GC-MS-BASED METABOLITE PROFILING OF WILD
AND IN VITRO GROWING PLANTS OF *SATUREJA*
MONTANA L.

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Abstract

Satureja montana L. is used as a traditional culinary spice in the Balkan Peninsula cuisine, as well as in the folk and traditional medicine for treatment of different diseases. The supply of secondary metabolites from naturally growing plants has many restrictions, such as biomass availability and variations in the chemical composition, which is most strongly affected by seasonal and environmental factors. These disadvantages could be overcome by cultivating plants in optimized and controlled in vitro conditions. The purpose of the current study was to compare the phytochemical compositions of newly developed in vitro plants and intact plants of *S. montana* used for production of secondary metabolites. The GC-MS analysis revealed the presence of a number of primary (carbohydrates, amino acids, organic acids and lipids) and secondary metabolites (phenolic acids) with pharmacological and biological activities beneficial to humans. The results revealed that in vitro *S. montana* plants are a potential biotechnological matrix for the production of valuable biologically active substances for the food, pharmaceutical and cosmetic industries.

Key words: *Satureja montana* L., winter savory, metabolite profiling, gas chromatography coupled to mass spectrometry (GC-MS)

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Introduction. *Satureja montana* L., also called winter or mountain savory, belongs to the genus *Satureja* (family Lamiaceae), which comprises 30 herbs, subshrubs, and shrubs distributed in temperate areas in the Mediterranean region and all over Europe [1,2]. *S. montana* is used as a traditional culinary spice in the Balkan Peninsula cuisine. Different extracts of the underground part of this plant are also used in folk and traditional medicine for treatment of respiratory diseases, stomach disorders, against intestinal parasites, and lymphatic system illnesses [2]. These health benefits are due to the presence of various biologically active constituents, such as thymol, p-cymene, carvacrol in the essential oil [3], triterpenes [4], and polyphenolic compounds [1,5]. Other bioactivities of the *Satureja* species, such as anti-HIV, antioxidant, antibacterial, antifungal, antidiabetic, etc., have also been reported [1,5-7].

The production of particular plant metabolites strongly depends on the species, climatic and geographical conditions, growth phase, harvest time, post-harvest factors, biotic and abiotic factors [8]. In this regard, metabolic profiling provides information about the phytochemical composition, allows the detection of chemically diverse bioactive molecules and unknown compounds, and provides functional information on metabolic phenotypes of plants [9]. One of the widely used and powerful approaches for metabolite profiling of highly complex biological matrixes such as plant extracts is gas chromatography coupled to mass spectrometry (GC-MS) [10].

The supply of secondary metabolites from naturally growing plants has many restrictions, such as biomass availability and variation in their chemical composition, which is most strongly affected by seasonal and environmental factors. These disadvantages could be overcome by cultivating the plants in optimized and controlled in vitro conditions. The advantages of plant in vitro cultures over in vivo plants, in terms of secondary metabolite production, are well known. In the laboratory, growth conditions can be controlled and, therefore reproducible yields of the target product can be obtained. Growth parameters, such as pH, temperature, medium components, and other microenvironmental factors, can be optimized to achieve metabolite production, preferably significantly higher than in in vivo plants. Separation of target compounds is also much easier due to lower complexity of the plant in vitro material. Furthermore, large-scale cultivation of in vitro plant cell, tissue and organ cultures as sources of secondary metabolites can be obtained in liquid media in bioreactors and ultimately commercialized [11]. In addition, the commercial cultivation of plant in vitro systems represents an alternative source of secondary metabolites, helping to preserve the genetic diversity of the wild populations of medicinal plants [12]. Therefore, the aim of the present study was to compare the phytochemical compositions of in vitro and the naturally growing plants of *S. montana*. It should be noted that this paper for the first time described the results of the investigation of *S. montana* in vitro culture.

Materials and methods. Plant material. Randomly selected wild growing plants of *S. montana* L. were collected during the flowering stage in August 2017 from their natural habitat in Beledie Han locality, Sofia region (geographical coordinates – 42°53'27.26"N, 23°9'26.25"E, at 740 m altitude). The botanical identification of the collected plant material was made by a binocular magnifying glass, according to the Identifier of the higher plants [13] and Flora of the Republic of Bulgaria [14] by Dr. Ina Aneva (co-author of the current study). The aerial parts of the collected samples were dried in the shade at ambient temperature and ground to powder using an electric mill.

Obtaining in vitro plants. In vitro *S. montana* plants were obtained from seeds of the wild growing population described above. Due to the small size (about 1 mm) and low survival of *S. montana* seeds, it was necessary to modify the standard procedure for seed sterilization.

An entirely new sterilization method was developed, based on the sequential implementation of the processes of surface sterilization, main sterilization and washing of the seeds in a sterile polypropylene filter holder. The sterilization procedure involved preliminary preparation of the seeds by soaking in distilled water with added 0.1% gibberellic acid (Sigma-Aldrich, Merck KGaA, Germany), at 4 °C for 3 hours. The seeds were then sucked from the aqueous solution into a sterile syringe and filtered through a sterilized polypropylene filter holder. A 25 mm polycarbonate syringe filter holder (Sartorius Stedim Biotech GmbH, Germany) was used, loaded with filter paper (20 mm, Whatman® qualitative filter paper, Grade 1) and sterilized by autoclaving at 121 °C for 30 min. After filtration, the seeds remained loaded in the filter holder. The whole filter was placed in a beaker with 70% ethanol and surface sterilization of the seeds was carried out by sucking the alcoholic solution through the syringe until the seeds were completely wet. The seeds were kept in 70% ethanol for 30 s, after that the alcohol was removed from the filter by means of a syringe. This was followed by sterilization with 4% solution of calcium hypochlorite (Sigma-Aldrich, Merck KGaA, Germany). The procedure was carried out as described, with a residence time and sterilization of the seeds of 20 min. After the hypochlorite was removed, the seeds were washed three times with distilled water, then the filter holder taken out of the syringe, disassembled and the sterile filter paper with the seeds on it was transferred using sterile precision tweezers (Duchefa Biochemie) into containers with semi-solid Murashige and Skoog (MS) medium with added 30 g.L⁻¹ sucrose and 5 g.L⁻¹ Plant agar (Duchefa Biochemie) for germination and production of sterile in vitro plants. Sterilized seeds germinated seven days after sowing and after 14 days of cultivation at 26 °C and a photoperiod of 16 hours light:8 hours dark germinated in sterile in vitro plants. Twenty percent seed germination was achieved through the sterilization procedure used.

Extraction procedure. Plant samples were extracted according to the procedure previously described [10].

GC-MS analysis. GC-MS analyses were carried out on gas chromatograph 7890A (Agilent Technology) coupled with mass detector 5975C (Agilent Technology) inert XL EI/CI MSD at 70 eV). Separation of compounds was on HP-5MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) at temperature programme: initial temperature 100 °C, hold for 2 min, increase from 100 to 180 °C at a rate of 15 °C per min, hold at 180 °C for 1 min, and increase from 180 to 300 °C at 5 °C per min, then hold at 300 °C for 10 min. The injector and detector temperatures were 250 °C, and the flow rate of the carrier gas (helium) was 1.0 mL min⁻¹. Electron-impact ionization was performed with electron energy of 70 eV. The spectra were acquired over a mass range of $m/z = 50 - 550$. The injection volume was 1 μ L with split mode (10:1 for polar compounds, 5:1 for phenolic compounds and 5:1 for nonpolar compounds). The results are presented as μ g of respective internal standard equivalent per g dry weight (DW).

Identification of the metabolites. Mass spectra were read using AMDIS software, version 2.64 (Automated Mass Spectral Deconvolution and Identification System, NIST Gaithersburg, MD, USA). The separated polar and non-polar compounds were identified by comparing their GC-MS spectra and Kovats retention index with reference compounds in The Golm Metabolome Database (<http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html>) and NIST'08 database (NIST Mass Spectral Database, PC-Version 5.0, 2008 from the National Institute of Standards and Technology, Gaithersburg, MD, USA). The RIs of compounds were recorded with a standard n-hydrocarbon calibration mixture (C₈–C₃₆, Restek, Teknokroma, Spain) using 2.64 AMDIS software.

Statistical analysis. Three independent extracts from each of the analyzed samples were prepared and each extract was analyzed by GC-MS in triplicate. The presented values are means with standard deviations ($\pm$ SD) calculated by Microsoft Office Excel[®] 2000.

Results and discussion. The GC-MS analysis of polar phase I and II and non-polar phase revealed the presence of 49 metabolites in in vivo plants and 40 metabolites in in vitro plants belonging to different classes of organic compounds: amino acids, carbohydrates, sugar alcohols, organic acids, fatty acids, and phenolic acids (Tables 1, 2, 3).

In polar fraction I, four essential amino acids (L-valine, L-Leucine, L-Isoleucine and L-phenylalanine) and six non-essential amino acids (L-serine, L-proline, L-glycine, L-aspartic acid, L-glutamic acid and L-glutamine) were identified, with L-glutamine being dominant in in vivo plants and L-glutamic acid in in vitro plants (Table 1). The in vitro and in vivo plants biosynthesized phenylalanine, which is a biosynthetic precursor of polyphenols via the phenylpropanoid pathway [11]. Five organic acids were identified, with the malic acid being predominant in both types of plants studied (Table 1). Two monosaccharides (isomers of fructose and glucose) and one disaccharide, sucrose (Table 1), were also identified in polar fraction I. Carbohydrates in the plant cell are involved in a variety of metabolic

T a b l e 1

GC-MS analysis of polar fraction I of in vivo and in vitro plants of *S. montana*

Metabolites in polar fraction I, $\mu\text{g.g}^{-1}$ DW	RI	In vivo plants	In vitro plants
Amino acids			
L-Valine	1219.8	16.8 ± 0.9	12.8 ± 0.8
L-Leucine	1280.9	14.1 ± 0.8	13.2 ± 0.7
L-Isoleucine	1300.7	29.9 ± 1.2	13.7 ± 0.8
L-Serine	1368.4	17.0 ± 0.9	16.4 ± 0.8
L-Proline	1591.3	85.7 ± 1.8	44.3 ± 1.5
L-Glycine	1575.1	n.d.*	13.4 ± 0.6
L-Aspartic acid	1525.6	12.3 ± 0.7	9.1 ± 0.5
L-Glutamic acid	1626.8	127.6 ± 2.3	151.1 ± 2.5
L-Glutamine	1596.2	138.5 ± 2.4	70.4 ± 1.6
L-Phenylalanine	1640.7	89.5 ± 1.7	34.3 ± 1.5
Organic acids			
Succinic acid	1324.5	22.5 ± 0.9	4.0 ± 0.5
Fumaric acid	1357.4	15.0 ± 0.8	14.1 ± 0.8
Malic acid	1488.0	112.7 ± 2.3	102.4 ± 2.1
γ -aminobutyric acid	1533.1	16.4 ± 0.8	13.0 ± 0.7
Pyroglutamic acid	1529.3	24.6 ± 0.9	12.4 ± 0.5
Sugar acids and sugar alcohols			
Glycerol	1292.8	18.9 ± 0.8	19.3 ± 0.8
Arabitol	1702.3	5.42 ± 0.4	9.6 ± 0.5
Glyceric acid	1352.7	26.9 ± 0.7	28.5 ± 0.7
Threonic acid	1567.1	17.1 ± 0.7	15.7 ± 0.6
myo-Inositol	2029.6	36.3 ± 0.8	35.5 ± 0.8
Carbohydrates			
Fructose isomer 1	1861.7	284.7 ± 4.8	250.3 ± 5.1
Fructose isomer 2	1868.4	417.4 ± 8.7	235.6 ± 4.3
Fructose isomer 3	1875.9	227.5 ± 4.1	144.8 ± 2.3
Glucose isomer 1	1889.3	118.8 ± 2.2	133.9 ± 2.1
Glucose isomer 2	1897.0	132.5 ± 2.0	125.8 ± 2.1
Glucose isomer 3	1908.1	240.1 ± 4.6	223.8 ± 4.5
Sucrose isomer 1	2653.6	18.8 ± 0.4	14.6 ± 0.5
Sucrose isomer 2	2761.4	156.2 ± 2.5	169.7 ± 2.6
Sucrose isomer 3	3017.2	80.5 ± 1.9	86.6 ± 1.8

n.d.* – not determined

T a b l e 2

GC-MS analysis of non-polar fraction of in vivo and in vitro plants of *S. montana*

Metabolites in non-polar fraction, $\mu\text{g}\cdot\text{g}^{-1}$ DW	RI	In vivo plants	In vitro plants
Tetradecanoic acid (Myristic acid)	1851.6	109.34 ± 2.5	95.1 ± 2.1
Hexadecanoic acid (Palmitic acid)	2046.5	68.9 ± 1.6	51.8 ± 1.5
9-Octadecenoic acid (E), (cis-oleinic acid)	2217.4	64.9 ± 1.7	60.7 ± 1.6
9-Octadecenoic acid (Z), (trans-oleinic acid)	2088.3	99.0 ± 2.3	48.6 ± 1.2
Octadecanoic acid (Stearic acid)	2243.0	131.2 ± 2.4	88.2 ± 1.9
9,12-Octadecadienoic acid, (Z,Z), (Linoleic acid)	2299.5	142.1 ± 2.7	72.5 ± 1.8
Arachidonic acid	2239.1	42.1 ± 1.1	30.9 ± 0.8
11-Eicosenoic acid, (Z), (gondoic acid)	2282.7	73.1 ± 1.8	n.d.*
13-Eicosenoic acid, (Z), (paulinic acid)	2311.5	69.1 ± 1.7	n.d.
1-Monopalmitylglycerol	2603.2	90.2 ± 2.0	n.d.
1-Monostearyl glycerol	2781.9	53.8 ± 1.3	n.d.

n.d.* – not determined

T a b l e 3

GC-MS analysis of polar fraction II of in vivo and in vitro plants of *S. montana*

Metabolites in polar fraction II, $\mu\text{g}\cdot\text{g}^{-1}$ DW	RI	In vivo plants	In vitro plants
2,3-dihydroxyphenylacetic acid	1844.1	55.1 ± 1.2	traces
3,4-dihydroxyphenylacetic acid	1869.4	40.8 ± 0.9	traces
Vanillic acid	1779.6	21.4 ± 0.6	n.d.*
Gentisic acid	1798.2	26.0 ± 0.7	25.27 ± 0.7
Caffeic acid	2153.5	31.1 ± 0.7	31.66 ± 0.7
Syringic acid	1821.5	23.8 ± 0.6	n.d.
Protocatechuic acid	1827.4	29.6 ± 0.7	n.d.
<i>p</i> -Coumaric acid	1947.3	20.0 ± 0.5	n.d.
Ferulic acid	2101.7	35.2 ± 0.6	n.d.

n.d.* – not determined

pathways, energy sources, and are also precursors for the biosynthesis of primary and secondary metabolites, such as organic acids, amino acids, phenolic acids, and aromatic components [20]. In addition to carbohydrates, the analyzed in vivo and in vitro *S. montana* plants also accumulated sugar acids and alcohols (Table 1).

Three saturated fatty acids, seven unsaturated fatty acids, and two esters of fatty acids with glycerol were identified in the non-polar fraction (Table 2). Linoleic acid was in the highest concentration in in vivo plants ($142.1 \mu\text{g}\cdot\text{g}^{-1}$ DW), while myristic acid ($95.1 \mu\text{g}\cdot\text{g}^{-1}$ DW) was the major fatty acid in in vitro plants. It should be noted that in vitro *S. montana* plants did not accumulate 11-

eicosanoic acid (Z), 13-eicosanoic acid (Z), paulinic acid, 1-monopalmitylglycerol, and 1-monostearyl glycerol.

GC-MS analysis of polar fraction II showed the presence of nine phenolic acids accumulated by the in vivo plants, with 2,3-dihydroxyphenylacetic and 3,4-dihydroxyphenylacetic acids predominating (Table 3). In vitro plants accumulated four phenolic acids, of which caffeic acid was the principal one ($31.1 \mu\text{g}\cdot\text{g}^{-1}$ DW) (Table 3).

This phenolic acid is a precursor for the biosynthesis for rosmarinic acid, a typical phenolic acid for the plants of the *Lamiaceae* family [17]. The concentration of rosmarinic acid in the analyzed plants was previously determined by the HPLC method [18] because the available gas chromatograph with mass spectrometer (GC-MS) has a scanning range of 50 to 550 m/z, and derivatized rosmarinic acid (5-trimethylsilylrosmarinic acid) is with m/z 720 [19]. In addition, as described in Materials and methods section, rosmarinic acid was degraded to its constituent metabolites, due to the consistent alkaline and acid hydrolysis to release ester- and glycoside bound aglycones. It should be mentioned that according to the HPLC analysis provided the newly developed in vitro plants are overproducers of rosmarinic acid ($22.14 \pm 0.18 \text{ mg}\cdot\text{g}^{-1}$ DW) that was almost 5 times higher than the quantity in in vivo plant [18].

Phenolic acids are the most abundant plant secondary metabolites with important physiological functions for plants and valuable biological activities for humans, such as antioxidant, anti-inflammatory, antimicrobial, anti-allergic, hepatoprotective, anticarcinogenic, antithrombotic, etc. [20].

Conclusion. According to the available scientific literature, this is the first detailed report describing the metabolite profiles of in vivo and in vitro plants of *S. montana*. The GC-MS analysis revealed the presence of a number of primary (carbohydrates, amino acids, organic acids and lipids) and secondary metabolites (phenolic acids). The identified primary metabolites are initial compounds for the biosynthesis of different plant secondary metabolites, such as polyphenols and flavour compounds with valuable pharmacological and biological activities for humans. The obtained line of in vitro *S. montana* plants is a potential biotechnological matrix for the production of valuable biologically active substances for the food, pharmaceutical and cosmetic industries.

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