

HISTO-ANATOMICAL RESEARCHES, POLYPHENOLIC PROFILE AND BIOLOGICAL ACTIVITIES OF THE AERIAL PARTS AND TUBERS OF *DACTYLORHIZA MACULATA* L. SOÓ. (ORCHIDACEAE)

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Abstract

The present study aimed to provide a histo-anatomical characterization, to evaluate the polyphenolic profile of the tubers and aerial parts belonging to *Dactylorhiza maculata* L. Soó. and to test their antioxidant capacity and antibacterial effects. Polyphenols were analysed by an HPLC-MS method. The antioxidant activity of the extracts was tested by TEAC and EPR assays. Antibacterial activity was assessed on five bacterial strains. Ethanolic extracts of aerial parts proved to contain mainly isoquercitrin, quercitrin, ferulic and *p*-coumaric acid, among the tested compounds. Assays of the biological activity revealed a significant antioxidant and antibacterial activity especially for the aerial parts. At the same time, the histo-anatomical features for the main constituents of aerial parts could be established. These results offer a new perspective on *D. maculata*, proving it as an important source of bioactive compounds.

Rezumat

Scopul acestui studiu a fost de a realiza o caracterizare histo-anatomică a speciei *Dactylorhiza maculata* L. Soó., dar și de a evalua profilul polifenolic al tuberculilor și părților aeriene aparținând speciei, precum și de a le testa potențialul antioxidant și antimicrobian. Analiza polifenolilor a fost realizată printr-o metodă HPLC-MS. Activitatea antioxidantă a extractelor a fost realizată prin metodele TEAC și EPR. Activitatea antimicrobiană a fost testată pe cinci tulpini bacteriene. Extractele etanolice ale părților aeriene au dovedit în principal prezența izoquercitrinei, urmată de quercitrină, acid ferulic și acid *p*-coumaric, dintre compușii analizați. Analizele activității biologice au evidențiat o activitate semnificativă antioxidantă și antibacteriană, în special pentru părțile aeriene. Totodată, a fost stabilită caracterizarea histo-anatomică a principalilor constituenți ai părților aeriene. Aceste rezultate oferă o nouă perspectivă asupra speciei *D. maculata*, dovedind că este o sursă importantă de compuși bioactivi.

Keywords: *Dactylorhiza maculata* L. Soó., optical microscopy, HPLC-MS, antioxidant capacity, antimicrobial activity

Introduction

Orchidaceae family represents one of the largest flowering plants families known worldwide, comprising over 20,000 species, which belong to more than 800 genera [3, 14]. Among these species, dactylorchids (species belonging to *Dactylorhiza* Necker ex Nevski genus) are a taxonomically important group which has been intensively studied from the molecular and morphological point of view [17, 21]. Members of this group are also known as the "digitate tuber" clade, as their stem morphology is an important feature that can help an accurate distinction between these species and the ones belonging to *Orchis* L. genus, which present bulbous tubers [5]. Species belonging to *Dactylorhiza* genus are distributed from the

Mediterranean area, throughout all the temperate areas of the European continent, northern Africa, eastern, western and northern America, but also south-western Africa [5, 17, 18, 21]. These species are known since ancient times for their medicinal uses that seem to be due to their content in polysaccharides, fibres, proteins, steroids and flavonoids [8, 9, 22] but, despite this fact, the scientific data on their chemical content and potential bioactivities remain scarce [3, 4, 7, 8, 14].

Dactylorhiza maculata L. Soó. is a widespread species throughout the western and northern parts of Europe [21]. The herbal medicinal product belonging to this species consists in the powdered tubers, which are used for the treatment of different disorders such as inflammatory intestinal pathologies, tuberculosis,

diarrhoea, cancer or Parkinson's disease [8]. Other scientific sources cite their use as aphrodisiacs [8, 14]. The few existing data on the chemical and biological profile of the species corroborated with the significant potential of the species makes the study of *Dactylorhiza* species an important research subject. In this context, the present study was undertaken in order to bring new data on the histo-anatomical features of *D. maculata*, but also on its polyphenolic profile and potential biological activities.

Materials and Methods

Chemicals and apparatus: Chlorogenic acid, p-coumaric acid, caffeic acid, rutin, apigenin, quercetin, isoquercitrin, quercitrin, hyperoside, kaempferol, fisetin, myricetol were purchased from Sigma (St. Louis, USA), ferulic acid, sinapic acid, gentisic acid, gallic acid, patuletin, luteolin from Roth (Karlsruhe, Germany), cichoric acid, caftaric acid from Dalton (Toronto, Canada). HPLC grade methanol, ethanol and all reagents for spectrophotometric assays were purchased from Merck (Germany). Reagents for antioxidant assays were purchased from Sigma-Aldrich (Germany). All spectrophotometric data were acquired using a SPECTROstar Nano Microplate Reader with 96-well plates (BMG Labtech, Ortenberg, Germany). The tested microorganisms were obtained from the Food Biotechnology Laboratory, Life Sciences Institute, University of Agricultural Sciences and Veterinary Medicine Cluj Napoca, Romania.

Vegetal material. The aerial and underground parts of *D. maculata* (Voucher No. 162.5.2.1) were collected from the spontaneous flora of Cluj County, Romania, and were identified by the Department of Pharmaceutical Botany of "Iuliu Hațieganu" University of Medicine and Pharmacy (Figure 1).



Figure 1.

D. maculata: A – aerial parts; B – tubers

Analysis of histo-anatomical features

Microscopic study of the powder: Samples of aerial parts were dried and ground using a Grindomix knife mill. The obtained powder was studied with the Olympus CX31 optical microscope, using as mounting medium the chloralhydrate 80% (m/V) [19].

Microscopic study of the histo-anatomical features of the vegetative organs: Stems were cross-sectioned using a Nahita 501 manual microtome. Sections were stained using alum carmine Alfa Aesar® and malachite green Merck® and studied using an Olympus CX31 optical microscope equipped with a digital camera [15, 19].

Analysis of polyphenols

Extraction procedure: Air-dried powdered tubers and aerial parts of *D. maculata* were macerated for 48 hours with ethanol 70% (v/v). The samples were then sonicated for 30 min and filtered through a 0.45 mm membrane filters.

HPLC/MS Analysis: 5 µL of the ethanolic extracts were analysed using an Agilent Technologies 1100 HPLC Series system. The detection of compounds was performed using UV and MS methods. The UV detector was set at 330 nm until 17.5 min, then at 370 nm. The MS system operated using an electrospray ion source in negative mode. The separation was performed on a Zorbax SB-C18 column (100 × 3.0 mm, i.d., 3.5 µm particle) with binary gradient consisting in a mixture of methanol and acetic acid 0.1% (v/v) at a flow rate of 1 mL/min. The elution started at 5% methanol and ended with 42% methanol, for the first 35 minutes and then passed at 42% methanol for the last 3 minutes. The MS signal was used only for qualitative analysis based on specific mass spectra of each polyphenol [6, 16].

Determination of Total Phenolic and Flavonoids content: The total phenolic content was determined using the Folin-Ciocalteu method, with some modifications and the results were expressed as mg gallic acid equivalents/g dry weight herbal product (mg GAE/g d.w.) [15]. The total flavonoid content was quantified using the method described in the Romanian Pharmacopoeia (10th edition) in the *Cynarae folium* monograph and was expressed as quercetin equivalents/g dry weight vegetal product (mg QE/g d.w.) [6, 16, 23].

Biological activity assays

Antioxidant Capacity: The antioxidant capacity was determined by the TEAC (Trolox Equivalent Antioxidant Capacity) assay and by the EPR (Electron Paramagnetic Resonance) spectroscopy measurements - Fremy's salt Equivalents [6, 10-13, 16].

Antibacterial activity: *Staphylococcus aureus* (ATCC 49444), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19114), *Salmonella typhimurium* (ATCC 14028) and *Escherichia coli* (ATCC 25922) strains were used for the bioassays. The bacterial strains were cultured on Muller-Hinton Agar, stored at 4°C and sub-cultured once a month. The five bacterial species were cultured overnight at 37°C in Tryptic Soy Broth (TSB) medium at 37°C. Bacterial cell suspensions were adjusted with sterile saline to a concentration of approximately 3×10^5 Colony Forming Units (CFU)/mL in a final volume of 100

$\mu\text{L}/\text{well}$. The inoculum was stored at $+4^{\circ}\text{C}$ for further use. Dilutions of the inoculum were cultured on solid Muller-Hinton (MH) for bacteria to verify the absence of contamination and to check the validity of the inoculum. Determinations of minimum inhibitory concentrations (MIC) were performed by a serial dilution technique using 96-well microtitre plates. Different solvent dilutions of ethanol: methanol extracts were carried out over the wells containing 100 μL of Tryptic Soy Broth (TSB) and afterwards, a volume of 10 μL of inoculum was added to all the wells. The microplates were incubated for 24 - 48 h at 37°C . The MIC of the samples were detected following the addition of 20 μL (0.2 mg/mL) of resazurin solution to each well, and the plates were incubated 2 h at 37°C . A change from blue to pink indicates the reduction of resazurin and therefore bacterial growth. The MIC was defined as the lowest drug concentration that prevented this colour change. The minimum bactericidal concentrations (MBC) were determined by serial sub-cultivation of a 2 μL volume into microtitre plates containing 100 μL of broth per well and further incubation for 48 h at 37°C . The lowest concentration with no visible growth was defined as MBC, indicating 99.5% killing of the original inoculum. Streptomycin (Sigma P 7794) (0.05 - 3 $\mu\text{g}/\text{mL}$) was used as positive control. A 10% solution of ethanol: methanol in water was used as negative control [2, 12, 13].

Results and Discussion

Histo-anatomical analysis: The cross section through the stem revealed the primary anatomical structure (Figure 2A), which is divided in three major areas: epidermis, cortex and stele. Under the polygonal cells that form the epidermis, the collenchymatic cortex is found and, after a few layers of parenchymatic cells, the circular multi-layered sclerenchymatic cortex appears, being formed of 4-5 layers of cells, with lignified walls (Figure 2B). In the stele, the collateral vascular bundles were identified (Figure 2C). Each vascular bundle is formed by xylem (xylemian vessels with lignified walls and xylemian parenchymatic tissue) and phloem (sieve tubes, phloemian parenchymatic tissue and companion cells). Pith cells are cylindrical and delineate inside a central lacuna. This is the first scientific evidence on the histo-anatomical features of the stems of the Romanian *D. maculata* species; different sources describe the similar anatomy of other species from the

same genus [1] or of the related species (*Orchis*) [20], particularly the morphology of the reproductive organs [5].

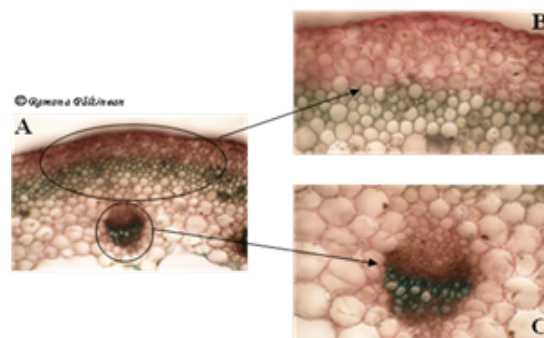


Figure 2.

Microscopic observation of the cross section through the stem of *D. maculata*: A – general view (x100); B, C – structural details (x200)

Micrographic analysis: The powder obtained from the aerial parts of *D. maculata* revealed the main features of vegetative and reproductive organs. The most important histological components found were: parts of secretory papillae (Figure 3B), xylem vessels and potassium oxalate crystals in form of raphides (Figure 3A). The features of the powder confirmed the features that were found in the anatomical structure of the stems.

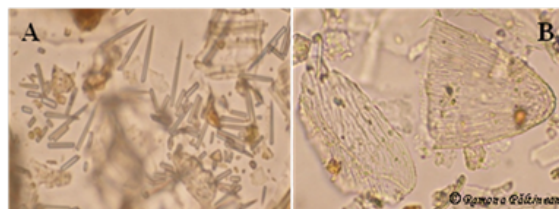


Figure 3.

Components of the powder of aerial parts of *D. maculata* (A, B - 200x)

Total Phenolic and Flavonoids Content: The total polyphenolic content for *D. maculata* extracts were quantified based on the linear equation obtained from gallic acid standard calibration curve (mg GAE/g d.w.). The calculation of total flavonoid content was carried out using the standard curve of quercetin (mg QE/g d.w.). The highest amounts of the total polyphenols and flavonoids were determined in the aerial parts of *D. maculata* extracts (Table I).

Table I

The content of total polyphenols and flavonoids in *D. maculata* extracts

Samples	Total phenolic content (mg GAE/g d.w.)	Total flavonoids (mg QE/g d.w.)
<i>D. maculata</i> tubers	2.76 ± 0.21	0.46 ± 0.05
<i>D. maculata</i> aerial parts	8.60 ± 0.79	2.69 ± 0.29

Each value represents the mean $\pm$ SD ($n = 3$).

HPLC/MS Analysis: In the ethanolic extract of *D. maculata* aerial parts, isoquercitrin was the compound found in the highest amount (518.6 ± 3.7 µg/g d.w.), being followed by quercitrin (474.6 ± 4.34 µg/g d.w.). Ferulic acid, *p*-coumaric acid, caffeic acid and kaempferol were also identified, but in lower amounts (Table II). The tubers proved to have no detectable amounts of the polyphenols studied in

the described analytical method. The results obtained hereby strongly correlate with the ones obtained in the case of the quantification of the total polyphenolic and flavonoidic contents. To the best of our knowledge, this is the first report of a kind that describes the quantification of certain phenolic compounds in the composition of the species.

Table IIPolyphenolic compounds content in *D. maculata* extracts (µg/g d.w.)

Polyphenolic compound	<i>D. maculata</i> aerial parts	<i>D. maculata</i> tubers
Caffeic acid	16.56 ± 0.12	NF
<i>p</i> -Coumaric acid	69 ± 0.54	NF
Ferulic acid	51.4 ± 0.45	NF
Isoquercitrin	518.6 ± 3.7	NF
Quercitrin	474.6 ± 4.34	NF
Kaempferol	3.6 ± 0.22	NF

NF - Not found. Each value represents the mean $\pm$ SD ($n = 3$).

Antioxidant Activity Assays: The ethanolic extracts from aerial and underground parts showed different antioxidant capacities by means of TEAC and EPR assays. The sample that proved to have the most significant capacity to reduce the ABTS cationic radical is the sample of aerial parts (Table III).

These results correlate with the results obtained for the qualitative and quantitative analysis of polyphenols, which clearly indicate the fact those aerial parts, might have an important potential as antioxidant agents, related to their important content in polyphenolic compounds.

Table IIIAntioxidant activity results obtained for the tested *D. maculata* extracts

Samples	TEAC (mg TE/g d.w.)	EPR (mg FSE/g d.w.)
<i>Dactylorhiza maculata</i> tubers	13.34 ± 0.79	25.04 ± 0.3
<i>Dactylorhiza maculata</i> aerial parts	40.88 ± 0.73	88.87 ± 0.9

Each value represents the mean $\pm$ SD ($n = 3$).

Antibacterial Activity Assays: All tested micro-organisms were susceptible to the plant extracts with minimum inhibitory concentration (MIC) ranging between 0.0078 - 0.031 mg/mL (Table IV) and minimum bactericidal concentration (MBC) ranging between 0.015 - 0.062 mg/mL (Table V). These results indicate that the bacterial strains: *L. monocytogenes*, *S. typhimurium* and *E. coli* were the most sensitive to the aerial parts, with MIC

0.078 mg/mL and MBC 0.015 mg/mL. The extracts of *D. maculata* tubers present an equal antimicrobial activity against *S. typhimurium* and *E. coli* with MIC 0.078 mg/mL and MBC 0.015 mg/mL, but a smaller activity against *S. aureus* and *B. cereus* with MIC 0.015 mg/mL and MBC 0.031 mg/mL, than *D. maculata* aerial parts extracts. The extracts obtained from the aerial parts showed the strongest antimicrobial activity.

Table IVMinimum inhibitory concentration (MIC) of *Dactylorhiza maculata* extracts

Samples	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Listeria monocytogenes</i>	<i>Salmonella typhimurium</i>	<i>Escherichia coli</i>
<i>Dactylorhiza maculata</i> tubers	0.015	0.015	0.015	0.0078	0.0078
<i>Dactylorhiza maculata</i> aerial parts	0.031	0.031	0.0078	0.0078	0.0078
Standard antibiotic/Streptomycin	0.03	0.015	0.015	0.06	0.12

Each value represents the mean $\pm$ SD ($n = 3$).

Table VMinimum bactericidal concentration (MBC) of *Dactylorhiza maculata* extracts

Samples	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Listeria monocytogenes</i>	<i>Salmonella typhimurium</i>	<i>Escherichia coli</i>
<i>Dactylorhiza maculata</i> tubers	0.031	0.031	0.031	0.015	0.015
<i>Dactylorhiza maculata</i> aerial parts	0.062	0.062	0.015	0.015	0.015
Standard antibiotic/Streptomycin	0.06	0.03	0.03	0.12	0.24

Each value represents the mean $\pm$ SD ($n = 3$).

Conclusions

The microscopic analysis allowed the identification of histological features and the anatomical structures of the stems. The polyphenolics content of the species brought important data on the polyphenolic profile of the aerial parts of the species.

Isoquercitrin, quercitrin, ferulic and *p*-coumaric acid were identified and quantified. Antibacterial and antioxidant activities proved important results for the aerial parts, which correlate to the polyphenolic content.

Together, all these results represent important criteria for the identification and characterization of the species. Furthermore, this study brings new perspectives for further investigation of the bioactive compounds of *D. maculata* species.

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