Ph.D. thesis

Ethnopharmacobotanical evaluation of Homoródalmás; histological, phytochemical and microbiological study of *Ononis arvensis* L.

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1. Introduction

Ethnobotany, as an interdisciplinary science including e.g. botany and ethnography, deals with the traditional knowledge on nature, the role and use of plants in human culture, and related beliefs and customs (Gub 1994). Medicinal plants, used in the traditional folk-therapy, play an important role in the everyday life of people of Transylvania. Recording the valuable diappearing data of folk medicinal knowledge is nowadays very important from an ethnobotanical, phytochemical and phytotherapeutic point of view. The preservation of ethnobotanical resources is vital for the future. The researches have be continued, wherewith these valuble data are disappearing these days.

In recent decades, the number of publications on European medico-ethnobotanical studies has been steadily increased published by several reshearch groups. There are numerous reasons for conducting medical ethnobotanical studies: a great number of locally gathered medicinal plants is still widely used in many households for local healthcare, some regions are hotspots for both biodiversity and cultural/ethnic/religious diversities, maintenance of ecoturisms and rural development, as well as the significance role of wild medicinal and food plants in many economic initiatives and programs.

The first herbal books have been published in the 16th century in Transylvania (Lencsés 1570; Melius 1578). Since the 1960s, several ethnobotanical surveys were carried out in several regions of the country, such as in Ghimes, Bucovina, Tara Călatei, Moldova, Gheorgheni, Maramureș, Uz valley, Cașin, and Homorod.

The main aim of our work is to summarize the used medicinal plants and local plant knowledge in Homoródalmás (Merești, Romania), focusing on the changes of the traditional ethnomedicinal knowledge of inhabitants. After comparing the current data with previous reports obtained from scientific databases (Scopus, PubMed, ScienceDirect), field restharrow (*Ononis arvensis* L.) was selected for further histological, phytochemical and microbiological studies. The herb of *O. arvensis* is known for diarrhea, liver and stomach disorders as a tea in the traditional human and veterinary medicine in Transylvania.

2. Aims of the study

1. The aim of our work was to document medicinal plants used in the ethnomedicine of Homoródalmás (local name, drug and application form), to document the materials of animals and other origin, as well as the magico-mythological data in the human and veterinary practice;
2. histological study of *Ononis arvensis* (root, stem, leaf, sepal, petal, pollen), which are selected and traditionally used in the area;
3. to identify and quantify the polyphenolic compounds in the aerial part of *O. arvensis*
4. to determine and compare the total phenol, flavonoid and tannin content of the studied parts (aerial part, stem, root and leaf, flower) of *O. arvensis*;
5. the antioxidant potential of the aerial part of *O. arvensis*, employing ECL, DPPH and ORAC methods, as well as the antioxidant potential of the aerial part, root, stem, leaf and flower applying ABTS method;
6. to investigate the antimicrobial activity of *O. arvensis* stem and leaf extracts against four bacterial and one fungal strain.

3. Materials and methods

3.1. Data collection

In our field work (2013–2016), 60 informants from Homoródalmás, aged between 11 and 95 years were randomly selected and asked for their ethnomedicinal knowledge inherited from their parents and grandparents. Collections were carried out by semi-structured interviews about the inhabitants' healing methods and knowledge of medicinal plant use. During the interviews the vernacular and scientific plant names, habitat, drug part, indication and application forms of plants were described which are used in human and veterinary medicine. The vegetables, fruit-bearing and ornamental plants of home gardens, and the cultivated species in agriculture were also documented. The food, fodder, ornamental and dyeing plants were classified. All remedies of plant, animal and other origin were documented for the mentioned indications, completed with magico-mythological elements related to plants and their application in the ethnomedicine. In addition, the interview questions covered the origin of the medical knowledge (e.g. read or heard data). Data were recorded with dictaphone (Olympus VN-4100 PC) (about 29 hours), handwritten

notes, and photos (Canon SX40HS) on drugs, plant habitats, and preparations (~1200 photos). Voucher specimens labelled with unique codes were deposited at the Department of Pharmacognosy, University of Pécs. Plants were identified as species with the identification key and scientific plant terminology of Király (2009).

3.2. Histological study of *O. arvensis*

Aerial part of the plant was harvested at a hayfield in Petreni, Homoród-valley in 2013, and at a road edge in Mereşti (Romania) in 2014. The studies were carried out at the Department of Pharmacognosy and the Institute of Biology, University of Pécs.

The leaf and flower parts were studied by leaf surface cast method. Leaf samples were cut into 0.5 × 0.5 cm pieces and boiled in 15 mL of distilled water (10 min), 15 mL of the mixture of cc. potassium hydroxide and 10% ethanol (4 min), 4 mL of 5% H_2O_2 (1 min), and thereafter they stayed at room temperature (10 min). Finally, they were boiled in 15 mL of 96% ethanol (10 min) to lose their colour. Cleared samples were placed on slides and covered with Neo-Mount®. Petals and sepals (5 pieces each) were soaked into 20-30 mL of water and 10 mL of 80% chloral hydrate. Finally, 2-3 drops of glycerine were but onto the surface of the samples, and they were covered. As a second method, the freshly collected root, stem, leaf, petiole, stipule, and flower were fixed in a mixture of 96% ethanol : glycerine : distilled water (1:1:1). Dehydration followed in ethanol series (30, 50, 70 and 96% ethanol for 12, 12, 24 and 3 h, respectively), then samples were embedded in synthetic resin (Technovit 7100; Realtrade Ltd., Hungary). Cross sections (10-15 μm) prepared by a rotation microtome (Anglia Scientific 0325) were stained in toluidine blue (0.02%, 5 min), placed consecutively into distilled water (5 sec, twice in 96%), ethanol (3 min each), isopropanol (2 min), and twice in xylol (3 and 10 min) (Sárkány and Szalai 1957). Preparations were covered with Neo-Mount®.

Pollen sampling was carried out by gel cubes (1-2 mm^3) touched to the anthers, then put on slides, warmed, covered and stained by fuchsin (Beattie 1971). Preparations were studied by Nikon Eclipse 80i, Olympus C01 stereo microscope, and Nicon Coolpix 4500 camera. Microphotos were taken by SPOT BASIC 4.0 program in each studied part.

3.3. Phytochemical studies

3.3.1. Thin-layer chromatography (TLC)

5.0 mL of ethanol: water (7:3) solvent mixture were added to 0.2 g of the powdered aerial part of the plant. The solutions were shaken at room temperature for 30 minutes (150 rpm, Edmund Bühler KL-2) and filtered. Reference solutions included 1.0 mg/mL rutin, hyperoside, chlorogenic acid and caffeic acid in methanol. Chromatography was performed on 20 cm × 20 cm silica gel 60 F_{254} aluminium sheet TLC plates (Merck, Darmstadt, Germany). 10 μL of plant extracts and 3-3 μL of reference solutions were applied with Minicaps capillary pipettes. After sample application, the plates were developed with mobile phase optimized for flavonoids (ethyl acetate : formic acid : acetic acid : distilled water = 100:11:11:27). Ascendant development chromatography was used in a saturated twin trough chamber. After chromatographic separation, the plates were dried, dipped into Naturstoff/PEG reagent (mixture of 10 g/L solution of diphenylboric acid aminoethyl ester in methanol and 50 g/L solution of PEG 4000 in methanol), dried at 105°C and examined at 365 nm.

3.3.2. LC-MS method

Polyphenols were extracted from homogenized samples (500 mg of aerial part) in 7.5 mL solvent mixture (methanol:water = 3:2 (v/v)) for 30 s. The mixture was sonicated for 20 min and centrifuged for 15 min (13.000 g, ambient). The extract was filtered through a 0.45-μm pore size syringeless filter (Mini-Uniprep, Whatman). The sample preparations were performed in triplicate ($n = 3$). Samples were stored in the dark at 4°C until the LC-MS analyses.

The concentrations of the quantified compounds were determined by an Agient 6530 Accurate-Mass Q-TOF LC/MS (Agilent, USA) with Jet stream ESI ion source coupled with an Agilent 1290 Infinity UHPLC (Agilent, USA) system. The MassHunter workstation software (Agilent, USA) was used to control the LC-MS system and for data processing.

Chromatographic separations were performed on an Ascentis Express C18 column (50 × 2.1 mm, 2.7 μm, Supelco, USA) and Supelquard Ascentis C18 precolumn (20 × 2.1 mm, 3.0 μm, Supelco, USA). For the separations, a gradient of mobile phase A (0.2 *v/v*% formic acid in water) and mobile phase B (0.2 *v/v*% formic acid in methanol) was used. The gradient profile was set as follows: 0.00 min 0% B eluent, 15.00 min 35% B eluent, 30.00 min 60% B eluent, 40.00 min 100% B eluent, 45.00 min 100% B eluent, 45.10 min 0% B eluent and 50.00 min 0% B eluent. The flow rate was 0.3 mL/min, the column temperature was set at 50 °C. The injection volume was 5 μL for the

extract and standard mixtures, as well. The column effluent passed through a diode array detector before arriving in the MS interface. The electrospray source operated in the negative ion mode with spectra acquired over a mass range of *m/z* 50-1100. The optimum values of ESI-MS parameters were: capillary voltage of +4.0 kV; dry gas temperature: 250 ^{o}C; dry gas flow: 8.0 l min-1; nebulizer pressure: 35 psig; sheath gas temperature: 230 ^{o}C; sheath gas flow: 11.0 l/min; and spectra rate 1 Hz. The limit of detection (LOD) was determined experimentally, and was taken as the concentration that produced a detector signal, which could be clearly distinguished from the baseline noise (3 times baseline noise). The limit of quantitation (LOQ) was taken as the concentration that produced a detector signal 10 times greater than the baseline noise. The optimization of the LC-MS conditions, the method validation and determination of polyphenolic components were performed.

3.3.3. Total polyphenol, flavonoid and tannin content

For total phenolic and flavonoid assays, 2.5 g of the aerial part, stem, root and leaf, as well as 1.25 g of the flower were powdered and extracted with 25.0 mL methanol solvent for 20 min. The extracts were placed in ultrasound bath (Nahita Digital Ultrasonic Bath) for 20 minutes at 40°C. Extracts were completed to 25.0 mL with the adequate solvents.

For the study of total polyphenolic content, 20 µL of each extract was placed into tubes with 1580 µL water and 100 µL Folin-Ciocâlteu reagent. In 5 min, 300 µL sodium carbonate (20% w/v) was added for each, then stored for 2 h at 20 ^{o}C. Spectrophotometric measurements (JKI UV/VIS-752N) were carried out at 765 nm for test solution compared with compensation solution (water and reagents). Polyphenol concentration was expressed in gallic acid equivalent (GAE) per 100 gram dry weight of plant.

Based on the monograph of Cynarae folium in Romanian Pharmacopoeia 10th, total flavonoid content was determined with slight modification. For 500 µL extracts, 1000 µL Na-acetate (100 g/L), 600 µL $AlCl_3$ (25 g/L), 1400 µL methanol, and 1500 µL water were added. After 15 min, absorbance was measured at 430 nm compared with compensation solution of extract mixture without reagents. Flavonoid concentration was expressed in quercetine equivalent (QE) per 100 gram dry weight of plant.

For tannin content, 0.75 g aerial part was powdered and added into 150 mL distilled water for heating. The cooled extracts were completed to 250 mL, filtered, then the first 50 mL the extract were used.

Total tannin content was detected based on the method of Pharmacopoeia Hungarica VIIIth applied skin powder and pyrogallol. 0.75 g of aerial parts were powdered and extracted with 150 mL solvent using distilled water for 30 min. Then the absorbance of total polyphenols, polyphenols not absorbed by skin powder and pyrogallol standard were determined. In each case the residue was diluted with distilled water, then phosphowolframic acid was added, and they were diluted with sodium carbonate (150 g/L). Spectrophotometric measurements were performed at 760 nm in 2 min compared with compensation solution of water (total polyphenol: A_1, polyphenols not absorbed by skin powder: A_2, pyrogallol as compensation solution: A_3). Each analysis was performed in triplicate. Tannin contents were calculated with formulas from Pharmacopoeia Hungarica VIIIth, expressed in pyrogallol as following:

$[62.5 \cdot (A_1-A_2) \cdot m_2] / A_3 \cdot m_1$

m_1 = weight of sample (g),

m_2 = weight of pyrogallol (g).

3.4. Antioxidant activity of *O. arvensis*

The analyses were performed at the Department of Laboratory Medicine, Medical School, University of Pécs, and at the Department of Pharmacognosy and Phytotherapy, University of Medicine, Pharmacy, Science and Technology "George Emil Palade" from Targu Mures. The antioxidant capacity of the aerial part of the plant was determined by three methods (ECL, DPPH, ORAC). The antioxidant capacity of the aerial part, leaf, root, stem and flower was examined by ABTS method.

0.25 g of each plant sample was ground, then 5 mL of 50% ethanol was added. The solutions were shaken at room temperature for 30 minutes (200 rpm). The extracts were filtered through a 0.45 μm pore-size filter (Mini-Uniprep, Whatman), then stored at -20°C until the analyses.

3.4.1. Chemiluminescence-based antioxidant assay (ECL)

Peroxidase and H_2O_2 working solutions:

POD stock (in phosphate buffer saline (PBS)) was diluted with BSA containing phosphate buffer and kept on ice (15 μU/mL). 10 M H_2O_2 was diluted with 0.1% citric acid to reach a concentration of 1360 μM and also kept on ice protected from light. The reagents were always prepared freshly prior to measurements.

ECL reagent: Luminol and p-iodophenol were prepared in 0.2 M boric acid/NaOH buffer (pH=9.6) and kept at 4°C (being stable for at least 1 month).

Trolox standard: 1 mM Trolox (dissolved in 50% ethanol) was prepared every week, and Trolox standards were diluted with the same solution used for the samples.

ECL antioxidant assay: The reaction was adapted to a plate reader using 96-well white optical plates (Perkin-Elmer). The enzyme working solution and the detection reagent were premixed in a ratio of 200 µL POD to 70 µL ECL reagent. Into each well, 20 µL Trolox/blank/sample and 270 µL of PODECL reagent were pipetted and the reaction was initiated by automated injection of 20 µL ice-cold H_2O_2 in citric acid. The final concentrations of the components in the wells were as follows: 0.97 µU/mL POD, 101.6 µM luminol, 406.4 µM p-iodophenol, 88 µM H_2O_2. Chemiluminescence signal was monitored for 20 min at 64 s intervals.

3.4.2. DPPH assay

For DPPH assay, 4 mg DPPH was prepared in 100 mL methanol (0.1 mmol/L) and kept in the fridge. Trolox standards were prepared in 50% ethanol. The assay was also adapted to a plate reader using standard 96-well plates (Sarstedt). Into each well, 20 µL Trolox/blank/sample and 180 µL DPPH solution were pipetted and the absorbance was read at 517 nm after 30 minutes of incubation in the dark at 25°C.

3.4.3. ORAC assay

4 µM Na-fluorescein (FL) stock was prepared in high purity water (stable for 1 week at 4°C). The working FL solution was made freshly by diluting the stock with 75 mM K-phosphate buffer (pH 7.5) at a 1:99 ratio (40 nmol/L FL working concentration). AAPH was also prepared before the measurements in phosphate buffer (9.22 mM). Trolox standards were used as described above. Into each well of black optical plates (Perkin-Elmer), 25 µL of blank/standard/ sample and 150 µL of diluted FL were used, and the plates were preheated to 37°C for 20 min. The outer wells of the plates were filled with 200 µL phosphate buffer, and only the inner 6 x 10 matrix was used for the assay. The reaction was initiated by automated injection of 25 µL AAPH solution into each well and fluorescence intensities were immediately monitored for 80 min (490/520 nm) at 150 s intervals. The final concentrations of the components in the wells were as follows: FL 30 nM, AAPH 1.15 mM.

3.4.4. ABTS radical scavenging activity

Extracts of the aerial part, leaf, root, stem and flower were prepared based on the description in the 3.3.3. subsection. Used this method, the antioxidant capacity of ethanol, methanol and water extracts was determined. ABTS oxidized with potassium persulfate resulted 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS●+), which was applied with slight modification using methanol. The concentration of ABTS●+ reduces affected by antioxidant compounds as proton donors.

ABTS radical cation (ABTS●+) was produced by reacting 7 mM ABTS solution with 16.5 g of 2.45 mM potassium persulfate, then 10 mg ABTS tablet was dissolved in 2.6 mL of the solution. The mixture was stored in the dark at room temperature for 12-16 h before use. The ABTS●+ solution was diluted with methanol to an absorbance of 0.90 (± 0.05) (meaning 50-100x dilutions) at 734 nm against ethanol as standard solution. The ABTS●+ solution and extracts were used for dilution series added 25, 30, 35, 40, 50 µL extracts to 2.6 mL ABTS●+ solution. Ascorbic acid (100 µM) was used as antioxidant standard for dilution series, similarly to the 2.6 mL ABTS●+ solution.

3.4.5. Equipments and data interpretation

The chemiluminescence-based antioxidant assay was performed using a Biotek Synergy HT plate reader equipped with programmable injectors. At each luminescence reading the measuring time/well was 0.2 s and the light output was followed for 20 min at 64 s measuring intervals. For data quantification, a calibration range of 0-150 µM Trolox was prepared in 50% ethanol, and a 32-fold dilution of the plant extracts was made in 50% ethanol (n = 12 replicates for each sample). The antioxidant capacity of the plant extracts was calculated from the regression equation obtained for the standards, multiplied by the dilution factor and expressed as µM Trolox equivalent (TE). TE was referred to 1 g of initial dry material for each plant part.

The DPPH assay was performed by a Perkin-Elmer EnSpire Multimode reader. Trolox standards were used in 50% ethanol in the range of 0 - 200 µM. Antioxidant capacities were calculated in two ways: a) using the equation of the calibration line; b) expressing the antioxidant activity of the extracts in % by this formula: $(A_{blank} – A_{sample}/A_{blank}) \times 100$ (Lu et al. 2014). TE values were also calculated for 1 g of dried plant material.

For the ORAC assay fluorescence intensities were monitored for 80 min at 150 s sampling intervals using the Biotek Synergy HT plate reader. The area under the curve (AUC) was determined for

the blank and this light output was subtracted from each standards/samples. In this way, net AUC (nAUC) was used for quantification of antioxidant capacity.

Spectrophotometric measurements (JKI UV/VIS-752N) were carried out for the ABTS assay. The absorbance value was measured after 6 minutes. We expressed the antioxidant activity of the extracts in % by this formula: $(A_{blank} – A_{sample}/A_{blank}) \times 100$.

3.4.6. Statistical analysis

Data of all determinations were performed in triplicate, expressed as mean ± standard deviation (mean ± SD), and they were analyzed by GraphPad Prism statistical program (paired t-test, Spearman-rank correlation). The value difference was statistically significant considered if $p \leq 0.05$.

3.5. Microbiological study

For microbiological analysis, the leaf and stem of the plant were ground (1 g each). Samples were suspended in 29 mL methanol in Erlenmeyer flasks and they were shaken at 150 rpm for overnight. Filtration and evaporation resulted altogether 6 phases including methanol, *n*-hexane, chloroform, ethyl-acetate, butanol, and aqueous extracts (Lee et al. 2014). The whole method was carried out in triplicate and the final different dried residues were dissolved in dimethyl sulfoxide (DMSO) to give the same concentration than in extraction solvents. The different extracts of plant stem and leaf were tested against *Staphylococcus aureus* (ATCC®25923), *Escherichia coli* (ATCC®25922), *Pseudomonas aeruginosa* (ATCC®27853), *Salmonella* Typhimurium (abbreviated scientific name of *Salmonella enterica* subsp. *enterica* (Le Minor and Popoff) serovar. Typhimurium, ATCC®14028), and *Candida albicans* (ATCC® 90028).

3.5.1. Tube dilution method for determination of minimum inhibitory concentration of plant extracts

The antimicrobial potential of each extracts were investigated based on the protocol of Clinical and Laboratory Standards Institute (CLSI). 100 µL of each extract were added to 1900 µL of Mueller-Hinton broth with 0.3% Tween 20, and twofold dilutions were performed in 1 mL of culture medium containing tubes. Mueller-Hinton broth alone was used for dilution of water extracts. Each inoculum was standardized to MacFarland 0.5 (~1.5 × 108 colony forming unit = CFU/mL) by spectrophotometer (NovospecTM Plus Amersham Biosciences), and each tube was inoculated. After inoculation they contained approximately 5 × 105 CFU/mL colony (CLSI 2012).

Following overnight incubation at 37 °C, each tube was examined for visible turbidity that means bacterial growth. Subcultures were performed from unturbid tubes checking the bactericid and/or bacteriostatic effects of extracts on Mueller-Hinton agar medium. The growth of colonies was checked on the medium after incubation for 24 h at 37 °C. The same method was applied in the investigation of extracts' effect for growth of *Candida albicans* in Sabouraud medium. Antimicrobial activity of solvent dilutions was also checked and their results were compared with the extracts' dilutions. Minimum inhibitory concentration (MIC) values were determined both in leaf and stem extracts. The study was carried out in triplicate.

3.5.2. Microdilution method for determination of minimum inhibitory concentration of plant extracts

Dried extracts were solved in 2% DMSO in concentration consistent to those of solvents, diluted by twofold dilution in Mueller-Hinton broth on 96 wells microplates, and inoculated by microorganisms similarly to tubes, so that final concentrations in microwells were less than 1% DMSO, which has no effect on bacterial growth. A 1% DMSO was used as control and was inoculated. Following overnight incubation each inoculated well was checked for turbidity. Each extract's dilution was checked in triplicate for each strain. MIC values were demonstrated by the comcentrations of dilutions, which did not showed the reproduction of microorganisms.

4. Results and discussion

4.1. Etnopharmacobotanical data in Homoródalmás

Majority of ethnomedicinal data collected from 60 informants aged between 11 and 95 was preserved mostly by women in the village. During our ethnobotanical collection, 161 plants and 2 fungi, 11 animals and 20 other medicinal treatments were recorded. Among plants, 97 medicinal taxa (human medicine: 92 taxa, veterinary medicine: 20 taxa), 45 food plants, 14 fodder plants and 7 dyeing plants were mentioned. The used 13 plant parts were the followings: root, rhizome, bulb, tuber, aerial part, branch / bud, resin, leaf, flower, fruit, pseudo-fruit, seed, and cortex, from which the most frequently mentioned was the aerial part. The treatment of gastrointestinal diseases was most frequently mentioned among the treated disorders. The selected species were used mainly as a tea, foment, syrup, tincture, cream, and bath. This knowledge is based on traditional observation

and experience of local people, but sometimes data origined from media sources and books were also observed.

After data comparison with literature data, 33 plants were found in the VIIIth Pharmacopoeia Hungarica (2003). Further 95 species were pharmacologically tested in earlier reports, but no data was found for 2 species. Based on the mentioned data, plants were reviewed by the following sections: scientific (written by *italics*) and official Hungarian name, scientific and Hungarian family name, remedy, local plant and drug names (*italics)* corresponded to Ph. Hg. VIIIth. Traditional observation and experience of people were summarized in the Ethnobotanical section, cited also the explanations of people written literally with *italics*. In *Application* section, relevant pharmacological studies and publications were randomly selected from the last 20 years, underlined the safety use of the species recently. Sample section see below:

***Arnica montana* L. / mountain arnica (Asteraceae/sunflower family)**

Local name: *árnyika*

Drug: *Arnicae flos* (Ph. Hg. VIIIth)

Remedy: tincture (in official form: *Arnicae tinctura*, Ph. Hg. VIIIth)

Ethnobotanical data: flower in alcohol or brandy to gargle and sore throat

Application: mouthwash, mainly external use, stimulation of epithelium development, for ointments (Szabó 2005); extracts with antibacterial, antitumour, antioxidant, anti-inflammatory, fungicid and immunomodulant activity (Kriplani et al. 2017).

4.2. Histological features of *O. arvensis*

In the secondary thickened root, under the rhizodermis, cortex, isodiametric parenchyma cells in the middle part, vascular elements form a contiguous ring with medullary rays in the xylem.

In the secondary thickened stem, the cells of single-layered epidermis are anisodiametric. The flattened cells of lamellar collenchyma form a cortex under the epidermis. Sclerenchyma cells extend in half moonshaped form on the upper part of collateral open bundles, which arrange in concentric circles. Medullary rays are found between the vascular bundles, while the central part consists of several loosely arranged parenchyma cells forming the pith. The thickening type of the stem can be classified into Ricinus type involving intra- and interfascicular cambium, which produce concentric vascular elements not only in, but among the bundles, as well.

The adaxial surface of the leaf is covered by unbranching non-glandular and capitate glandular trichomes including 3 cells in the neck part. Both the adaxial and abaxial surfaces are covered by a thin cuticle layer. The epidermis is single-layered consisting of flattened cells. The leaf structure is dorsiventral, the heterogeneous mesophyll includes palisade (2 layers) and isodiametric spongy cells (4-6 layers), and several intercellular spaces. The collateral closed bundles are surrounded by sclerenchyma cells formed a protective sheath. Mesomorphic stomata are found on the abaxial side.

Stipules were observed at the base of the leaves surrounding the stem. The epidermis is one-cell-layered on both sides. The mesophyll consists of only spongy cells with intercellular spaces. Similar to the leaves, stomata are in mesomorphic position on the abaxial side.

The petiole is surrounded by a single-layered epidermis on both sides. In the mesophyll, collateral closed bundle is surrounded by isodiametric parenchyma cells and intercellulars, which also extend to the terminal parts of the petiole.

In the flower, sepals are covered by unicellular non-glandular hairs called bristles. The stomata are mesomorphic. The tracheas can be characterized by spiral cell wall thickening both in sepals and petals. The epidermis cells of petals are anisodiametric forming a single layer on both sides. The middle part is composed of 4-5 layers of isodiametric parenchyma cells and collateral closed bundles. The opolar and prolate pollen grains are 20-25 μm of diameter. The 3 apertures are combined including both colpus and porus (tricolporate aperture), like ectocolpus and one or more endoaperture.

4.3. Phytochemical study of *O. arvensis*

Using TLC method, caffeic acid, chlorogenic acid, rutin and hyperoside were identified in the aerial part of the plant.

During LC-MS analyses, for the separation and identification of polyphenols, a sensitive method of HPLC coupled with diode-array detector and electrospray ionization mass spectrometry was optimized. The optimized method was validated for the investigated polyphenolic compounds in terms of linearity, limit of detection, limit of quantification, precision, and accuracy. Altogether 19 polyphenolic compounds were identified and quantified by this method in *O. arvensis*. The dominant compound was eriodictiol. In addition, five phenolic acids (gallic acid, caffeic acid, chlorogenic acid, ferulic acid, and *p*-coumaric acid), two flavanones (eriodictiol, naringenin), four

flavonols (dihydro-quercetin, rutin, quercetin, kaempherol), two flavones (luteolin, apigenin), two flavan-3-ols (catechin, epicatechin), a dilactone (ellagic acid), a dihydrochalcon (phloridzin), and two stilbenoid derivatives (piceid, *trans*-resveratrol) were detected.

Among the studied polyphenols the dominant eriodictiol, naringenin, phloridzin, piceid and *trans*-resveratrol were detected as new compounds in large amount in the species.

Total polyphenol, flavonoid and tannin content was determined from various parts of the plant. Among the studied parts, the 50% ethanol extract of the flower showed the highest polyphenolic content, while the 50% ethanol extract of the flower and the aerial part showed the highest total flavonoid content. The total tannin concentration of the aerial part had a low value.

4.4. Antioxidant activity of *O. arvensis*

Antioxidant activity of *O. arvensis* using *in vitro* ECL, DPPH, ORAC and ABTS methods was measured for the first time. The 50% ethanolic extracts of the aerial part of the plant exerted a similar radical-scavenging effect measured by DPPH and ECL, however, considerably higher values were measured by ORAC method which data did not correlate with the results of the ECL and DPPH assays. Using ABTS method, the flower has the highest antioxidant capacity in all extracts (ethanol, methanol, water), while the aqueous extract of the root has an extremely low antioxidant value. Ethanol and methanol proved to be more effective solvents for the extraction of all studied plant parts, resulted markedly higher antioxidant capacities of these extracts. The total flavonoid and ABTS antioxidant values showed significant correlation. It is necessary to identify and characterize the active components, which could be responsible for the antioxidant activity. In conclusion, based on these preliminary results, *O. arvensis* could be a potential source of new antioxidant agent which should be further analyzed.

4.5. Antimicrobial activity of *O. arvensis*

The chloroform extract of the leaf showed strong inhibitory effect against *Escherichia coli* and *Candida albicans* (MIC = 51 and 12.75 μg/mL, respectively). Leaf hexane extract was effective only against *C. albicans* (MIC = 8 μg/mL), whereas ethyl acetate extract inhibited the growth of *Staphylococcus aureus* and *C. albicans* in 74 μg/mL and 37 μg/mL concentration. Ethyl acetate extract of the stem was effective against *S. aureus* and *C. albicans* in low concentrations (MIC = 16 and 8 μg/mL). Chloroform stem extract with 43.5 μg/mL had an impact only on the growth of

Pseudomonas aeruginosa. Almost the same concentration (46.5 μg/mL) of butanol extract had inhibitory effect on the growth of *Salmonella* Typhimurium. Minimum inhibitory concentrations of these extracts were higher against the investigated microorganisms than that of any other effective antibiotics or antifungal agents, which have MIC values between 0.03 and 8 μg/mL. We failed to detect any antimicrobial effect with methanol and aqueous plant extracts, the hexane stem extract, or with any extracts in DMSO. Other extracts of the plant showed similar antimicrobial activity than in the diluted extraction solvents in the same concentration. Certain extracts of *O. arvensis* in low concentration can evoke or increase the antimicrobial activities of low concentration extraction solvents. In conclusion, our results emphasize the promising degree of antimicrobial activity of *O. arvensis* against the tested microorganisms. These preliminary data should be supported by further large-scale studies in the future.

5. Novel findings

- The ethnobotanical fieldwork in Homoródalmás, Harghita County, Transylvania was carried out for the first time. During our collection we recorded ethnopharmacobotanical data of 97 medicinal plants / 161 plants used in human and veterinary medicine.
- After comparison with literature data, 33 species are described in the Pharmacopoeia Hungarica VIIIth. In addition, 95 species were noted mainly in pharmacological publications, but no data was found for 2 species.
- Local knowledge of medicinal plants plays a significant role in traditional primary health care in the human and veterinary medicine. The results highlight the ethnobotanical and ethnopharmacological relevance of the role of plants in the everyday life of the indigenous people. The ethnomedicinal practices are a valuable source of knowledge for different diseases, highlighting the relevance of field work in the selected region.
- Documentation, evaluation and comparison of ethnobotanical data and earlier reports, further species can be selected to investigate their phytochemical and pharmacological values, as in the case of field restharrow (*Ononis arvensis*) selected in this work.
- Histological features of the root, stem, leaf, sepal, petal, and pollen of *O. arvensis* were described for the first time.
- Caffeic acid, chlorogenic acid, rutin and hyperoside were identified in the aerial part of the plant by TLC.

- For the separation and identification of polyphenols, a sensitive method of LC-MS was optimized and validated, resulted 19 polyphenolic compounds identified and quantified in the aerial part of *O. arvensis*.
- Among the studied polyphenols the dominant eriodictiol, naringenin, phloridzin, piceid and *trans*-resveratrol were detected as new compounds in the species for the first time.
- Total polyphenol, flavonoid and tannin content were determined in various parts of the plant.
- We described the antioxidant activity of *O. arvensis* using *in vitro* ECL, DPPH, ORAC and ABTS methods for the first time.
- Antimicrobial effects of the stem and the leaf extracts were studied against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella* Typhimurium and *Candida albicans* for the first time.
- In conclusion, based on our preliminary results, further studies are required to complete the phytochemical and antimicrobial analysis in the case of field restharrow.

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7. List of publications

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