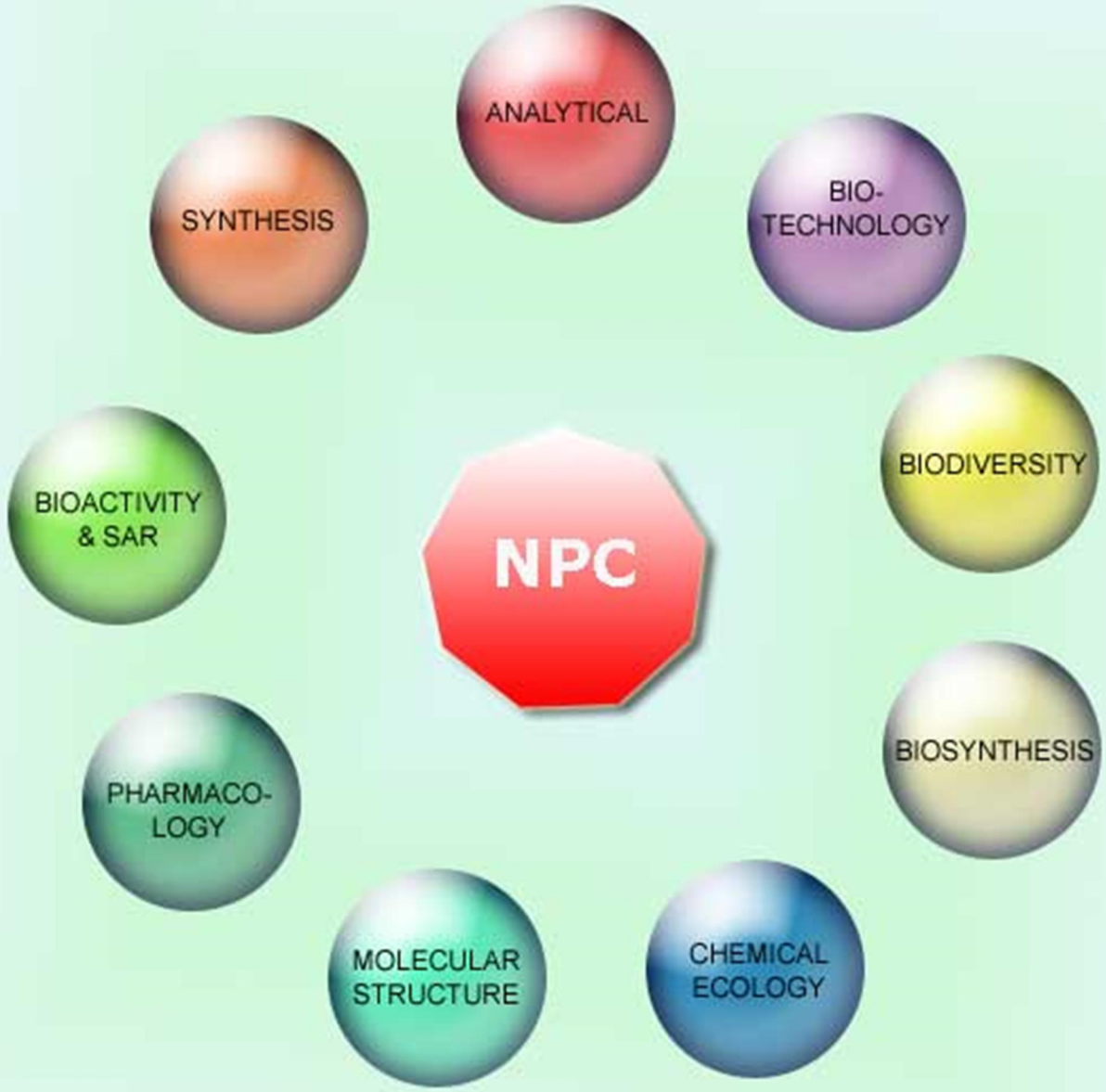
NATURAL PRODUCT COMMUNICATIONS

An International Journal for Communications and Reviews Covering all Aspects of Natural Products Research



This Issue is Dedicated to
Dr. Pawan K. Agrawal
On the Occasion of his 60th Birthday

Volume 10. Issue 6. Pages 823-1140. 2015
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

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NPC Natural Product Communications

2015
Vol. 10
No. 6
839 - 841

Bioactive Compounds in Wild, *In vitro* Obtained, *Ex vitro* Adapted, and Acclimated Plants of *Centaurea davidovii* (Asteraceae)

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Received: December 2nd, 2014; Accepted: March 9th, 2015

In vitro cultures were initiated from a single seed of *Centaurea davidovii*. Whole plantlets were regenerated and cultivated for several months on agar-solidified nutrient media differing by their composition: basal MS medium, MS medium supplemented with plant growth regulators, and liquid MS medium. Plantlets were *ex vitro* adapted and successfully acclimated to open-air conditions; flowering was observed in some individuals in the first summer, and mass flowering during the second summer. The contents of the total flavonoids and the total phenolic compounds were determined spectrophotometrically in the leaves of the *in vitro* plantlets cultured on different media, and then compared with those in the leaves of the wild plants and in the leaves of the acclimated plants of the field plot. The sesquiterpene lactone 8α-(5'-hydroxyangeloyl)-salonitenolide was determined by HPLC in leaf samples of *C. davidovii* wild plants, *in vitro* obtained plantlets and *ex vitro* acclimated plants in the greenhouse and on the experimental field plot. The composition of the nutrient medium influenced the contents of all studied bioactive substances. The highest concentrations of all tested secondary metabolites were detected in the leaves of the acclimated plants during mass flowering, the content of the lactone reaching 56.2 mg/g DW, which was several times more than in the other leaf samples. The obtained results revealed both the effectiveness of biotechnological methods for propagation and conservation of rare and endangered plant species, and the possibility to use *C. davidovii* plants *ex vitro* acclimated to field conditions as a source of secondary metabolites with potential biological activity.

Keywords: *Centaurea*, *in vitro* Cultures, Sesquiterpene lactones, Flavonoids, Polyphenols.

The genus *Centaurea* s. l. (family Asteraceae) is represented in the Bulgarian flora by more than 70 species and is the richest in endemics (ca. 30%). One of them, the endemic *C. davidovii* Urum., is represented by only a few and very fragmented populations in the floristic region of Stara planina Mt. Its limited distribution requires some *in situ* and *ex situ* conservation actions [1].

Previously, a biotechnological method for *in vitro* propagation of *C. davidovii* was developed [2]: plantlets were micropropagated through direct and indirect regeneration, on basal MS medium [3] either free of plant growth regulators or on media supplemented with cytokinins and/or auxins (Figure 1-A). Then, plants have been successfully *ex vitro* adapted using a special climate chamber, and several dozens of them have been acclimated consecutively to greenhouse and to open-air conditions on the experimental field plot. Some of the plants flowered and set seeds in the first summer, while mass flowering was observed in the second year with multiple florets per individual (Figure 1-B).

It is worth noting that plants cultivated under controlled conditions, i.e. regularly watered and weeded, on a sunny field plot, were very large and uniform, with numerous stems reaching heights of 90 to 100 cm, while the size of those growing in the natural populations varied much and their height was between 15 and 60 cm.

Phenolic compounds (phenolic acids, flavonoids, coumarins, etc.) and sesquiterpene lactones are characteristic constituents of *Centaurea* species [4a,b]. Due to the suggested medicinal properties of *C. davidovii*, and the influence of cultivation conditions on the content of biologically active compounds, it is important to evaluate the biosynthesis of the desired secondary metabolites in the corresponding plant extracts. Thus, the aim of this study was to estimate the total phenolic, flavonoid and sesquiterpene lactone content in plant extracts derived from wild, *in vitro* obtained and *ex vitro* acclimated plants of *C. davidovii*.



Figure 1: A) *In vitro* regenerated *C. davidovii* plantlet; B) Acclimated *C. davidovii* plants on the experimental field plot at mass flowering.

The total phenolic content (**TPC**) was determined by the Folin-Ciocalteu assay and expressed in gallic acid equivalents [5a], and the flavonoid content (**FC**) was obtained by a spectrophotometric method in catechin equivalents [5b]. The contents of total phenolics and flavonoids in the extract from leaves of wild plants were 40.6±0.4 mg/g and 5.7±0.2 mg/g, respectively, and decreased significantly in the extracts from *in vitro* plantlets (Table 1). Similar results have been reported in the literature [6]. The high content of phenolics and flavonoids in the extracts from wild plants is due to the fact that these secondary metabolites serve as a natural defense

of the plants to oxidative stress and UV radiation, and protect plants from microorganisms, insects and herbivores [7]. *In vitro* plantlets are grown under sterile ambient conditions with a uniform light and temperature regime all year, which can explain the abrupt reduction of TPC and FC biosynthesis.

Table 1: Content of total phenolic (TPC) and flavonoid (FC) compounds in *C. davidovii* leaves from wild plants, *in vitro* plantlets regenerated on different media, and *ex vitro* acclimated plants at the first and second year after their transfer to the experimental field plot.

Leaves of *C. davidovii*	TPC [mgGAE/g DM]	FC [mgCE/g DM]
Wild (flowering)	40.6±0.4	5.7±0.2
In vitro (MS)	11.2±0.1	1.2±0.1
In vitro (C2)	28.7±0.4	3.4±0.1[a]
In vitro (MS/C7)	16.6±0.2	2.0±0.2
In vitro (MS/liquid)	20.7±0.3	2.6±0.1
Ex vitro (flowering 1Y)	30.1±0.2	3.4±0.1[a]
Ex vitro (flowering 2Y)	63.1±0.5	6.9±0.2

Values with the same letter are not significantly different, $p \leq 0.05$. Standard deviations were calculated on the base of three samples.

Furthermore, the medium composition significantly influenced the ability of the *in vitro* plantlets to biosynthesize phenols and flavonoids. The amounts of these bioactive substances increased in the presence of plant growth regulators (**PGR**) in the medium (up to 28.7 mg/g phenolics and 3.4 mg/g flavonoids in the case of medium C2, containing 0.5 mg/L BAP), and remained relatively high even after the transfer of the culture to a PGR-free medium (MS/C7). This could be a plant answer aiming to avoid the negative influence of the exogenous BAP on the cell structures, which has been observed histologically, especially when this cytokinin was applied alone, without auxins [8]. Similarly, *in vitro* plantlets increased the contents of phenolics and flavonoids when cultured in liquid basal MS medium which causes water stress. The relationship between the medium composition and the contents of TPC and FC confirmed the protective role of these secondary metabolites in plants grown under *in vitro* conditions.

After the successful transfer of the plantlets to *ex vitro* conditions, first in pots with soil mixture and then to the open-air field plot, the contents of phenolic compounds and flavonoids increased considerably, reaching 30.1 mg/g and 3.4 mg/g, respectively, during the first flowering stage (July 2013). Due to the transfer stress, most of the plants did not bloom and the contents of TPC and FC were lower compared with those in the wild plants (Table 1). The favorable growing conditions on the experimental field plot: regular watering and weeding, led not only to intensive plant growth but also to mass flowering in July 2014 during the second flowering season (flowering 2Y). All plants bloomed, forming multiple stems and florets, while in the natural populations some of the plants did not flower. This is the reason for the highest contents of the phenolic compounds and flavonoids in the leaves of the well-acclimated blooming plants after two years of cultivation on the field plot. The contents of TPC and FC in the leaf extracts of these plants significantly exceeded the respective contents in the extract of the wild plants: 63.1 mg/g and 6.9 mg/g, compared with 40.6 mg/g and 5.7 mg/g.

The IR spectrum of the chloroform extract obtained from the leaves of wild-growing *C. davidovii* had an absorption band at 1760 cm^{-1}, characteristic for sesquiterpene lactones. HPLC confirmed the presence of one main lactone, 8α-(5'-hydroxyangeloyl)-salonitenolide (SL), the structure of which was elucidated by spectroscopic (^1H and ^{13}C NMR) and MS methods [9]. Due to its insufficient amount and low stability, the content of this sesquiterpene lactone (SL) was determined by HPLC using cnicin as external standard; the obtained results are presented at Figure 2.

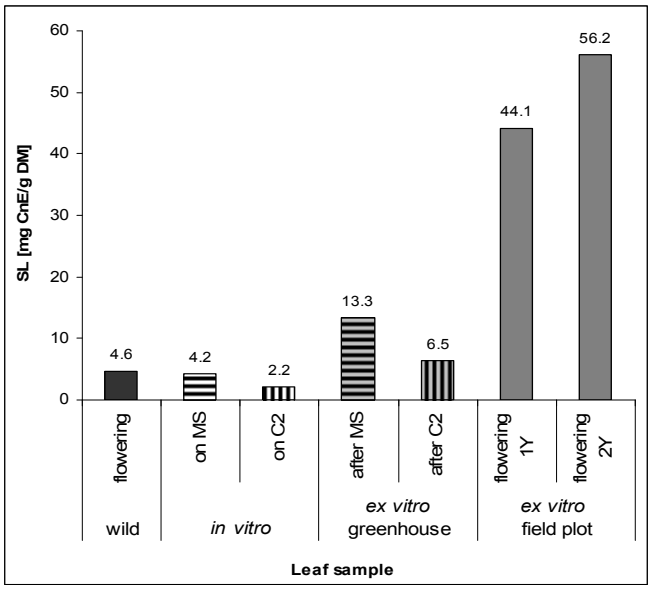


Figure 2: Content of SL in *C. davidovii* leaves from wild plants, *in vitro* plantlets regenerated on different media, *ex vitro* adapted in the greenhouse, and *ex vitro* acclimated plants on the experimental field plot.

The functions of sesquiterpene lactones in plants are similar to those of TPC and FC; they provide numerous health benefits to people as well [10]. The highest content of the main lactone, 8α-(5'-hydroxyangeloyl)-salonitenolide, was detected in the leaf extracts of the plants cultivated for two years under controlled field conditions after their *ex vitro* acclimation (56.2 mg/g during the mass flowering stage). As for TPC and FC, the difference between the contents of SL during the first and the second year on the field plot was obviously due to the flowering rate: in 2013 both flowering and non-flowering individuals were observed while in the second summer in 2014 all plants formed numerous flower stems with multiple florets. Unexpectedly, SL in the extract from wild plants was only 4.6 mg/g, which was commensurable with its value in the extract of *in vitro* plantlets grown on MS basal medium.

The content of SL was lowest in the leaf extracts from the *in vitro* plantlets grown on medium C2. Unlike phenolic compounds and flavonoids, sesquiterpene lactones were reduced by the presence of BAP in the nutrient medium (up to 2.2 mg/g for medium C2 compared to 4.2 mg/g for basal MS medium). Since all plantlets were regenerated from a single initial seed, i.e. they were genetically identical, and the cultivation conditions such as temperature and light regime were the same, the presence of cytokinins in the medium was the sole factor influencing the biosynthetic rate of the tested secondary metabolites. Apparently, the contrary influence of BAP on the contents of TPC and FC on the one hand and of SLC on the other, is related to the different biosynthetic paths of these secondary metabolites.

Once the *in vitro* multiplied *C. davidovii* plantlets were transferred to pots with soil mixture, the content of SL in their leaves began to increase and tripled in a short time; however, the effect of BAP still manifested itself during the next months (6.5 mg/g in the leaf extracts of the *ex vitro* adapted plants multiplied on the medium containing BAP, compared with 13.3 mg/g in those of *ex vitro* adapted plants multiplied on basal MS medium).

The amount of all bioactive substances tested in the leaf extracts of *C. davidovii*, phenolic compounds, flavonoids, and sesquiterpene lactones, rose significantly when plants were cultivated under

controlled field conditions. The biosynthetic enhancement was the strongest for the lactone 8α-(5'-hydroxyangeloyl)-salonitenolide, the content of which increased several times compared with the leaf samples of the wild and the *in vitro* obtained plants.

The obtained results are a good example of the successful application of biotechnological methods for rapid plant propagation and conservation of rare and endangered species. The *ex vitro* acclimated *C. davidovii* could be mass cultivated under controlled field conditions as a source of secondary metabolites with potential biological activity.

Experimental

Plant material: Secondary metabolites were extracted from leaves of wild plants in the flowering stage, leaves of *in vitro* obtained and multiplied plantlets, and leaves of *ex vitro* adapted and acclimated plants to greenhouse and to experimental field plot; the latter taken twice, during the flowering stage of 2 consecutive years (2013 and 2014). *C. davidovii* was collected from Stara planina Mt in Bulgaria during flowering. *In vitro* cultures were initiated from a single seed of *C. davidovii*. Whole plantlets were regenerated and cultivated for several months on agar-solidified nutrient media differing by their composition: **MS** (basal MS medium) [3], MS media supplemented with cytokinins - **C2** (0.5 mg/L BAP), **C7** (1.4 mg/L BAP and 3.5 mg/L TDZ), and **MS liquid** (liquid MS medium), as described previously [2]. To facilitate root formation, some of the plantlets regenerated on C7 were transferred to MS (**MS/C7**). Plantlets were rooted *in vitro* and successfully adapted *ex vitro* in a phytotron under a controlled temperature, light, and humidity regime, and then consecutively acclimated to greenhouse and to open-air conditions on the experimental field plot, where flowering was observed in some individuals in the first summer (***ex vitro* flowering 1Y**) and mass flowering with multiple florets per individual in the second year (***ex vitro* flowering 2Y**).

Extraction and isolation: Dried and finely powdered plant material was successively extracted with either 80% aq. CH_3OH (for total phenolics and flavonoids) or $CHCl_3$ (for sesquiterpene lactones) in an ultrasonic bath at 25ºC. Then, the extract was dried *in vacuo* and frozen at – 20ºC prior to analysis. A portion of the $CHCl_3$ extract (20 mg) obtained from wild-growing *C. davidovii* was submitted to CC (Silica gel) using $CHCl_3$-CH_3OH, 20:1, and prep. TLC ($CHCl_3$-CH_3OH, 20:1) to give 1.5 mg of 8α-(5'-hydroxyangeloyl)-salonitenolide.

Determination of the total phenolic compounds: Total phenolics were determined spectrophotometrically by the Folin-Ciocalteu method and expressed as mg gallic acid equivalents per g dry plant material (DM) [5a].

Determination of the total flavonoids: Total flavonoids were estimated spectrophotometrically by a modified Christ-Mueller method and expressed as mg catechin equivalents per g dry plant material (DM) [5b].

HPLC determination of sesquiterpene lactones: An HPLC 1200 Series (Agilent Technologies) with DAD was used. Separations were made on a Zorbax XDB-C18 column (RRHT, 50 × 4.6 mm i.d., 1.8 μm), thermostated at 40°C using a combination of isocratic and gradient modes of elution H_2O (A)-CH_3CN (B): 0-6 min -90% A, 6-12 min – 70% A, 12-20 min – 50% A, 20-25 min - 20% A, 25-29 min – 90% A . Chromatograms were recorded at 210 nm (and used for quantification), and all spectra were stored in the range of 190-450 nm. The amount of 8α-(5'-hydroxyangeloyl)-salonitenolide (SL) was calculated from peak areas (t_R = 12.44 min) in the HPLC chromatograms with reference to cnicin (external standard, t_R = 8.94 min, y = 12916.31683x + 226.96522, R^2 = 0.99954) and expressed as mg of cnicin equivalents per g of dry plant material (mg CnE/g DM).

Acknowledgements - The authors are grateful to Assoc. Prof. Svetlana Bancheva, PhD, for supplying leaves of *C. davidovii* wild plants from one natural population.

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Natural Product Communications
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