).

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} (10 x 20 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). Plant extracts were diluted to a concentration of 1 mg mL^{-1}, and aliquots of 0.5 mL were mixed with 2.5 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of Na_2CO_3 (6%). After 1 h at room temperature, the absorbances of the samples were measured at 765 nm on spectrophotometer versus blank sample. Total phenols were determined as gallic acid equivalents (mg GA per g of extract) by the following formula:

$$C = c \times V / m$$

where C is total content of phenolic compounds, mg g^{-1} plant extract, in GAE; c is the concentration of gallic acid established from the calibration curve in μg mL^{-1}; V is the volume of extract in mL; m is the weight of pure plant methanolic extract in grams.

Determination of total flavonoid content. Total flavonoid content was determined according to Miliauskasa et al. (2004), using rutin as a reference compound. One mL of plant

extract in methanol (10 g L^{-1}) was mixed with 1 mL aluminium trichloride in ethanol (20 g L^{-1}) and diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min at room temperature. Blank samples were prepared from1 mL plant extract and 1 drop acetic acid and diluted to 25 mL. The absorption of rutin solutions was measured under the same conditions. Standard rutin solutions were prepared from 0.05 g rutin. All determinations were carried out in duplicate. The amount of flavonoids in plant extracts in rutin equivalents (RE) was calculated by the following formula:

$$C = Ab_{sample} \times m_{control} \times 10 / Ab_{control} \times m_{sample}$$

where C is flavonoid content (mg g^{-1} plant extract) in RE; Ab_{sample} is the absorption of plant extract solution; $Ab_{control}$ is the absorption of standard rutin solution; m_{sample} is the weight of plant extract in grams; $m_{control}$ is the weight of rutin in the solution in grams.

DPPH radical scavenging activity. The effect of methanolic extracts on DPPH radical was estimated according to CHOI et al. (2002) and STANOJEVIĆ et al. (2009). Different concentrations of plant extract (10, 20, 50, 100 and 200 μg/mL), in methanol were added at an equal volume (2.5 mL) to methanol solution of DPPH (0.3 mM, 1 mL). After 30 min at room temperature, the Ab values were measured at 517 nm on a spectrophotometer (Jenway 6320D) and converted into the percentage antioxidant activity using the following equation:

$$\text{DPPH antiradical scavenging capacity } (\%) = [1-(Ab_{sample}-Ab_{blank})/Ab_{control}] \times 100$$

Methanol (1.0 mL) plus plant extract solution (2.5 mL) was used as a blank, while DPPH solution plus methanol was used as a control. The IC_{50} values were calculated by sigmoid non-linear regression model using plots, where the abscissa represented the concentration of tested plant extracts and the ordinate the average percent of scavenging capacity (Software Prizm 3.00). IC_{50} values denote the concentration of sample required to scavenge 50% of DPPH radical.

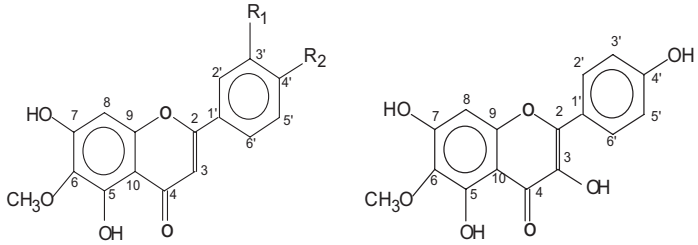
Statistical analysis

Statistical analysis was carried out using excel. All experiments were performed in triplicate. Results were presented as a value ± standard deviation (SD). Significant levels were defined at $p < 0.05$ as analyzed by t-test.

Results

Surface flavonoid aglycones

Four surface flavonoid aglycones were detected in the acetone exudates of the studied samples (Fig. 1): scutellarein 6-methyl ether (hispidulin) (1), scutellarein 6,4'-dimethyl ether (pectolinarigenin) (2), 6-OH luteolin 6-methyl ether (3) and kempferol-6-methyl ether (4). Three of detected flavonoid structures belong to the flavone class of flavonoids and one belongs to the flavonol class. Thin layer chromatographic data – R_F (rate of flow) and color of detected flavonoid aglycones are presented in table 1. The flavonoid aglycones (1) and (2) were the main flavonoids in all studied exudates. The flavonoid aglycones (3) and (4)



(**1, 2, 3**) (**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 (**3**)
(**4**) kempferol-6-methyl ether

Fig. 1. Structures of the identified flavonoid aglycones 1–4.

Tab. 1. Thin layer chromatographic data on detected flavonoid aglycones in the studied samples of *Arnica Montana*

Flavonoid aglycones	Thin layer chromatographic data – R_F (rate of flow) and color		
	S_1	S_2	S_3
Scutellarein 6,4'-dimethyl ether (pectolinarigenin)	0.52 (brown/ brown)	0.57 (brown/ brown)	0.67 (brown/ brown)
Scutellarein 6-methyl ether (hispidulin)	0.33 (brown/ brown)	0.21 (brown / brownish-yellow)	0.52 (brown/ brown)
Kempferol-3-methyl ether	0.39 (brown / brownish-yellow)	0.15 (brownish-yellow / yellow)	0.28 (brownish-yellow / yellow)
6-OH luteolin 6-methyl ether	0.16 (brown / orange)	0.06 (brown / yellow)	0.21 (brown / Light orange)

S_1 – sorbent=silicagel, eluent=toluene:dioxan:acetic acid (90:25:4); S_2 – sorbent=polyamid, eluent=toluene:methylethylketone:methanol (60:25:15); S_3 – sorbent – cellulose, eluent=30% acetic acid

were detected in trace amounts in the leaf exudates while in the flower exudates they were present in high quantities. Differences among the leaf exudates of studied samples were not observed.

Total phenolic content

The results of total phenolic content determination in the methanol extracts of studied samples, evaluated using Folin – Ciocalteu method, are presented in table 2. The content of phenols in extracts of in vitro samples AGM and AGR expressed as gallic acid equivalents (GAE) was 23.65 to 24.78 mg g^{-1} of dry extract, respectively. There were no significant differences in content of total phenols ($p > 0.05$) among the methanol extracts of in vitro

Tab. 2. Polyphenol content and free radical scavenging activity of samples of *in vitro*, *ex vitro* and *in vivo* grown *Arnica montana* plants

Sample	Plant part	Total phenols* mg GAE/g extract	Total flavonoids* (mg RE/g extract)	DPPH scavenging activity IC_{50} (μg/mL)
AGM in vitro	Leaves	23.65±1.6[a]	1.48±0.8[d]	>200[k]
AGR in vitro	Leaves	24.78±1.1[a]	1.49±0.7[d]	>200[k]
AG1_L ex vitro	Leaves	38.13±3.5[b]	4.20±1.4[f]	64.01±5,51[h]
AG1_F ex vitro	Flower heads	36.41±5.9[b]	9.21±1.1[e]	85.73±8,11[j]
AG2_L in vivo	Leaves	57.80±4.2[c]	4.54±1.6[f]	33.79±6,32[g]
AG2_F in vivo	Flower heads	51.25±2.6[c]	9.5±2.7[e]	60.22±7,51[h]

AGM – *in vitro* sample multiple stage; AGR – *in vitro* sample, plant rooting stage; AG1_L – *ex vitro* grown plants, leaves, AG1_F – *ex vitro* grown plants, flower heads; AG2_L – *in vivo* grown plants, leaves; AG2_F – *in vivo* grown plants, flower heads; GAE- gallic acid equivalents; RE – rutin equivalents. * values represent mean ±SD, n=3. Values with the same letter are not significantly different, $p \geq 0.05$

samples. The leaf extracts of ex vitro and in vivo (AG1 L and AG2 L) developing plants contain higher levels of phenols compared to extracts of in vitro samples, respectively 38.13 and 57.80 mg g^{-1} of dry extract. The extracts from flower heads of the same samples (AG1 F and AG2 F) contained phenols of respectively 36.41 and 51.25 mg g^{-1} of dry extract. The extracts of in vivo (AG2 L and AG2 F) samples contain higher phenols than extracts of ex vitro samples (AG1 L and AG1 F). The marked differences were statistically significant ($p < 0.05$). There were no significant differences in the phenolic content ($p > 0.05$), between the extracts of flower heads and leaves of a separate samples.

Total flavonoid content

Flavonoid contents is expressed as rutin equivalents: mg RE per g of dry extract. The amount of flavonoids was the lowest in the leaf extracts from in vitro grown plants AGM and AGR – 1.48 and 1.49 mg g^{-1} (Tab. 2). No differences in the content of total flavonoids among leaf extracts from ex vitro and in vivo grown plants (AG1 L and AG2 L) were observed – 4.20 and 4.54 mg g^{-1} of dry extract, respectively. However, a significant difference ($p < 0.05$) was found in the content of flavonoids between extracts of leaves and flowers of a sample. The extracts of flower heads of ex vitro and in vivo grown plants (AG1 F and AG2 F) contain flavonoids of respectively 9.21 and 9.50 mg g^{-1} of dry extract.

DPPH free-radical scavenging activity

The degree of discoloration of violet colour of DPPH·, as it gets reduced, indicated the radical scavenging potential of the antioxidant. Results of the DPPH scavenging activity of studied samples, expressed as IC_{50} value that represent the concentration of the sample required to scavenge 50% of DPPH radicals, are given in table 2.

It was found that the leaf extracts of ex vitro and in vivo samples had the strongest radical scavenging activity, respectively $IC_{50} = 64.01$ and 33.79 μg mL^{-1}. The extracts of in

vitro samples showed low activity and their IC_{50} values were above 200 μg mL^{-1}. The extracts of flower heads of ex vitro and in vivo samples showed slightly lower activity than the corresponding leaf extracts IC_{50} = 85.73 and 60.22 μg mL^{-1} respectively.

Discussion

The commercial importance of polyphenols has led to attempts to develop alternative systems for their production. The advantage of using the tissue and organ cultures is more stable production of secondary metabolites than cultures of undifferentiated cells, such as cells in callus or suspension culture (RAMACHANDRA RAO and RAVISHANKAR 2002). In the present study surface flavonoids, total phenols and flavonoids as well as the antioxidant potential of extracts from in vitro, ex vitro and in vivo grown plants of *Arnica montana* were analyzed. It was found that there was no difference in the synthesis of surface flavonoids among in vitro, ex vitro and in vivo grown plants. Differences were observed in the composition of surface flavonoids between the exudates of leaves and flower heads. The exudates of flower heads contained a high quantity of the flavonoids 6-OH luteolin 6-methyl ether and kempferol-6-methyl ether. This is in accordance with reports of other authors for differences in surface flavonoid composition in different plant organs (WILLIAMS et al. 1999).

The results of quantitative analysis showed that the total content of phenols and flavonoids in the extracts of in vitro cultures were lower than in the extracts of ex vitro and in vivo grown plants. The differences in the content of these secondary metabolites might be due to the different growth conditions and stages of plant development. The lower level of phenols in extracts of in vitro samples compared to extracts of field grown plants has been already reported (STANLY et al. 2001, RAMES et al. 2009, PARSAEIMEHR et al. 2010, SAGWAN et al. 2011, SINGH et al. 2011). RAMESH et al. (2009) explained the increased synthesis of phenols in natural conditions as a defensive reaction to environmental stress.

The higher content of total phenols in *in vivo* grown plants than in those from ex vitro adapted plants could be explained by the different altitudes at which they were grown, respectively Central Rhodopes – Beglica 1500 m a.s.l. and greenhouse, Sofia region 553 m a.s.l. The positive correlation between altitude and the content of caffeic acid derivatives in *A. montana* cv Arbo flower heads was reported (SPITALER et al. 2006, 2008). Higher levels of flavonoids in the extracts of flower heads of ex vitro and in vivo grown plants were observed compared to corresponding leaf extracts. This result is in agreement with other studies reporting flavonoid concentration in different plant parts (SAVICKIENE et al. 2002, TOMCZYK and GUDEJ 2003, KHATIWORA et al. 2010)

The extracts of ex vitro and in vivo samples had a radical scavenging activity greater than that of the extracts from in vitro samples. Higher activity of the extracts of these samples corresponds to a high content of phenols in them. A positive correlation between total phenolic contents and antioxidant activity has been reported by many authors (CAI et al. 2004, SHAN et al. 2005, WONG et al. 2006, WU et al. 2006, MOLDOVAN et al. 2011, MAIZURA et al. 2011). Moreover, the results presented confirm earlier observations that the antioxidant activity of extracts is correlated more strongly with the content of phenolics than that of flavonoids (MILIAUSKASA et al. 2004, NIĆIFOROVIĆ et al. 2010, NIKOLOVA 2011). To the best of our knowledge the present study is the first report of polyphenol content and free radical scavenging activity of in vitro grown plants of *Arnica montana.*

In conclusion, four surface flavonoid aglycones were identified in the acetone exudates of the studied samples of *A. montana*. No differences were observed in the synthesis of surface flavonoids among the extracts of in vitro, ex vitro and in vivo samples. But there was a difference between different organs – the extracts of the flower heads were richer in surface flavonoids than leaf extracts.

The antioxidant capacity and total flavonoid and phenolic content of the studied samples increased in the order in vitro, ex vitro and in vivo grown plants of *A. montana*. The extracts of flower heads were the richest in total flavonoids, but leaf extracts had the highest content of phenols and exhibited the highest free radical scavenging activity. *A. montana* plants obtained by in vitro technique and grown under natural conditions could be an appropriate source of antioxidants. The selection of highly productive lines as well as the optimization of cultivation environments is a prerequisite for the application of this system.

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