GRIGORE T. POPA UNIVERSITY OF MEDICINE AND PHARMACY IASI

THE CHEMICAL AND BIOLOGICAL STUDY OF SOME ENDEMIC *PTERIDOPHYTAE* SPECIES IN THE AREA OF MOLDOVA

DOCTORAL THESIS ABSTRACT

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PhD Univ. Prof. Monica HĂNCIANU

Doctoral Student,
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(married BATÎR MARIN)

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This Doctoral Thesis **"The chemical and biological study of some endemic *Pteridophytae* species in the area of Moldova"** contains 180 pages, of which 44 are dedicated to the General Part, 119 to the Personal Part and 17 to the Bibliography.

The thesis contains 107 figures, 37 tables and 309 bibliographic references.

In this Summary, the selected figures, tables and bibliographic references keep the same number as the one assigned in the text of the Doctoral Thesis.

Keywords: ***Equisetum pratense*** **Ehrh.,** ***Equisetum sylvaticum*** **L.,** ***Equisetum telmateia*** **Ehrh., polyphenols, flavones, antioxidant, antimicrobial, silver nanoparticles, neuroprotective, antitumor.**

CONTENTS

THE PERSONAL PART

MOTIVATING THE CHOICE OF THE THESIS SUBJECT, PURPOSE AND OBJECTIVES PURSUED

People have used natural resources, especially spontaneous flora, both as a food source and for maintaining health. Worldwide, research in the recent decades has shown an increased interest in pharmaceuticals with herbal extracts, conditioned in various forms, both for internal and topical use.

Several studies have shown that such products are the first choice in various conditions of varying severity, as they help to normalize the functions and metabolism of the body without having any toxic or side effects. Also, by the antioxidant effect of the extracts, they protect the body against oxidative stress, generated in the context of diseases such as: atherosclerosis, ischemic heart disease, cancer, Alzheimer's disease, Parkinson's disease and even in infectious diseases (123).

Mechanisms of abnormal protein aggregation or excess formation of reactive oxygen species determine neuronal apoptosis. In such neurodegenerative pathologies, endogenous antioxidant systems (superoxide dismutase or catalase) fail to fight the overproduction of the reactive oxygen species, leading to the installation of oxidative stress. This process causes peroxidation of membrane lipids of proteins and nucleic acids, and brain tissue remains exposed to oxidative aggression.

The experimental data published so far and the data in this work show that the polyphenolic derivatives that have been isolated from *Equisetum* species may provide indirect protection by activating the antioxidant transcription in the promoter regions of the genes that induce oxidative stress. Moreover, polyphenols can modulate both the cellular signal involved in cell proliferation and the cell development itself by inhibiting inflammation and initiating the process of apoptosis of the damaged cells (127, 128).

The study of cytotoxicity and antimicrobial activity of plant extracts is important for medical practice. Bacterial resistance encountered in current bacterial infections requires finding new agents with complementary action or synergistic effect with antibiotic therapy.

Staphylococcus aureus is known to be an etiologic agent for many skin and mucosal infections, especially for those caused by S. aureusmeticilino – resistant to patients with low or postoperative immunity. *Escherichia coli* is frequently described as a cause of chronic and recurrent urinary tract infection while *Candida albicans* is a fungus that may overlap to the bacteria by sterilizing the saprophytic flora given by the antibiotic, thus aggravating the existing disease (132–134).

By their chemical composition, ferns may represent a promising plant resource for the production of plant extracts or the separation of components with important biological value.

The *Pteridophytae* species extracts have an increased content of flavonic derivatives and polyphenolicarboxylic acids that show important antioxidant and antimicrobial action, without generating the side effects specific to the allopathic treatments. This aspect justifies the promotion of the study of the bioactive components of the species of the *Equisetum* genus for discovering new prevention and treatment opportunities with the help of phytopreparations with a higher pharmacotoxicological profile. This is why many researchers have started from traditional medicine and have exploited the natural therapy string that proves to be inexhaustible and always surprising.

The *Pteridophytae* phylum is divided into four large classes: *Psilotopsida, Equisetopsida, Maratiopsida and Polypodiopsidace* totaling over 9,000 species. The current study was conducted only at the level of the *Equisetopsida* class, *Equisetaceae* family. Of the 9 species existing on the territory of our country, three were chosen, less studied from a chemical and biological point of view, *Equisetum pratense* Ehrh., *Equisetum sylvaticum* L. and *Equisetum telmateia* Ehrh., endemic in the Moldavian area (3, 11).

The purpose of this work is to highlight the chemical composition of the extracts obtained from the *Equisetum* species and to investigate the biological action (cytotoxic, antioxidant and antimicrobial) both *in vitro* and *in vivo*.

In this context, the main objectives of the research presented in this doctoral thesis focused on carrying out phytochemical and biological studies on three *Equisetum* species previously less investigated and that grow spontaneously on the territory of Romania.

Thus, I followed:

- The investigation of the histo-anatomical characters of the aerial parts of the species of *E. pratense* Ehrh., *E. sylvaticum* L. and *E. telmateia* Ehrh.;
- The evaluation of the silicon and heavy metals content in the aerial parts of the species of *E. pratense* Ehrh., *E. sylvaticum* L. and *E. telmateia* Ehrh.;
- A qualitative and quantitative chemical analysis of the bioactive components present in the methanol and ethanol extracts obtained (polyphenol and flavone content);
- The physico-chemical characterization and the photocatalytic determination of the silver nanoparticles synthesized based on ethanol extracts of the *Equiseum* species*;*
- The *in vitro* evaluation of the antioxidant and antimicrobial activity for both polar extracts and synthesized nanoparticles obtained;
- The *in vitro* investigation of anti-tumor activity for the nanoparticles obtained from the *Equisetum* species;
- The *in vivo* evaluation of the neuroprotective activity of plant extracts obtained from the species of *E. pratense* Ehrh., *E. sylvaticum* L. and *E. telmateia* Ehrh.

CHAPTER III. Macro- and microscopic pharmacognostic analysis of the *Equisetum* species studied (*E. pratense* Ehrh., *E. sylvaticum* L. and *E. telmateia* Ehrh.)

III.1. Species identification; morphological characters

After drying, the plant product was sorted so that we obtain the aerial part free of impurities and other parts of plants. For all the products the predominant green color was observed along with slightly lignified fragments of the brown stem.

The Equisetum pratense herba is identified by: a large friable mass, rough when touched; a low, characteristic odor, close to that of the hay; a mild astringent taste, slightly bitter; leaves: twisted, with the appearance of scales, slightly widened, dark green; thick, articulated stem fragments with longitudinal ribbing, ranging from green to purplish-brown. For the species *Equisetum pratense*, approx. 14 kg were harvested, but, after drying, approx. 3 kg remained, i.e. 21.43%, losing, by drying, 78.57% water.

Equisetum sylvaticum herba has the following characteristics: large friable mass, lighter, with a smooth texture, finer than the *Equisetum pratense* species; slightly aromatic odor, reminiscent of honeycombs and beeswax; mild astringent taste at first, then sweetish, mucilaginous; filamentous thin twisted leaves, extremely crumbling, in shades of light green; the stem fragments are thinner, and the predominant color is brown-red. For the species *Equisetum sylvaticum* approx.11 kg were harvested, but, after drying, approx. 2 kg remained, i.e. 18.18%, losing, by drying, 81.82% water.

Equisetum telmateia herba is identified by: a large friable mass, rough when touched; a low, characteristic odor, close to that of hay; a mild astringent taste at first, slightly sour, then sweetish; scaly leaves, slightly widened in the basal part, dark green; thick, articulated stem fragments with longitudinal ribbing, ranging from green to dark brown. For the species *Equisetum telmateia* approx. 13 kg were harvested, but, after drying, approx. 3 kg remained, i.e. 23.08%, losing, by drying, 76.92% water.

III.2. Investigation of the histo-anatomical characters of the aerial parts of the species *E. pratense* Ehrh., *E. sylvaticum* L., *E. telmateia* Ehrh.

The histo-anatomical analyzes performed with both the optical microscope and the scanning electron microscope revealed common features specific to the *Equisetum* species studied, but also some peculiarities by which they differ.

Equisetum pratense Ehrh.

The stem (main axis): the contour of the cross-section is circular-costal, with slightly prominent ribs, separated by shallow valleculas. The epidermis has isodiametric cells, with all the walls thickened, and there are stomates from place to place. The bark is differentiated into: collenchyma type mechanical tissue; uni- or bistratified palisade tissue; parenchyma consisting of large cells, with thin walls, forming a thick cortical area. The central cylinder (the star) is very thick, consisting of two areas: an external one, in which the conducting beams are located; an internal one, representing a large aeriferous cavity (17) (Figure III.4).

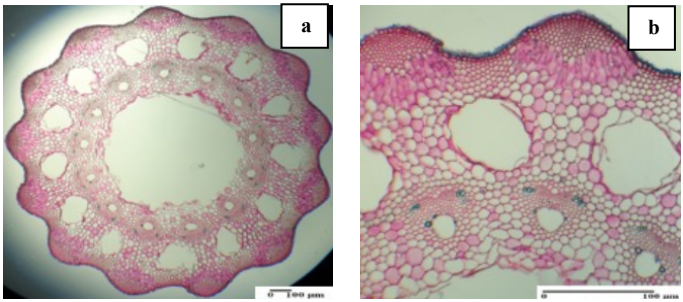


Figure III.1. Cross section of the *Equisetum pratense* stem: overview (a); structure detail (b).

The stem branch from the nodal whorl: the contour of the cross-section is cross-shaped (Figure III.6). The epidermis has slightly elongated cells with all the walls silicified. The bark shows collenchymatic tissue at the tip of the ribs and parenchymatic tissue consisting of very long cells with thin walls along them. The central

cylinder starts with an unistratified pericycle, supporting 4 conducting beams and a small aquiferous cavity, radially elongated, elliptical or triangular in shape in cross-section.

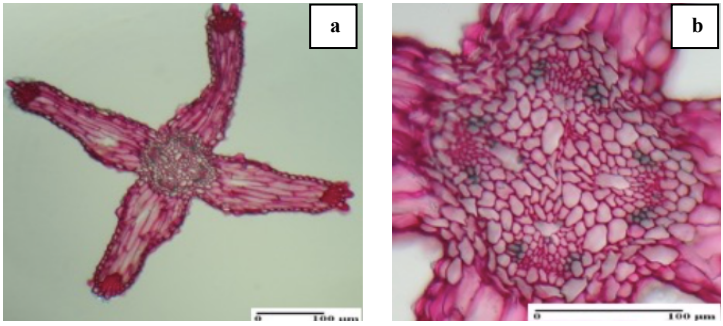


Figure III.2. Cross section of an *Equisetum pratense* stem branch from the nodal whorl: overview (a); central cylinder detail (b).

In Figure III.7, the scanning electron microscope shows the intercostal epidermal cells between which numerous stomates are visible. The epidermal cells on the ribs take the appearance of very short trichomes, with rounded tip.

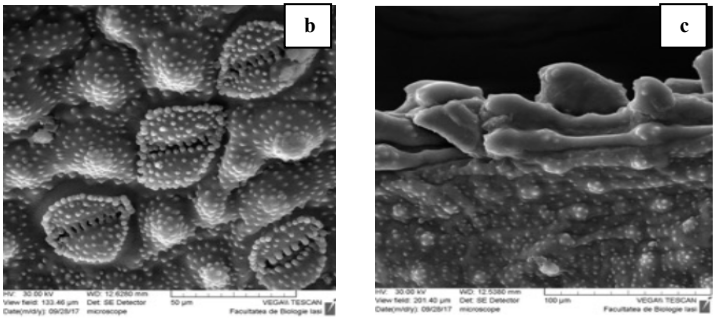


Figure III.3. *Equisetum pratense* stem branch from the nodal whorl front view: stomates and epidermal cells (b); trichomes on the ribs (c).

The leaf (scale-shaped): all the leaves from the nodes of the stem axis are grouped in whorls, joining at the base and forming a sheath (figure III.8). The epidermis has slightly elongated cells tangentially with the external wall visible thickened and silicified. The fundamental parenchyma (mesophile) has isodiametric cells. In the middle of each

leaf there is a conducting beam with few liberian and woody elements (189).

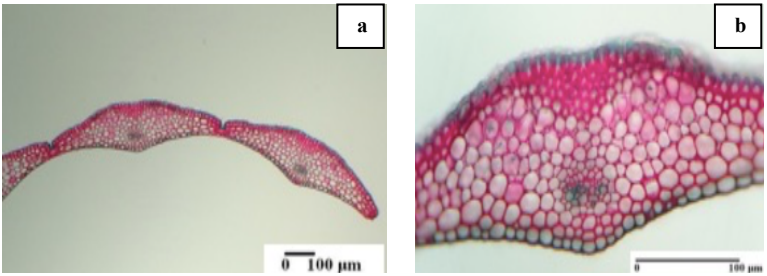


Figure III.4. Cross-section of the *Equisetum pratense* leaf: overview with leaves joining in sheaths (a); leaf lamina structure (b).

***Equisetum sylvaticum* L.**

The stem: the contour of the cross-section is circular, costal, with shallow valleculas. The epidermis has isodiametric cells, with all the walls thickened, strongly silicified. The outer bark has mechanical cords of collenchyma in the ribs, and, at the base, valleculas, forming 1-2 layers. The inner bark is composed of large, isodiametric cells. Compared to *E. pratense*, there are no aquiferous cavities in the bark, which reflects the environment type in which the plant lives (185). The central cylinder starts with an unistratified pericycle, it continues with a ring of conducting beams; and the center is occupied by a large aeriferous cavity (18, 190). All these details described herein may be seen in figure III.10.

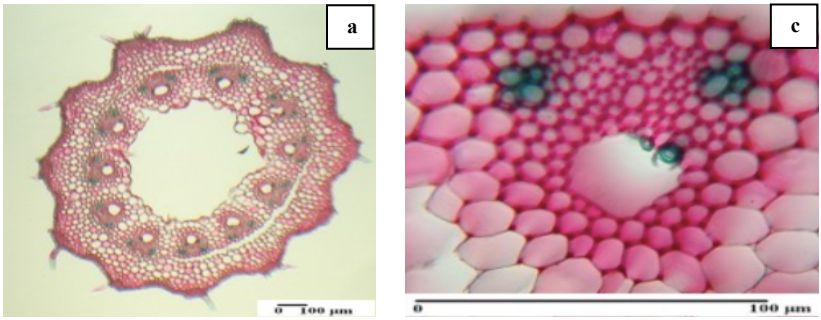


Figure III.5. Cross-section of the *Equisetum sylvaticum* stem: overview (a); conducting beam (c).

The stem branch from the nodal whorl: the contour of the cross-section is starred, with 5 ribs, and 5 valleculas between them. The epidermis has isodiametric cells. The bark: in all 5 ribs there is a cord of collenchymatic cells with non-lignified walls. The central cylinder starts with a unistratified pericycle, supporting 5 conducting beams. The center of the stem branch lacks an aeriferous gap (185). The details described herein may be observed in figure III.12.

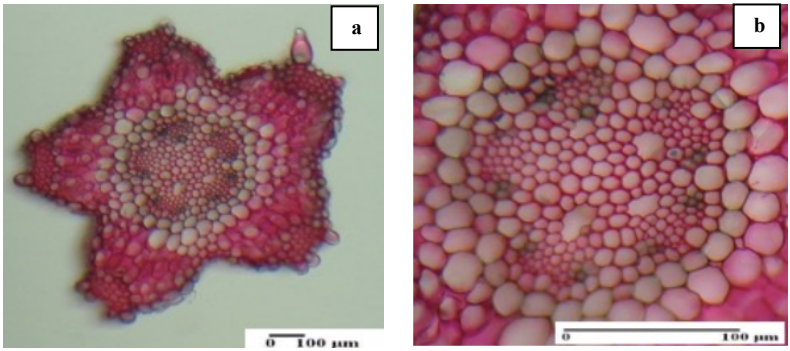


Figure III.6. Cross section of an E.*sylvaticum* stem branch from the nodal whorl: overview (a); central cylinder detail (b).

The leaf is joined at the base (17). The epidermis has slightly elongated cells tangentially, with all the walls slightly thickened. The fundamental parenchyma of the leaf has a very small conducting beam made up of few discontinuous cells and wood vessels. Under the electron microscope (figure III.15), single-cell protective trichomes are observed, relatively long and very numerous (191).

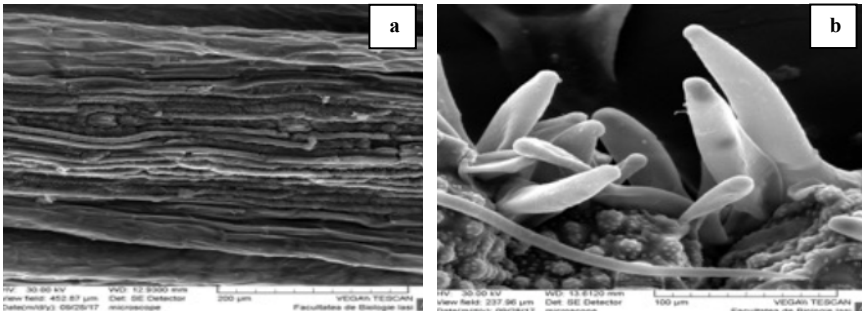


Figure III.7. *Equisetum sylvaticum* leaf epidermis front view: overview (a); trichomes (b).

***Equisetum telmateia* (sin. *Equisetum maximum* Lam.)**

The stem: the contour of the cross-section is circular, without ribs and valleculas (figure III.16). The epidermis has isodiametric cells, and stomates are observed from place to place. The bark is composed of three sub-areas: an outer parenchyma; a very thick middle area where approx. 18 aeriferous cavities are observed; an internal area, representing the endoderm. The central cylinder shows a large number of liberian-wood beams.

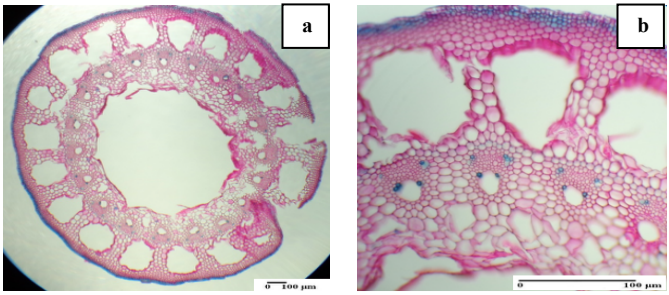


Figure III.8. Cross section of a *Equisetum maximum* stem: overview (a); bark and central cylinder detail (b).

The stem branch from the nodal whorl: the contour of the cross-section is circular-costal, which can be seen in figure III.18. The epidermis has cutinized isodiametric cells. The bark is thick comprising collenchymatic cords and a thick area with parenchymal cells presenting 4-5 large aeriferous cavities. The central cylinder contains 5 conducting beams.

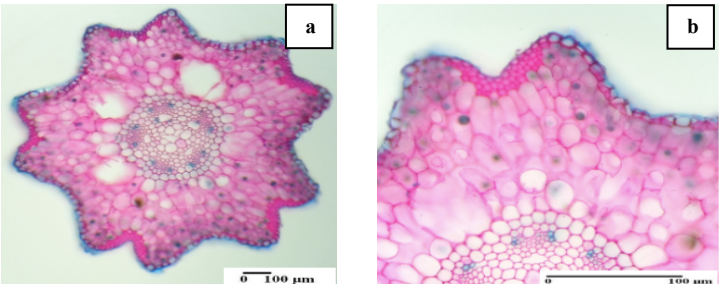


Figure III.9. Cross section of an *Equisetum telmateia* stem branch: overview (a); bark and central cylinder detail (b).

The leaf is scale-shaped and is joined at the base through a thin sleeve. There are noted: The external epidermis and the internal epidermis are made up of tangentially elongated cells. The mesophile is homogeneous, parenchymatic type; liberian-wood small conducting beams are observed.

CHAPTER IV. Evaluation of the silicon and heavy metals content in the aerial parts of the *Equisetum* species (*E. pratense* Ehrh., *E. sylvaticum* L. and *E. telmateia* Ehrh.)

IV.1. Spectrophotometric determination of silicon

For the quantification of the silicon found at the level of the main stem and of the stem branch from the nodal whorl, spectrophotometric analyzes were performed. This assessment is of particular importance because the *Equisetum* species are silicon accumulating plants. The concentrations calculated for the plant products are presented in table IV.2.

Table IV.1. Silicon concentration for the *Equisetum* species

Plant product	Concentration SiO_2 mg/1g dry sample	Concentration Si% (g/100g) dry sample
E. pratense main stem	101.50	4.745 %
E. pratense stem branch	132.84	6.210 %
E. sylvaticum main stem	95.12	4.446 %
E. sylvaticum stem branch	146.96	6.870 %
E. telmateia main stem	127.55	5.962 %
E. telmateia stem branch	162.10	7.578 %

Quantitative determinations of silicon from the dry plant product revealed that in the stem branch from the nodal whorl there is a higher concentration of silicon than in the main stem for all three species analyzed (182). The species containing the highest concentration in silicon (main stem and stem branch) is *E. telamateia* (5.962% and 7.578% respectively) while *E. pratense* and *E. sylvaticum* showed similar concentrations.

IV.2. Heavy metal determination by atomic absorption spectroscopy

The heavy metals, Cu^{+2}, Cd^{+2}, Ni^{+2} and Pb^{+2} were determined by atomic absorption spectroscopy of the dry plant material, respectively from the main stem and the stem branch from the nodal whorl of the *Equisetum* species. Such determinations have been made to see if the assessed species accumulate heavy metals.

The content in heavy metals (mg/100g plant product) for plant products is presented in table IV.5.

Table IV.2. Heavy metal content mg/100 g dry plant product

Plant product	mg/100 g dry plant product			
	Cu^{+2}	**Cd^{+2}**	**Ni^{+2}**	**Pb^{+2}**
E. pratense main stem	8.32	4.01	3.50	0
E. pratense stem branch	6.42	3.90	3.20	0
E. sylvaticum main stem	2.48	present *	present *	present *
E. sylvaticum stem branch	2.05	present *	present *	present *
E. telmateia main stem	3.83	0	present *	0
E. telmateia stem branch	3.55	0	present *	0

* present below the detection limit of the method

Quantitative determinations of heavy metals in dry plant products have shown that *E. pratense* has the ability to accumulate both Cu^{+2}, Cd^{+2} and Ni^{+2}, without these metals interfering with the plant development (210).

For the trace element Cu^{+2} it can be observed that in the main stem of all species there is a more intense accumulation than in the stem branch from the nodal whorl. The highest amount of Cu^{+2} was recorded in the main stem of the *E. pratense* species (8.32mg/100g plant product), and the lowest was obtained in the stem branch of the *E. sylvaticum* species (2.05mg/100g plant product).

The element Cd^{+2} was found in large quantities in the *E. pratense* species. Research has shown that silicon increases resistance to toxic metals, including Cd^{+2}. Also, silicon enhances the rigidity of cell walls, forming a natural mechanical barrier for Cd^{+2} ions (195, 212).

Although Ni^{+2} is essential for plant growth and development, in *E. pratense* it far exceeds the limits admitted by WHO at the level of both the main stem and the stem branch. This result suggests good tolerance to soil pollution with nickel (212).

The analyzed *Equisetum* species were not noted to accumulate Pb^{+2} except for *E. sylvaticum* where the recorded values were below the detection limit of the method.

CHAPTER V. Chemical and biological evaluation of the extracts obtained from the species of *Equisetum pratense* Ehrh., *Equisetum sylvaticum* L. and *Equisetum telmateia* Ehrh.

V.1. Obtaining extracts

In order to evaluate the extractability, it was calculated the ratio between the mass of the dry and minced plant product used to obtain the extracts and the mass of the dry extract obtained from solvent evaporation.

Table V.3. Extractability values

Extract	Extractability
Ep_m	10:1.05
Es_m	10:1.13
Et_m	10:1.06
Ep_e	10:1.15
Es_e	10:0.99
Et_e	10:1.04

V.3. Quantitative chemical analysis of bioactive components present in methanol and ethanol extracts

V.3.1. Quantitative determination of total polyphenols by the spectrophotometric method

The results obtained from the quantitative determination of total polyphenols by the spectrophotometric method are graphically represented in figure V.3.

The highest concentration in total polyphenols, 236.17mg gallic acid/g dry extract, was obtained for the *E. telmateia* species (ethanol extract), followed by *E. sylvaticum* (methanol extract), with a concentration of 196.50mg gallic acid/g dry extract.

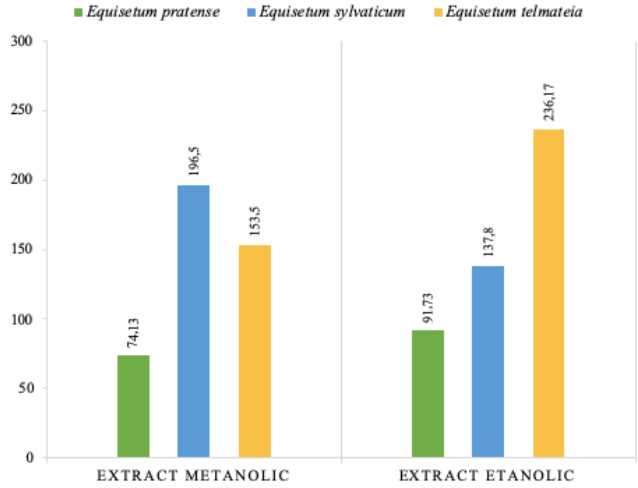


Figure V.10. Content of total polyphenols (mg gallic acid/g dry extract) of the extracts of the *E. pratense*, *E. sylvaticum* and *E. telmateia* species

V.3.2. Quantitative determination of flavonoids by the spectrophotometric method

The quantitative determination of flavonoids from the extracts made with 70% methanol was higher than the one made with 70% ethanol, being shown in Figure V.5 that the extract obtained from *E. sylvaticum* has the highest content in flavonoid derivatives (170.65mg quercetol/g dry extract).

Comparatively, the methanol extract of *E. pratense* has a concentration approx. two times lower (94.05mg quercetol/g dry extract) and the methanol extract of *E. telmateia* has a concentration of approx. 3 times lower (45.03mg quercetol/g dry extract).

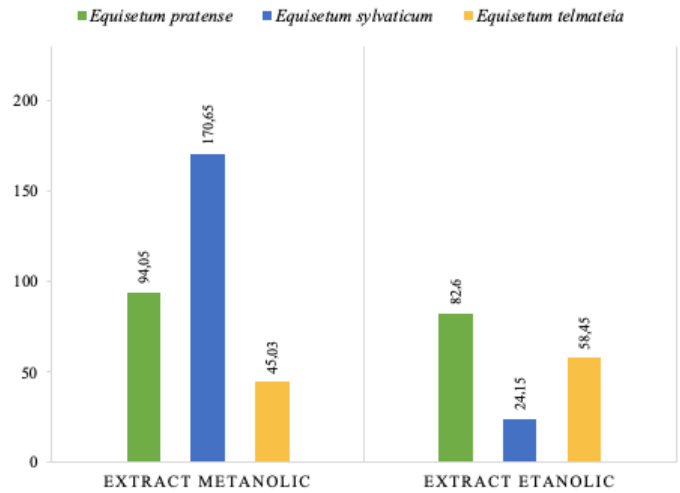


Figure V.11. Flavonoid content (mg quercetol/g dry extract) of the extracts of *E. pratense*, *E. Sylvaticum* and *E. telmateia* species

V.4. Semi-quantitative and qualitative determination of the polyphenolic compounds (UHPLC)

The presence of the following polyphenolic derivatives in the two types of extracts (methanol and ethanol) obtained from the three investigated *Equisetum* samples was confirmed by the liquid chromatography technique: chlorogenic acid, caffeic acid, ferulic acid, as well as glucosidated derivatives of quercetol and luteolin. Such active principles have also been identified by other authors in extracts obtained by various methods from *Equisetum arvense* (20, 129, 220).

The chromatograms recorded for the analysis of methanol extracts are shown in figures V.12, V.13 and V.14, the images being reproduced for the three species at three wavelengths characteristic of polyphenolcarboxylic acids and flavonoids.

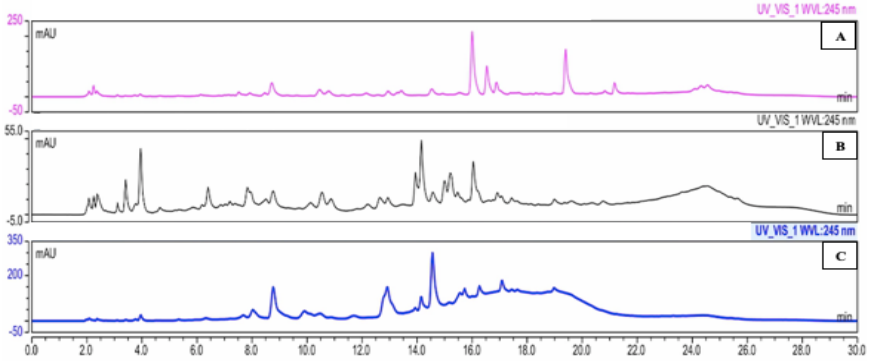


Figure V.12. Chromatograms for methanol extracts at 245nm: Es_m(A), Ep_m (B), Et_m(C)

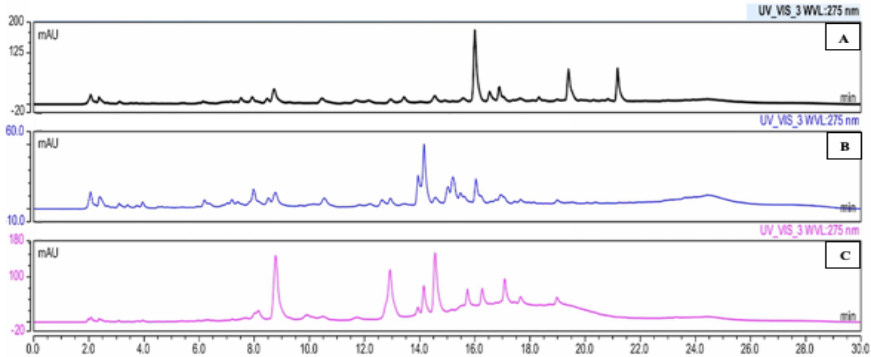


Figure V.13. Chromatograms for methanol extracts at 275nm: Es_m(A), Ep_m (B), Et_m(C)

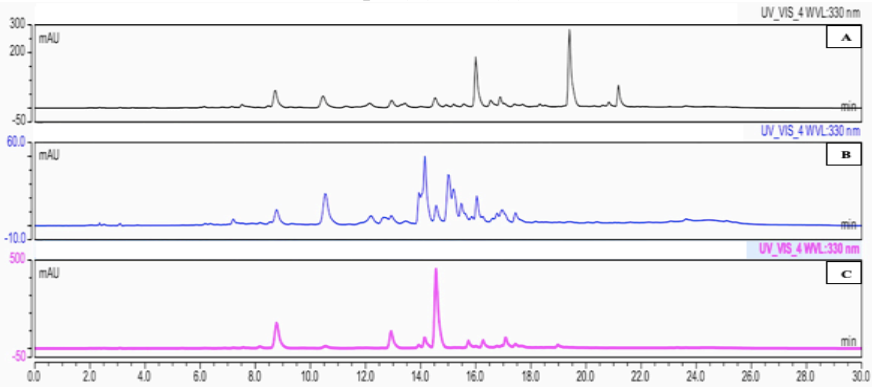


Figure V.14. Chromatograms for methanol extracts at 330nm: Es_m(A), Ep_m (B), Et_m(C)

According to the spectral and quantitative analysis, the flavonoid components quantified in the samples are presented briefly in table V.8.

Table V.4. Flavonoids identified and quantified in the investigated samples

Compound expressed in mg/g plant product	Sample (70% methanol extract)		
	E. sylvaticum	***E. pratense***	***E. telmateia***
epicatechin	0.9010	0.5925	1.1967
quercetin-3-D-glucoside	1.6995	8.2442	23.4765
luteolin-glucoside	11.0456	1.8562	5.6447
apigenin-7-glucoside	2.3249	2.2947	27.7463
luteolin	0.6150	0.0344	0.4214
quercetol	0.0658	0.0059	0.4013
apigenol	0.0716	0.6260	0.9571
kaempferol	1.4997	0.9781	1.4417

Quantitatively, *E. telmateia* and *E. sylvaticum* are richer in the flavonoid fraction than *E. pratense*, being evident that the glycosidated derivatives of quercetol, luteolin and apigenol are predominant in all three samples.

The concentrations of the four identified polyphenolic acids are shown in table V.9.

Table V.5. Polyphenolcarboxylic acids identified in the methanol extracts of *Equisetum* species

Compound expressed in mg/g plant product	Sample (methanol extract)		
	E. sylvaticum	***E. pratense***	***E. telmateia***
neochlorogenic acid	0.2639	0.0730	0.6381
chlorogenic acid	4.3322	0.7491	10.1162
caffeic acid	0.1468	0.2363	0.8590
ferulic acid	0.6414	0.2295	1.0245

The polyphenolic acid with the highest content is chlorogenic acid, regardless of the investigated sample, but the maximum content is found in *E. telmateia* (approx. 10 mg/g), while the proportion decreases in half in *E. sylvaticum* and less than 7%. in *E. pratense.* The investigation of the extracts made with ethyl alcohol 70% followed the same steps as in the case of the chromatographic analysis for the methanol extracts (figures V.15, V.16, V.17).

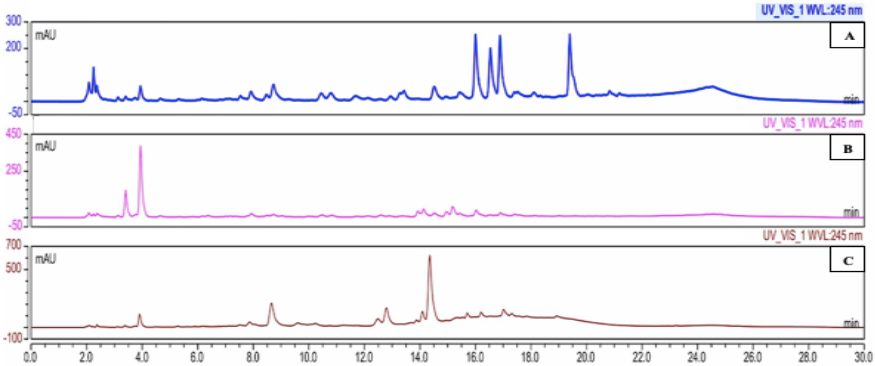


Figure V.15. Chromatograms for ethanol extracts at 245nm: Es_e(A), Ep_e (B), Et_e(C)

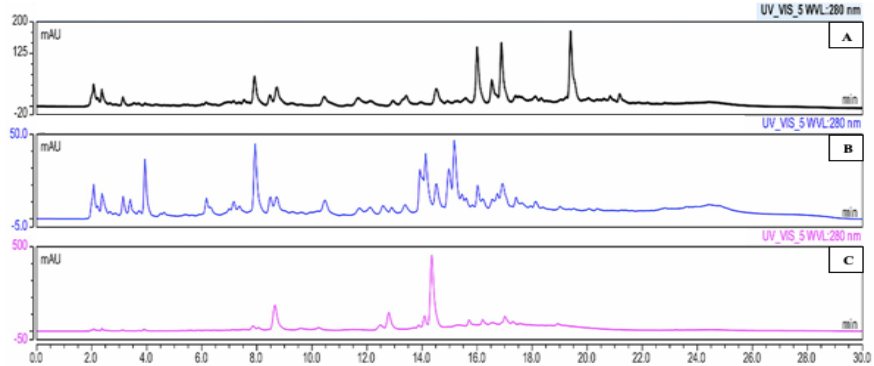


Figure V.16. Chromatograms for ethanol extracts at 280nm: Es_e(A), Ep_e (B), Et_e(C)

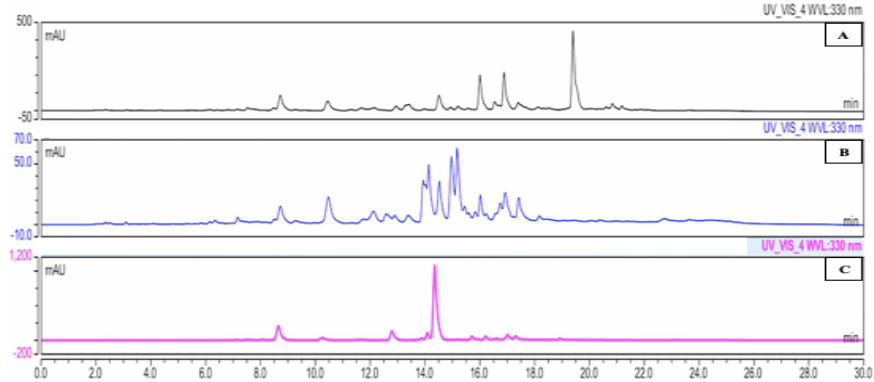


Figure V.17. Chromatograms for ethanol extracts at 330nm: Es_e(A), Ep_e (B), Et_e(C)

The results obtained for the qualitative and semi-quantitative analysis of the ethanol extracts of the three species of the field horsetail are included in table V.10.

Table V.6. Flavonoids identified and quantified in ethanol extracts of *Equisetum* species

Compound expressed in mg/g plant product	**Sample (ethanol extract)**		
	E. sylvaticum	***E. pratense***	***E. telmateia***
epicatechin	0.1586	0.0263	0.7079
quercetin-3-D-glucoside	41.9429	3.8440	13.6175
luteolin-glucoside	3.7656	0.0378	1.2254
apigenin-7-glucoside	7.8806	1.4626	21.0042
luteolin	0.4069	0.0076	0.1455
quercetol	0.0255	0.0071	0.1369
apigenol	0.0591	0.1359	0.3758
kaempferol	0.0216	0.2022	0.7609

It is noted the low solubility of the flavonoid aglycones in the hydroalcoholic solution, but similar to the studies of other researchers, the extraction yield also varies depending on the chemical structure of each component.

Quercetol glycosides are predominant in the *E. sylvaticum* sample, and, in the *E. telmateia* sample, quercetol and apigenol glycosides represent the majority.

The concentration in polyphenolic acids is shown in table V.11.

Table V.7. Polyphenolcarboxylic acids identified in ethanol extracts of *Equisetum* species

Compound expressed in mg/g plant product	**Sample (ethanol extract)**		
	E. sylvaticum	***E. pratense***	***E. telmateia***
neochlorogenic acid	0.4838	0.1879	0.3059
chlorogenic acid	3.6744	07362	14.3814
caffeic acid	0.6624	0.1922	0.3244
ferulic acid	5.7962	0.5559	1.0439

In general, the amount of polyphenolcarboxylic acids is higher in hydroalcoholic extracts than in methanol extracts, but each compound has a different extractability.

V.5. *In vitro* evaluation of the antioxidant activity of the plant extracts obtained from the three species of the *Equisetum* genus

V.5.1. The chelating capacity of the ferrous ion determination

The chelating capacity of ferrous ions is an important indicator for the evaluation of antioxidant activity. The results obtained in this test are shown in figures V.19 and V.20.

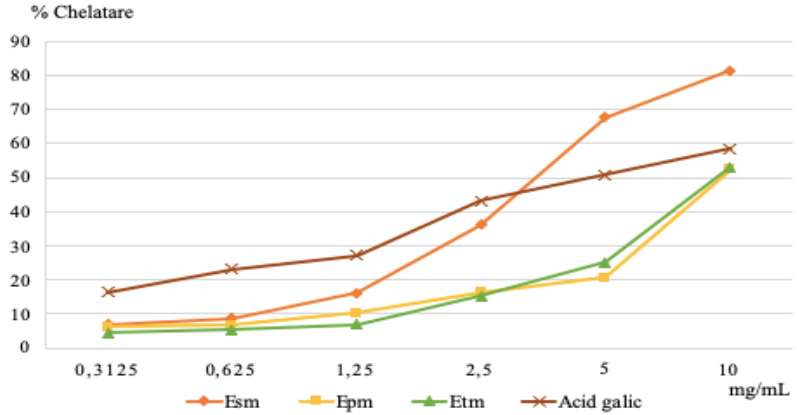


Figure V.18. Graphical representation of the iron chelating capacity (%) depending on the concentration of the methanol extracts used

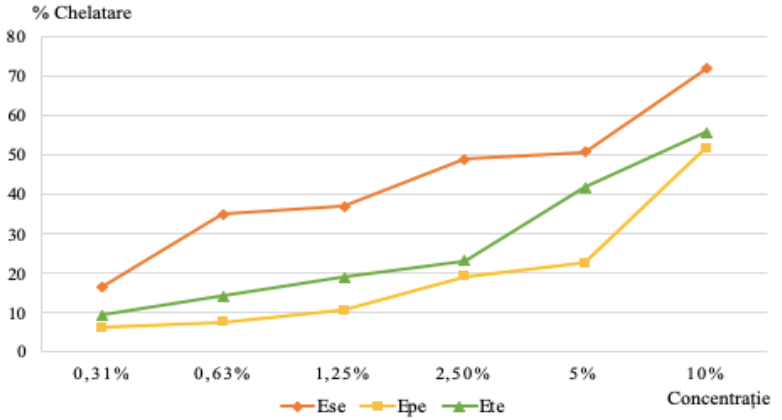


Figure V.19. Graphical representation of the iron chelating capacity (%) depending on the concentration of the ethanol extracts used

Methanol extract 70% of *E. sylvaticum* is more active compared to the gallic acid, this time the phyto-complex present in the extract achieving an addition synergy compared to a single compound.

The analysis of ethanol extracts 70% indicates again the superiority of the extract obtained from *E. sylvaticum*, but, unlike the methanol one, it is not the richest in polyphenols of the three extracts analyzed. This time the difference in efficacy between the extracts of *E. telmateia* and *E. pratense* increases in favor of the first extract.

V.5.2.Determination of the lipoxygenase inhibition capacity

The results obtained for the evaluation of the lipoxygenase inhibition capacity are shown in figures V.21 and V.22.

Comparing for each plant species analyzed, the two types of extracts, it is observed that the methanol extracts are more active than the ethanol ones for *E. pratense*, while, for the other two species, the ethanol extracts are more active.

For both methanol and ethanol extracts, the best antioxidant activity determined for this test was obtained for the *E. sylvaticum* species, the values recorded especially for the ethanol extract being slightly higher, but comparable to those of gallic acid, used as reference.

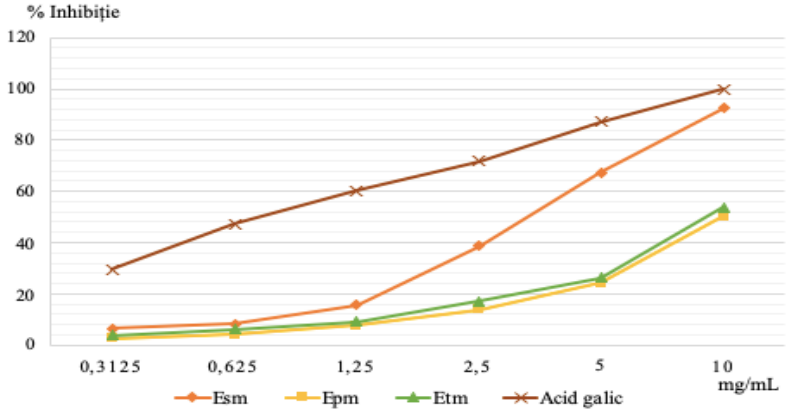


Figure V.20. Graphical representation of the lipoxygenase inhibition capacity (%) depending on the concentration of the methanol extracts used

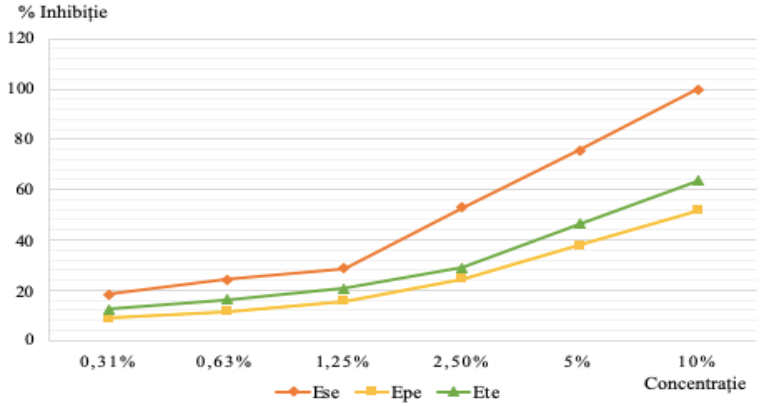


Figure V.21. Graphical representation of the lipoxygenase inhibition capacity (%) depending on the concentration of the ethanol extracts used

V.5.3. The scavenger action of the hydroxyl radical determination

The results obtained when evaluating the scavenger capacity of the hydroxyl radical are presented in Figures V.23 and V.24.

The methanol extract of the species *E. sylvaticum* showed the most important action, being slightly higher than that of the reference substance, the gallic acid.

The lowest scavenger activity of the hydroxyl radical was recorded for the hydro-alcoholic extracts of *E. pratense*.

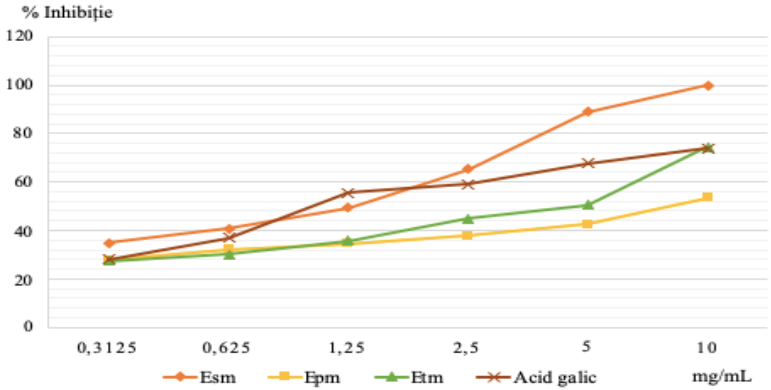


Figure V.22. . Graphical representation of the scavenger capacity of the hydroxyl radical (%) depending on the concentration of the methanol extracts used

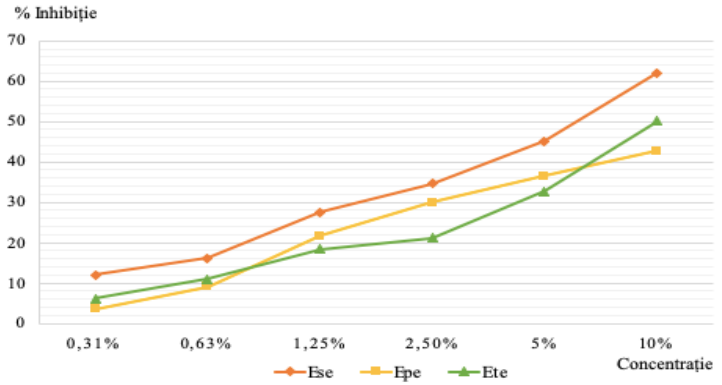


Figure V.23. Graphical representation of the scavenger capacity of the hydroxyl radical (%) depending on the concentration of the ethanol extracts used

V.5.4. The scavenger capacity of the superoxide anion determination

The results obtained when evaluating the scavenger capacity of the superoxide radical anion are presented in figures V.25 and V.26.

It is observed that the antioxidant efficiency for this test is closer as a value for the methanol extracts of *E. sylvaticum* and *E. telmateia* as compared to the reference substance, the gallic acid. As with all other determinants of the antioxidant action, *E. pratense* showed the lowest action for both methanol and ethanol extracts.

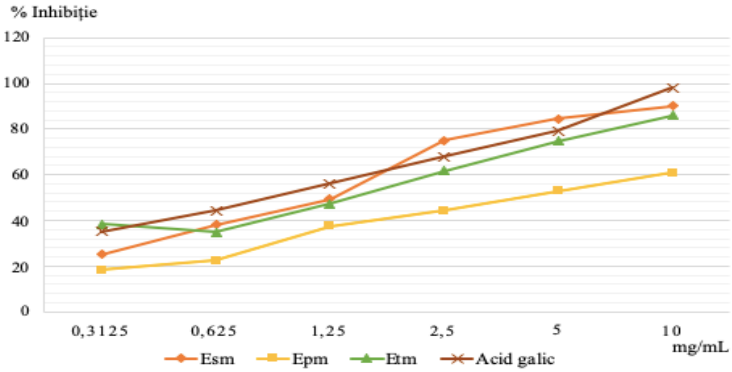


Figure V.24. Graphical representation of the scavenger capacity of the superoxide radical anion (%) depending on the concentration of the methanol extracts

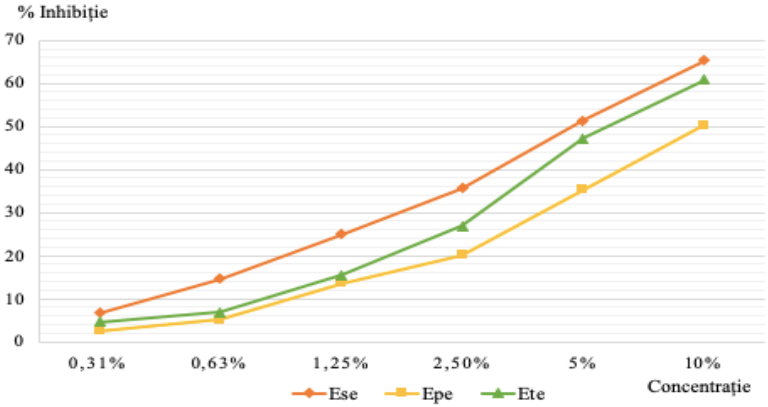


Figure V.25. Graphical representation of the scavenger capacity of the superoxide radical anion (%) depending on the concentration of the ethanol extracts

V.6. *In vitro* evaluation of the antimicrobial activity of the ethanol and methanol extracts of the *Equisetum* species studied

The results of the *in vitro* antimicrobial tests on the methanol and ethanol extracts of the field horsetail species were determined by measuring the inhibition diameter (mm) using the diffusimetric method. These are presented in table V.20.

Table V.8. Antibacterial and antifungal activity of extracts obtained from *Equisetum* species

Sample	Diameter of inhibition zones (mm)			
	S. aureus **(ATCC 25923)**	***E. coli*** **(ATCC 25922)**	***Pseudomonas aeruginosa*** **(ATCC 27853)**	***Candida albicans*** **(ATCC 90028)**
Ep_m	14	0	0	12
Ep_e	13	0	0	21
Es_m	10	0	0	11
Es_e	11	0	0	20
Et_m	16	0	0	16
Et_e	11	0	0	15

Ciprofloxacin	28	30	28	-
Fluconazole	-	-	-	30
Voriconazole	-	-	-	30

For *Staphylococcus aureus*, the largest inhibition diameter was observed in the methanol extract of *E. telmateia* (16mm). This result is lower than the positive control represented by ciprofloxacin.

All the compounds showed a good activity on the *Candida albicans* strain. Ethanol extracts obtained from *E. pratense* and *E. sylvaticum* had the highest inhibition diameter, proving the most pronounced antifungal action (21 mm and 20 mm respectively).

The results obtained are consistent with previous studies that reported the antimicrobial activity of *Equisetum* species, especially on gram positive bacteria, but also on *Candida albicans* strains (20, 104, 134).

V.7. *In vivo* evaluation of the neuroprotective activity of the plant extracts obtained from the three species of *Equisetum*

V.7.3. Behavioral tests

The behavioral tests carried out followed the effects of the ethanol extracts of the three species of *Equisetum* on the anxious behavior (the Swimming Pool Test) and on short-term memory (the Y Labyrinth Test).

V.7.3.1. The Swimming Pool Test (NTT)

The behavior observed during the Swimming Pool Test is used to assess the level of anxiety and the desire to explore. An anxiolytic agent increases the time spent in the upper part of the tank as well as the number of entries.

For each type of *Equisetum* extract (Ep_e, Es_e, Et_e), individual tests were performed at both 0.5mg/L and 1mg/L ethanol extract.

The test results are shown in figures V.28, V.29 and V.30.

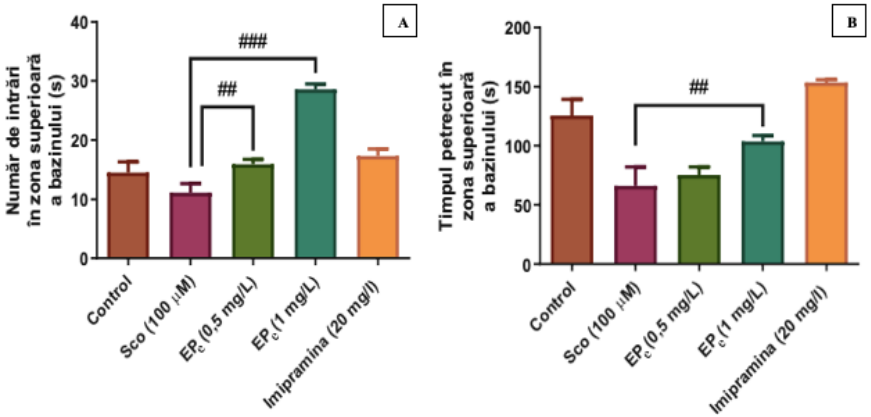


Figure V.26. Effects of ethanol extract of *E.pratense* (0.5mg/L and 1mg/L) on the number of entries to the upper part of the basin (A) and the time spent in the upper area of the basin (B) in zebra fish treated with scopolamine (100μM) in the NTT test. The values are means ± E.S.M. for the Tukey post hoc test multiple comparisons: p <0.0001 vs. Sco (100μM).

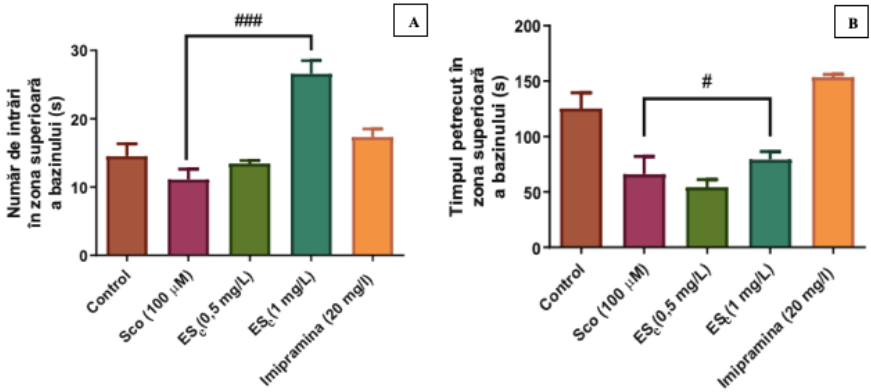


Figure V.27. Effects of ethanol extract of *E.sylvaticum* (0.5mg/L and 1mg/L) on the number of entries to the upper part of the basin (A) and the time spent in the upper area of the basin (B) in zebra fish treated with scopolamine (100μM) in the NTT test. The values are means ± E.S.M. for the Tukey post hoc test multiple comparisons: p <0.0001 vs. Sco (100μM).

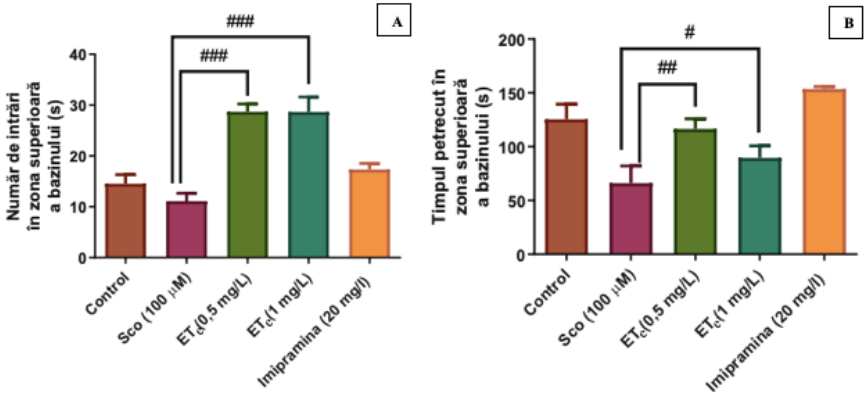


Figure V.28. Effects of ethanol extract of *E.telmateia* (0.5mg/L and 1mg/L) on the number of entries to the upper part of the basin (A) and the time spent in the upper area of the basin (B) in zebra fish treated with scopolamine (100μM) in the NTT test. The values are means ± E.S.M. for the Tukey post hoc test multiple comparisons: $p < 0.0001$ vs. Sco (100μM).

Ethanol extracts from the three species of *Equisetum* had positive effects in assessing anxious behavior. Zebra fish exposed to concentrations of 1mg/L ethanol extract of *E. pratense* and *E. sylvaticum* exhibited anxiolytic and antidepressant effects higher than the concentration of 0.5mg/L. For the ethanol extract from *E. telmateia* it can be observed that the time spent in the upper area of the basin is higher for the concentration of 0.5mg/L compared to 1mg/L.

Compared to the reference substance, the imipramine, the number of entries to the upper area of the basin for the zebra fish was higher at the concentration of 1mg/L, while at the concentration of 0.5mg/L, the number of entries was lower, but comparable with it.

V.7.3.2. The Y Labyrinth Test

The test is based on the tendency of zebra fish to explore a new environment they are in. The percentage of spontaneous alternation is defined as the ratio of the number of alternations performed and the number of possible alternations. This behavior reflects spatial working memory which is a form of short-term memory (265). For each type of *Equisetum* extract (Ep_e, Es_e, Et_e) individual tests were performed at both

0.5mg/L and 1mg/L ethanol extract. The test results are presented in figures V.32, V.33 and V.34.

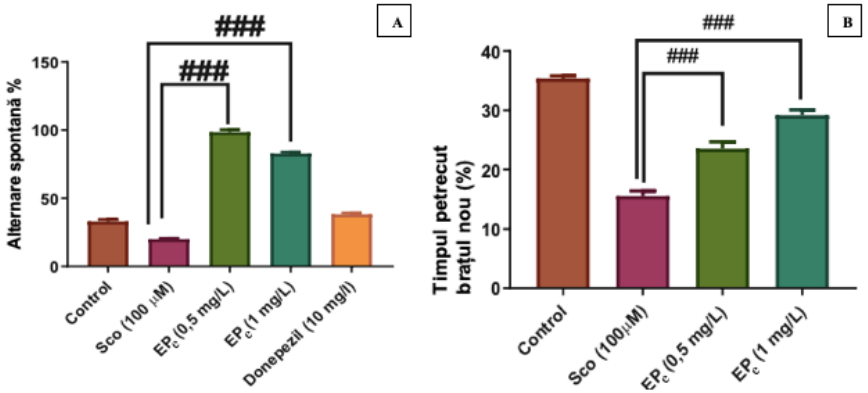


Figure V.29. Effects of ethanol extract of *E.pratense* (0.5mg/L and 1mg/L) on the percentage of spontaneous alternation (A) and the time spent in the new wing (B) in zebra fish treated with scopolamine (100μM) in the Y labyrinth test. The values are means ± ESM for the Tukey post hoc test multiple comparisons: p <0.0001 vs. Sco (100μM).

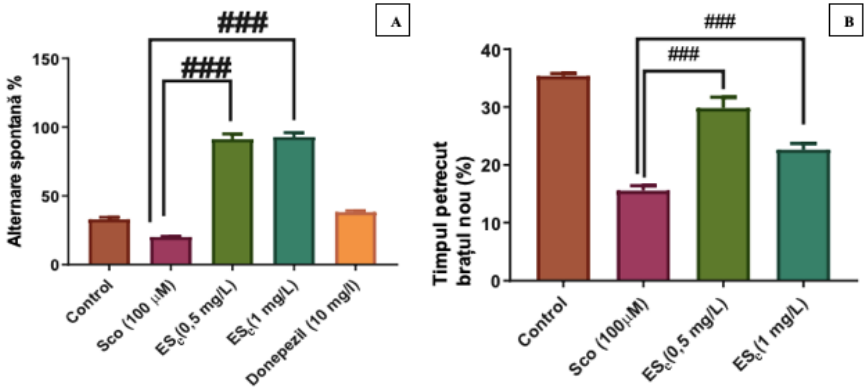


Figure V.30. Effects of ethanol extract of *E.sylvaticum* (0.5mg/L and 1mg/L) on the percentage of spontaneous alternation (A) and the time spent in the new wing (B) in zebra fish treated with scopolamine (100μM) in the Y labyrinth test. The values are means ± ESM for the Tukey post hoc test multiple comparisons: p <0.0001 vs. Sco (100μM).

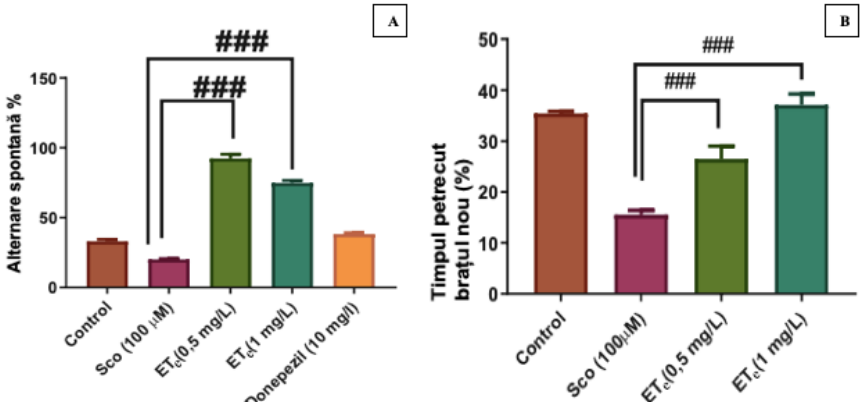


Figure V.31. Effects of ethanol extract of *E.telmateia* (0.5mg/L and 1mg/L) on the percentage of spontaneous alternation (A) and the time spent in the new wing (B) in zebra fish treated with scopolamine (100µM) in the Y labyrinth test. The values are means ± ESM for the Tukey post hoc test multiple comparisons: $p < 0.0001$ vs. Sco (100µM).

Ethanol extracts from the three species of *Equisetum* had positive effects, improving short-term memory. The differences between the ethanol concentrations of 0.5mg/L and 1mg/L were not significant, both with the role of improving memory performance in a dose-dependent manner.

Compared to the reference substance, the donepezil, both the 0.5 mg/L and the 1 mg/L concentrations of the three species caused an even twofold stimulation of short-term memory by increasing the contribution of spontaneous alteration.

When correlating the chemical composition with the biological tests performed *in vitro* (antioxidant action, enzymatic inhibitors, radical scavenger), there is a direct reciprocity relationship between the increased concentration of flavonosids, the doses administered and the anxiolytic and antidepressant intensity in the fish.

Moreover, considering the high level of silicon (water soluble silicon derivatives) of the species *E. telmateia*, it was observed that the efficacy of the extracts is below the level of the other two samples. When evaluating the effect on short-term memory, significant differences are observed that reconfirm the great effectiveness of the *E. pratense* and *E. telmateia* extracts compared to *E. sylvaticum*.

CHAPTER VI. Chemical and biological evaluation of the silver nanoparticles synthesized based on the ethanol and aqueous extracts of the *Equisetum* species (*E. pratense* Ehrh., *E. sylvaticum* L. and *E. telmateia* Ehrh.)

VI.2. Physico-chemical characterization of the AgNPs obtained from the extracts of *Equisetum* species studied

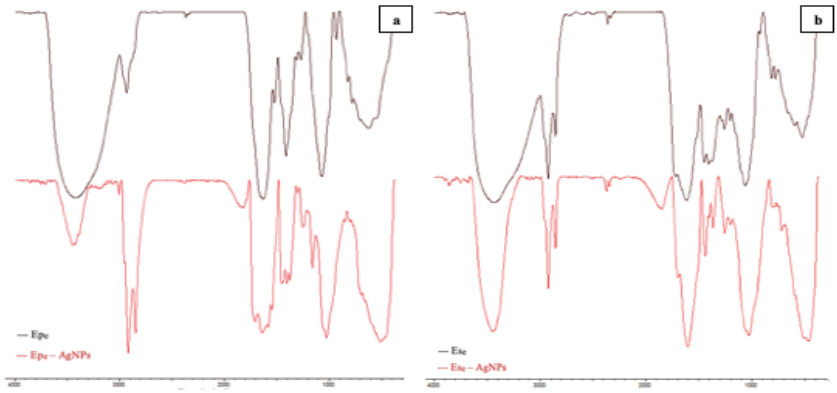
The following methods were used to characterize the AgNPs obtained: FTIR, EDX and DLS spectroscopy.

- **DLS characterization and determination of Zeta potential for colloidal solutions obtained:**

The dimensional distribution and the mean value of the AgNPs diameter were determined by DLS. The mean diameter of the AgNPs is: 124.9nm, with a polydispersity index of 0.587 for Ep_e– AgNPs, 74.4nm and 0.659 for Es_e– AgNPs, 314nm and 0.623 for Et_e– AgNPs and 136.7nm with polydispersity index of 1.116 for Es_a– AgNPs. The Zeta potential values are: **-23.76** for Ep_e– AgNPs; **-29,54** for Es_e– AgNPs; **-20,72** for Et_e– AgNPs and **-23,99** for Es_a– AgNPs.

- **FTIR characterization of the AgNPs of the extracts obtained:**

In order to demonstrate the formation of AgNPs and to see the chemical composition of the AgNPs surface, the FTIR spectra of the extract and the corresponding AgNPs were drawn (Figure VI.11).



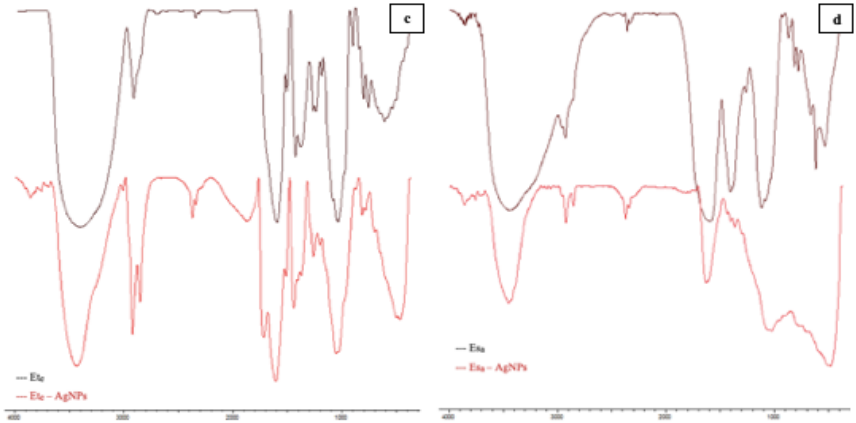


Figure VI.32. Superimposed FTIR spectra for extracts and corresponding AgNPs: Ep_e/Ep_e– AgNPs (a); Es_e/Es_e– AgNPs (b); Et_e/Et_e– AgNPs (c); Es_a/Es_a– AgNPs (d).

For the absorption bands in the FTIR spectra, the types of vibration presented in table VI.3 were assigned.

Table VI.9. Assignment of absorption bands from FTIR spectra

Ep_e	Es_e	Es_e	Es_a	Vibration type
3433	3440	3427	3442	H-O hydrogen bonds from alcohols, phenols (100)
2929, 2364	2924, 2854	2927, 2362	2925, 2356	C-H stretching vibrations of CH_3 and CH_2 (alkanes)
1629	1614	1616	1591	stretching vibrations C=O, stretching vibrations C-N (amide I), asymmetrical stretching vibrations COO^-
1516	1519	1523	-	N-H (amide II) deformation vibrations and aromatic domain
1408, 1307	1408, 1375	1444, 1402	1406	C-O (amide) stretch vibrations and C-C stretching vibrations of phenyl groups, COO^- symmetric stretching vibrations, CH_2 bond vibrations
1265	1259	1284, 1259	1261	C-O stretching vibrations of alcohols, ethers, esters, carboxylic acids
1060	1060	1056	1116	C-O and C-C stretching vibrations from carbohydrates (100)
923 - 621	921 - 520	923 - 630	869 - 536	C-H bond vibrations outside the plane (alkenes) (100, 283)

In case of the AgNPs, the displacement of the absorption bands or the division of the absorption band into smaller intensity peaks has been observed:

- **Ep_e– AgNPs:** → 3448; → 3005 - 2848; → 1706 - 1543; → 1456 – 1375; → 1257; → 1024; 852 – 511
- **Es_e– AgNPs:** → 3448; → 2922 – 2852; 2372 → 1604; → 1436, 1367; → 1257, 1199; → 1026; → 802 – 459
- **Et_e– AgNPs:** → 3433; → 2920, 2850, 2372, 2342; → 1718, 1608; → 1514; → 1438 – 1373; → 1261, 1203; → 1055; 812 – 466
- **Es_a– AgNPs:** → 3450; → 2922; 2852; → 2370, 2341; → 1627; → 1402; 1024; 480

Also, the emergence of new bands is observed:

- **Ep_e – AgNPs:** 1834; 1157;
- **Es_e – AgNPs:** 1851;
- **Et_e – AgNPs:** 1876.

The emergence of bands in the FTIR spectra of the AgNPs can be explained by the attachment to the surface of the AgNPs of some groups from the extracts compounds.

- **EDX characterization of the AgNPs:**

In order to highlight the presence of silver in the AgNPs, the EDX spectra of the synthesized nanoparticles were determined below.

From a quantitative point of view, silver was found in a proportion of: 60.68 (m%) in the case of Ep_e– AgNPs, 63,08 (m%) for Es_e– AgNPs, 42.40 (m%) for Et_e– AgNPs and 74.75 (m%) for Es_a– AgNPs.

In addition to silver, characteristic peaks of carbon, oxygen and nitrogen elements could also be observed:

- for Ep_e –AgNPs: carbon 20.97%, nitrogen 1%, oxygen 3.04%;
- for Es_e–AgNPs: carbon 18.93%, nitrogen 1.52%, oxygen 4.25%;
- for Et_e–AgNPs: carbon 39.47%, nitrogen 1.91%, oxygen 9.42%;
- for Es_a–AgNPs: carbon 11.56%, nitrogen 1.20%, oxygen 3.66%.

VI.4. *In vitro* evaluation of the antioxidant activity of the AgNPs obtained from the *Equisetum* species

The evaluation of the antioxidant action consisted in the use of the extracts obtained from the three plant products and the AgNPs synthesized using plant extracts. The synthesized AgNPs respected the environmentally friendly technologies that are recommended for their realization.

VI.4.1. The chelating capacity of the ferrous ion determination

In the presence of ferozine, Fe^{2+} forms a pink complex with maximum absorbance at 562nm (229).

Table VI.10. The chelating capacity of the ferrous ion of the AgNPs obtained from the extracts

Samples	Solution concentration						CE_{50} (µg AgNPs/ mL)
	2.5 mg/mL	1.25 mg/mL	0.625 mg/mL	0.3125 mg/mL	0.15625 mg/mL	0.078125 mg/mL	
Es_e – AgNPs	83.74 ± 0.15	78.54 ± 0.19	59.18 ± 0.16	41.70 ± 0.07	26.71 ± 0.05	19.16 ± 0.09	86.86 ± 0.39
Ep_e – AgNPs	82.97 ± 0.40	69.49 ± 0.24	32.89 ± 0.10	22.26 ± 0.36	18.77 ± 0.45	12.71 ± 0.11	172.83 ± 0.54
Et_e – AgNPs	85.44 ± 0.24	68.14 ± 0.26	39.74 ± 0.54	27.43 ± 0.08	21.43 ± 0.12	20.78 ± 0.17	160.56 ± 1.72
Es_a – AgNPs	82.50 ± 0.55	72.76 ± 0.52	41.14 ± 0.26	20.31 ± 0.15	16.92 ± 0.15	19.16 ± 0.09	151.80 ± 1.11

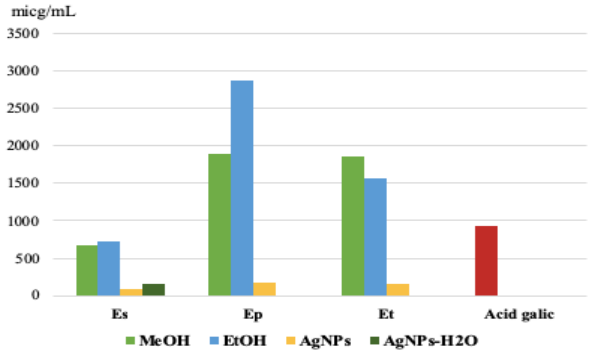


Figure VI.33. Graphical representation of the EC_{50} value determined when evaluating the chelating capacity of the ferrous ion

In table VI.4 and figure VI.17 it can be observed that the AgNPs synthesized from the *E. sylvaticum* extract are only twice as active compared to those prepared with *E. pratense* and *E. telamteia* extracts, while for extracts the difference activity is 4 times (*E. pratense*), respectively twice (*E. telamteia*) higher. Also, methyl alcohol allows better extraction of polyphenols, and the chelating capacity of the ferrous ion is comparable to that of the gallic acid.

VI.4.2. Determination of the lipoxygenase inhibition capacity

The active compounds present in the extracts block 15-lipoxygenase by blocking the oxidation of linoleic acid and reducing the absorbance to 234nm (235).

Table VI.11. Lipoxygenase inhibition capacity of the AgNPs obtained from the extracts

Samples	Solution concentration						CE_{50} (µg AgNPs/ mL)
	2.5 mg/mL	1.25 mg/mL	0.625 mg/mL	0.3125 mg/mL	0.15625 mg/mL	0.078125 mg/mL	
Es_e – AgNPs	74.37 ± 2.36	65.26 ± 1.66	53.24 ± 1.42	35.93 ± 1.57	28.03 ± 0.85	12.16 ± 0.63	9.14 ± 0.53
Ep_e – AgNPs	53.58 ± 1.73	38.15 ± 1.52	28.10 ± 0.91	19.98 ± 1.67	15.14 ± 0.48	8.30 ± 0.27	35.56 ± 2.68
Et_e – AgNPs	56.91 ± 1.98	42.63 ± 1.84	34.01 ± 1.59	25.35 ± 1.71	18.40 ± 0.81	8.73 ± 0.41	29.88 ± 2.77
Es_a – AgNPs	50.73 ± 2.02	37.22 ± 2.23	30.68 ± 0.26	27.11 ± 1.53	5.54 ± 1.76	3.60 ± 1.48	40.22 ± 4.18

From the AgNPs prepared it can be observed (table VI.5) that the reduced efficacy of lipoxygenase inhibition is present Es_a – AgNPs followed by Ep_e – AgNPs. The species *E. sylvaticum* has the most important inhibitory capacity of lipoxygenase, both for methanol and ethanol extracts, but also for Es_e – AgNPs. The AgNPs prepared from the ethanol extract of *E. sylvaticum* showed efficacy comparable to the gallic acid CE_{50} (figure VI.19).

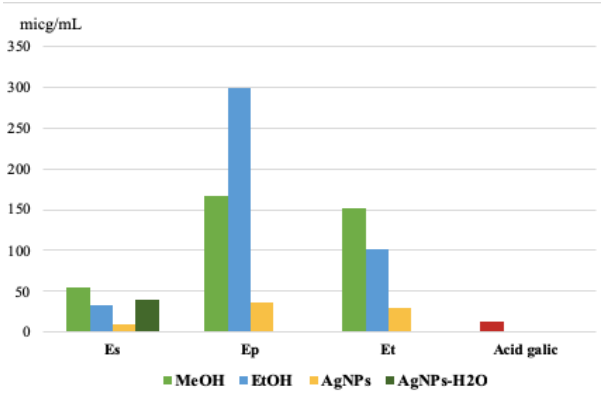


Figure VI.34. Graphical representation of the EC_{50} value (µg/mL final solution) determined when evaluating the lipoxygenase inhibition capacity

VI.4.3. The scavenger capacity of the hydroxyl radicals determination

The hydroxyl radical, formed in the reaction between the ferrous ion and the hydrogen peroxide, will hydroxylate the salicylic acid with the formation of a pink-purple compound with maximum absorbance at 562nm (238).

Table VI.12. The scavenger capacity of the hydroxyl radical of the AgNPs obtained from the extract

Samples	Solution concentration						CE_{50} (µg AgNPs/ mL)
	2.5 mg/mL	1.25 mg/mL	0.625 mg/mL	0.3125 mg/mL	0.15625 mg/mL	0.078125 mg/mL	
Es_e – AgNPs	88.90 ± 1.63	65.14 ±1.31	49.17 ± 1.47	40.87 ± 0.85	34.70 ± 0.90	31.19 ± 0.47	60.79 ± 3.85
Ep_e – AgNPs	42.43 ± 0.87	37.72 ± 0.91	34.62 ± 0.78	32.05 ± 0.65	27.87 ± 0.35	22.61 ± 0.26	-*
Et_e – AgNPs	51.24 ± 1.07	45.47 ± 0.65	35.57 ± 0.86	33.13 ± 0.73	27.35 ± 0.69	23.70 ± 0.38	204.04 ± 24.21
Es_a – AgNPs	37.18 ± 0.74	29.25 ± 0.36	21.08 ± 0.17	17.95 ± 0.32	14.08 ± 0.18	10.52 ± 0.11	-*

For the neutralization of the hydroxyl radical, the presence of hydrogen donor groups is required. It can be observed (table VI.6 and figure VI.21) that the Es_e – AgNPs presented the most significant scavenger capacity of the hydroxyl radical, comparable to the gallic acid. For Ep_e – AgNPs and for Es_a – AgNPs the effective concentration

could not be calculated because at the maximum concentration tested, no scavenger capacity of the hydroxyl radical greater than 50% was obtained.

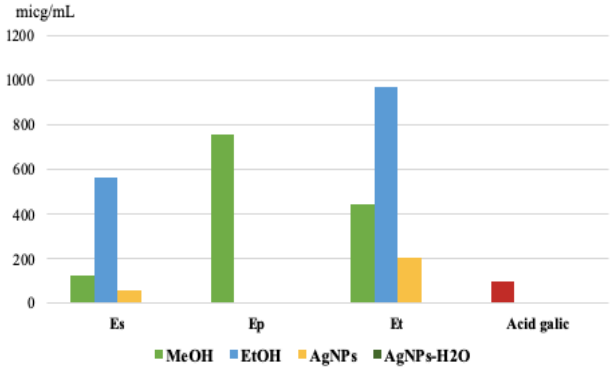


Figure VI.35. Graphic representation of the CE_{50} value determined when evaluating the scavenger capacity of the hydroxyl radical

VI.4.4. The scavenger capacity of the superoxide radical anion determination

The superoxide radical generated by the nicotinamide adenine nucleotide reduced-phenazine methosulfate system reduces nitroblue tetrazole to a violet-blue formazan with absorbance at 560nm (240).

Table VI.13. The scavenger capacity of the superoxide radical anion of the AgNPs obtained from the extracts

Samples	Solution concentration						CE50 (µg AgNPs/ mL)
	2.5 mg/mL	1.25 mg/ mL	0.625 mg/ mL	0.3125 mg/ mL	0.15625 mg/ mL	0.078125 mg/ mL	
Es_e – AgNPs	84.25 ± 2.04	60.28 ± 0.97	47.53 ± 1.18	37.62 ± 0.71	28.56 ± 0.54	21.45 ± 0.39	178.80 ± 11.07
Ep_e – AgNPs	50.96 ± 1.28	32.14 ± 0.84	26.89 ± 0.94	21.62 ± 0.85	12.23 ± 0.41	4.65 ± 0.10	604.15 ± 28.00
Et_e – AgNPs	62.25 ± 1.82	50.82 ± 1.05	39.25 ± 0.59	28.57 ± 0.60	20.69 ± 0.70	15.23 ± 0.12	298.36 ± 18.23
Es_a – AgNPs	33.25 ± 0.45	27.58 ± 0.23	20.08 ± 0.36	17.85 ± 0.28	10.95 ± 0.41	5.68 ± 0.28	-*

*CE50 was not calculated because at the maximum concentration tested, no scavenger capacity of the hydroxyl radical greater than 50% was obtained.

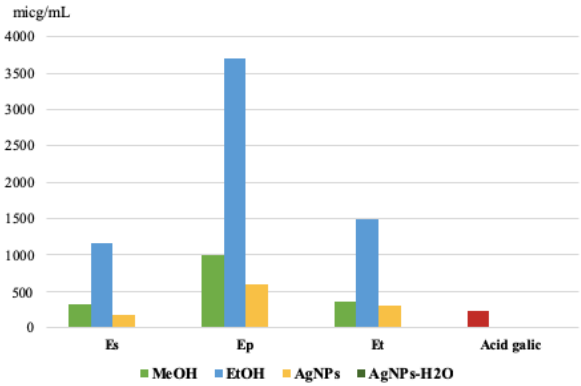


Figure VI.36. Graphic representation of the CE_{50} value (µg/mL final solution) determined when evaluating the scavenger capacity of the superoxide radical anion

The nanoparticles obtained from the ethanol extracts proved to be less effective in this test compared to the first three tests performed. It can be observed (table VI.7 and figure VI.23) that, in this test also, the Es_e – AgNPs showed the highest scavenger capacity of the superoxide anion, while, the Ep_e – AgNPs, as in all other antioxidant tests performed, obtained the weakest results. It can also be noted that the antioxidant efficiency of this test is closer in value to the Es_e – AgNPs and the Et_e – AgNPs.

If the presence of hydrogen donor groups in the scavanger molecule is required for the neutralization of the hydroxyl radical, both hydrogen donor groups and functional groups capable of neutralizing the anion charge are required to neutralize the superoxide radical anion.

VI.5. *In vitro* evaluation of the antimicrobial activity of the AgNPs obtained from the extracts of the *Equisetum* species

VI.5.2. Determination of the minimum inhibitory concentration using EUCAST instructions

The *in vitro* sensitivity test was performed following EUCAST 7.1 instructions for yeasts and EUCAST 5.1 instructions for antimicrobial sensitivity testing of bacteria and the evaluation of efficacy on the synthesized AgNPs from the four extracts is determined

by the minimum inhibitory concentration. The results are presented in table VI.9.

Table VI.14. Minimum inhibitory concentration of the AgNPs synthesized from the *Equisetum* species

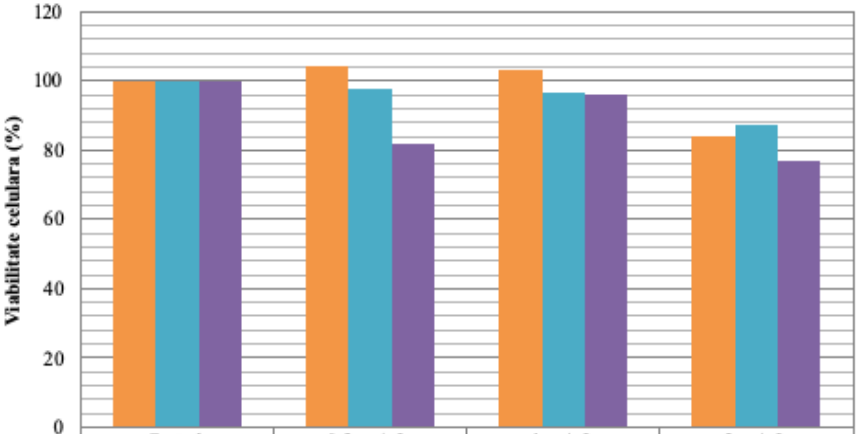
Sample	Minimum inhibitory concentration (CMI) (µg/mL)			
	***Staphylococcus aureus* MRSA (ATCC 43300)**	***Escherichia coli* (ATCC 25922)**	***Pseudomonas aeruginosa* (ATCC 27853)**	***Candida albicans* (ATCC 90028)**
Ep_e – AgNPs	512	512	512	256
Es_e – AgNPs	512	> 512	512	256
Es_a – AgNPs	512	> 512	512	512
Et_e – AgNPs	512	512	512	256

Following the tests performed, it was established that all the analyzed extracts had antimicrobial activity. It has been observed that the synthesized AgNPs from the *Equisetum* species had a greater antifungal activity than the antibacterial one, as demonstrated by the diffusimetric method presented above. The Es_a – AgNPs recorded the lowest minimum inhibitory concentration.

As for the antibacterial activity, for both gram positive and gram negative bacteria it can be observed that the CMI obtained for the AgNPs was 512µg/mL.

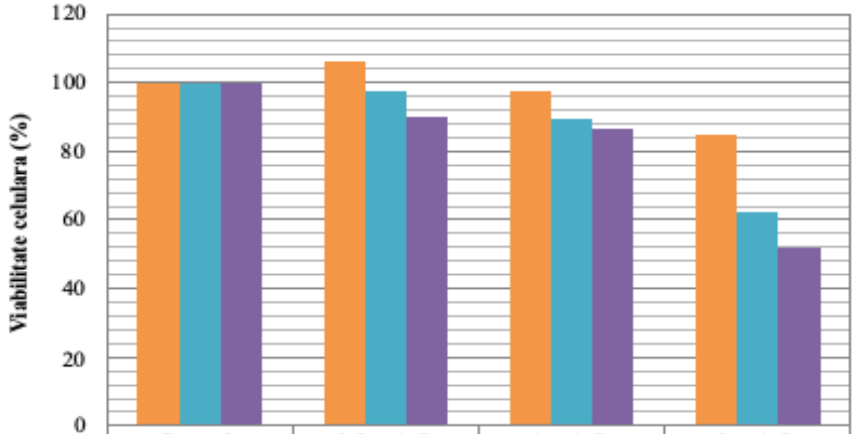
VI.6. *In vitro* evaluation of the antitumor action of the AgNPs obtained from the extracts of the *Equisetum* species

The presence of viable cells in the cultures incubated with extracts was evaluated by the MTT method at 24 hours, 48 hours and 72 hours after incubation. In figures VI.26, VI.27, VI.28 and VI.29 are presented the results of cell viabilities calculated and reported to the witness for the Ep_e – AgNPs, Et_e – AgNPs, Es_e – AgNPs and Es_a – AgNPs studied.



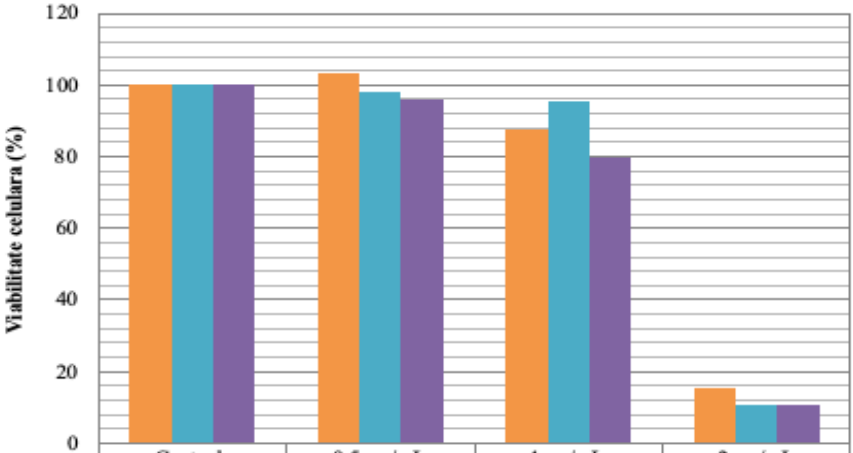
	Control	0,5 mg/mL	1mg/mL	2mg/mL
24 ore	100	104,53	102,99	84,03
48 ore	100	97,57	96,85	87,19
72 ore	100	81,78	96,03	77,00

Figure VI.37. Cell viability determined by the MTT test for Ep_e – AgNPs



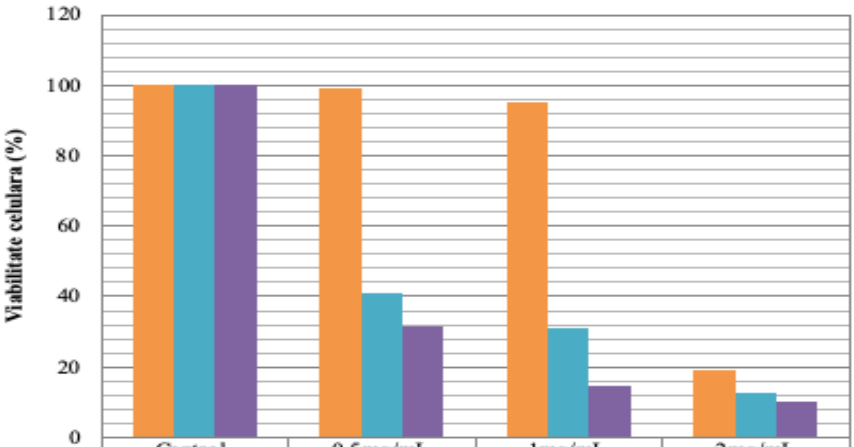
	Control	0,5mg/mL	1mg/mL	2mg/mL
24 ore	100	105,96	97,42	84,82
48 ore	100	97,76	89,64	62,19
72 ore	100	90,23	86,52	51,75

Figure VI.38. Cell viability determined by the MTT test for Et_e – AgNPs



	Control	0,5mg/mL	1mg/mL	2mg/mL
24 ore	100	103,15	87,48	15,35
48 ore	100	98,29	95,28	10,62
72 ore	100	95,99	80,01	10,51

Figure VI.39. Cell viability determined by the MTT test for Es_e – AgNPs



	Control	0,5mg/mL	1mg/mL	2mg/mL
24 ore	100	98,99	95,42	19,27
48 ore	100	41,04	31,14	12,87
72 ore	100	31,67	14,53	9,95

Figure VI.40. Cell viability determined by the MTT test for Es_a – AgNPs

From the analysis of all the charts it can be easily observed that the species *E.sylvaticum* L., both the AgNPs synthesized from the ethanol extract, but also the aqueous one, had the lowest cell viability corresponding to a high cytotoxicity.

The determinations concluded that the lowest concentration that produced a change below DL_{50} for the analyzed cell lines is obtained for the Es_a – AgNPs (0,5mg/mL and 48 hours).

The highest cell viability, even at the highest concentration (2mg/mL) and for the longest time (72 hours) was recorded for the Ep_e – AgNPs, the result being slightly below 80%.

CHAPTER VII. General conclusions, the degree of originality and research perspectives

General conclusions

The macro- and microscopic pharmacognostic analysis allowed the study of the morphological characteristics and the highlighting of the histo-anatomical elements specific to the three species of *Equisetum* studied. It was observed the existence of specific features depending on the geographical conditions and climate, regarding the main stem and the stem branch, which helps in the correct identification of the three species. From a microscopic point of view, all species are characterized by the presence of numerous stomates on whose walls there are numerous granules of silicon dioxide. *E. sylvaticum* has no gaps in the bark, while for *E. pratense* 13 aeriferous cavities are identified, and for *E. telmateia* approx. 18 gaps.

The evaluation of the silicon and heavy metals content of the aerial parts of the *Equisetum* species consisted of spectrophotometric analyzes that showed that the *E. telmateia* species has the highest silicon concentration for both the main stem and the stem branch from the nodal whorl (5.962% respectively 7.578%). The quantitative determinations of heavy metals from the dry plant product highlighted *E. pratense* as the species with the highest capacity to accumulate Cu^{+2}, Cd^{+2} and Ni^{+2}, without these products interfering with the development of the plant.

The qualitative chemical analysis of the bioactive components allowed the approximation of several polyphenolic and flavonoid acids

from the ethanol and methanol extracts of the *Equisetum* species studied: chlorogenic acid, gallic acid, caffeic acid, apigenol-7-glucoside, isoquercetin.

The quantitative chemical analysis by spectrophotometric methods of the methanol and ethanol extracts obtained from *E. pratense* Ehrh., *E. sylvaticum* L. and *E. telmateia* Ehrh. allowed the determination of the total polyphenols content (mg gallic acid/g dry extract) and flavonoids (mg quercetol/g dry extract). Of all the extracts analyzed, the highest concentration in total polyphenols, 236.17mg gallic acid/g dry extract, was obtained for the *E. telmateia* species (ethanol extract), and the highest concentration in flavonoid derivatives was recorded for *E. sylvaticum* (methanol extract), that is 170.65mg quercetol/g dry extract.

The UHPLC analysis of methanol and ethanol extracts highlighted the presence of the following components: chlorogenic acid, caffeic acid, ferulic acid, neochlorogenic acid, epicatechin, quercetol, quercetol-3-D-glucoside, luteolin, luteolin-glucoside, apigenol, apigenin-7-glucoside and kaempferol. Of all the polyphenolic acids, chlorogenic acid was found in a significant amount in both extracts for *E. sylvaticum* and *E. telmateia* species. As for the flavonoid derivatives, in the methanol extract of the *E. sylvaticum* species luteolin-glucoside (11.05mg/g) is noted, while for the ethanol extract, the quercetol glycosides (41.94mg/g) are noted. For *E. telmateia*, in both extracts, quercetol and apigenol glycosides represented the majority. *E. pratense* did not present numerous flavonoid fractions or phenolic acids in the extracts studied. One possible explanation may be that this plant is a very good heavy metal accumulator.

The synthesis of silver nanoparticles was performed from ethanol and aqueous extracts.

The physico-chemical characterization of the AgNPs was performed by the following methods:

- **FTIR spectroscopy** led to the identification of the bonds type O-H, C-H, C=O, C-O, respectively C-C.
- **EDX spectroscopy** was used to highlight the presence of silver in the AgNPs. From a quantitative point of view, silver was found in the highest proportion in the case of Es_a – AgNPs, 74.75 (m%), and the smallest amount was recorded for Et_e – AgNPs, 42.40 (m%).

- **DLS spectroscopy and Zeta potential determination** provide information on the mean diameter of the AgNPs and the colloidal solution stability obtained. The Et_e – AgNPs recorded a mean diameter of 314nm and a polydispersity index of 0.623, and the Zeta potential value of -20.72.

The photocatalytic activity of the AgNPs synthesized from the *Equisetum* species has proven that they can be an ecological, rapid and economical choice to remove organic dyes from the environment.

The antioxidant action of the methanol, ethanol extracts and nanoparticles obtained from the three *Equisetum* species analyzed was highlighted by four tests:

The determination of the chelating capacity of the ferrous ion indicated that the methanol, ethanol extracts and the AgNPs synthesized from the *E. sylvaticum* species was the most active, being comparable to the gallic acid.

The determination of the lipoxygenase inhibitory capacity showed that methanol extracts are more active than ethanol extracts only for *E. pratense.* The best antioxidant activity was obtained for the *E. sylvaticum* species. As for the AgNPs, it can be observed that the lower efficacy of lipoxygenase inhibition is represented by the aqueous extract of the *E. sylvaticum* species, followed by the ethanol extract of the *E. pratense* species. The best efficiency is recorded in the Es_e – AgNPs.

The determination of the scavenger capacity of the free radicals indicated that both methanol extract and synthesized AgNPs of the *E. sylvaticum* species showed the most important action, being comparable to the reference substance, the gallic acid.

The determination of the scavenger capacity of the superoxide radical anion shows that the antioxidant efficiency is closer in value to the methanol extracts of *E. sylvaticum* and *E. telmateia* compared to the reference substance, the gallic acid. Also, the determinations made on the synthesized AgNPs indicated that *E. sylvaticum* has the highest scavenger capacity of the superoxide anion, whereas, *E. pratense* obtained the weakest results.

The antimicrobial action of the methanol, ethanol extracts and the nanoparticles obtained from the three species of *Equisetum* analyzed was highlighted by two methods:

The diffusimetric method characterizes the antibacterial activity of the methanol extracts 70%, of the ethanol extracts 70% and of the AgNPs obtained using gram positive, gram negative bacteria and fungi. All analyzed extracts of all species exhibited more important antifungal activity than antibacterial activity. Also, only the AgNPs obtained from the aqueous extract of *E. sylvaticum* developed inhibition diameter on gram negative bacterium, *E. coli*. Regarding the action on the gram-positive bacterium *S. aureus*, it was established that the methanol extract caused the greatest inhibition diameter to occur in both the *E. pratense* and *E. telmateia* species.

The determination of the minimum inhibitory concentration was performed only for the AgNPs synthesized from the *Equisetum* species. The AgnPs synthesized from the *Equisetum* species have been shown to have greater antifungal activity than antibacterial.

The *in vitro* evaluation of the antitumor action of the AgNPs obtained from the *Equisetum* species revealed an important concentration and time-related cytotoxicity for the *E. sylvaticum* species, both for the aqueous and ethanol extracts.

The *in vivo* evaluation of neuroprotective activity was performed by following up the effects produced by the ethanol extracts of the three species of *Equisetum* above zebra fish in terms of anxious behavior (the Swimming Pool Test) and short-term memory (the Y Labyrinth Test).

The Swimming Pool Test (NTT) analyzed the number of entries to the upper area of the basin and the time spent in the upper area of the tank. The 1 mg/L concentration in *E. pratense* and *E. sylvaticum* had anxiolytic and antidepressant effects higher than the 0.5 mg/L concentration. Regarding the ethanol extract of *E. telmateia*, the time spent in the upper area of the basin is higher for the concentration of 0.5mg/L compared to 1mg/L.

The Y Labyrinth Test analyzed the percentage of spontaneous alternation and the time spent in the new wing. The differences between the ethanol concentrations of 0.5mg/L and 1mg/L were not significant for the analyzed species.

The degree of originality

The originality of this study consists of:

- the investigation for the first time in our country of the species *E.pratense* Ehrh. and *E.sylvaticum* L.;
- the study of the morphological characteristics and highlighting the specific histo-anatomical elements for the species *E.pratense* Ehrh., *E.sylvaticum* L. and *E.telmateia* Ehrh.;
- the analysis of the silicon and heavy metals content in the aerial parts of the *Equisetum* species*;*
- the synthesis and physico-chemical characterization of the AgNPs obtained from the ethanol and aqueous extracts of the *Equisetum* species;
- the evaluation of the antioxidant and antimicrobial activity of the AgNPs synthesized from the *Equisetum* extracts*;*
- the *in vitro* determination of the antitumor action for the AgNPs obtained from the *Equisetum* species studied using MG63 cell lines;
- highlighting the neuroprotective effects of the plant extracts obtained from the *Equisetum* species on *Danio rerio* by evaluating the anxious behavior (the Swimming Pool Test) and short-term memory (the Y Labyrinth Test).

Research perspectives

The results of the research carried out within the doctoral thesis justify the continuation of the studies in the following directions:

- the evaluation of the *in vivo* neuroprotective effects on animal models of Alzheimer's disease for *Equisetum* extracts*;*
- the isolation of certain flavonosids subfractions and the evaluation of their complex biological potential;
- the follow-up of the impact of the silicon and other minerals accumulation level on the extractability of the different classes of chemical compounds present in the plant material;
- the investigation of the pharmacotoxicological potential of different types of nanoparticles and inclusions in cyclodextrins made from *E. sylvaticum*.

SELECTIVE BIBLIOGRAPHY

3. Margulis L, Schwartz K. *Five Kingdoms: an Illustrated Guide to the Phyla of Life on Earth*. New York: WH Freeman and Company, 2001.
11. Alexan M, Bojor O, Crăciun F. *Flora Medicinala a Romaniei Vol. I*. București: Ceres, 1988.
17. Simpson MG. *Evolution and Diversity of Vascular Plants*. 2012. Epub ahead of print 2012.
18. Husby C. Biology and Functional Ecology of Equisetum with Emphasis on the Giant Horsetails. *Bot Rev* 2013; 79: 147–177.
20. 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.
100. Altameme H, Hameed I, Biol NA-S-, 2015 U. Analysis of bioactive phytochemical compounds of two medicinal plants, Equisetum arvense and Alchemila valgaris seed using gas chromatographymass. *ResearchgateNet* 2015; 44: 47–58.
104. Radojević ID, Stanković MS, Stefanović OD, Topuzović MD, Čomić LR, Ostojić AM. Great horsetail (Equisetum telmateia Ehrh.): Active substances content and biological effects. *EXCLI J* 2012; 11: 59–67.
123. Al-Snafi AE. The pharmacology of Equisetum arvense- A review. *IOSR J Pharm* 2017; 07: 31–42.
127. Calliste CA, Trouillas P, Allais DP, Simon A, Duroux JL. Free radical scavenging activities measured by electron spin resonance spectroscopy and B16 cell antiproliferative behaviors of seven plants. *J Agric Food Chem* 2001; 49: 3321–3327.
128. Štajner D, Popović BM, Čanadanović-Brunet J, Boža P. Free radical scavenging activity of three Equisetum species from Fruška gora mountain. *Fitoterapia* 2006; 77: 601–604.
129. Pallag A, Jurca T, Pasca B, Sirbu V, Honiges ANA, Costuleanu M. Analysis of Phenolic Compounds Composition by HPLC and Assessment of Antioxidant Capacity in Equisetum arvense L . Extracts. *Rev Chem* 2016; 67: 1623–1627.

132. Niculae M, Spînu M, Şandru CD, Brudaşcă F, Bolfă P. Studii preliminare asupra potențialului antimicrobian al unor extracte vegetale. *Ann SNBC* 2008; 13: 312–317.
133. Alavarce RAS, Saldanha LL, Almeida NLM, Porto VC, Dokkedal AL, Lara VS. The Beneficial Effect of Equisetum giganteum L. against Candida Biofilm Formation: New Approaches to Denture Stomatitis. *Evidence-Based Complement Altern Med* 2015; 2015: 1–9.
134. Yeganegi M, Tabatabaei Yazdi F, Mortazavi SA, Asili J, Alizadeh Behbahani B, Beigbabaei A. Equisetum telmateia extracts: Chemical compositions, antioxidant activity and antimicrobial effect on the growth of some pathogenic strain causing poisoning and infection. *Microb Pathog* 2018; 116: 62–67.
182. Sandhu NS, Kaur S, Chopra D. Equietum arvense: Pharmacology and phytochemistry - a review. *Asian Journal of Pharmaceutical and Clinical Research*.
185. Schweingruber FH, Börner A. *The Plant Stem*. 2018. Epub ahead of print 2018.
189. Sârbu I, Stefan N, Oprea A. *Plante vasculare din România. Determinator ilustrat de teren*. București: Victor B. Victor, 2013.
190. Zajączkowska U, Kucharski S, Nowak Z, Grabowska K. Morphometric and mechanical characteristics of Equisetum hyemale stem enhance its vibration. *Planta* 2017; 245: 835–848.
191. Jackson BP SD. *Atlas of microscopy of medicinal plants, culinary herbs and spices*. London: Belhaven Press, 1992.
195. Kirkham MB. Cadmium in plants on polluted soils: Effects of soil factors, hyperaccumulation, and amendments. *Geoderma* 2006; 137: 19–32.
210. Vrinceanu N, Motelica D, Dumitru M, Gamet E, Toti M, Tanase V, Preda M. Estimation of some heavy metals accumulation in plants and soils from copsa mica area. *USAMV Bucharest* 2009; LII: 76–81.
212. Mai J, Yang Q, Zhang Y, Zeng X, Zhong Y, Liu D. The Enrichment and Transfer of Heavy Metals for Two Ferns in Pb-Zn Tailing. *MATEC Web Conf* 2017; 100: 04030.

220. Čanadanović-Brunet JM, Ćetković GS, Djilas SM, Tumbas VT, Savatović SS, Mandić AI, Markov SL, Cvetković DD. Radical scavenging and antimicrobial activity of horsetail (Equisetum arvense L.) extracts. *Int J Food Sci Technol* 2009; 44: 269–278.
229. Dinis TCP, Madeira VMC, Almeida LM. Action of Phenolic Derivatives (Acetaminophen, Salicylate, and 5-Aminosalicylate) as Inhibitors of Membrane Lipid Peroxidation and as Peroxyl Radical Scavengers. *Arch Biochem Biophys* 1994; 315: 161–169.
235. Malterud KE, Rydland KM. Inhibitors of 15-Lipoxygenase from Orange Peel. *J Agric Food Chem* 2000; 48: 5576–5580.
238. Jeong JB, Chul Hong S, Jin Jeong H. 3,4-Dihydroxybenzaldehyde purified from the barley seeds (Hordeum vulgare) inhibits oxidative DNA damage and apoptosis via its antioxidant activity. *Phytomedicine* 2009; 16: 85–94.
240. Wang Z, Luo D. Antioxidant activities of different fractions of polysaccharides purified from Gynostemma pentaphyllum Makino. *Carbohydr Polym* 2007; 54–58.
265. Hritcu L, Foyet HS, Stefan M, Mihasan M, Asongalem AE, Kamtchouing P. Neuroprotective effect of the methanolic extract of Hibiscus asper leaves in 6-hydroxydopamine-lesioned rat model of Parkinson's disease. *J Ethnopharmacol* 2011; 137: 585–591.
283. Topală CM, Tătaru L., Ducu. ATR-FTIR spectra fingerprinting of medicinal herbs extracts prepared using microwave extraction. *Arab J Med Aromat Plants* 2017; 3: 1–9.