Research Article

ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., Suppl. 11, 2018: 91-94

GC/MS based metabolite profiling of five populations of *Glaucium flavum* (Ranunculales: Papaveraceae) from the Black Sea coast of Bulgaria

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Abstract: Metabolite profiles of methanolic extracts of leaf samples of five Bulgarian *Glaucium flavum* Crantz. (Papaveraceae) populations were analyzed by gas chromatography-mass spectrometry (GC/MS). Compounds belonging to alkaloids, fatty and phenolic acids, fatty alcohols, carbohydrates, alkanes and others were identified. In the alkaloid fraction, glaucine was the main alkaloid with the exception of the samples from Shkorpilovtsi where the isocorydine was the most abundant one. In the alkaloid profile of the samples from Sinemorets salutaridine has been identified in relatively high quality. Octadecane (alkane), hexadecanol (fatty alcohol), fatty acids: hexadecanoic acid (C16:0) and octadecatrienoic acid (C18:3) were the predominant components of the lipid fraction. In the polar fraction, the major chromatographic peaks were determined as sucrose, fructose and myo-inositol. Ferulic and *p*-hydroxycinnamic acids were the main components of the phenolic fraction. Based on the alkaloid composition of the leaves of the studied populations, three profile types were observed. No significant differences were found regarding the other metabolites.

Key words: Yellow horned poppy, alkaloids, carbohydrates, fatty and phenolic acids

Introduction

Yellow horned poppy (*Glaucium flavum* Crantz., Papaveraceae) is a medicinal plant which curative effects are due to the alkaloid glaucine. Its populations in Bulgaria are scarce, mainly along the Black Sea coast and in the Tundzha Hilly Plane and the Danube Plain. The aboveground part of the plant (Herba Glaucii flavi) is used in the pharmaceutics for production of several medicines mainly as a remedy of pulmonary diseases. The main alkaloid of *G. flavum* is glaucine, and several related alkaloids as dehydroglaucine, isocorydine, cataline, protopine, isoboldine, corunin, chelidonine, sanguinarine, corydine, α-aldriptopine were also reported (Israilov et al. 1979, Daskalova et al. 1988, Kuzmanov et al. 1992). Based on the qualitative and quantitative alkaloid composition, different chemotypes of the species have been found in Iran, Israel, Sardinia (Lalezari et al. 1976, Shafiee et al. 1977, Peled et al. 1988, Petitto et al. 2010). In Bulgaria, three chemotypes were reported regarding the main alkaloid and the types of the other alkaloids (Doncheva et al. 2016).

Extensive metabolic profiling of root of *G. flavum* was done by using different analytical methods: *nuclear magnetic resonance* (*NMR*), *high-performance-liquid chromatography* with *ultraviolet detector* (HPLC-UV), liquid chromatography-mass spectrometry (LC-MS), ultrahigh-performance liquid chromatography- fourier-transform mass spectrometry (UPLC-FTMS). Over 100 lipid derivatives, sugar, amino and organic acids, phenolic acids (gallic, caffeic cinnnamic, syringic, ferulic and sinapic acids), flavonoids (rutin, myricetin, quercetin, luteolin, naringenin, kaempferol, isorhamnetin,

catechin) have been identified (Hagel et al. 2015). Data for metabolite in the aboveground part of the plant are insufficient; furthermore, Bulgarian populations of *G. flavum* have been studied mainly for alkaloid content (Daskalova et al. 1988, Kuzmanov et al. 1992, Doncheva et al. 2016, Doycheva et al. 2017).

This study presents comparative analysis of five *G. flavum* localities along the Black Sea shores, concerning the metabolite profiles of the leaves determined by gas chromatography-mass spectrometry (GC/MS), as a part of a larger research on the Bulgarian populations of the species.

Material and Methods

Plant material

Five *G. flavum* populations along the Bulgarian Black Sea shore were chosen, consisting of ten or more individuals, as most convenient for the analyses, near the villages of Shabla (N 43.54282, E 28.60612), Shkorpilovtsi (N 42.95834, E 27.89775), Pomorie (N 42.58634, E 27.63191), Varvara (N 42.13460, E27.89718) and Sinemorets (N 42.06564, E 27.97379). Samples were gathered at random in July 2015, during the flowering stage, one leaf per plant, ten samples per locality, and were dried at room temperature.

Methanolic extracts

Methanolic extract was obtained in the following way: 100 mg of plant material with internal standards of 50 μg of nonadecanoic acid, 50 μg of ribitol and 50 μg of 3,4 dichloro-4-hidroxy benzoic acid were placed in 2 mL Ependorf tube and extracted with 1 mL of MeOH for 2 h at room temperature. Aliquot of 800 μL was transferred into another Ependorf tube, to which 500 μL H_2O and 500 μL of $CHCl_3$ were added, and after 2 min vortexing the mixture was centrifuged. The chloroform fraction was separated, evaporated and transmethylated with 2% of H_2SO_4 in MeOH at 60ºC for 18 h. Then lipids were extracted with *n*-hexane (2x500 μL) dried with anhydrous Na_2SO_4 and were evaporated to obtain a *lipid fraction*. An aliquot of 100 μL from the aqueous fraction was placed in a glass vial and evaporated to obtain a *polar fraction*. The rest of aqueous fraction was hydrolysed with 0.5 mL of 1N NaOH for 18 h at 60ºC. After acidification to pH 1-2 with conc. HCl, the phenolic compounds were extracted with EtOAc (2x500 μL) which was dried with anhydrous Na_2SO_4 and evaporated to obtain a *phenolic fraction*. Lipid, polar and phenolic fractions were silyated with 50 μL of N,O-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) in 50 μL of pyridine for 2 h at 50°C.

Alkaloid fraction – 50 mg of dried plant material was macerated in a screw-top Ependorf tube (1.5 mL of volume) with 1 mL of methanol and 50 μg of codeine as an internal standard (IS). Two-hour extraction was conducted at room temperature with an acceleration by ultrasonic bath for 15 min, which was repeated every 30 min. After that the samples were centrifuged at 10,000 rpm for 1 min. Aliquot of 500 μL was transferred into another Eppendorf tube and 500 μL of 2% sulfuric acid in distilled water was added. The neutral compounds were eliminated by duplicate extraction (vortexing) with 500 μL chloroform. The mixtures were basified with 200 μL 25% ammonia and the alkaloids were extracted in triplicate with 500 μL chloroform. The organic solvent was evaporated and the dry extract was dissolved in 300 μL chloroform for further GC–MS analysis without derivatization.

GC–MS analysis

The GC–MS spectra were recorded on a Termo Scientific Focus gas chromatograph coupled with Termo Scientific dual stage quadrupole (DSQ) mass detector operating in electron ionization (EI) mode at 70 eV. ADB-5MS column (30 m x 0·25 mm x 0·25 μm) was used. The temperature program was: 100-180°C at 15°C x min^{-1}, 180-300 20 at 5°C x min^{-1} and 10 min hold at 300°C. The injector temperature was 250°C. The flow rate of carrier gas (Helium) was 0·8 mL x min^{-1}. The split ratio was 1:10; 1 μL of the solution was injected. The metabolites were identified as TMSi derivatives comparing their mass spectra and Kovats Indexes (RI) with those of an on-line available plant specific database. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. RI of the compounds were recorded with standard n-hydrocarbon calibration mixture (C9-C36) (Restek, Cat no. 31614, supplied by Teknokroma, Spain) using AMDIS 3.6 software.

Results

Metabolite profiles of methanolic extracts of leaf individual samples from the five studied populations of *G. flavum* were determined by GC/MS. Fatty acids (saturated and unsaturated: linolenic and linoleic acids), phenolic and organic acids, carbohydrates, fatty alcohols and alkaloids were identified (Table 1). In the alkaloid fractions, glaucine was the main alkaloid with exception of the samples from Shkorpilovtsi, where isocorydine was the most abundant one. In the

Table 1. Metabolites identified in *Glaucim flavum* leaf samples from five natural Bulgarian populations.
Legend: Gf1 – Shabla village population; Gf2 – Shkorpilovtsi village population; Gf3 – Pomorie village population; Gf4 – Sinemorets village population; Gf5 – Varvara village population. The response ratios were calculated for each analyte relative to the internal standards using the calculated areas for both components.

Compounds	**RI**	**Gf1**	**Gf2**	**Gf3**	**Gf4**	**Gf5**
Alkaloid fraction						
Salutaridine	2656	3,1		6,88	87,3	8,7
Isocorydine	2845	3,4	987,8	3,99	12,7	6,5
Glaucine	2908	609,1	134,0	293,2	211,6	692,9
Protopine	3006	7,9	80,7	27,9	14,3	22,4
Lipid fraction						
Alkanes						
Hexadecane	1600	15,5	53,7	32,8	26,9	19,5
Octadecane	1800	18,5	60,2	49,6	33,0	25,8
Nonadecane	1900	1,5				2,5
Icosane	2000	8,9	24,9	22,7	14,4	12,9
Docosane	2200	4,6	10,2	9,3		6,2
Fatty alcohols						
Tetradecanol	1768	14,1	24,6	28,8	28,8	20,0
Hexadecanol	1965	33,0	61,8	47,0	61,1	61,0
Octadec-9Z-enol	2143		8,2		8,6	8,3
Octadecanol	2163	13,8	40,0	21,6	39,4	
Fatty acids						
Octanoic acid (C8:0)	1575	15,3	19,8	23,5	28,1	22,6
Dodecanoic acid (C12:0)	1534	5,3				4,4
Tetradecanoic acid (C14:0)	1732	8,2	5,6	7,0	8,1	
Hexadecanoic acid (C16:0)	1933	106,0	127,1	216,7	152,0	131,3
Octadecadienoic acid (C18:2)	2100	25,8	15,9	43,1	26,5	40,5
Octadecatrienoic acid (C18:3)	2107	77,6	49,4	91,6	125,5	139,3
2-Hydroxyhexadecanoic acid	2128	8,0	9,3		9,2	9,1
Octadecanoic acid (C18:0)	2135	35,5	52,2	64,8	40,0	42,2
Phenolic fraction						
Protocatechuic acid	1813	8,2			1,8	
Quinic acid	1849	2,8	6,2	7,3	5,4	5,7
trans p-Hydroxycinnamic acid	1934	55,2	44,8	4,8	64,6	2,6
trans Ferulic acid	2086	30,4	45,0		21,6	
Polar fraction						
Glycerol	1262	10,6	36,4	13,6	24,2	5,3
Organic acids						
Succinic acid	1302	17,3	24,5	20,0	10,5	17,1
Glyceric acid	1315				12,4	
Malic acid	1471	12,1	35,8	28,9	38,0	
Pyroglutamic acid	1515	34,9	12,6	49,4	31,9	59,6
Carbohydrates						
Fructose 1	1876	15,1	13,5	103,1	21,7	24,8
Fructose 2	1964	25,9	29,4	102,5	24,3	37,3
Sucrose	2634	16,7	29,7	24,3	73,4	25,3
Polyols						
Myo-Inositol	2084	87,7	170,7	57,2	120,1	56,8

alkaloid profiles of the samples from Sinemorets the alkaloid salutaridine was identified in relatively high quantity. Octadecane (alkane), hexadecanol (fatty alcohol), fatty acids: hexadecanoic acid (palmitic acid, C16:0) and octadecatrienoic acid (linolenic acid, C18:3) were the predominant components of the lipid fraction. In the polar fraction, the main chromatographic peaks were identified as myo-inositol, sucrose, fructose and pyroglutamic acid. Ferulic and *p*-hydroxycinnamic acids were the main components of the phenolic fraction.

Discussion

The present study reports data on the metabolite composition of leaf samples collected during the flowering stage from five Bulgarian populations of *Glaucium flavum*. No significant differences were found between studied populations regarding detected metabolites with exception to the alkaloid composition. Three types of alkaloid profiles were determined. The first of them, which is most widespread type, contains glaucine as its main alkaloid. In the alkaloid pattern of the second type, isocorydine is the main alkaloid, while the third type contains morphinane alkaloid salutaridine in a relative high quantity. The obtained data are in agreement with the reports by Doncheva et al. (2016) and Doycheva et al. (2017). The results of the present study which point that the population of Sinemorets accumulate in large amount the alkaloid salutaridine are complementary to those of Doncheva et al. (2016). The alkaloid protopine was found in all studied leaf samples. Bournine et al. (2013) reported that this alkaloid was predominant in the roots of the plant and was related to their anticancer properties.

The obtained results will be completed with further studies on the genetic diversity between the five concerned *G. flavum* populations and with details on the environmental conditions in their habitats, which will allow the optimal conservation of this valuable medicinal plant.

Acknowledgements: This research was supported by the Bulgarian National Science Fund, Bulgarian Ministry of Education and Science (project DFNI-B02/18).

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