HISTOLOGICAL AND ANTIMICROBIAL STUDY
OF *ONONIS ARVENSIS* L.

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In this study field restharrow (*Ononis arvensis*) was investigated for histological and antimicrobial features. The aerial part and the root were embedded in synthetic resin and investigated following sectioning by a rotation microtome. The antimicrobial activity and minimum inhibitory concentration of the solvent fractions of the aerial part were studied against four bacterial strains and one fungus. According to histology, the root covered by rhizodermis contains contiguous vascular elements, which are surrounded by sclerenchyma cells. The epidermis cells are anisodiametric in the stem, sepal, and petal. The bundles of the stem form a Ricinus type thickening. The adaxial side of the heterogeneous leaf is covered by unbranching non-glandular and capitate glandular trichomes. The stipule, petiole, sepals and petals are isolateral having mesomorphic stomata. Pollen grains are tricolpate. The different extracts of the herb showed antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella* Typhimurium, *Staphylococcus aureus*, and *Candida albicans*. Data show that the extracts of the leaf contain compounds which may be responsible for the antifungal effect, while extracts obtained from display against the tested bacteria, except *Escherichia coli*. Further studies are required to complete the phytochemical analysis and identify the antimicrobial compounds of extracts.

Keywords: Field restharrow – histology – antimicrobial activity – minimum inhibitory concentration – bacteria – yeast

INTRODUCTION

The majority of recent medications derives originally from ancient herbal traditions. Medicinal plants with therapeutic value have been used for centuries. Numerous remedies, which have antifungal, antibacterial, and antiprotozoal activities, can be used systemically or locally both in human and veterinary medicine [17]. Plant based antimicrobials represent an outstanding undiscovered source for medicines because of their therapeutic potential. They are effective in the treatment of infectious diseases, and they simultaneously mitigate side effects, which are often associated with synthetic antimicrobials [18]. The exploration of these antimicrobials requires further analyses, which highlight, e.g. the importance of histological, phytochemical, and chemotaxonomical study of herbal drugs to identify their potential in the actual phytotherapy.

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Ononis genus (restharrows) is a large genus belonging to Fabaceae family. Many members of the genus were investigated for their phytochemical and pharmacological potential. Biochemical characterisation and identification of isoflavones and phenolic acids were described in *O. spinosa* L. [14, 19], more than 20 flavonoid aglycones in *O. fruticosa* L., *O. natrix* L., *O. tridentate* L. [38], and phenolic constituents in the root of *O. vaginalis* Vahl [1]. Isoflavones like ononin, and phytoestrogens, e.g. formonetin were identified in the root of *Ononis angustissima* L. [16]. Composition of the essential oil of *Ononis viscosa* L. subsp. *breviflora* (DC.) was identified by Erdemgil et al. [13]. Many *Ononis* species have been proven to have antibacterial, antifungal, antipyretic, and anti-inflammatory activities [1–3, 8, 23]. Decoctions from these plants have been reported to apply in gout, skin and rheumatic diseases [22]. The aqueous and ethanolic extracts of the aerial part of *O. macrosperma* Hub.-Mor. have remarkable wound healing and anti-inflammatory activities [31]. The styrylcoumarin derivatives of the root of *O. vaginalis* have shown antiviral activity against herpes simplex virus type 1 and weak cytotoxicity [1]. The dimethyl sulfoxide (DMSO) extracts of *O. natrix* [2, 24], and *Ononis viscosa* were assessed against some bacterial strains [12]. *Ononis spinosa* extracts have exhibited inhibitory effect similar to ofloxacin against *Listeria monocytogenes* ()*, L. ivanovii, L. innocua,* and *L. murrayi* in agar diffusion and macrodilution assay [3], and also have antimicrobial activity against other Gram-positive and Gram-negative bacteria, and *Candida* species [4, 8, 23].

In our study field restharrow (*Ononis arvensis* L., syn.: *Ononis hircine* Jacq.) was selected for analyses based on a few earlier reports. The plant is a perennial shrub living in the humid fields of Europe. The 50–100 cm high erect stem is covered by trichomes. It has elliptical leaves and pink flowers [37]. The stem, leaf and root of the plant were studied for main histological features like multilayer tissues, cell wall thickening of vascular elements, crystals, stomata and trichome types [26]. In contrast to the commonly used root of *O. spinosa*, the aerial part of *O. arvensis* has been studied for essential oil [10], hydroxycinnamic acid [28], flavonoid [20, 27, 29, 33–36], isoflavon, triterpene (e.g. α-onocerin), and coumarine (scopolin, scopoletin) content [26, 30].

To complete earlier anatomical description of the species [26], the aim of this study was to characterize the histological features of the root, stem, leaf, petiole, stipule, flower parts, and pollen. The goal was also to provide information on the antimicrobial effect of the extracts made of flowering stem and leaf against four bacterial strains and a yeast.

MATERIAL AND METHODS

Data analysis and sample collection

The analysis of data obtained from scientific databases (PubMed, Science Direct and Scopus) focused on the histological, phytochemical, antimicrobial, and pharmacological data 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. Herbarium specimens have been deposited and labelled with unique code at the Department of Pharmacognosy, University of Pécs, Hungary.

Histological preparation

The root, stem, leaf, petiole, stipule, and flower were fixed in a mixture of 96% ethanol : glycerine : distilled water (1:1:1). Ethanol and glycerine were purchased from Molar Chemicals Ltd., Hungary. Dehydration followed in ethanol series (in 30, 50, 70 and 96% for 12, 12, 24 and 3 hours, respectively), then plant materials 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), in isopropanol (2 min), and twice in xylol (3 and 10 min) [25]. Preparations were covered with Canada balsam purchased from Merck Ltd., Hungary.

The leaf and flower parts were studied by leaf surface cast method. Leaf samples were cut into 2×2 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 there after 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 Canada balsam. 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. Pollen grains were stained by fuchsin based on Beattie's work [6].

Leaves were studied by Nikon Eclipse 80i, while flower parts by IOR Bucharest ML-4M microscope, Olympus C01 stereo microscope, and Nicon Coolpix 4500 camera. Microphotos were taken by SPOT BASIC 4.0 program in each studied part.

Preparation of extracts for microbiological study

For microbiological analysis, the leaf and stem were grounded in a mortar (1 g each). Samples were suspended in 29 mL methanol in Erlenmeyer flasks and they were agitated at 150 rpm for overnight. The extracts were filtered and evaporated at room temperature, then they were resuspended in 2 mL methanol. One mL of these extracts were mixed with 5 mL distilled water and 5 mL of *n*-hexane. After phase separation, *n*-hexane layers were collected and evaporated, and dry materials were measured. The aqueous phases were further extracted by the same method using chloroform, ethyl acetate, and butanol. Each extract was redissolved in 5 mL appropriate extraction solvents. This method was described previously by Lee et al. [21]. The whole method was carried out in triplicate and the final different dried residues were dis-

solved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to give the same concentration than in extraction solvents.

Microbial strains and culture media

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). Standard Mueller-Hinton broth and agar without and with 0.3% Tween 20 were used as a bacterial culture media, as well as Sabouraud broth and agar without and with 0.3% Tween 20 were applied for cultivation of *C. albicans* strain.

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

A 100 μL of each extract in appropiate extraction solvent were given 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 standardised to MacFarland 0.5 ($\sim 1.5 \times 10^8$ colony forming unit = CFU/mL) by spectrophotometer (Novospec™ Plus Amersham Biosciences), and each tube was inoculated. After inoculation they contained approximately 5×10^5 CFU/mL [9].

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 bactericidal 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.

Microdilution method for determination of minimum inhibitory concentration of plant extracts

Same concentration of each extract in 2% DMSO than in extraction solvents was applied and diluted by twofold dilution in Mueller-Hinton broth on 96 wells microplates and was inoculated by microorganisms similarly to tubes, so that final concen-

trations 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 triplicate for each strain.
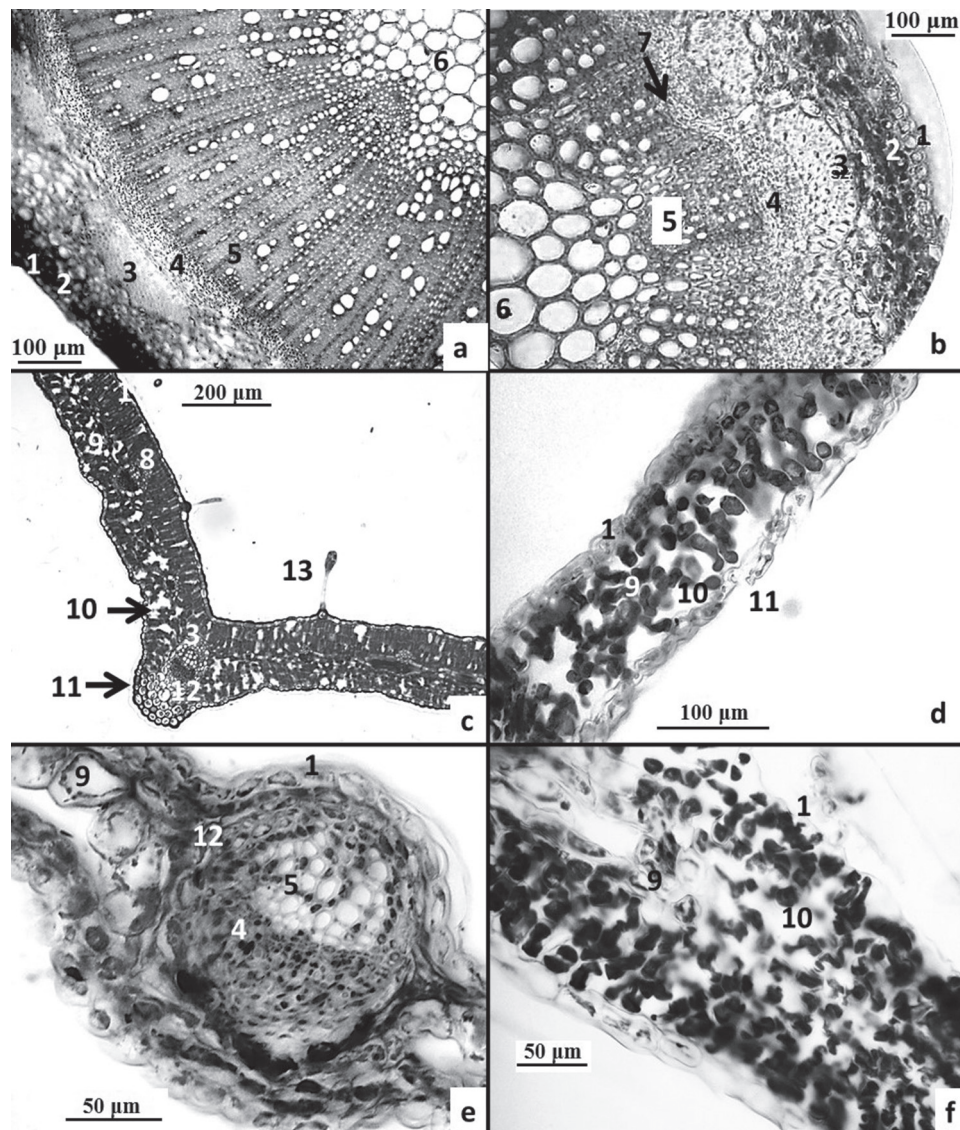

Fig. 1. Histological features of the vegetative parts of *Ononis arvensis*. (a) root, (b), stem, (c) leaf, (d) stipule, (e–f) petiole. In each photo: 1. epidermis, 2. lamellar collenchyma, 3. sclerenchyma, 4. phloem, 5. xylem, 6. parenchyma, 7. cambium, 8. palisade parenchyma, 9. spongy parenchyma, 10. intercellular space, 11. mesomorphic stomata, 12. collateral closed bundle, 13. capitate glandular trichome
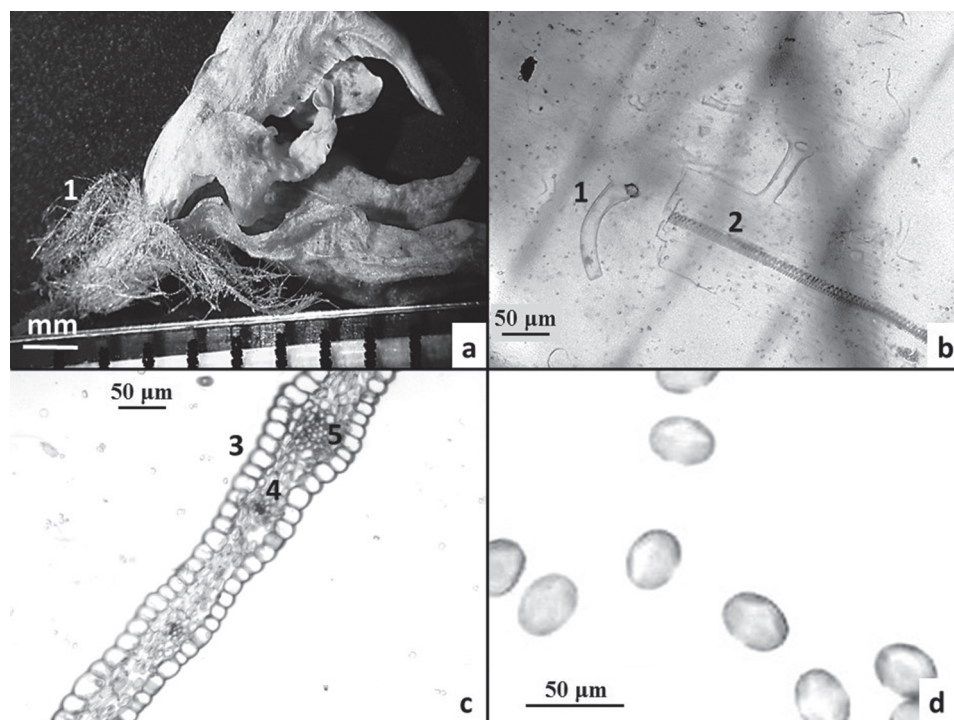
Fig. 2. Histological features of generative parts of *Ononis arvensis.* (a) flower (by stereo microscope), (b) cleared sepal, (c) petal, (d) tricolpate pollen grains. In each photo: 1. non-glandular trichomes, 2. spiral wall thickening of tracheas, 3. epidermis, 4. spongy parenchyma, 5. collateral closed bundle

Table 1

Fraction concentration of *Ononis arvensis* leaf extracts after evaporation redissolved in solvent and applied for dilution and determination of MIC value

Solvents	Weight of extract after evaporation (mg)	Concentration after redissolved in 5 mL solvent (mg/mL)	Weight in 100 μL solvent (μg)	Started concentration for dilution (μg/mL)
Methanol	185.6	18.56	1856	928
Hexane	0.8	0.16	16	8
Chloroform	10.2	2.04	204	102
Ethyl acetate	7.4	1.48	148	74
Butanol	59.5	11.9	1190	595
Water	21.5	4.3	430	215

RESULTS

Histological features

A lamellar collenchyma consisting of 3–4 cell layers was observed under the rhizo-dermis in the root (Fig. 1a). The vascular elements surrounded by sclerenchyma cells form a contiguous ring with medullary rays in the xylem. Isodiametric parenchyma cells can be found in the centre.

In the stem, the cells of single-layered epidermis are anisodiametric (height/width falls within the range of 1.5–3.0) (Fig. 1b). The flattened cells of lamellar collen-chyma form a cortex under the epidermis. Sclerenchyma cells extend in half moon-shaped 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 thicken-ing type of the stem can be classified into Ricinus type involving the intra- and inter-fascicular 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 capi-tate glandular trichomes with 3 cells in the neck. Both the adaxial and abaxial sur-faces are covered by a thin cuticle layer. The epidermis is single-layered consisting of flattened cells. The leaf structure is dorsiventral (bifacial). The heterogeneous mesophyll includes palisade (2 layers) and isodiametric spongy cells (4–6 layers), and several intercellular spaces (Fig. 1c). The collateral closed bundles are surround-ed by sclerenchyma cells which form a protective sheath. Mesomorphic stomata are found on the abaxial side (hypostomatic leaf).

Stipules were observed at the base of the leaves surrounding the stem. The epider-mis is one-cell-layered on both sides. The mesophyll consists of only spongy cells (isolateral structure) with intercellular spaces. Similar to the leaves, stomata are in mesomorphic position on the abaxial side (Fig. 1d).

The petiole is surrounded by a single-layered epidermis on both sides (Fig. 1e–f). In the mesophyll, collateral closed bundle is surrounded by isodiametric parenchyma cells and intercellulars (Fig. 1e), which also extend to the terminal parts of the petiole (Fig. 1f).

In the flower, sepals are covered by unicellular non-glandular hairs called bristles (Fig. 2a–b). 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 (isolateral structure) and collateral closed bundles (Fig. 2c). The opolar and prolate pollen grains are 20–25 μm of diam-eter. The 3 apertures are combined including both colpus and porus (tricolpate aper-ture) (Fig. 2d).

Table 2
Minimum inhibitory concentration of leaf extract fractions (µg/mL) with solvents

Solvents	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Salmonella typhimurium*	*Candida albicans*
Methanol 2% DMSO	928< (5%) 928<	928< (5%) 928<	928< (5%) 928<	928< (5%) 928<	928< (5%) 928<
Hexane 2% DMSO	8< (5%) 8<	8< (5%) 8<	8< (5%) 8<	8< (5%) 8<	8* (5%) 8<
Chloroform 2% DMSO	**51*** (2.5%) 102<	102≤ (5%) 102<	102≤ (5%) 102<	51≤ (2.5%) 102<	**12.75*** (0.625%) 102<
Ethyl acetate 2% DMSO	74≤ (5%) 74<	74≤ (5%) 74<	**74*** (5%) 74<	74≤ (5%) 74<	**37*** (2.5%) 74<
Butanol 2% DMSO	595≤ (5%) 595<	148≤ (1.25%) 595<	595≤ (5%) 595<	595≤ (5%) 595<	287≤ (2.5%) 595<
Water	215<	215<	215<	215<	215<

*Antimicrobial effect of extract; ( ): extraction solvent concentration.
≤ Effects of concentration of diluted control solvent and that of the extract in the same concentration of solvent are equal (started concentrations of solvents were 5% in each extract and solvent control dilution).
< No antimicrobial effect of extract in this concentration.

Table 3
Fractions concentration of *Ononis arvensis* stem extracts after evaporation redissolved in solvent and applied for dilution and determination of MIC value

Solvents	Weight of extract after evaporation (mg)	Concentration after redissolved in 5 mL solvent (mg/mL)	Weight in 100 µL solvent (µg)	Started concentration for dilution (µg/mL)
Methanol	91.6	9.16	916	458
Hexane	0.6	0.12	12	6
Chloroform	8.7	1.74	174	87
Ethyl acetate	1.6	0.32	32	16
Butanol	9.3	1.86	186	93
Water	9.8	1.96	196	98

Minimum inhibitory concentration of plant extracts

The quantities of dry residues were different after evaporation (Tables 1, 3). Each extract was redissolved in 5 mL extraction solvents and in appropriate volume of DMSO to use as stock solutions for MIC determination. The stock solutions concen-

trations of the same extracts were equal, but the concentrations of different extracts' stock solution were not the same.

The chloroform extract of the leaf with extraction solvent showed strong inhibitory effect against *Escherichia coli* and *Candida albicans* (MIC values were 51 µg/mL and 12.75 µg/mL, respectively). Leaf hexane extract was effective only against *Candida albicans* (MIC value 8 µg/mL), whereas ethyl acetate extract inhibited growth of *Staphylococcus aureus* and *C. albicans* in 74 µg/mL and 37 µg/mL concentration (Table 2). 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 impact only on the growth of *Pseudomonas aeruginosa*. Almost the same concentration (46.5 µg/mL) of butanol extract had inhibitory effect on growth of *Salmonella typhimurium* (Table 3). Minimum inhibitory concentrations of these extracts were higher against the investigated microorganism (Tables 2, 4) 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 methanolic and aqueous plant extracts, or with the hexane stem extract with extraction solvent, or with any extracts in DMSO. Other extracts of the plant showed similar antimicrobial activity to the diluted extraction solvents in the same concentration.

Table 4
Minimum inhibitory concentrations of stem extract fractions (µg/mL) with solvents

Solvents	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Salmonella typhimurium*	*Candida albicans*
Methanol 2% DMSO	458< (5%) 458<	458≤ (5%) 458<	458< (5%) 458<	458< (5%) 458<	458< (5%) 458<
Hexane 2% DMSO	6< (5%) 6<	6< (5%) 6<	6< (5%) 6<	6< (5%) 6<	6< (5%) 6<
Chloroform 2% DMSO	87≤ (5%) 87<	43.5* (2.5%) 87<	43.5≤ (2.5%) 87<	87≤ (5%) 87<	43.5≤ (2.5%) 87<
Ethyl acetate 2% DMSO	16≤ (5%) 16<	16≤ (5%) 16<	16* (5%) 16<	16≤ (5%) 16<	8* (2.5%) 16<
Butanol 2% DMSO	93≤ (5%) 93<	23.25≤ (1.25%) 93<	93≤ (5%) 93<	46.5* (2.5%) 93<	46.5≤ (2.5%) 93<
Water	98<	98<	98<	98<	98<

*Antimicrobial effect of extract; ( ): extraction solvent concentration.
≤ Effects of concentration of diluted control solvent and that of the extract in the same concentration of solvent are equal (started concentrations of solvents were 5% in each extract and solvent control dilution).
< No antimicrobial effect of extract in this concentration.

DISCUSSION

The observed histological features of the root of field restharrow are now fully described for the first time involving collenchyma and sclerenchyma, completing the anatomical data reported earlier [26]. In the stem, Ricinus type thickening and sclerenchyma cells were observed as new data besides flattened cells of lamellar collenchyma documented earlier [26].

Based on previous reports [11], non-glandular and capitate glandular trichomes were also found on the adaxial surface of the leaf, which we complemented with 3 cells in the neck of the trichomes. The heterogeneous mesophyll of dorsiventral leaf [11, 26] is composed of palisade and spongy cells, and collateral closed bundles encompassed by sclerenchyma cells [26]. Stipules, which are characteristic for to Fabaceae family [37], can be found at the base of the leaves. Histological structure of the petiole, the sepals and petals involving cell shape of epidermis, bundle, stomata and trichome types, as well as pollen characteristics were documented for the first time.

The antimicrobial activities of *Ononis* species have been reported in many studies [1–3, 5, 7, 8, 23, 24]. In our study, antibacterial effects were detected in all stem and leaf extracts compared to extraction solvents' effect in the same concentration. Antimicrobial effects of these extracts were noticed against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella typhimurium* at the first time. It was surprising that the extracts dissolved in DMSO did not show any antimicrobial activity in the same concentrations, which have inhibition for bacterial growth. The low concentrations can explain that ineffectivity. Certain extracts of *O. arvensis* in low concentration can evoke or increase the antimicrobial activities of low concentration extraction solvents.

Our results were compared with data of other *Ononis* species, mainly the more frequently investigated *O. spinosa,* which also inhibited the growth of *S. aureus, E. coli, P. aeruginosa,* and *Bacillus subtilis* [8]. Certain leaf and stem extracts of *O. arvensis* have shown antifungal activity in low concentration against *Candida albicans* similar to the aqueous and ethanolic extracts of *O. spinosa* (MIC = 1.25 μg/ mL) [4, 23], which were also effective against *C. glabrata* (only *ethanol extract,* MIC = 5.00 μg/mL) *and C. krusei,* but were not effective against other clinical *Candida* species [4, 8, 23]. The ethyl acetate extract of *Ononis natrix* inhibited the growth of *Bacillus subtilis* and *B. brevis* (MIC 12.5–50 μg·mL^{-1}) [5], while ethanol, hexane, acetone and methanol extracts were described to have small inhibitory activity against *S. aureus, Staphylococcus epidermidis, S. saprophiticus* similar to *O. arvensis,* but they were ineffective against *E. coli, E. faecalis, Shigella flexneri, Enterobacter cloaceae, Klebsiella pneumonia, Aeromonas hydrophila,* and *Serratia marcescens,* as well as for *P. aeruginosa,* which was more resistant to antimicrobial agents [2, 7]. The ethanolic extracts of *O. hirta* and *O. sicula* have potential antimicrobial activity (in more than 1000 μg/mL concentration) against MRSA, *Bacillus cereus, E. coli, P. aeruginosa, S. typhimurium,* C. *albicans,* and *Aspergillus niger.* The *n*-hexane extract of *O. hirta* aerial parts showed stronger activity against *P. aerugi-*

nosa (MIC of 125 μg/mL) [32]. In addition to plant extracts, the essential oil of *O. angustissima* L. exerted significant antibacterial effect against *Enterococcus faecalis* (MIC = 0.07 mg/mL) and *P. aeruginosa* (MIC = 0.31 mg/mL) [15].

In the present study the histological and antimicrobial results provide new data for the possible medicinal use of *Ononis arvensis*. However, the minimum inhibitory concentrations of plant extracts with small amount of extraction solvents were higher than the MIC values of antibiotics, which are applied for treatment of infections, they showed strong antimicrobial activity against the tested microorganisms. Despite the effective extracts contain small concentration of extraction solvents they can be applied with these in the topical treatment of superficial infection if their cytotoxicity is checked in human tissue culture. It is known the phenol is harmless and cytotoxic, but in small concentration (1.4%) is used in topical treatment. Determining the exact chemical compounds or components of the extracts, which have antimicrobial effect requires further investigation with more advanced techniques and other microorganisms. The isolated components without toxic solvents might have on effect against bacterial and fungal pathogens in very low concentration, similarly to antibiotics.

In conclusion, our results emphasize the promising degree of antimicrobial activity of *Ononis arvensis* against the tested microorganisms. Therefore these preliminary data should be supported by further large-scale studies in the future.

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