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Sesquiterpene Patterns of the Leaves and Roots in Local Populations of Medicinal Plant Petasites hybridus (L.) from Bulgaria

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Abstract. Biosynthesis of biologically active substances, products of secondary plant metabolism is highly sensitive to various geographic, ecological and phenological factors. Clarification of these relationships is essential to define their impact on the efficacy and therapeutic potential of phytochemical preparations of medicinal plants. The aim of this study was to investigate the variation in the pattern of the main biologically active compounds of *Petasites hybridus* (L.) (Butterbur), classified as sesquiterpene esters of petasin and iso-petasin, in natural population from different habitats in Bulgaria. The results from TLC analysis confirmed the petasin chemo-type of Butterbur plants from the investigated areas. HPLC analysis revealed qualitative and quantitative differences of the six main sesquiterpene esters found in leaf and subterranean parts extracts. The investigated Bulgarian populations of *Petasites hybridus* have a relatively high content of petasin in the roots and are relatively poor in petasin content in the leaves. The present results indicate modulations in the profile and accumulation of secondary metabolites in *Petasites hybridus*, reflecting adaptive responses to specific environmental conditions in their natural habitats.

Key words: *Petasites hybridus*, methanol extracts, HPLC, sesquiterpene esters, TLC.

Introduction

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant (Briskin, 2000). Plant secondary metabolites are a group of naturally occurring compounds known to play a major role in the adaptation of plants to their biotic and abiotic environment (Sun et al., 2007). Although medicinal plants are the subject of intensive research to identify specific secondary metabolites and to characterize the potential sites and modes of action of biologically active substances (BAS), many of the pathways for their biosynthesis and regulatory mechanisms remain unclear. It is well known that changes in the biosynthetic profile and the content of BAS are determined by the species, genotype, physiology, developmental stage and environmental factors (Kutchan, 2001; Gobbo-Neto & Lopes, 2007; Isah, 2019). Much information has been accumulated regarding the effects of biotic stress (e.g., pathogenic attacks or herbivore attacks) on the synthesis of secondary metabolites (Wink, 2010), but relevant information on how abiotic stress alters secondary metabolism is limited. Abiotic factors that determine plant habitats and inhibit the growth, photosynthesis and other processes of primary metabolism in stressful conditions, such as drought, salinization, high light intensity, UV radiation etc. can affect the profile, accumulation and antioxidant potential of secondary metabolites (Gobbo-Neto & Lopes, 2007; Fine et al., 2006).

The genus *Petasites* Mill (Asteraceae) is widely vegetated in Europe, Northwest Asia and North America and has a long history of use in alternative medicine. Taxonomic survey of *Petasites* reveals the existence of 18 species of this genus. Representatives of genus *Petasites* are shown to be potential sources of high levels of bioactive substances with very promising aspects of therapeutic utility (Aydın et al., 2012). In Bulgaria, three *Petasites* taxa can be naturally found: *P. hybridus* (L.) P. Gaertn., B. Mey. & Scherb (*P. officinalis* Moench), *P. albus* (L.) Gaertn. and *P. kablikianus* Tausch ex Bercht. The subject of the present study is *P. hybridus* (common Butterbur), the most common species and the main medicinal plant in *Petasites* genus, used in European phytotherapy. The plant grows most plentifully in humid thickets, marshy meadows, along the banks of streams, ravines and rivers, preferably in partial shade. The main active substances of *P. hybridus* include sesquiterpene esters (Debrunner et al., 1995). Plants also contains low amounts of pyrrolizidine alkaloids, mainly senecionine and intergerrimine, with hepatotoxic and carcinogenic effects, the amount of which does not depend on the content of sesquiterpenes (Wildi et al., 1998). The refined reparations of the extracts of *P. hybridus* root, free of pyrrolizidine alkaloids are finding an increasingly widespread pharmacological application for the prophylaxis and treatment of migraine, bronchial asthma and allergic rhinitis - diseases affecting a large number of people (Lipton et al., 2004). Extracts are also successfully used for the prevention of gastric ulcer, urinary tract irritation and respiratory problems (Ziolo & Samochowiec, 1998).

There are no systematic studies of the effects of ecological and phenological factors on the metabolic profile of the biologically active components of the Bulgarian populations of *Petasites hybridus*. This study aimed to investigate the variation in the pattern of the pharmacologically most relevant biologically active compounds of *Petasites hybridus* in natural population from some local habitats in Bulgaria, differing in altitude and specific climate conditions. Clarification of these relationships is essential to define their impact on the efficacy and therapeutic potential of phytochemical preparations of medicinal plants.

Material and Methods

Study area. Four natural locations of *Petasites*, in florogeographical regions, differing in altitude and soil-climatic conditions were selected (Table 1). Sampling was made in the middle of May 2019 after the flowering period and when water supply was non-limiting and temperature was moderately high. The environmental conditions during the measurement period were characterized by average minimum and maximum temperatures of 5 and 17 °C for Devil's bridge Kokaliane region and 6.2 and 17 °C for Rila Monastery Nature Park, respectively. For the plants growing in Sofia University Botanical Garden, St.St. Constantine and Helena, the corresponding temperature values in May was 15.0 and 23.0 °C and for Gorno Sahrane plants population 16 and 25 °C, respectively. There was non-limiting water supply (75.7 - 78 mm average monthly amount of precipitation) for all the investigated areas. The analysis of plant water status of investigated samples determined by measuring the hydration of leaves (gH_2O/gdw) show values of about

5.75 – 6.5. Similar values were obtained in preliminary studies in two-year period (2015-2016) (Yordanova et al., 2017). Averaged samples of leaves and subterranean plant parts (rhizomes, roots and runners) of 3 - 4 plants were collected.

Plant extraction and Quantitation. Dried and finely powdered leaves and subterranean parts were exhaustively extracted by ultrasonication with methanol at room temperature, according to Debrunner & Neuenschwander (1994). The extracts were concentrated under vacuum using a rotary evaporator and stored at -20 °C for later use. For the determination of the sesquiterpene type of *Petasites* plants we used a qualitative TLC method. 6 μL of the subterranean parts of the plant extracts were applied on a silica gel 60 F 254 HPLC plates (Merck, Germany) and the chromatogram was developed with toluene/ethyl acetate (93:7, v/v). Detection was performed with Vanillin/H_2SO_4 reagent and heating at 120 °C for 10 minutes. The spots containing separated petasines were visualized in UV light at 365 nm.

Chromatographic conditions. The HPLC analysis was performed on system Waters Alliance 2695 with 4 channel degasser, quaternary pump, autosampler with 100 μL loop, column thermostat and UV-VIS detector Waters 2998 PDA. The analysis was performed on column Venusil XBP C 18 (250 mm × 4.6 mm × 5 μm) (Agela Tech, USA) with particle characteristics: particle size 5 μm, pore size 150 Å and surface area 200 m^2/g. The isocratic mode was used with methanol, acetonitrile and water (32:31:37, v/v/v), as described by Wildi et al. (1998) at 30 °C temperature of column. The eluent flow rate was 1.0 mL/min in isocratic mode. A content of the six main sesquiterpene esters was quantified against an external petasin standard, measuring the peak area of the sesquiterpene esters at 235 nm. Software for system control, data acquisition and data processing Waters Empower 3 v7.20 00 00 was used.

Results and Discussion

Among many secondary metabolites of *Petasites* such as polyphenol acids, flavonoids, tannins and small concentrations of pyrrolizidine alkaloids, the pharmacological effects are mainly attributed to eremophilane (petasin) type sesquiterpenes (Debrunner & Neuenschwander, 1994; Wildi et al., 1998). A second chemotype which cannot be distinguished by morphological characteristics, furanopetasine type (with furanoeremofilanes) is also known (Novotný et al., 1966) and is considered as non-suitable for pharmaceutical purposes (Chizzola et al., 2006). The determination of the sesquiterpene type of the plants from the investigated areas was done by thin layer chromatography. The methanolic extracts from the subterranean plant parts show mostly blue and green-blue fluorescent zones from the start up to the solvent front, due to more than 20 sesquiterpenes (Wagner & Bladt, 1996). The sesquiterpenes petasin and iso-petasin are found as blue UV absorbing spots in the Rf range from 0.4 to 0.45 (Tzoneva et al., 2021-in press). Due to the limited number of sampling plants the existence of furanopetasine type plants in the investigated local populations can not be ruled out.

The main active substances of *P. hybridus* are esters of 3-isomeric sesquiterpene alcohols (petasole, iso-petasole and neopetasole) and include six main sesquiterpene esters: petasin and its isomers (i.e., iso-petasin and neopetasin) and s-petasin and its isomers, iso-s-petasin and neo-s-petasin (Debrunner & Neuenschwander, 1994). Among them, petasin has the highest antispasmodic activity (Debrunner & Meier, 1998). Differences in sesquiterpene profiles and the average content of petasin in different populations of the species have been established (Debrunner et al., 1995; Debrunner & Neuenschwander, 1994; Wildi et al., 1998) in dependence of geographical origin, seasons, altitude, diurnal cycles, the time of collection, parts of the plant, etc. (Ozarowski et al. 2013).

The comparative HPLC analysis of the methanol extracts from the subterranean parts of *P. hybridus* (Figure 1) show the presence of all six main sesquiterpene components, with petasin being dominating compound at all the investigated habitats. Gorno Sahrane is the richest population showing petasin values of 12.2 mg/g dried weight and relatively higher values of neo-petasin (2.58 mg/g) and iso-petasin (1.39 mg/g) but relatively lover contents of s-petasin and its isomers. The amount of iso-s-petasin is lower in all studied populations while the content of the other two components shows an upward increase in the order of neo-s-petasin, s-petasin, except for the population growing in Gorno Sahrane.

Table 1. Geographical coordinates of the studied areas.

Sampling sites	Locality	Altitude	Climate	Coordinates
Sofia University Botanical Garden, St.St. Constantine and Helena	Varna Municipality	58 m	Black Sea climate	N 43◦14″9′ E 28◦0″19′
Gorno Sahrane	StaraZogora	385 m	Transitional continental	N 42◦38″38′ E 25◦12″36′
Devil's Bridge, Kokalyane Region	Sofia Capital Municipality	667 m	Moderate continental	N 42◦33″37′ E 23◦25″18′
Rila Monastery, Nature Park	Rila Municipality	1710 m	Mountain	N 42◦8″27′ E 23◦21″6′

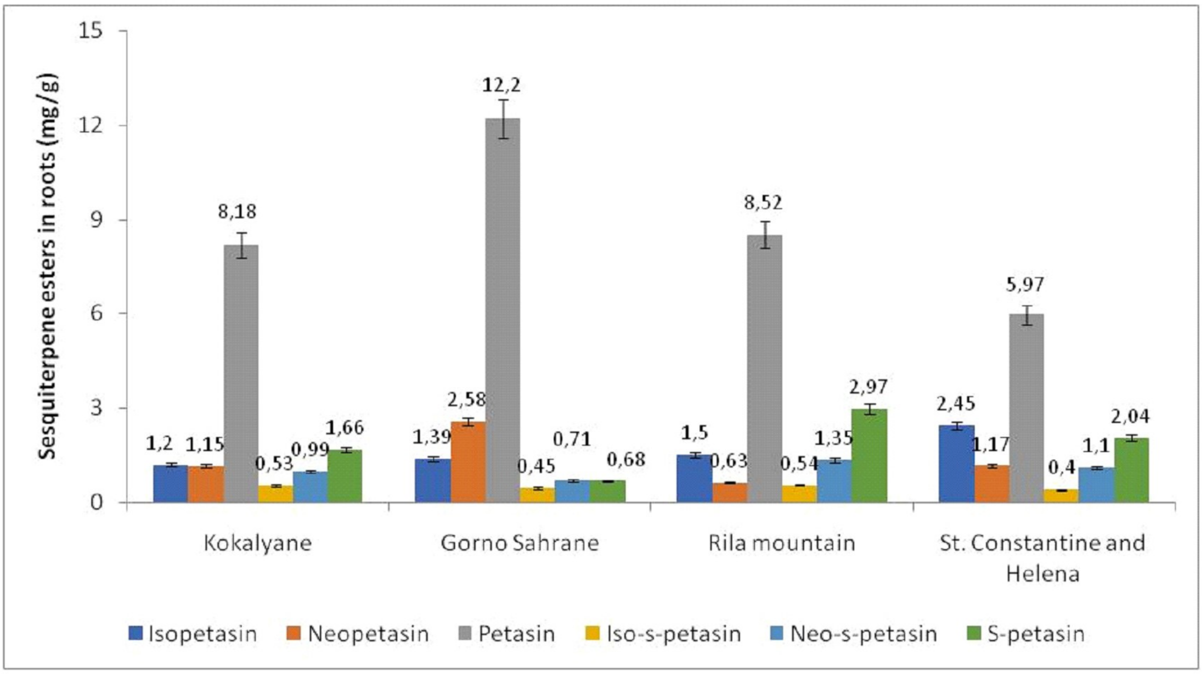

Fig. 1. Contents of the six main sesquiterpene esters (mg/g) found in the extracts from the subterranean parts of *P. hybridus* at different habitats by HPLC analysis.

HPLC-chromatograms of the leaf extracts from the plants growing in Gorno Sahrane and Kokaliane are comparable with that of subterranean parts in regard to sesquiterpene distribution but the content of petasin was considerably lover (Fig. 2). The contents of the other esters vary only slightly. The leaves of the plants from Black Sea area are very poor in petasines while the plants from highland population show unusual sesquiterpene pattern with very high contents of s-petasin. petasin was considerably lover (Figure 2). The contents of the other esters vary only slightly. The leaves of the plants from Black Sea area are very poor in petasines while the plants from highland population show unusual sesquiterpene pattern with very high contents of s-petasin.

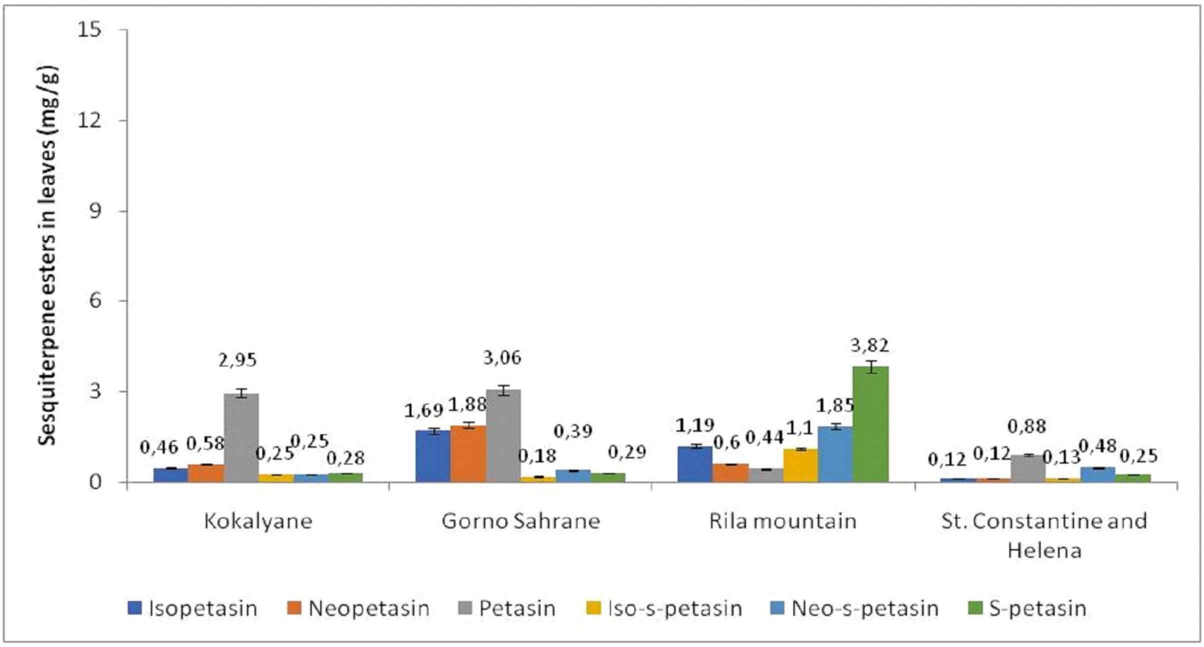

Fig. 2. Contents of the six main sesquiterpene esters in (mg/g) found in the extracts from the leaves of *P. hybridus* at different habitats by HPLC analysis.

Conclusions

The production of secondary metabolites by plants growing in natural populations is conditioned by environmental factors. Properties of photosynthetic apparatus may contribute to a great extent to plant habitat separation and adaptation to environmental factors. Our previous research (Yordanova et al., 2017), has shown a close relation of photosynthetic performance of *P. hybridus* plants to the specific climatic conditions in some natural habitats in Bulgaria, differing in altitude thus suggesting some changes in the accumulation and sesquiterpene profiles of this important Asteraceae medicinal species. We therefore continued our research by studying the peculiarities in the sesquiterpene patterns and contents in *Petasites* plants from the populations at the same florogeographical regions. Samples of leaves and subterranean plant parts (rhizomes, roots and runners) were collected on May, taking into account the data from the comprehensive research of Debrunner et al. (1995) over a complete vegetation period of *Petasites* showing the highest amounts of the six most important sesquiterpene esters in spring time. Our comparative studies have shown that the total amount of six main sesquiterpene esters in the subterranean parts of *P. hybridus* plants at different habitats varies from 13.22 mg/g to 17.33 mg/g, while in the leaves the differences are much more significant and the values vary from 1.98 mg/g to 9 mg/g. Given the data on the average content of petasin in subterranean parts of *P. hybridus* ranging from of 7.4 to 15.3 mg/g and from 3.3 to 11.4 mg/g in the leaves (Aydın et al., 2013) it could be concluded that the investigated Bulgarian populations of *Petasites hybridus* have a relatively high content of petasin in the roots and are relatively poor in petasin content in the leaves. The present results indicate modulations in the profile and accumulation of secondary metabolites in *Petasites hybridus*, reflecting specific adaptive responses to fluctuations in environmental factors in their natural habitats. Under field condition these responses can be synergistically or antagonistically modified by the superimpositions of different environmental constrains.

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