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Sideritis romana L. subsp. purpurea (Tal. ex Benth.) Heywood, a new chemotype from Montenegro

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ABSTRACT
A study on essential oil fractions of the Western Balkan endemic Sideritis romana L. subsp. purpurea (Tal. ex Benth.) Heywood collected in Montenegro is reported. The 24-h systematic steam distillation extraction procedure was performed. The gas chromatographic/mass spectrometric (GC/MS) analysis of the fractions showed y-elemene and spathulenol as two main constituents, revealing a new chemotype of this plant species. Although varying in the content of these two main compounds, which makes the fractions quite different between each other, evaluation of the anti-Candida activity showed the lack of any significant efficacy.

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1. Introduction

The genus Sideritis comprises around 150 species distributed in north temperate regions, occurring mainly in the Mediterranean area. The genus name is derived from the Greek word sideros (iron) in reference to the property of these plants to heal wounds caused by iron...
weapons (Koutsaviti et al. 2013; Venditti et al. 2016). The results of various investigations of plants belonging to this genus have revealed plant-derived compounds of particular pharmacological and nutritional interest (Tadić et al. 2012a).

*S. romana* L. subsp. *purpurea* (Tal. ex Benth.) Heywood (SP) (Figure S1 in Supplementary Material) is an annual Lamiaceae species that grows up to 30 cm, with eglandular stems, oblong-ovate, dentate or crenate-dentate leaves, and purple (or rarely white) flowers (Tutin et al. 1968). It is an endemic of the Western Balkan where inhabits dry, rocky places or abandoned meadows and arid grasslands, but can also be found on the edges of irrigation ditches or alleys, usually in the lower altitudes. To the best of our knowledge, there is no particular report on any traditional use of this subspecies. However, different biological activities of other *Sideritis* species have been investigated by numerous authors (Aboutabl et al. 2002; Basile et al. 2006; Charami et al. 2008; Güvenç et al. 2010; Demirtas et al. 2011; Tadić et al. 2012a, 2012b). These plants have been traditionally used as teas for feeding, as flavouring agents and in folk medicine in the Mediterranean and Balkan regions as anti-inflammatory, antilulcerative, antimicrobial, anticonvulsant, antispasmodic, antioxidant, vulnerary, algiesic and carminative agents (González-Burgos et al. 2011).

A study on essential oil (EO) chemical composition and antimicrobial activity of SP has been reported just recently (Tadić et al. 2017). In line with that study, a new approach based on 24-h steam distillation extraction procedure has been applied on SP collected in Montenegro (Figure S2 and Section S1 in Supplementary Material) (Božović et al. 2017b). Chemical analysis of obtained EO fractions has revealed a new chemotype. The results, followed by the ones concerning related antifungal activity, are presented herein.

2. Results and discussion

2.1. EO extraction

Dried aerial parts of SP were subjected to fractionated steam distillation process and the EO was collected at different intervals (1, 2, 3, 6, 12 and 24 h). The oil yield rapidly decreased after the first hour of the extraction process, increasing in the last 21 h, with the same last fraction yield as of the first one showing a U-shaped sketch (Figure S3 in Supplementary material). This is an unusual profile, as normally majority of EO is reported to be extracted in the first 3 or 4 h. The overall EO amount increased from 0.1 to almost 0.4 grams per kg of plant subjected to steam distillation indicating a low EO content in this species. Interestingly, the higher amount of EOs were obtained in the first and sixth fractions, accounting for about 28 and 29% of extracted EO (Table S1 in Supplementary Material).

2.2. EO chemical analysis

The GC/MS analysis of six EO fractions showed the presence of 23 different constituents with varying concentrations in different fractions (Table S2 in Supplementary Material). In general, the most abundant compounds were sesquiterpenes γ-elemene (13) and spathulenol (20). In all cases, these two compounds never disappeared, being present in smaller but significant amounts in the last two or three fractions. Based on the extraction time, their percentages reached the maximum during the first 3 h of the extraction process but diminished during the last three phases. γ-elemene is one of four structural isomers that contribute to the floral
aromas of some plants and are used as pheromones by some insects (Sections 2 and 3 in Supplementary material). It reached the maximum of 25.2% in the second fraction, with no significant difference in the first one (22.2%). Spathulanol, an azulenic sesquiterpene alcohol (Sections 2 and 3 in Supplementary material), reached its maximum in the second and the third fraction (27.7% and 26.7%, respectively).

Appearance of other constituents is related to the extraction time, but some of them are always present in significant amounts. For instance, pulegone (11) was present in each fraction and gradually increased its amount with the process duration. It reached the maximum in the last two fractions (15.7 and 16.1%, respectively) and that amount was even three times higher than in the first hour. Cis-sabinol (16) showed the same quantity evolution. On the contrary, elixene (3), isoledone (12) and apiol (23) decreased its amount during the process of extraction, while myristicin (22) was present in every fraction with the relatively same amount.

Regarding other chemical components, some are present only in particular fractions, such as δ-cadinol (7) in the first one (6.5%). Some others appeared with the extraction progress: e.g. thymol (21) from the second and verb-none (14) from the third fraction, slightly increasing in its amount, while p-cymen-8-ol (16) is characteristic only of the last three extraction phases (6–24 h) with more or less the same amount. In general, the complexity of SPEO chemical composition was more or less the same in the first three h (14–17 compounds), layer becoming higher with 21–22 different constituents. Taking into account the increasing amount of some ingredients, or even later appearance of others, as well as the significant decrease in the amount of γ-elemene and spathulanol with the process progress, it is obvious that EO composition of SP changes greatly with the extraction time. Thus, the 12 and 24 h fractions remarkably differ in their chemical content.

In Table S2, RIs values were inserted as a confirmation of the peak assignment. Generally, there must be correspondence between the experimentally calculated values and those reported in literature. For some compounds, i.e. 4, 14, 19, 21, 22, 23, the difference between calculated and literature RI values could be argued. Nevertheless, in the present work we used different temperature programmes and column length than those reported that could account for the non-ideal RIIs match. Differently, for some compounds the RIIs were not available for polar GC columns, such as the one used in this work.

To the best of our knowledge, there is only one report for SP regarding material collected in Greece (Koutsaviti et al. 2013). In that study, analysis showed predominance of sesquiterpene fraction (83.6%) with bicyclogermacrene (48.9%), caryophyllene (12.7%) and γ-murolene (11.9%) as major components. Monoterpenes were present in 16.2% with β-pinene (7.9%) being the main metabolite. The chemical profile of SPEO from Montenegro has been reported just recently (Tadić et al. 2017), mainly confirming the previous report from Greece. Thus, as the major components were found bicyclogermacrene (23.8%), germacrene D (8%), (E)-caryophyllene (7.9%) and spathulanol (5.5%). Sesquiterpenoid group was found to be the most dominant one (64.8%), with mono- and diterpene fractions being less important (4.4 and 5.7%, respectively). It is also worth noting that the other subspecies romana was found to be rich in oxygenated monoterpenes with thymol (24.9%) being the most abundant in the material from Turkey (Kirimer et al. 2000) and carvacrol (20%) in the Italian-origin material (Flamini et al. 1994).

The results presented herein on SP harvested from another Montenegrin locality differ to a great extent, thus, highlighting the different environmental conditions impact. However,
some authors have marked spathulenol as an artefact, observing that bicyclogermacone is easily converted to spathulenol by standing at room temperature, and this transformation also occurred during the extraction process (Toyota et al. 1996). Additionally, these two compounds are present together in many cases, and according to some authors, spathulenol can be considered as an artifactual formation produced from bicyclogermacone by autoxidation (Asakawa et al. 2009). Even spathulenol content is the result of the above-cited conversion process, herein presented Montenegrin SP sample can be considered as quite unique belonging to a particular chemotype. The most common terpene group of constituents among Sideritis species is almost absent, since some species are rich in monoterpenoids, while others are characterised by the prevalence of sesquiterpenoid fraction, or even diterpenes (Kirimer et al. 2000; Koutsaviti et al. 2013). Montenegrin SP can thus, be described as the sesquiterpenoids-rich one. A peculiar aspect is the important decrease of the sesquiterpenoid fraction after the first 3 and 6 h, followed by an increase in the monoterpenoid content (pulegone, sabinol, verbenone and thymol). Consequently, the later fractions, 12 and 24 h, were rich in monoterpenoids, displaying quite different chemical profiles of the oils.

2.3. Anti-Candida activity

The in vitro anti-Candida albicans activities of six SPEO fractions are reported in Table S3 (Supplementary material). The majority of the fractions was not active in the concentration range used. For one fraction, the MIC value was 12.48 mg/mL.

According to the results of the chemical analysis, SPEO belong to the unique chemotype rich in γ-elemene and spathulenol. Although varying in the content of these main compounds, which makes the fractions quite different between each other, the antifungal assay results presented herein showed the lack of any significant activity for this plant species. γ-elemene is a monocyclic sesquiterpene with a fruity odour that was first isolated from the rhizomes of Chinese Curcuma wenyujin Chen & Ling. Numerous studies have proved the antitumor properties of this plant (Xia et al. 2015; Zhou et al. 2015). However, according to them, this activity is mainly associated with β-elemene. Spathulenol is a tricyclic sesquiterpene that is used as fragrance and flavouring agent for drinks, in aromatising compositions for food and in sophisticated perfumes; its application covers different cosmetic and cleaning products and preparations (Mendes et al. 2008). It is present in different EOs showing antimicrobial, antinflammatory and anti-inflammatorv activities (Araújo et al. 2015). It was found to have immunosuppressive effect and to inhibit MDR protein 1 in vitro, presenting moderate cytotoxicity, which makes it a good adjuvant candidate to be used in chemotherapy of MDR cancer (Martins et al. 2010). Additionally, some authors have reported its moderate activity against most of the tested strains of human carcinogenic cells (Pullas et al. 1994). Its spasmylytic effect in a concentration dependent manner in uterus smooth muscle has been showed (Perez-Hernandez et al. 2008), as well as its insecticidal activity (Cantrell et al. 2005).

3. Conclusions and Future Perspectives

Steam distillation is the most common technique to isolate EO from plant material. Classical procedure is usually reported to be completed in 2–4 h. In line with our previous research (Božović et al. 2017a, 2017b; Garzoli et al. 2015, 2017), herein we report a systematic 24-h
EO extraction procedure applied to SP. The study has included chemical analyses of obtained EOs, as well as the related antifungal activity. The extraction method applied gave fractions that differ greatly in their chemical compositions. The GC/MS analysis of the EO fractions showed sesquiterpenoids γ-elemene and spathulenol as two main constituents, revealing a new, unique chemotype of this plant species. Although the main characterising compounds are usually present in every fraction, variations in their amount are particularly evident between the first three fractions and the last ones (after 12 or 24 h). Furthermore, some compounds appear only with the development of the extraction process, and gradually increase in amount, being significantly present only in the last few fractions. The majority of SPEO fractions were not active against Candida albicans in the tested concentration range. Further investigations are currently ongoing in order to highlight other biological activities and potential application.

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