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BIOCHEMICAL RESEARCH OF THE SPECIES *ORCHIS MORIO* L. FROM ZLATAR

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Abstract: Salep tuber is sourced from young tubers of plants belonging to genera such as *Orchis (Orchis morio* L, *Orchis militaris* L, *Orchis mascula* L, *Orchis latifolia* L), *Ophrys* (*Ophrys fuciflora* (Cr) Haller), *Platanthera* (*Platanthera bifolia* (L) Rich,), *Anacamptis* (*Anacamptis pyramidalis* (L) Rich), and *Gymnadenia* (*Gymnadenia conopsea* (L) RBr) from the Orchidaceae family. Ph.Yug.II categorizes *Orchis morio* L as salep, aligning with CITES convention and IUCN categories for vulnerable species. As a mucilaginous drug, salep is used as an enema for intestinal catarrh and added to drugs with local irritating effects due to its soothing impact on mucous membranes. Qualitative gas chromatographic-mass spectrometric (GH/MS) analysis of Orchis morio tubers from Zlatar mountain's dry meadows reveals compounds like cinnamaldehyde, α-terpinyl acetate, coumarin, α-muurolol, pogostol, caryophyllene, n-hexadecanoic acid, linoleic acid, pentacosane, heptacosane, nonacosane, campesterol, cyclolartenol, sitosterol, and predominantly β-sitosterol. The elevated β-sitosterol content supports the use of salep in treating benign prostate diseases.

Keywords: *Orchis morio* L., Salep tuber, β-sitosterol

Introduction

The source of the drug Salep tuber are young tubers of plants from the Orchidaceae family. The plant species we used are *Orchis morio* L., *Orchis militaris* L., *Orchis mascula* L., *Orchis latifolia* L., *Ophrys fuciflora* (Cr.) Haller *Platanthera bifolia* (L.) Rich, Anacamptis pyramidalis (L.) Rich, and *Gymnadenia conopea* (L.) R B. The following species are listed on the CITES list of protected

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plants in Serbia and Montenegro: *Anacamptis pyramidalis* (L) Rich, *Gymnadenia conopsea* (L) RBr, *Orchis militaris* L, *Orchis morio* L According to EU laws on trade in wild plants, these species are protected and listed for SCG (EU Regulation on trade in wild animals Protection of wild fauna and flora species by regulating trade in them). The Ph.Yug. II mentions the *Orchis morio* L. type as salep, which is in accordance with the CITES convention and IUCN vulnerable species categories. It is widespread in the following areas: Europe, northern part of England, southern part of Norway and Estonia, southeastern and central Russia, which are areas rich in this plant species. *Orchis morio* L. belongs to the Mediterranean-Submediterranean-Pannonian Atlantic-Western Sarmatian floristic element (Obratov-Petkovic, et al., 2007; Bogosavljević et al., 2014). It is widespread in Europe, areas rich in this plant species are the northern part of England, southern part of Norway and Estonia, southeastern and central Russia. In southern Europe, this mesothermic species is widespread in mountainous areas. In Serbia it is represented on mountains Goč, Kopaonik, Tara, Golija, Javor, Mučanj, Ovčar-Kablar gorge, gorge of Svrljiški Timok, Carska bara - old Begej (altitude 75-85m).

Orchis morio L. is a perennial herbaceous plant that inhabits extremely dry habitats, but can also be found in mesophilic phytocenoses, which is why it is classified as a subxerophyte ecological group. The soil on which it grows has neutral to weakly acidic pH values, and a small amount of nitrogen in the soil is sufficient for its growth and development. The results of the research pointed to the harmful consequences of even small amounts of fertilizers (containing N, P, K and Mg) and it is not recommended to use either organic or inorganic fertilizer in meadows where it is necessary to preserve *Orchis morio* L. (Silvertown et al., 1994). It has been determined by analyzing the buds of European orchids that they contain cyanidinglucoside (orchicyanin). As the concentration of this anthocyanin is specific to each species and genus, identification of orchids can be performed by determining the concentration of anthocyanin during flower development (Uphoff, 1981).The tuber *Orchis morio* L. are round, rarely oval. The tuber contains 50% mucus, 25% starch, 5% protein, 2% cellulose, 1% sugar, a little bitter substances, fats and tartaric acid. Research indicates that salep samples obtained from tubers of *Orchis anatolica*, *Orchis italica*, *Orchis morio*, *Orchis tridentata* and *Serapias vomeracea ssp orientalis* contain higher amounts of mucus and have higher viscosity than those obtained from tubers of *Dactylorhiza osmanica* var *osmanica*, *Ophrys mammosa*, *Orchis coriophora*, *Orchis palustris*, *Orchis simian* (Tekinşen & Güner, 2010).

Salep as a typical mucous drug (Mucilago) is used as an enema in the intestinal catar and as an adjunct to drugs with a locally irritating effect because it soothes mucous irritation (Tekinşen & Güner, 2010).

Materials and methods

Plant material (tubers) was collected from dry meadows on The Zlatar Mountain. After identification, the sample was left to dry naturally, and then packed in a paper bag. For the purposes of analysis, the plant is protected.

Gas chromatography-mass spectrometry analysis (GC/MS) was performed using an Agilent 6890 gas chromatograph connected to an Agilent 5973 Network mass selective (MSD) and flame ionization detector (FID) in positive electron impact (EI) ionization mode. The separation was carried out on the Agilent 19091S-433 HP-5MS capillary column, measuring 30 m x 0.25 mm i.d. and film thickness 0.25 μm. GC/MS analysis was performed under the following conditions: temperature range of 60-285°C (4.3°C/min); carrying helium gas with a flow rate of 1 ml/min, measured at 210 °C; injector temperature 250 ° C, and injection in splitless mode. Conditions of MS analysis: source temperature 200 °C; interface temperature of 250°C; ionization energy of 70 eV; mass shooting range 40–350 amu (atomic mass units).The quantification of individual components was performed using a flame ionization detector. By comparing the areas of the graph obtained by analyzing the sample and the internal standard, the relative proportion of each component was calculated. Component identification was performed by comparing the results of GH/MS analysis with spectra (NIST and Wiley and Adams library). The ^{1}H NMR spectrum was recorded on a Varian Gemini 2000 r (200 MHz) camera. DMSO-d6 and TMS were used as an internal standard.

Results and discussion

The results of the qualitative gas chromatography-mass spectrometry analysis are shown on the chromatogram (Figure 1). Signals from the detector on the graph are shown as a series of peaks, each representing an individual component from the mixture of the tested sample, while the area under the peak represents the amount of the present component in ppm (parts per million, mg/l).

Signals on a chemical shift from 3.00 to 5.20 δ indicate the presence of sugar, and signals between 6.8 and 8.1 δ the presence of compounds with aromatic rings. The results of the qualitative analysis are shown in Figure 2.

Figure 2. Amounts of components present in the *Orchis morio* L. tuber sample in ppm

Component mixtures after separation show different retention times -times for each component in the column. Compounds shown in Table 1. were identified by their detection, retention time is given for each component, as well as the relative proportion in the sample of the plant drug salep.

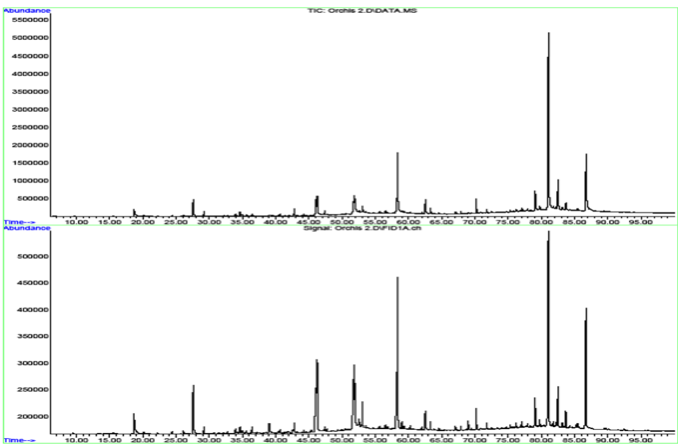

Figure 1. Chemical components in tubers of the species *Orchis morio* L.

Identification compounds of the peaks in the chromatogram shown in Figure 1 and 2 are represented in Table 1. The results show that of all compounds, the highest relative proportion is β-sitosterol and linolenic acid. Sterols are essential components of the cell membrane and animal and plant cells. Pharmacological screening of β-sitosterol has been established to possess numerous activities such as antimicrobial, anti-inflammatory, anticancer, antifertility, antioxidant, antidiabetic (Chanioti at al., 2021; Le Goff et al., 2019; Ambavade at al., 2014). Several studies and clinical trials suggest the use of β-sitosterol against prostate, colon and breast cancer, experimentally these studies confirm the important protective role of β-sitosterol in cancer prevention (Chanioti at al., 2021; Le Goff et al., 2019). An in vitro study shows that β-sitosterol effectively inhibits the growth of HT-29 cancer cells tumors (a line of human colon cancer cells) (Jones & Abumweis 2009). Due to its positive properties, β-sitosterol is used in herbal therapy and in the treatment of benign prostatic hypertrophy). Alone or in combination with similar phytosterols β-sitosterol also reduces the level of cholesterol in the human blood, hence it can be used in the treatment of hypercholesterolemia, inhibiting cholesterol absorption in the intestinal tract (Matsuoka et al., 2008).

Table 1. Identification compounds in a salep obtained from *Orchis morio* L. Tubers

Jedinjenja Compound	Retenciono vreme Retention time	Relativni udeo Relative abundance
Cinamaldehid	18.68	3.077
α-terpinil acetat	22.16	0.187
Kumarin	26.03	0.731
α-murolol	34.59	0.890
Pogostol	34.93	0.449
Cariofilen	35.60	0.298
n-heksadekanska kiselina	46.23	10.458
Linolenska kiselina	51.79	15.300
ND	51.98	5.578
Pentakosan	61.95	0.221
ND	62.5	2.256
Heptakosan	67.07	0.290
Nonakosan	71.78	trag
Campesterol	79.12	3.924
β-sitosterol	80.94	28.266
Cikloartenol	82.46	4.172
Sitostenon	83.67	1.773

Another component that is found in a large concentration is linoleic acid. Linoleic acid enters the membrane of plants, for animals and humans is an essential fatty acid, necessary for a number of metabolic processes and the normal functioning of the body and the preservation of health as a whole. Linoleic acid deficiency causes hair loss, poor wound healing. Its consistent intake is associated with a reduced risk of atherosclerosis, hypercholesterolemia (Das, 2021; Ramsden et al., 2021). and other chronic health conditions.

Conclusion

Based on the performed qualitative gasnochromatographic-mass spectrometric (GH/MS) analysis, it can be concluded that tubers of the species *Orhis morio* L. collected from the dry meadows of The Zlatar Mountain contain:

cinnamaldehyde, α-terpinyl acetate, coumarin, α-murolol, pogostol, caryophyllene, n-hexadekanic acid, acid, pentacosan, heptacosan, noncosan, campesterol, cycloartenol, sitostenone, and in the largest quantities β-sitosterol and linolenic acid. Large amounts of β-sitosterol sytosterol indicate the justification of the use of salep in pharmacotherapy in the treatment of benign prostate diseases, but also certain types of cancers such as prostate, colon and breast cancer.

References

Ambavade S.D., Misar A.V., Ambavade P.D. (2014). Pharmacological, nutritional, and analytical aspects of β-sitosterol: a review. Orient Pharm Exp Med 14, 193–211.

Bogosavljević S., Zlatković B., Ranđelović V. (2014). Flora klisure Svrljiškog Timoka 9th Symposium on Flora of Southeastern Serbia and Neighbouring Regions, 41-54.

Chanioti S., Katsouli M., Constantina T. (2021). β-Sitosterol as a functional bioactive., A Centum of Valuable Plant Bioactives, pp.193-212.

Das U.N. (2021). Bioactive lipids and vascular disease. Eur. J. Clin. Nutr. 75:1528–1531.

Jones P.J.H., Abumweis S.S. (2009). Phytosterols as functional food ingredients: Linkages to cardiovascular disease and cancer. Current Opinion in Clinical Nutrition and Metabolic Care, 12(2), 147–151.

Le Goff M., Le Ferrec E., Mayer C., Mimouni V., Lagadic-Gossmann D., Schoefs B. (2019). Microalgal carotenoids and phytosterols regulate biochemical mechanisms involved in human health and disease prevention, Biochimie, 167, 106–118.

Matsuoka K., Nakazawa T., Nakamura A., Honda C., Endo K., Tsukada M. (2008). Study of Thermodynamic Parameters for Solubilization of Plant Sterol and Stanol in Bile Salt Micelles. Chem. Phys. Lipids, 154, 87-93.

Obratov-Petkovic D., Popovic I., Dajic-Stevanovic Z., (2007). Diversity of the vascular flora of Mt. Zlatar (Southwest Serbia), EurAsia J BioSci 5, 35-47.

Ramsden C.E., Zamora D., Faurot K.R., MacIntosh B., Horowitz M., Keyes G.S., Yuan Z.X., Miller V., Lynch C., Honvoh G., et al. . (2021). Dietary alteration of n-3 and n-6 fatty acids for headache reduction in adults with migraine: Randomized controlled trial. BMJ.; 374:n1448.

Silvertown J., Wells A.D., Gillman M., Dodd E.M., Robertson H., Lakhani H.K., (1994). Short-term effects and long-term after-effects of fertilizer application on the flowering population of green-winged orchid Orchis morio, Biological Conservation. 69, 191-197.

Tekinşen K.K., Güner A. (2010). Chemical composition and physicochemical properties of tubera salep produced from some Orchidaceae species Food Chemistry, Volume 121, Issue 2, Pages 468-471.

Uphoff W. (1981). Identification of European orchids by determination of the anthocyanin concentration during development of the blossoms Cellular and Molecular Life Sciences Volume 38, Number 7, 778-780.