Research into Microscopic Structure and Essential Oils of Endemic Medicinal Plant Species Satureja Subspicata Bartl. Ex Vis. (Lamiaceae)

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Abstract

In this study we looked into the cells and histological organization of leaves (*Saturejae folium*) as well as a phyto-chemical composition of overground parts (*Saturejae herba*) of endemic species *Satureja subspicata* Bartl. ex Vis. (*Lamiaceae*) collected during year 2003 on south slopes of mountain Velez in Herzegovina. Microscopic organization was analyzed in wet slides using light microscope. Estimation of stomata index was done according to Ph. Yug. IV. Chemical composition of overground material extracts was determined by thin layer chromatography (TLC) using thymol as a reference. In our research we found the following: Leaf structure of the analyzed species *Satureja subspicata* points at numerous specificities in anatomical and histological sense. In histological sense, leaf is of ventral type, with differentiated upper and lower epidermis and palisade and spongy tissue in between. Stoma index assigned according to Ph. Yug. IV leads to a conclusion that it is the case of diastitic stomata, which is common feature of most species from *Lamiaceae* family. Comparative qualitative analysis of essential oils in species *Satureja subspicata* showed similarities with other species from Lamiaceae family such as *Thymus* L. (thymol). In fact, we found more common substances that are part of the species *Satureja montana* L. extract, but in different concentrations.
KEY WORDS: plant anatomy, essential oil, thin layer chromatography (TLC), Bosnia and Herzegovina, endemic plant, stomata index, thymol.
NOMENCLATURE: Flora Europea (1)
PROOF MATERIAL: Herbaria specimens of the studied plant material are deposited into Herbarium Centre for ecology and natural resources (HCEPRES) at Faculty of Science University of Sarajevo.

Introduction

The application of medicinal plants with essential oils is continuously rising. Aromatic plants and essential oils are used in pharmaceutical and chemical industries but also in pharmacies for mixing of various sanative preparations. One of the advantages of essential oils therapy is the simplicity of their application. It can be part of regular care (bath, face care etc.) without changes in everyday routine (2, 3, 4, 5). Identification of pharmaco-dynamic characteristics and antimicrobial activity of isolated plant components are set up as main tasks, which imply successful phytochemical research. Today, those are undertaken in order to find new cures from nature, especially from chemically low examined or unknown plants (6, 7, 8, 9, 10, 11). Essential oils are mixtures of aliphatic, aromatic and hydro-aromatic substances as well as various carbohydrates, alcohols, aldehydes, ketones, esters, phenols, acids etc. (12, 13) (Figure 1). Two very important phenols, thymol and carvacrol, are known to possess antiseptic properties for a long time. They can be found in *Thymus serpyllum* L. s. lat., *Origanum vulgare* L., *Ocimum basilicum* L. and *Satureja hortensis* L (14, 15, 16, 17, 18, 19). Certain extent of antimicrobial activity is experimentally demonstrated for citral, citronellal, cinnamon and oil-aldehyde, cineole, some gumerzines and other ingredients. Genus *Satureja* L. is represented with several species in the flora of B&H. Many of those are endemic and relic elements. They are widely applied in ethno-medicine and ethnobotany of old. Significant proportion are officinal plants that have important role in the contemporary pharmaceutical industry. Due to their content of various essential oils and antimicrobial effects they are widely used in various types of phyto-therapies and cosmetics. However, many species are not thoroughly studied. One of them is *Satureja subspicata*. It is an endemic Balkan species, which, in our country, is found in the area of sub-Mediterranean rocks (20). Therefore, we designed this study in order to conduct basic biological and phytochemical research using applicable methods. The amount of reference data in this area demonstrates an increasing interest in exploration of biological, phyto-chemical, pharmaceutical and pharmaceutical/technological properties of the most common species of this type (21, 22, 23).

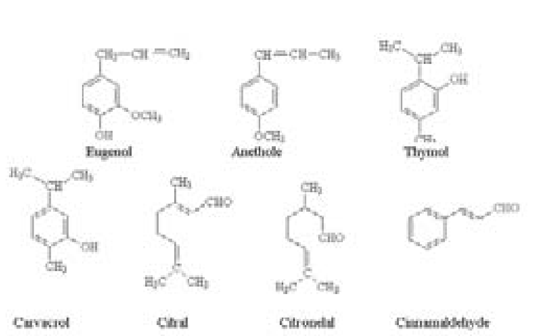


FIGURE 1. The most common ingredients of essential oils

Material and Methods

BOTANICAL AND SYSTEMATIC CHARACTERISTICS OF THE STUDIED PLANT SPECIES

Description of plant: Vertical, half-laid or half-risen half-bush 8-20 cm high (Figure 2). The plant is four-partite, smooth, and almost completely bare. The leaves are thick, leathery, with glands at the edges or very rough. The flowers are composed into levels and make a false ear. The calyx is wide, bell-like, dark green or dirty pink, containing numerous oil glands and 10 distinguishable nerves. It blooms from August till October.

THE FIELD STUDY

The process of collecting and drying herbs

Plant material of species *Satureja subspicata* was collected at two locations in sub-Mediterranean area of mountains Prenj and Velez, where this species grows on geological foundation and lower grounds, mostly of neutral or alkali reaction. The material was collected from several populations in various phenological phases. Part of the material was deposited in herbarium, and part preserved for microscopic analysis. After the drug was dried an extract was made, which was used for other chemical and microbiological analysis.

LABORATORY RESEARCH

Methods of microscopy

Standard light microscopy was used for the analysis of leaf cross section (*Saturejae folium*) as well as for the analysis of leaf vertical section (*Saturejae folium*), which was necessary for the purpose of stoma index determination in accordance with Ph. Yug. IV (25).

PRODUCTION OF HERBAL EXTRACT

Procedure of hydro-distillation:

90 g of chopped up overground part of drug (*Satureja subspicata*) was mixed with 400 ml of water in a distilla-

tion flask. The flask was heated over asbestos net to the boiling point and distilled. The distillate was collected in Erlenmeyer flask during that time. Before distillation, apparatus was washed with water, followed with dichrome-sulfuric acid and again with water. The harvested distillate was transferred into separation funnel. Then, this solution was extracted with dichloromethane four times. The layer of etheric oil was collected in the glass until there was no layer between oil and watery phase. Anhydrous Na_2SO_4 was added to etheric oil extract in order to remove residual water. Subsequently it was removed by filtration. Extracted and purified oil was then left to absorb steam (Figures 3, 4 and 5).

(Family *Lamiaceae /=Labiatae*); (Syn.: *Satureja illyrica* Host; *Satureja montana* L. subsp. *illyrica* Nyman); Local names: crveni vrijesak, klasoliki vrijesak, klasoliki vrisak
The balmy part of the plant (drug): upper part with flower *Saturejae herba*. Chemical composition: essential oils, tannins, slime.
Medicinal effect: for stomach discomfort, carminative, expectorant, anti-diarrhoeal, aromatics. Most commonly used as infusion (21).

FIGURE 2. Studied species Satureja subspicata Chaix (24)

THIN-LAYER CHROMATOGRAPHY OF ESSENTIAL OILS (TLC)

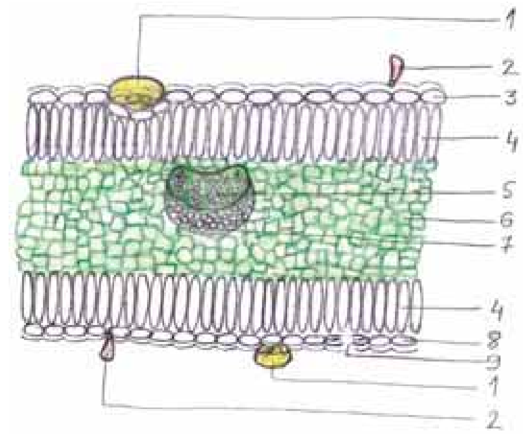
Procedure:

On a fictitious start line drawn on previously activated chromatography panel (absorbance: silica gel 60 F_{254}; Merck, Germany; 30 minutes at 110°C) we applied etheric oil extract of over ground part of herb Satureja subspicata in two different concentrations, solution of thymol in ethanol (standard 1) (Thymol, $C_{10}H_{14}O$, Kemika Zagreb) and essential oil extract of herb *Satureja montana* (standard 2). After the stains were dried, the panel was placed vertically in a chromatography chamber coated with filter paper and saturated with solvent (eluant) vapor (rising chromatography). Nevertheless, we ensured that the eluant level was 1-2 cm below the start line. Developing solution (eluant) was a mixture of toluene-ethyl-acetate (93:7). When the analyzed solution reached the desired level, the panel was removed from the chamber and dried. After that, the position of separated substances was detected by illuminating the panel with UV lamp at 254 and 365 nm (UV lamp Spektroline (model CM-10 ENF-260 C/F producer: Spectronics corporation, Westbury, New York, USA). The substances were visualized by spraying the panel with universal and specific reagents, which develop characteristic color with the analyzed substances. Iodine vapor was used as a universal reagent for the detection of organic chemical compounds. The panel was exposed to iodine vapor in ex-

FIGURE 3. Extraction of essential oil from distillate with CH2Cl2 (orig.)

FIGURE 4. Organic phase of essential oils + CH2Cl2 (orig.)

FIGURE 5. Drying of essential oil with anhydrous Na2SO4 (orig.)



Key:
1. Glands with etheric oils (type Lamiaceae), 2. Cuneiform hairs,
3. Upper epidermis, 4. Palisade tissue, 5. Tracheae, 6. Spongy tissue,
7. Sieve like pipes, 8. Lower epidermis, 9. Stoma pores

FIGURE 6. *Saturejae folium*-cross section (original)

TESTED MATERIAL	QUANTITY
Sample	16 drops
Sample	18 drops
Standard 1	12 drops
Standard 1	14 drops
Standard 2	16 drops
Standard 2	18 drops

TABLE 1. Quantity of samples and standards applied on the chromatography panel

icer and as a result, characteristic brown stains appeared. Reagents specific for components of essential oils are:

a) 1% solution of vanillin in ethanol (solution 1)

b) 10% solution of sulfuric acid in ethanol (solution 2)

The panel was sprayed first with solution 1, then with solution 2 and first, he board was sprayed with solution 1, and then with solution 2 and subsequently heated at 110°C for about 10 minutes in a dryer. The result was characteristic coloring.

Results

PLANT MICROSCOPIC ANALYSIS

The leaf of analyzed species *Satureja subspicata* has differentiated upper and lower epidermis with palisade and spongy tissue between them (Figure 6). Mesophyll consists of two series of palisade at both upper and lower side, with regularly lined cells. Central part consists of spongy tissue with isodiametrically shaped cells (there is no stressed intercellular area). Collateral vascular bundles with approximately balanced proportion of phloem and xylem cells are also located in the central part. Epidermis is built of thick compressed cells with no intercellular area and chloroplasts and with stressed external inner side. The lower epidermis contains stomas of *Helleborus type*. Epidermis also contains numerous glands with etheric oils and many multicellular hairs. Vertical section of leaf of the studied plant species (*Saturejae folium*) was analyzed under microscope in order to estimate stoma index, which was defined on the basis of the number of stomas and number of epidermis cells according to formula (25):

Stoma index=100xS/E+S

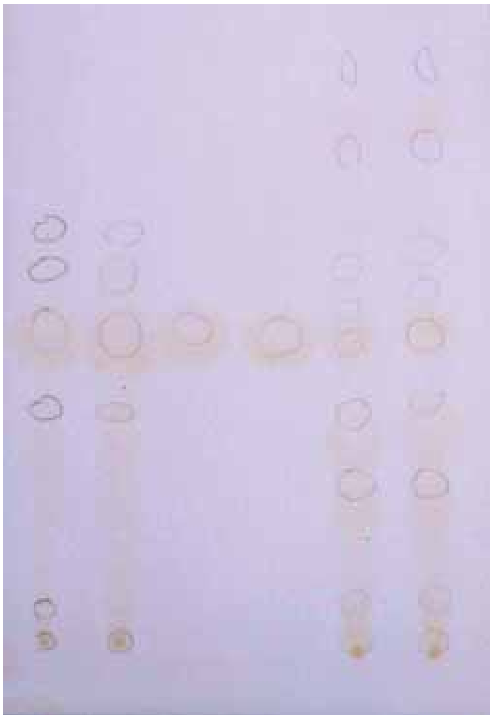
S–number of stomas per leaf area unit = 9

E–number of epidermis cells per leaf area unit = 18

Stoma index (SI) =33, 33

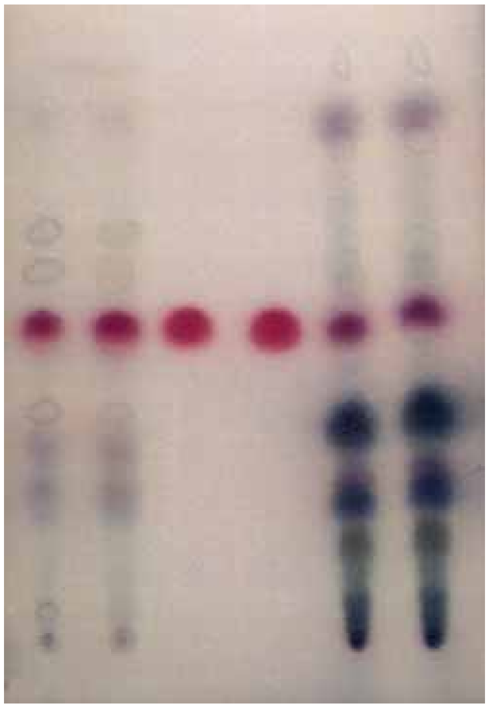
ESSENTIAL OILS

Figure 7 shows chromatogram where a mixture of toluene ethyl-acetate (93:7) was used a developing agent. For the purpose of detection it was exposed to iodine vapor. In every sample yellow zones (stains) appeared or, to be more precise, one stain for thymol standard and four stains in *Satureja montana* etheric oil standard. For the purpose of detection, chromatogram was, before being exposed to iodine vapor, examined under UV lamp and extinguishing of fluorescence appeared at 254 nm in some zones. Four zones, where extinguishing of fluorescence appeared, were detected for sample. One zone was detected for thymol standard, and eight zones, where extinguishing of fluorescence appeared, for standard of essential oil of species *Satureja montana*. Later, chromatogram was sprayed with specific reagents vanillin-sulfuric acid (solution 1+solution 2) for the purpose of visualization. Then, the panel was heated at 110°C in a dryer for 10 minutes (Figure 8). During heating, three colored stains developed in the sample (x_2 is blue,



SAMPLE STANDARD 1 STANDARD 2

FIGURE 7. Chromatogram 1 (orig.)



SAMPLE STANDARD 1 STANDARD 2

FIGURE 8. Chromatogram 2 (orig.)

d_1	ANALYZED SAMPLES		STANDARD 1	STANDARD 2			
d_1	4,6	6,1	6,2	0,8	3,1	4,3	6,2
Rf	0.315	0,418	0,425	0,055	0,212	0,294	0,425

TABLE 2. Comparative overview of Rf values

x_3 is violet, x_5 is pink), and x_5 showed extinguishing of fluorescence at 254 nm, too. Thymol standard showed one pink stain, and extinguishing of fluorescence appeared at the same place, too. The second standard containing essential oil of species *Satureja montana* resulted in five colored stains (x_1 is brown, x_2 is light green, x_3 is blue, x_4 is dark green, x_5 is violet), and extinguishing of fluorescence appeared in all of them, except x_2. Rf values (Table 2) were counted for all zones visible in chromatogram according to the formula (13):

Rf=d1/d2

d1–distance of chromatographic substance in cm

d2–distance of eluant in cm

d2=14, 6 cm

Discussion

PLANT MICROSCOPY

Anatomical-histological picture of leaf species *Satureja subspicata* points at numerous specificities in comparison with other dicotyledonous plants, even the species from the same family, *Lamiaceae*. According to its cytological differentiation, it would be a form of dorsal-ventral equalfacial leaf. The existence of two layers of palisade tissue (epipalisade and hypo-palisade tissue), which is a result of plant adaptation to sunlight utilization leads us to that conclusion. Namely, increase in palisade tissue compensates for a quite small leaf area. Such palisade tissue organization is an important feature of this plant's microscopic organization and can be successfully used for identification. Also, the leaf has some elements of sclerophilly. Not only palisade, but also spongy tissue illustrate that. Namely, there is no intercellular space, and that is clear characteristic of this histological product inside of spongy tissue. Structure of epidermis hairs and cytological organization of gland apparatus can be used as important characteristics for this plant's microscopic identification. In comparison with histological differentiation of close species *Rosmarini folium* and *Salviae folium* (26) numerous differences were noticed. Primarily, the organization of the tested leaves is more sclerophyllic. The analysis of stoma index according to Ph. Yug. IV (25) *Satureja subspicata* has diacitical stomas, like most of the species from Lamiaceae family. Stomas are surrounded by two accompanist cells that make right angle with adductor cells.

ESSENTIAL OILS

Similarities with other species from *Lamiaceae* family are underlined through our study of qualitative structure of essential oils of species *Satureja subspicata* (27, 28, 29). That was enabled by comparison between Rf values of etheric oil of *Satureja subspicata* and Rf values of thymol and etheric oil of species *Satureja montana*, which were used as standards. Based on the results we can ascertain that the sample was not heterogeneous. Component with the highest contents was thymol, and that was verified by comparing thymol as a standard on the basis of Rf value and characteristic stains. Comparison with etheric oils of species *Satureja montana* revealed 4-5 shared components, but their concentrations are different (30, 31, 32, 33, 34, 35). The latest study (13) describes phytochemical profile and antimicrobial activity of Satureja subspicata Vis. essential oils, collected in Dalmatia (Croatia). Three samples of essential oils were obtained from the aerial parts of the plant by hydrodistillation and analyzed by GC–MS. From the 24 compounds representing 97.47% of the oils, carvacrol (16.76%), α-pinene (13.58), p-cymene (10.76%), γ-terpinene (9.54%) and thymol methyl ether (8.83%) appear as the main components. The oils also contained smaller percentages of myrcene, linalool, β-caryophyllene, limonene, geranyl acetate, 1-Octen-3-ol, nerol, thymol and borneol (13). Etheric oils of related species *Satureja* and *Satureja montana* are very complex. Very complex structure of essential oil was determined by gas chromatography – spectrometric analysis in the samples of *Satureja montana* from different regions of Serbia, B&H and Macedonia. There are more than 20 different components (28) in its structure. Among those are thymol and carvacrol and their quantity varies depending on the region (36, 37, 38, 39, 40). Evaluation of antimicrobial activity of *Satureja subspicata* oil was evaluated using agar diffusion and broth microdillution methods. Testing of antimicrobial activity showed that the oils had a great potential against all 13 bacterial and 9 fungal strains. Gram-positive bacteria are more sensitive to the examined oil, with a range of 0.09 to 6.25 μl/ml comparing to a significantly higher range of 1.56 to 25.00 μl/ml for Gram-negative bacteria. Results presented here suggest that the essential oil of *S. subspicata* possesses antimicrobial properties, and is therefore a potential source of antimicrobial ingredients for the food and pharmaceutical industry (13).

Conclusion

Based on the findings of microscopic study and the analysis of essential oils of endemic Dinaric medicinal plant *Satureja subspicata* it is possible to reach the following conclusions:

- The leaf of the analyzed species in anatomical-histological sense pints at numerous specificities. In histological sense, leaf is of ventral type, with differentiated upper and lower epidermis and palisade and spongy tissue in between. It is unique type of sclerophillic leaf and important for microscopic identification of this species;
- Stoma index assigned according to Ph. Yug. IV. leads to the conclusion that the stoma is diactitic, which is common feature of most species from family *Lamiaceae*;
- Comparative qualitative analysis of essential oils in species *Satureja subspicata* showed similarities with other species from Lamiaceae family such as *Thymus* L. (thymol). In fact, we found more common substances that are part of the species *Satureja montana* L. extract, but in different concentrations.
- Results of this study indicate that the essential oils and other components of *S. subspicata* may be a great resource in pharmaceutical industry and modern aromatherapy.

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