FULL PAPER

Developmental and Environmental Effects on Sesquiterpene Lactones in Cultivated *Arnica montana* L.

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The amount of sesquiterpene lactones and the lactone profile of *Arnica montana* L. in flowering and seed formation stages *in vitro* and *in vivo* propagated from seeds of German, Ukrainian, and Austrian origin and grown in two experimental fields were studied. It was found that *in vitro* propagated 2-year plants in full flowering stage accumulated higher amount of lactones in comparison to *in vivo* propagated 3-year plants and to the seed formation stage, respectively. Helenalins predominated in *in vivo* propagated 2-year or *in vitro* propagated 3-year plants. 2-Methylbutyrate (2MeBu) was the principal ester in the samples with prevalence of helenalins, while isobutyrate (iBu) was the major one in the samples with predominance of 11,13-dihydrohelenalins. The results revealed that the environmental conditions on Vitosha Mt. are more suitable for cultivation of *A. montana* giving higher content of lactones.

Keywords: *Arnica montana*, Asteraceae, Sesquiterpene lactones, Helenalins, 11,13-Dihydrohelenalins, Developmental and environmental factors.

Introduction

Arnica montana L. is an endemic species to Europe where it is found from Norway to the Balkans and from Spain to Ukraine [1]. In some countries, the populations of the species are still stable, while in others, the areas of distribution decreased because of large consumption or other human intervention in natural habitats [2], and hence, it is included in the lists of threatened and endangered species [3]. The existence of this species in Bulgaria in Rila Mountain was described earlier but the location was not specified. Unfortunately, its survival through the years is not confirmed [4]. To avoid the risk of extinction, as well as to ensure standardized raw material for pharmaceutical industry, many experiments for cultivation have been carried out [5 – 9].

Arnica montana is applied to people with arthritis, rheumatic disorders, inflammation bruises, sprains, soreness, and swelling/muscle spasms from sports activity; arthritis set off by seasonal change; and general muscle and joint pain [10][11]. It is used as tincture, cream, or ointment for external applications mainly [12] and as homeopathic pills [13]. Phytochemical research revealed that this species is rich in sesquiterpene lactones of helenalin and 11,13-dihydrohelenalin types which are responsible for the biological activity [14][15]. It has been shown that sesquiterpene lactones are responsible for anti-inflammatory activity [16] and inhibition of transcription factors NF-κB and NF-AT [11][14][17]. Minimum content of 0.4% sesquiterpene lactones is required by the European Pharmacopoeia [18] in *Arnica flos* as a medicinal plant. In addition, antioxidant and antimicrobial activities of the herb are attributed to flavonoids and phenolic acids [19].

Attempts for cultivation of *A. montana* are directed toward optimizing the conditions for improving the quality of the herb, *i.e.*, increasing the content of sesquiterpene lactones, especially the content of helenalins, which are reported as the most active lactones in the species [11][15]. This work aims to assess the influence of different developmental and environmental factors on the accumulation of these secondary metabolites, the lactone profile, and helenalins/11,13-dihydrohelenalins ratio (H/DH) in *A. montana*. To achieve the goal of this study, the following factors were investigated: plant age (two and three years), stage of development (full flowering and seed formation), origin of seeds for propagation (Germany, Ukraine, and Austria), method of propagation (*in vitro* and *in vivo*), and environmental conditions (experimental fields – Beglika on Rhodope Mts. and Zlatni mostove on Vitosha Mt.).

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Results and Discussion

In this study, the sesquiterpene lactone profile of 18 plant samples of *A. montana* (*Table 1*) was analyzed and the quantity of individual components was determined by GC using santonin as a standard. Acetyl (Ac), isobutyryl (iBu), methacryloyl (Meac), 2-methylbutyryl (2MeBu), isovaleryl (iVal), and tigloyl (Tig) esters of helenalin and 11,13-dihydrohelenalin (*Fig. 1*, *Table 2*) were detected in all investigated samples.

The influence of different developmental and environmental factors on the total content of sesquiterpene lactones (TSL), the lactone profile, and the ratio between helenalins (H) and 11,13-dihydrohelenalins (DH) are described below.

Influence of the Seeds Origin

Comparison of the lactones in the plants of the same age and other conditions being equal (*G3*, *G4*, *U3*, *U4*, *A1*, and *A2*) showed that the seeds origin is relevant for the chemical composition of the plants (*Fig. 2*). In this case (*in vitro* samples), the sample from Austria was the most productive of sesquiterpene lactones, followed by those from Germany and Ukraine. The lactone content in the Austrian sample was almost twofold higher than that in Ukrainian.

Influence of the Stage of Development

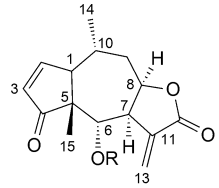
It was found that the stage of development affected only the total content of lactones. Thus, it was higher in the flowering stage in comparison with the seed formation stage (*Fig. 2*). The lactone profile and H/DH did not depend on this factor. Earlier, *Douglas et al.* [20] reported an increase in the total sesquiterpene lactones from 5.12 mg/g in buds to 9.43 mg/g in withered flowers. Thus, the full flowering is the proper stage for collection of the plant material.

Influence of the Plant Age

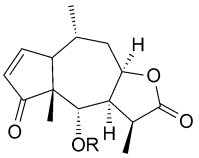
The lactone profiles of 2- and 3-year *in vitro* propagated plants of German (*G1* – *G4*) and Ukrainian (*U1* – *U4*) origins and those grown in one experimental field (Rhodope

Table 1. Plant samples

Sample	Seed origin	Stage of development	Plant age [Year]	Propagation	Experimental field	Voucher specimen
G1	Germany	Full flowering	2	*in vitro*	Rhodopes Mts.	169538
G2	Germany	Seeds formation	2	*in vitro*	Rhodopes Mts.	
G3	Germany	Full flowering	3	*in vitro*	Rhodopes Mts.	
G4	Germany	Seeds formation	3	*in vitro*	Rhodopes Mts.	
G5	Germany	Full flowering	2	*in vivo*	Rhodopes Mts.	16937
G6	Germany	Seeds formation	2	*in vivo*	Rhodopes Mts.	
G7	Germany	Full flowering	2	*in vivo*	Vitosha Mt.	168516
G8	Germany	Seeds formation	2	*in vivo*	Vitosha Mt.	
U1	Ukraine	Full flowering	2	*in vitro*	Rhodopes Mts.	169535
U2	Ukraine	Seeds formation	2	*in vitro*	Rhodopes Mts.	
U3	Ukraine	Full flowering	3	*in vitro*	Rhodopes Mts.	
U4	Ukraine	Seeds formation	3	*in vitro*	Rhodopes Mts.	
U5	Ukraine	Full flowering	2	*in vivo*	Rhodopes Mts.	16939
U6	Ukraine	Seeds formation	2	*in vivo*	Rhodopes Mts.	
U7	Ukraine	Full flowering	2	*in vivo*	Vitosha Mt.	168517
U8	Ukraine	Seeds formation	2	*in vivo*	Vitosha Mt.	
A1	Austria	Full flowering	3	*in vitro*	Rhodopes Mts.	169536
A2	Austria	Seeds formation	3	*in vitro*	Rhodopes Mts.	



Helenalin (H) R = H
6-*O*-Acetylhelenalin (H-Ac) R = Ac
6-*O*-Isobutyrylhelenalin (H-iBu) R = iBu
6-*O*-Methacryloylhelenalin (H-Meac) R = Meac
6-*O*-(2-Methylbutyryl)helenalin (H-2MeBu) R = 2MeBu
6-*O*-Isovalerylhelenalin (H-iVal) R = iVal
6-*O*-Tigloylhelenalin (H-Tig) R = Tig



11α,13-Dihydrohelenalin (DH) R = H
6-*O*-Acetyl-11α,13-dihydrohelenalin (DH-Ac) R = Ac
6-*O*-Isobutyryl-11α,13-dihydrohelenalin (DH-iBu) R = iBu
6-*O*-Methacryloyl-11α,13-dihydrohelenalin (DH-Meac) R = Meac
6-*O*-(2-methylbutyryl)-11α,13-dihydrohelenalin (DH-2MeBu) R = 2MeBu
6-*O*-Isovaleryl-11α,13-dihydrohelenalin (DH-iVal) R = iVal
6-*O*-Tigloyl-11α,13-dihydrohelenalin (DH-Tig) R = Tig

Fig. 1. Sesquiterpene lactones in *A. montana*.

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Table 2. Content of individual sesquiterpene lactones in *A. montana* samples [mg/g DW]

Sample[a])	Content of sesquiterpene lactones[b])											
	H-Ac[c])	H-iBu	H-Meac	H-2MeBu	H-iVal	H-Tig	DH-Ac	DH-iBu	DH-Meac	DH-2MeBu	DH-iVal	DH-Tig
G1	0.11 ± 0.01^{a}	0.25 ± 0.02^{a}	0.14 ± 0.01^{a}	0.81 ± 0.03	0.25 ± 0.02^{a}	0.13 ± 0.01^{a}	0.68 ± 0.03	1.44 ± 0.01	0.63 ± 0.02	1.34 ± 0.01	0.40 ± 0.03^{a}	0.47 ± 0.03
G2	0.13 ± 0.01^{ab}	0.24 ± 0.01^{a}	0.14 ± 0.01^{a}	0.52 ± 0.03^{a}	0.09 ± 0.01^{b}	0.06 ± 0.01	0.46 ± 0.02	0.86 ± 0.02	0.44 ± 0.02^{a}	0.67 ± 0.02	0.23 ± 0.01^{b}	0.29 ± 0.0^{a}
G3	0.43 ± 0.01	1.03 ± 0.01	0.74 ± 0.01^{b}	1.26 ± 0.01^{b}	0.34 ± 0.02^{c}	0.52 ± 0.02^{b}	0.23 ± 0.03^{a}	0.32 ± 0.02	0.26 ± 0.03^{b}	0.18 ± 0.03^{a}	0.36 ± 0.02^{c}	0.18 ± 0.03^{b}
G4	0.32 ± 0.02^{c}	0.90 ± 0.01	0.62 ± 0.01	1.30 ± 0.01^{bc}	0.32 ± 0.03 ^{cd}	0.52 ± 0.02^{b}	0.19 ± 0.03^{ab}	0.27 ± 0.03^{a}	0.16 ± 0.04^{c}	0.15 ± 0.03^{ab}	0.33 ± 0.02 ^{cd}	0.14 ± 0.03^{bc}
G5	0.30 ± 0.02^{cef}	0.43 ± 0.01	0.30 ± 0.02	0.67 ± 0.01 ^{fg}	0.18 ± 0.02	0.22 ± 0.02	0.19 ± 0.01^{bfgi}	0.15 ± 0.01^{bd,ef}	0.10 ± 0.02^{fi}	0.11 ± 0.01^{efghj}	0.10 ± 0.01 ^{lm}	0.09 ± 0.02^{kmop}
G6	0.23 ± 0.02^{d}	0.34 ± 0.01	0.23 ± 0.02	0.40 ± 0.01 ^{g}	0.11 ± 0.03^{bg}	0.13 ± 0.02^{ac-e}	0.13 ± 0.01^{j}	0.11 ± 0.01^{ceg}	0.09 ± 0.02^{fi}	0.08 ± 0.02^{efhij}	0.13 ± 0.01	0.07 ± 0.02^{kmop}
G7	0.55 ± 0.02	0.81 ± 0.01	0.87 ± 0.01	1.05 ± 0.01	0.60 ± 0.02^{hi}	0.72 ± 0.01 ^{g}	0.18 ± 0.02^{bf}	0.16 ± 0.02^{b}	0.17 ± 0.01^{cde}	0.09 ± 0.02^{e}	0.23 ± 0.01^{bef}	0.16 ± 0.01^{bcl}
G8	0.65 ± 0.01	0.54 ± 0.02	0.53 ± 0.01	0.74 ± 0.01	0.25 ± 0.02^{aej}	0.37 ± 0.01^{f}	0.19 ± 0.01^{bfg}	0.11 ± 0.01^{c}	0.10 ± 0.01^{f}	0.07 ± 0.03^{ef}	0.20 ± 0.01^{bfg}	0.07 ± 0.02 ^{km}
U1	0.11 ± 0.02^{ab}	0.28 ± 0.01	0.18 ± 0.02	0.59 ± 0.01^{d}	0.18 ± 0.03^{f}	0.12 ± 0.02^{ac}	0.63 ± 0.01	1.28 ± 0.01	0.69 ± 0.02	0.94 ± 0.01	0.32 ± 0.02^{di}	0.43 ± 0.01^{d}
U2	0.07 ± 0.02	0.14 ± 0.01	0.08 ± 0.02	0.48 ± 0.01^{ae}	0.25 ± 0.02^{ae}	0.15 ± 0.02^{ad}	0.50 ± 0.01	1.06 ± 0.01	0.48 ± 0.02	1.04 ± 0.01	0.30 ± 0.02^{di}	0.41 ± 0.02^{d}
U3	0.22 ± 0.02^{d}	0.57 ± 0.01	0.56 ± 0.02	0.59 ± 0.01^{d}	0.10 ± 0.04^{bfg}	0.15 ± 0.03^{ac-e}	0.32 ± 0.02^{c}	0.38 ± 0.02	0.43 ± 0.01^{a}	0.48 ± 0.01^{c}	0.33 ± 0.02 ^{cdhi}	0.31 ± 0.03^{ae}
U4	0.17 ± 0.03	0.49 ± 0.01	0.33 ± 0.02	0.51 ± 0.02^{ae}	0.05 ± 0.04^{b}	0.37 ± 0.02^{f}	0.26 ± 0.02^{d}	0.25 ± 0.03^{a}	0.28 ± 0.02^{b}	0.47 ± 0.01^{c}	0.26 ± 0.03^{be}	0.27 ± 0.03^{aef}
U5	0.48 ± 0.02	0.59 ± 0.01	0.74 ± 0.01^{bde}	0.86 ± 0.01	0.46 ± 0.02	0.82 ± 0.01	0.28 ± 0.01^{deh}	0.21 ± 0.02	0.22 ± 0.02^{dg}	0.18 ± 0.03^{abd}	0.21 ± 0.02^{bfgk}	0.28 ± 0.01^{aefn}
U6	0.29 ± 0.02^{cf}	0.38 ± 0.01	0.41 ± 0.01^{f}	0.63 ± 0.01^{df}	0.32 ± 0.02^{cd}	0.49 ± 0.01	0.15 ± 0.01^{fij}	0.13 ± 0.01^{bcefg}	0.14 ± 0.02^{ceh}	0.09 ± 0.02^{efhi}	0.12 ± 0.02 ^{lm}	0.14 ± 0.01^{bcjklo}
U7	0.73 ± 0.01	0.65 ± 0.01	0.80 ± 0.01	1.22 ± 0.01^{b}	0.62 ± 0.02^{i}	1.07 ± 0.01	0.29 ± 0.01^{cdeh}	0.17 ± 0.02^{bd}	0.24 ± 0.02^{bg}	0.13 ± 0.02^{bg}	0.25 ± 0.01^{befk}	0.28 ± 0.01^{aefn}
U8	0.33 ± 0.02^{ce}	0.32 ± 0.01	0.41 ± 0.01^{f}	0.65 ± 0.01^{f}	0.23 ± 0.02^{aej}	0.42 ± 0.01	0.18 ± 0.01^{bfghi}	0.12 ± 0.02^{ce}	0.16 ± 0.01^{ceh}	0.08 ± 0.02^{efh}	0.22 ± 0.01^{bfgk}	0.11 ± 0.02^{cjkmo}
A1	0.62 ± 0.01	1.66 ± 0.01	0.74 ± 0.01^{bcd}	1.89 ± 0.01	0.56 ± 0.02 ^{h}	0.70 ± 0.01 ^{g}	0.27 ± 0.03^{ade}	0.49 ± 0.03	0.32 ± 0.03	0.26 ± 0.03	0.37 ± 0.02^{acj}	0.15 ± 0.04^{bcg}
A2	0.51 ± 0.02	1.37 ± 0.01	0.73 ± 0.01^{bce}	1.31 ± 0.01^{c}	0.37 ± 0.02^{c}	0.46 ± 0.02	0.23 ± 0.03^{d}	0.42 ± 0.02	0.20 ± 0.03^{ad}	0.19 ± 0.03^{ad}	0.38 ± 0.02^{acj}	0.11 ± 0.04^{cjk}

[a]) For the abbreviations of the samples, see *Table 1*. [b]) Means with the same letter (in the columns) are not significantly different from each other based on *Duncan*'s test ($P > 0.05$). [c]) For the compound codes, see *Fig. 1*.

Mts.) demonstrate that the older plants produced lower amounts of lactones (*Fig. 2*). However, 11,13-dihydrohelenalins prevailed in the 2-year plants, while accumulation of helenalins increased in the 3-year plants with exception of *U3*. In the last sample, H and DH are in approximately equal concentrations. Principal components in the studied samples are iBu and 2MeBu esters of helenalin or 11,13-dihydrohelenalin (*Table 2*). In a previous study, it was found that the content of 11,13-dihydrohelenalins in leaves of *in vitro* cultivated *A. montana* increased from the beginning to the sixth week of vegetation and remains constant for a long time [21]. The transformation of sesquiterpene lactones from helenalin into 11,13-dihydrohelenalin type was supported by the enzyme hydrogenase, which reached its maximum activity several weeks after leaf formation. Surprisingly, the analysis of flowers collected from 2- and 3-year plants (derived from *in vitro* multiplication) showed that 2-year plants contained predominately 11,13-dihydrohelenalin esters, while the 3-year plants contained mainly helenalin esters. Obviously, further research is needed to understand which factors activate the enzymes responsible for the hydrogenation/dehydrogenation of the lactone ring during the plant aging.

Influence of the Method of Propagation

The influence of the method of propagation on the lactone composition was also studied using 2-year plants of German (*G1*, *G2*, *G5*, and *G6*) and Ukrainian (*U1*, *U2*, *U5*, and *U6*) origins grown in the same environmental and developmental conditions (*Fig. 2*). Thus, TSL was higher in the *in vitro* than in the *in vivo* propagated plants. In addition, 11,13-dihydrohelenalins prevailed in the *in vitro* propagated plants (H/DH < 1), which was also demonstrated earlier [21], while the helenalins dominated (H/DH > 1) in the *in vivo* propagated plants. DH-iBu was a principal component in the *in vitro* propagated plants, followed by DH-2MeBu (*Table 2*). In contrary, the *in vivo* propagated samples produced mainly H-2MeBu. Further, the higher concentration of H-Tig in comparison with H-iBu, distinguished the *in vivo* propagated Ukrainian samples (*U5* and *U6*) from the German ones (*G5* and *G6*). The observed differences could be due to the conditions of the early development of plants, effect of plant growth regulators, *etc*.

Influence of the Environmental Conditions

The influence of the environmental conditions on the accumulation of sesquiterpene lactones was also investigated. For this purpose, the plants were *in vivo* propagated and grown in two experimental fields – Vitosha Mt. and Rhodope Mts. (*G5 – G8* and *U5 –U8*). The total amount of sesquiterpene lactones (*Fig. 2*) showed that the ecological conditions on Vitosha Mt. were more favorable for the biosynthesis of the target compounds. The content of helenalins in these samples exceeded more than three

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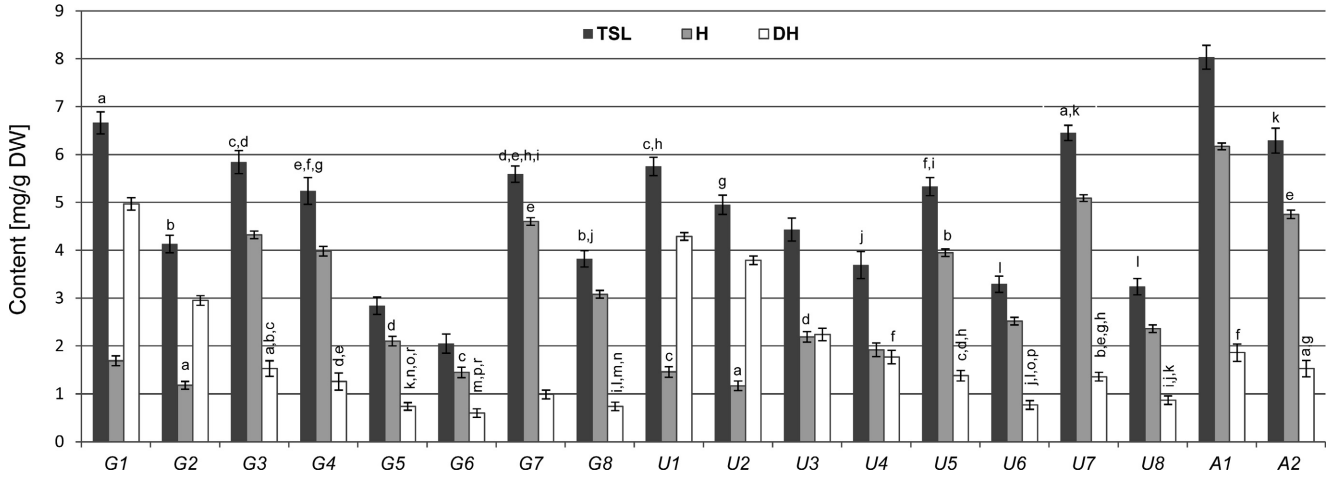


Fig. 2. Content of total sesquiterpene lactones (TSL), helenalins (H), and 11,13-dihydrohelenalins (DH) in *A. montana* samples depending on plant age, stage of development, seeds origin, method of propagation and environmental conditions – Rhodope Mts. and Vitosha Mt. For the abbreviations of the samples, see *Table 1*. Means with the same letter are not significantly different from each other based on *Duncan*'s test ($P > 0.05$).

times that of 11,13-dihydrohelenalins. It should be noted that H/DH ratio (2.4 – 3.3) was higher than the ratio of 1.5 reported earlier [22]. The higher TSL and H/DH ratio in plants grown in the experimental field on Vitosha Mountain might be due to higher content of humus and N, lower pH of the soil, and lower contents of P_2O and K_2O compared to the values of these parameters in Rhodope Mts. The influence of the specific environmental and ecological factors will be the subject of future work. The samples of Ukrainian origin compared to these of German origin, independent of the location of farming – Vitosha or Rhodope Mts., are characterized by predominant biosynthesis of H-Tig instead of H-iBu (*Table 2*).

The literature survey revealed that the ratio between helenalins and 11,13-dihydrohelenalins in flower heads of *A. montana* varied according to geographical distribution, depending on the altitude, specific soil, and climatic factors [22 – 24]. The analysis of flower heads from 39 different taxa showed that the Central European type *A. montana* contained principally H, while Spanish collections contained predominantly DH [23]. The presence of chemotypes in the Spanish population was reported later [24]. The flower heads collected from heathlands at high altitude (1330 – 1460 m) were with high levels of helenalin esters, while flower heads from meadows and peat bogs at lower altitudes (420 – 1215 m) showed high levels of 11,13-dihydrohelenalin esters.

Conclusions

The results from the analyses of sesquiterpene lactones in flowers of *A. montana* showed that the lactone content in the analyzed samples varied in a wide range 2.05 – 8.03 mg/g dry weight. With exception of one sample (*G5*), the lactone content exceeded the required value of 4 mg/g dry weight as recommended in the European Pharmacopoeia. Helenalins dominate in *in vivo* propagated plants, although the total amount of sesquiterpene lactones is lower in comparison to *in vitro* propagated, in which 11,13-dihydrohelenalins prevailed. TSL was higher in full flowering stage compared to the seed formation period. Among the studied samples, *A1* (the richest in lactones) and *G7* (containing 4.6 times more H than DH) were the most promising for commercial purposes. It was also observed that H-2MeBu was the principal component in the samples with domination of helenalins, while DH-iBu was the major one in the samples with higher concentration of 11,13-dihydrohelenalins. Thus, different developmental and environmental conditions could be selected in order to increase the content of lactones and to manipulate the H/DH ratio.

Experimental Part

Plant Material

Seeds of *A. montana* used for propagation were obtained from the Botanical Garden in Chemnitz (Germany); Natural habitat in Carpathian Mts., Ridge Chernogora, Rahoevski region, Zakarpatie area (Ukraine), and Botanical Gardens in Vienna (Austria). The samples (*Table 1*) were cultivated in the experimental fields in Rhodope and Vitosha mountains, and flower heads of each sample were collected in full flowering and seeds formation stages. Voucher specimens (SOM) of the plants propagated *in vivo* and *in vitro* (*Table 1*) were deposited with the Herbarium of the Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences.

Propagation and Growth

This study was carried out with plant materials obtained *in vivo* and *in vitro* and cultivated in two experimental

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fields in different geographic regions – location Beglika (Rhodope Mts.): 1500 m ASL, N 41.849332°, E 24.117781°, brown forest soil with humus content 2.84%; pH 6.5; N% 0.15; P_2O mg/100 g 19.83; K_2O mg/100 g 16.60 and location Zlatni Mostove (Vitosha Mt.): 1404 m ASL, N 42.614948°, E 23.234493°, brown forest soil, humus content 6.98%; pH 5.9; N% 0.35; P_2O mg/100 g 5.64; K_2O mg/100 g 11.90.

Propagation in vitro

Biotechnological tools: Nodal segments from *in vitro* seedlings were used for initiation of micropropagation. The shoots were multiplied on *Murashige* and *Skoog* (MS) medium [25] supplemented with 1 mg/l 6-benzylaminopurine (BAP) and 0.1 mg/l indole-3-acetic acid (IAA). The propagated plants were rooted on half-strength MS medium contained 0.5 mg/l indole-3-butyric acid (IBA). After successful *ex vitro* adaptation in greenhouse, the plants were planted in the experimental field Beglika in Rhodope Mts. in June 2010 in rows with distance 40/30 cm.

Propagation in vivo

Seeds of *A. montana* were germinated in March 2011 under greenhouse conditions at 20 °C by directly sowing of 3 – 4 seeds/cell into cell trays and after germination thin to leave only one seedling per cell. The substrata composed of a mixture of garden soil (containing pear, sand, pine bark, and compost). After 10 weeks (in May 2011), the seedlings formed rosettes characterized by 4 – 6 leaves and were planted in the field plots in rows with distances 40/30 cm in the experimental fields in Vitosha Mt. and Rhodope Mts.

Sample Preparation and GC Analysis

Air-dried flowers of *A. montana* (1 g) were extracted with $CHCl_3$ (4 × 10 ml) for 10 min at 25 °C in an ultrasonic bath. The solvent was removed under vacuum and the obtained total extract was worked up, as described previously [26], to give the crude lactone fraction for GC analysis. A quantitative analysis of sesquiterpene lactones in *A. montana* was carried out on a *Trace* ultra gas chromatograph fitted with a *Tri-Plus* autosampler (*Thermo Scientific*, Bremen, Germany) and *DB 225* capillary column (*J&W Scientific*, Folsom, CA, USA) 60 m × 0.25 mm ID, 0.25 μm film thickness. He was used as the carrier gas at a constant flow rate of 1 ml/min. A split injection was used at a split ratio of 1:10, and inlet temp. of 260 °C. The oven was programmed from initial temp. 60 – 220 °C at 15 °C/min and to 240 °C at 1 °C/min, and held at final temp. for 10 min. Flame-ionization detection was used with a detector temp. of 260 °C. The sesquiterpene lactones were identified by GC/MS (same conditions, 70 eV, EI mode). Retention times (t_R) and mass spectral fragmentation patterns (MS) were compared with standards previously isolated in our laboratory [27]. The absolute calibration method was used for the quantification using santonin as a standard.

Statistical Analysis

All measurements were performed in triplicate and are presented as arithmetic mean ± standard deviations. The data were analyzed with one-way analysis of variance (ANOVA) followed by *Duncan*'s multiple range tests using XLSTAT software (*Addinsoft Inc.,* New York, NY, USA).

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