ORIGINAL ARTICLE

COMPARATIVE HISTO-ANATOMICAL RESEARCHES ON THE VEGETATIVE ORGANS AND ASSESSMENT OF ANTIOXIDANT CAPACITY OF TWO SPECIES FROM *EQUISETUM GENUS*

ANNAMARIA PALLAG, BIANCA PAŞCA, TÜNDE JURCA, RAMONA SUCIU*, SEBASTIAN NEMETH, LAURA VICAŞ

University of Oradea, Faculty of Medicine and Pharmacy, Oradea, Romania

**corresponding author: dr_ramona_suciu@yahoo.com*

Manuscript received: December 2015

Abstract

This paper presents a series of researches conducted on two species belonging to the *Equisetum genus, Equisetum arvense L.* and *Equisetum telmateia* Ehrh., by establishing the amount of flavonoids, phenolic acids, antioxidant capacity and histo-anatomical characters. In the Romanian Pharmacopoeia 10th edition and in the European Pharmacopoeia (Ph. Eur. 7) only *Equisetum arvense L.* is mentioned as a source for *Equiseti herba*. The investigations showed differences regarding the histo-anatomical structures and the level of substances with antioxidant capacity which could be considered as a criterion for identifying possible substitutions.

Rezumat

În această lucrare sunt prezentate cercetările întreprinse asupra speciilor din genul *Equisetum*, respectiv *Equisetum arvense* L. și *Equisetum telmateia* Ehrh, privind cantitatea de flavonoide, acizi fenolici, capacitatea antioxidantă precum și caracterele histo-anatomice. În Farmacopeea Română, ediția a X-a și în Farmacopeea Europeană este prevăzută numai specia *Equisetum arvense* L., ca sursă pentru *Equiseti herba*. În urma cercetărilor s-au evidențiat diferențe histoanatomice, precum și la nivelul conținutului de substanțe cu capacitate antioxidantă, acestea putând constitui criterii de identificare a posibilelor substituții și confuzii.

Keywords: *Equisetum arvense* L., *Equisetum telmateia Ehrh*., antioxidant capacity, histo-anatomical characters

Introduction

Equisetum arvense L., field horsetail *and Equisetum telmateia* Ehrh. (sin. *Equisetum maixmum* Lam.), great horsetail, from *Equisetaceae* family is herbaceous perennial ferns, that throughout their life cycle exists as a pale yellowish non-photo-synthetic spore-bearing fertile stem, produced in early spring. Green photo-synthetic sterile stems are produced in late spring and persist to late autumn. They are heavily branched [16, 17].

Medicinal plants belonging to the *Equisetum genus* are often used in traditional medicine, for tea and other therapeutic products. They are highly efficient in treating urinary tract infections, cardiovascular diseases, respiratory tract infections and medical skin conditions [4, 16].

In the Romanian Pharmacopoeia 10th edition [21] and in European Pharmacopoeia (Ph. Eur. 7) [22] only *Equisetum arvense* L. is mentioned as a source for *Equiseti herba*. Sterile stems are used as a treatment of diseases such as inflammation, anaemia, diabetes, ulcers, cancer, convulsions, anxiety and depressive disorders [8, 10, 11].

Equisetum telmateia Ehrh. (sin. *Equisetum maixmum* Lam.), great horsetail, is a fern with many traditional uses. Relevant literature review has shown very little data about the biological activity of the great horsetail [3]. In recent years, many researches have proven that inorganic acids, salts, phenolic acids, flavonoids, alkaloids and volatile components are major biologically active compounds with diuretic, antiseptic, anodyne, cardiac, carminative, galactagogue, diaphoretic, anticancer, neuroprotective, antiulcerogenic, anti-microbial and antioxidative properties [3, 8, 10, 11, 14].

Therefore, the purpose of this study was to initiate comparative researches for these two species, to determine the total phenolic content, the concentrations of flavonoids, using spectrophotometric methods, antioxidant capacity, together with histo-anatomical characters and to evaluate these two species as new potential sources of natural antioxidants.

Phenolic compounds, especially flavonoids from plants, are a very important group of natural compounds with antioxidant and antimicrobial activity, with large applications in medicine and pharmacy [5, 13].

Materials and Methods

Plant materials

Sterile stems of *Equisetum arvense* L., *Equisetum telmateia* Ehrh. (sin. *Equisetum maximum* Lam.) were used. They were collected from different and

unpopulated geographical areas of Bihor County, in April and September 2015. From the collected sterile stems and ramifications, we made microscopically sections, in order to perform the chemical analysis. They were dried at a median temperature of 40°C, for 96 h.

Determination of polyphenols content

Total phenolic content was determined by the Folin Ciocalteu method. Using Folin-Ciocalteu method the OH groups can be evaluated from the studied sample, in an alkaline medium (adjusted with sodium carbonate). The absorbance, measured at the wave-length of 765 nm, increases proportionally with the number of the polyphenols OH groups. The extract solution (0.1 mL) containing 1000 µg of the extract was mixed with 46 mL distilled water in a volumetric flask and 1 mL Folin-Ciocalteu (Merck) reagent was added, and the flask was thoroughly shaken. The mixture was allowed to react for 3 min and 3 mL aqueous solution of 2% Na_2CO_3 was added. At the end of the 2 hours incubation, at room temperature, the absorbance of each mixture was measured at 765 nm using a Shimadzu UV-1700 Pharmaspec UV-VIS Spectrophotometer. The same procedure was also applied to the standard solutions of gallic acid, and a standard curve was obtained [7, 9, 17, 20]. The calibration curve (Figure 1) was obtained with gallic acid solutions of known concentrations (0-60 ppm), and the concentration of polyphenol extracts was calculated from the regression equation and expressed as mg gallic acid equivalents (GAE) / 100 g dry sample.

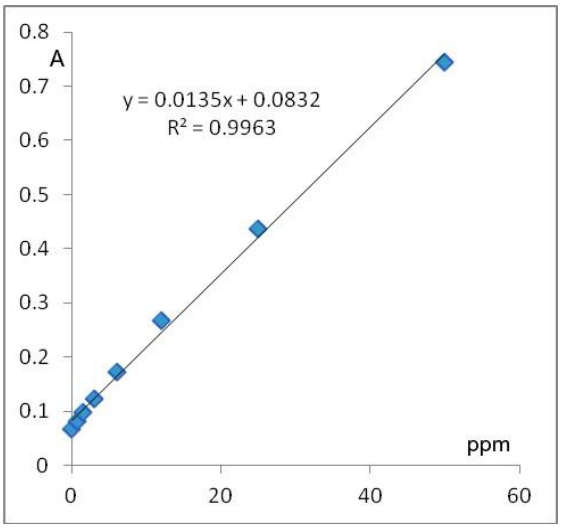

Figure 1.
The calibration curve made with gallic acid for Folin-Ciocalteu method in alcoholic medium

The total flavonoid content was determined using a previously described colorimetric method [5, 7, 18]. 1 mL sample (containing 0.1 mg/mL dry substance) was mixed with 4 mL water and inserted in a 10 mL volumetric flask.

Firstly, 3 mL 5% $NaNO_2$ solution were added, after 5 minutes 0.3 mL 10% $AlCl_3$ and after 6 minutes 2 mL 1 M NaOH. The flask was filled up to its calibration mark with distilled water. The solution was mixed and its absorbance was detected at 510 nm [5] in a Shimadzu UV-1700 Pharmaspec UV-VIS Spectrophotometer. The calibration curve (Figure 2) shall create its standard using quercetin (QE).

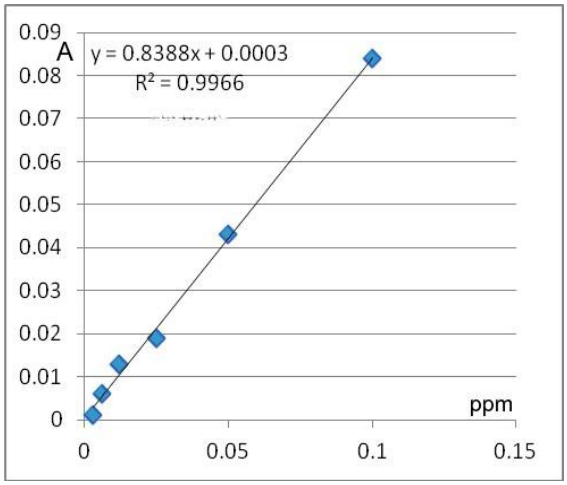

Figure 2.
Calibration curve made with quercetin in alcoholic medium (surroundings, environment)

The antioxidant capacity of sterile stems extracts

FRAP method (ferric reducing antioxidant power)

FRAP method is a simple spectrophotometric method, that assesses the antioxidant power of the studied samples, being based on the reduction of ferric tripyridyltriazine complex [Fe(III)-TPTZ] using a reductant, at an acid pH. The stock solutions included: 300 mM acetate buffer; 270 mg $FeCl_3 \cdot 6\ H_2O$ dissolved in 50 mL distilled water; 150 mg TPTZ and 150 µL HCl, dissolved in 50 mL distilled water. The working FRAP solution was freshly prepared *ex tempore* by mixing 50 mL acetate buffer, 5 mL $FeCl_3 \cdot 6\ H_2O$ solution and 5 mL TPTZ solution. Trolox was used as a standard solution, the calibration curve was made for concentrations between 0 and 300 µM, having a correlation coefficient $R^2 = 0.9956$ and the regression equation $y = 0.0017x + 0.0848$, where *y* represents the absorbance detected at 595 nm. The results are expressed as µmol Trolox equivalents (TE) / 100 µL extract [1, 2, 3, 19].

DPPH method

The radical scavenging activity of plant extracts against stable 2,2-diphenyl-2-picryl-hydrazyl-hydrate (DPPH) was determined by the slightly modified method of Brand-Williams *et al.* 1995 [2, 18]. DPPH reacts with an antioxidant compound, which can donate hydrogen, and reduce DPPH. The change in color (from deep

violet to light yellow) was measured at 517 nm on a UV visible light spectrophotometer. The $6 \cdot 10^{-5}$ M DPPH in methanol solution was prepared *ex tempore* daily, before UV measurements. The samples were kept in the dark for 15 minutes at room temperature and the decrease in absorbance was measured. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula:

$$\% \text{ Inhibition} = [(AB - AA) / AB] \times 100$$

where AB = absorption of blank sample (t = 0 min), AA = absorption of test extract solution (t = 15 min) [1, 8, 15, 18].

Cupric ions (Cu^{2+}) reducing-Cuprac assay

In order to determine the cupric ions (Cu^{2+}) reducing antioxidant capacity the method proposed by Karaman *et al.* was used with slight modifications [2]. To this end, 0.25 mL $CuCl_2$ solution (0.01 M), 0.25 mL ethanolic neocuproine solution (7.5×10^{-3} M) and 0.25 mL CH_3COONH_4 buffer solution (1 M) were added to a test tube, followed by mixing with the plants extracts. Then, total volume was adjusted to 2 mL with distilled water, and thoroughly mixed. The tubes were stoppered and kept at room temperature. Absorbance was measured at 450 nm against a reagent blank 30 min later. Increased absorbance of the reaction mixture indicates increased reduction capability [2, 18, 19, 20].

Results and Discussion

Microscopic analysis

In case of *E. arvense* L. the sterile stems, in the studied populations, have 6-18 edges. In the edges, under the silicified collenchyma tissue develops assimilated palisade. In the valecules are extensive aeriferous zones, in the forms of some channels, gaps, circulars, all arranged orderly. The central cylinder begins with a pericycle, the cells are small closely joined together. In the fundamental parenchyma of the marrow, there are numerous vascular bundles; all arranged in a circle, the phloem tissue being situated just below the pericycle and it is more developed than the xylem tissue (Figure 3).

In the xylem tissue, the protoxilem and metaxilem elements can be differentiated. In the right of protoxilem vessels, there is an aeriferous gap.

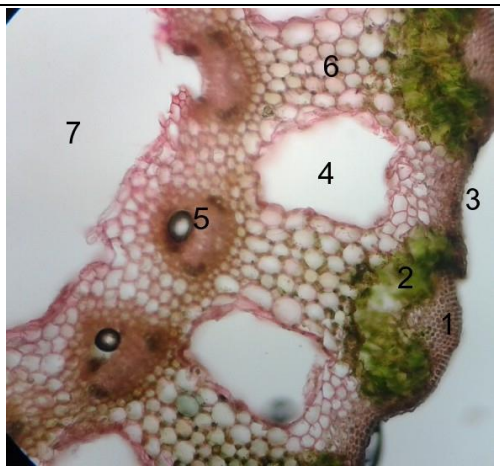

Figure 3.
Cross section through the *E. arvense* L. main stem (100X)
1- silicified collenchyma; 2- assimilated palisade parenchyma; 3- silicified collenchyma in valecules; 4- aeriferous zones in ground tissue; 5- leading bundles; 6- cortical parenchyma, 7- main gap marrow

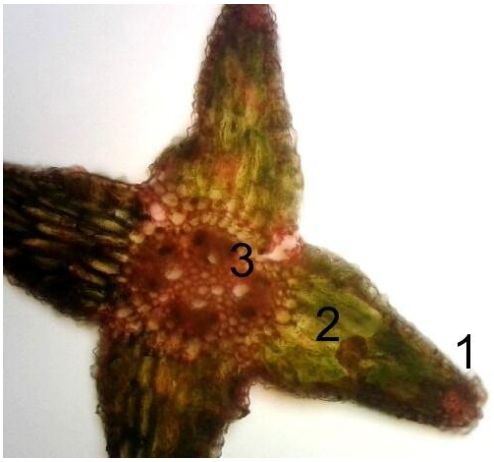

Figure 4.
Cross section through the *E. arvense* L. ramification with 4 edges (100X)
1- silicified colenchima; 2- assimilated palissade parenchyma; 3 -leading bundles

The ramifications were usually 4 deep edges, with silicified collenchyma on the top, and well developed palisade assimilation tissue. The central cylinder presents four vascular bundles, without central lacuna, according to the literature description (Figure 4). In some studied populations, there were identified individuals with unusual branching, with 5 or 6 edges (Figure 5 and 6). In case of those with 5 edges, on the top of the edges is present the silicified collenchyma. The central cylinder presents five vascular bundles and lacks central lacuna.

In the case of specimens with 6 deep edges, the silicified collenchyma in present at the top of each

edge. The central cylinder presents six vascular bundles and a small central lacuna.

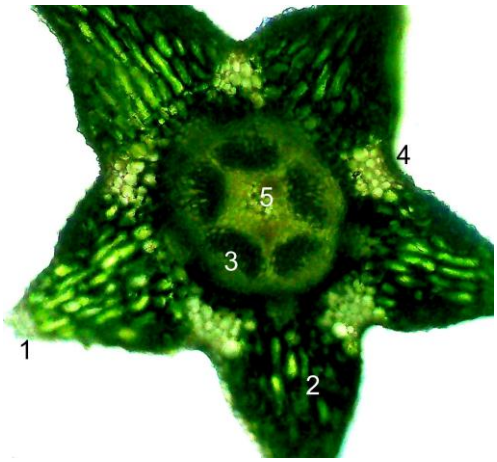

Figure 5.
Cross section through the *E. arvense* L. ramification with 5 edges
1- silicified collenchyma; 2- assimilated palissade parenchyma; 3 -leading bundles; 4- silicified collenchyma in valecules; 5 – central marrow (100X)

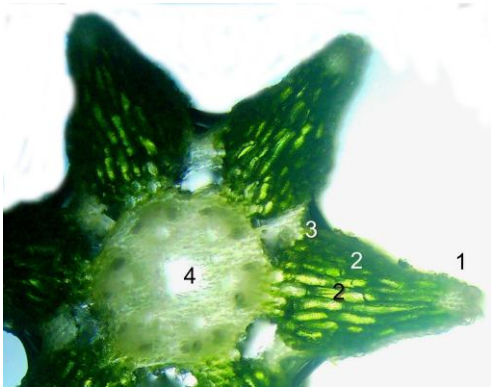

Figure 6.
Cross section through the *E. arvense* L. ramification with 6 edges
1- silicified collenchyma; 2- assimilated palissade parenchyma; 3- silicified collenchyma in valecules; 4 - main gap marrow (100X)

In the case of *E. telmateia* Ehrh., the edges from the main stem are no obvious, the valecules being not deep, the areas of silicified collenchyma are less obvious. The lacunas are more numerous and larger than in case of *E. arvense*, as well as the number of vascular bundles. The central lacuna is large and obvious (Figure 7).
Each lateral ramification has 8 edges on all samples, the edges present an underrepresented silicified collenchyma, in the central cylinder there are 4 directing libero-woody beams, without a central lacuna (Figure 8).

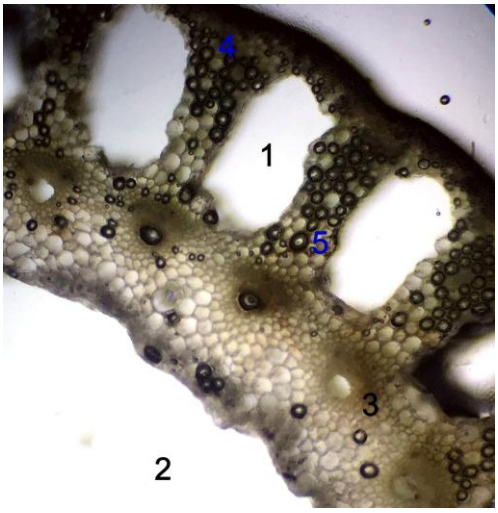

Figure 7.
Cross section through the *E. telmateia* Ehrh. main stem
1 - aeriferous aeras in ground tissue; 2 - main gap marrow; 3 - leading bundles; 4 - valecules; 5 - assimilated parenchyma (100X)

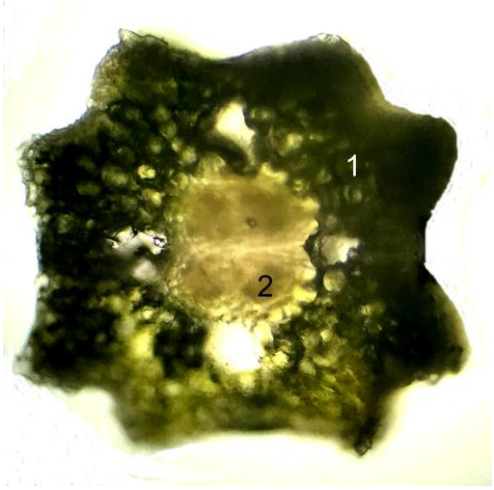

Figure 8.
Cross section through the *E. telmateia* Ehrh. ramification with 8 edges
1- assimilated parenchyma; 2- leading bundles (100X)

Quantitative analysis of total polyphenols and flavonoids
The results of quantitative analysis of total polyphenols and flavonoids are shown in Table I. We can observe that, in the case of *E. arvense* L., the values are higher than in case of *E. telmateia* Ehrh., for both stems collected in April and in September.

Table I

Total polyphenols, flavonoids and antioxidant capacity for *Equisetum arvense* L. and *Equisetum telmateia* Ehrh. dried extracts

Sample	Total polyphenolic content (mg GAE / 100 DW	Total flavonoid content (mg QE / 100 DW)	Per cent of total flavonoids in dried drugs (%)	FRAP (µmol Trolox equivalent / g DW)	DPPH (%)	Cuprac (µmol Trolox equivalent / g DW)
Equisetum arvense L. sterile stems April 2015	86.45 ± 0.12	75.66 ± 3.27	0.76	87.70 ± 0.081	95.30 ± 0.073	46.17 ± 0.089
Equisetum telmateia Ehrh (*E. maximum Lam.*) sterile stems April 2015	52.11 ± 0.27	29.72 ± 1.41	0.29	63.40 ± 0.079	57.50 ± 0.093	31.25 ± 0.143
Equisetum arvense L. sterile stems September 2015	47.37 ± 0.55	42.59 ± 2.63	0.42	34.90 ± 0.155	91.90 ± 0.213	34.55 ± 0.126
Equisetum telmateia Ehrh (*E. maximum Lam.*) sterile stems September 2015	24.84 ± 0.18	14.69 ± 0.97	0.15	18.40 ± 0.257	46.70 ± 0.066	12.48 ± 0.210

We converted the flavonoid amounts into percentages, so that they can be compared with the values provided by the Pharmacopoeia. Both in the Romanian and European Pharmacopoeia, a minimum content of 0.3% flavonoids are provided for the dried sample. In Table I it can be observed that, in case of *E. arvese* L., harvested in April, in the beginning of the vegetation period, the flavonoids amount is far higher compared to the plants harvested in September. In *E. telmateia* Ehrh., in the same way the amounts are higher in April than in September, but they are far more reduced compared to *E. arvense* L.

In other scientific papers published in this field, high values of antioxidants in *Equisetum telmateia* Ehrh. are described [3]. Our results showed that, in studied populations of *Equisetum arvense* L., total polyphenols and flavonoids were able to be detected in large quantities, confirming the results of other studies [8, 10, 11].

Antioxidant capacity of Equisetum arvense L. and Equisetum telmateia Ehrh. extracts

Antioxidant properties of the studied plants were determined by three methods, FRAP, DPPH and Cuprac. The results are shown in Table I. The antioxidant capacity of extracts was assayed using FRAP and Cuprac methods and were expressed as Trolox equivalents. The scavenging effect of sterile stem extracts was determined by DPPH method, and expressed as percentage of inhibition (%).

It can be observed that, in the case of plants harvested in April, the antioxidant capacity is higher compared to those harvested in September. In parallel, it may be seen that *E. arvense* L. has a higher antioxidant capacity than *E. telmateia* Ehrh.

Conclusions

On the macroscopic level, especially in the beginning of the vegetation period, the studied species may be easily confused, especially because they are not cultivated in Romania, the medicinal product coming from traditional collection centres. From the histo-anatomical point of view, the differences from the observed species appeared in cross sections of the sterile stems and ramifications. On the stems levels, the main differences are the amount of silicified collenchyma on the edges and valecules. In case of *E. telmateia* Ehrh. the central lacuna is more pronounced than in case of *E arvense* L. In case of *E. telmateia* Ehrh. the main stem's ramifications presented 8 edges, in the central cylinder being 4 vascular bundles.

In case of *E. arvense* L., the characteristic ramifications had 4 deep edges [4, 17, 21, 22],

respectively 4 vascular bundles, but we found populations with more edges, in which the number of vascular bundles corresponded with the number of edges. By studying the amounts of polyphenols, flavonoids and the antioxidant capacity, there were registered significant differences between the two species, *E. arvense* L. being richer in these principles. The anti-inflammatory action is due to these components. Our studies have shown that the principles with antioxidant activity are accumulated in large quantities at the beginning of the vegetation period, but only in *E. arvense* L. the percentages of flavonoids arrived and exceeded the minimum content provided in Pharmacopoeia. Therefore, for a correct identification of the *E. arvense* L. species, and in order to avoid confusion, the histo-anatomical analysis is necessary. Substitutions with other *Equisetum* species may not be acceptable.

References

1. Benzie I.F., Strain J.J., The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power". The FRAP assay. *Anal. Biochem.*, 1996; 239: 70-76.
2. Brand-Williams W., Cuvelier M.E., Berset C., Use a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft and Technologies Food Science and Technology*, 1995; 28: 25-30.
3. Correia H., Gonzalez-Paramas A., Amaral M.T., Santos-Buelga C., Batista M.T., Characterisation of polyphenols by HPLC-PAD-ESI/MS and anti-oxidant activity in Equisetum telmateia. *Phytochem. Anal.*, 2005; 16: 380-387.
4. Crăciun F., Bojor O., Alexan M., Farmacia naturii, Editura Cereș, București, 1976ș 53-56.
5. Cushnie T.P.T., Lamb A.J., Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents*, 2005; 26: 343-356.
6. Gholivand M.B., Piryaei M., Total phenols, flavonoids, anthocyanins, ascorbic acid contents and antioxidant activity of *Rhamnus kurdica* Boiss for flower and leaves in flowering and pre-flowering stages. *African Journal of Biotehnology*, 2014; 13(10): 1131-1135.
7. Kim D., Chun O., Kim Y., Moon H., Lee C., Quantification of Polyphenolics and Their Antioxidant Capacity in Fresh Plums. *J. Agric. Food Chem.*, 2003; 51: 6509-6515.
8. Kukrić Z., Topalić-Trivunović L., Pavičić S., Žabić M., Matoš S., Davidović A., Total phenolic content antioxidant and antimicrobial activity of *Equisetum arvense* L. *Chemical Industry & Chemical Engineering Quarterly*, 2013; 19(1): 37-43.
9. Merkl R., Hrbdkovb I., Filip V., Šmidrkal J., Antimicrobial and antioxidant properties of phenolic acids alkyl esters. *Czech J. Food Sci.*, 2010; 28: 275-279.
10. Milovanović V., Radulović N., Todorović Z., Stanković M., Stojanović G., Antioxidant, antimicrobial and genotoxicity screening of hydro-alcoholic extracts of five Serbian equisetum species. *Plant Foods Hum. Nutr.*, 2007; 62: 113-119.
11. Mimica-Dukic N., Simin N., Cvejic J., Jovin E., Orcic D., Bozin B., Phenolic compounds in field horsetail (*Equisetum arvense* L.) as natural antioxidants. *Molecules*, 2008; 13: 1455-1464.
12. Păltinean R., Crișan G., Balica G., Ielciu I., Ștefănescu C., Tămaș M., Histo-anatomical researches on the vegetative organs of five Romanian *Fumaria* species. *Farmacia*, 2015; 63(2): 262-266.
13. Popovici I., Lupuleasa D., Tehnologie farmaceutică, vol 1, Ed Polirom, Iași, 2011.
14. Singh A., Sharma P.K., Garg G., Natural products as preservatives. *Int. J. Pharma. Bio Sciences*, 2010; 1: 601-612.
15. Stylianou N., Gakasz V., Anghel I.A., Istudor V., Research regarding *Taraxacum Officinale* (L.) Weber II optimum extraction parameters of caffeic acid derivates using ultrasound- assisted extraction. *Farmacia*, 2014; 62(6): 1223-1129.
16. Tămaș M., Oniga I., Produse fitoterapeutice românești, Ediția a 2-a, UMF Iuliu Hațieganu, Cluj Napoca, 2001.
17. Toma C., Rugină R., Anatomia plantelor medicinale, Atlas, Editura Academiei Române, București, 1998; 55-56.
18. Vicaș L., Teușdea A., Vicaș S., Marian E., Jurca T., Mureșan M., Gligor F., Assessment of antioxidant capacity of some extracts for further use in therapy. *Farmacia*, 2015; 63(2): 267-274.
19. Yuan W., Zhou L.J., Deng G.R., Wang P., Creech D., Li S.Y., Anthocyanins, phenolics and anti-oxidant capacity of *Vaccinium* L. in Texas, USA. *Pharm. C.*, 2011; 2: 11-13.
20. Zia-Ul-Haq M., Ahmad S., Stankovici M.S., Sultan M.T., Imran I., Velter V., Badiu D., Halichidis S., Hangan T., Antimicrobial and antioxidant potential if *Ipomoea hedereacea*. *Farmacia*, 2014; 62(6): 1181-1190.
21. *** Farmacopeea Română, Ediþia a X-a, Editura Medicalã, Bucuresti, 1993.
22. *** European Pharmacopoeia 7th edition, 2012; 1121-1122.