

Comparative Study of Polyphenols, Flavonoids and Chlorophylls in *Equisetum arvense* L. Populations

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Equisetum arvense L. is known as horsetail. *E. arvense* extracts are considered important areas in drug development, with numerous pharmacological activities, in many countries. This species is not cultivated in Romania, the medicinal product is coming from traditional collection centers. In this study we followed the presence and quantity of polyphenols, flavonoids and chlorophyll from the sterile stems coming from different areas and different growing seasons. We used spectrophotometric methods to determine the antioxidant activity in the sterile stems of *Equisetum arvense* L., collected from different regions of Bihor and Arad County, between May and September of 2014. Our results show significant differences and we concluded that, for obtaining a medicinal product rich in substances with antioxidant activity, plants should be harvested from forested areas and mainly in May and June period.

Keywords: polyphenols, flavonoids, chlorophylls, comparative studies, *Equisetum arvense* L.

Equisetum arvense L., field horsetail or common horsetail is a herbaceous perennial fern, native throughout the arctic and temperate regions of the northern hemisphere. Horsetail has been used in medicine as far back as ancient Greece. It is a widely used medicinal species, and is considered an authentic living fossil, which in many countries are protected plants [1, 2].

The *Equisetum arvense* L. appears in the Romanian Pharmacopoeia [3] and in the European Pharmacopoeia [4] too. This species is not cultivated in Romania, the medicinal product is coming from traditional collection centers and from other countries, so the exact origin of the product is difficult to follow. Whole or cut, dried sterile aerial parts, *Equiseti herba*, of *Equisetum arvense* L. can be used in phytotherapy [3, 4].

The chemical content of the *Equiseti herba*: inorganic compounds (especially silica), caffeic acid derivatives, flavonoids, alkaloids, saponins [5-7]. The extracts possess free radicals scavenging activity [7]. Flavonoids (flavones and flavonols) are generally present in a glycoside form. Flavonoids and phenolic acids have antioxidant activity. The important active ingredients of the field horsetail are flavonoids, their content affect the quality of the drug [6-12]. The traditional uses and therapeutic activity of *Equisetum arvense* have been established through modern testing and evaluation in different disease conditions. These investigations, place this indigenous drug, as a novel candidate for bio prospection and drug development for the treatment of several diseases as anemia, inflammation, cancer, convulsions, diabetes, ulcers, anxiety and depressive disorders [7, 13-18].

The anti-inflammatory, antioxidant and antiproliferative activities, are the subject of many current studies [10, 19-20]. The medicinal applications of this plant and countless possibilities for investigation still remain in relatively newer areas of its function [16-20].

To the best of our knowledge, there are no information's about the variation in chemical composition, antioxidant properties of *Equisetum arvense* L. populations, from Romanian spontaneous flora. It has a great ecological spreading, from the plains to the mountains. Traditionally, in Romania, horsetails sterile stems are collected from July to September [2-4].

The aim of this study was to evaluate the amount of flavonoids, phenolic acids and chlorophylls in *Equisetum arvense* L. collected from different areas, in different periods of the year, in order to assess in which pedological, climatically conditions and period it accumulates highest quantities of active principles.

Experimental part

We performed a comprehensive study, in which we followed, during 2014, in five different areas, from two counties of Romania, the spontaneous *Equisetum arvense* L. populations.

There were studied the sterile stems of *Equisetum arvense* L., collected from five different regions of Oradea area (O1, O2), Beius area (B1, B2), Cefa area (C1, C2), from Bihor County, Macea area (M1, M2), Santana area (SA1, SA2) and from Arad County.

The area O1, O2, B1, B2, M1, M2, are in wooded regions, with clayey soil. The area C1, C2, SA1, SA2 are in sandy, wet soil. During five months, respectively between May and September in 2014, in each area we delimited 0.5x0.5 m zones, in which we measured the number of sterile stems and the stems lengths, monthly. In the same time, we harvested the sterile stems. We used the fresh stems to determine the amount of chlorophylls (*chlorophyll A* and *chlorophyll B*). We used dried sterile stems to measure the total quantity of phenolic acids and flavonoids [21].

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Determination of chlorophylls contents

All the determination were made with the Shimadzu UV-1700 Pharmaspec UV-VIS Spectrophotometer and alcohol was used as the control sample. We measured the absorbance at 646.6 nm wavelength, at which absorption is maximum for *chlorophyll B*, respectively 663.6 nm wavelength, at which absorption is maximum for *chlorophyll A*.

From all samples, there were taken 1 g of leaves, that were grinded in the mortar with 10-15 mL 70% ethanol, then filtered. The filtrates represents the samples. The chlorophyll quantity from the samples was calculated using the Porra R.J. formulas [22]:

$$[\text{Chl a}] = 12.25 E_{663.6} - 2.55 E_{646.6} \mu\text{g/mL}$$

$$[\text{Chl b}] = 20.31 E_{646.6} - 4.91 E_{663.6} \mu\text{g/mL}$$

$$[\text{Chl a+b}] = 17.76 E_{646.6} + 7.34 E_{663.6} \mu\text{g/mL}$$

$$[\text{Chl a/b}] = 12.25 E_{663.6} - 2.55 E_{646.6} / 20.31 E_{646.6} - 4.91 E_{663.6} \mu\text{g/mL}$$

Determination of total phenolic contents - Folin-Ciocalteu method

The alcoholic extract solutions, known as tinctures, were prepared by maceration in ethylic alcohol of 70%, at room temperature (20°C) for 10 days. The residue was removed by decantation.

Total phenolic contents were determined by using the Folin-Ciocalteu reagent, according to the method of Singleton and Rossi [23] by using gallic acid as standard, with some modifications. The extract solution (0.1 mL) containing 1000 µg 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% Na₂CO₃ was added. At the end of the 2 h incubation at room temperature, the absorbance of each mixture was measured at 765 nm in Shimadzu UV-1700 Pharmaspec UV-VIS Spectrophotometer. The same procedure was applied to the standard solutions of gallic acid, and a standard curve was obtained.

With the Folin-Ciocalteu method, we measured the OH groups of the samples taken into study in alkaline conditions (adjusted with sodium carbonate). The absorbance at the 765 nm wavelength increases proportional with the number of OH groups of the anthocyanins. The calibration curve (fig. 1) was obtained with a solution of known concentration of gallic acid (20-100 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 [23].

Determination of total flavonoid contents

The content of total flavonoids was determined by using a colorimetric method, which has been described previously [24]. The sample of 1 mL (containing 0.1 mg/mL dry weight) is mixed with 4 mL water and placed in a 10 mL volumetric flask.

First 0.3 mL ground NaNO₂ 5% is added, after 5 min 0.3 mL AlCl₃ 10% and after 6 min 2 mL NaOH 1M. The volumetric flask is filled to the mark with distilled water. The solution is mixed in the volumetric flask and it is read the absorbance at 510 nm in the Shimadzu UV-1700 Pharmaspec UV-VIS Spectrophotometer. The calibration curve (fig. 2) shall create its standard using quercetin (QE) [24, 25].

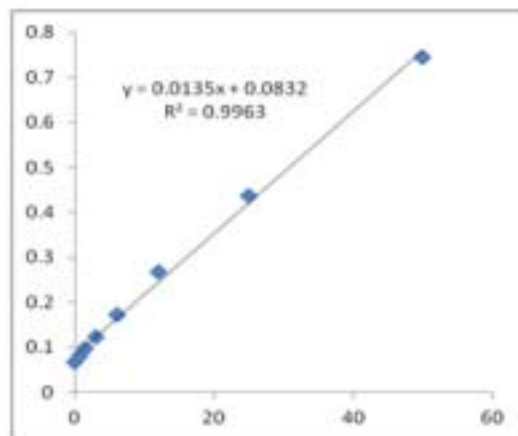


Fig. 1. Calibration curve made with gallic acid (Folin-Ciocalteu method) in ethanol medium

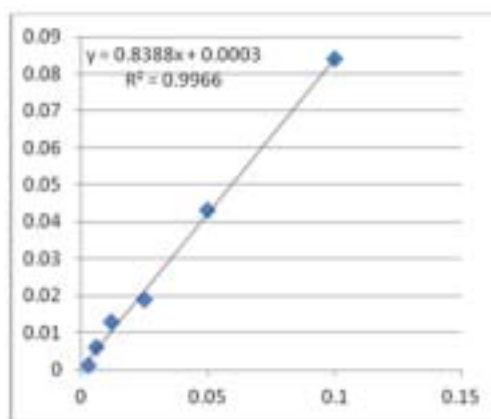


Fig. 2. Calibration curve made with quercetin in ethanol medium (surroundings, environment)

Results and discussions

The number and length of the sterile stems are presented in table 1 and 2. Maximum length of sterile stems was achieved in August and September in all studied areas, with some differences. The number of sterile stems increased from month to month. It can be seen that, in the areas where the field is wet (C1, C2, SA1, SA2), the number of the sterile stems was smaller, but the average value of the length of the sterile stems, was bigger than in other areas.

Chlorophylls contents

The obtained results were presented in table 3 and 4 and in figure 3 and 4. The quantity of chlorophyll A is lower in C1, C2, SA1 and SA2 areas. Between the quantities of chlorophyll B, there are no differences.

We observed that from month to month, the quantity of chlorophylls does not vary much. We calculated and compared the average values (fig. 3) and the ratio between *chlorophyll A* and *chlorophyll B* values (fig. 4). In C1, C2, SA1 and SA2 areas, values are lower, compared to other areas.

Total phenolic contents

The amounts of total phenols in the alcoholic extracts of *Equiseti herba* for different vegetative growth stage and different areas were determined spectrometrically according to the Folin-Ciocalteu procedure and calculated as gallic acid equivalents. Gallic acid is a water-soluble polyhydroxyphenolic compound that can be found in various natural plants. The amounts of total phenols found in the ethanolic extract of sterile stems are shown in figure 5.

Period 2014	The number of sterile stems									
	O1	O2	B1	B2	C1	C2	M1	M2	SA1	SA2
May	32	30	40	48	25	28	45	41	19	22
June	38	47	38	52	27	28	49	43	25	24
July	45	65	42	54	32	31	56	55	35	32
August	60	68	47	54	33	35	71	68	33	30
September	58	66	48	56	36	35	72	70	34	32

Table 1
EQUISETUM ARVENSE STERILE STEMS
NUMBER (IN 0.25 m²)

Period 2014	Length average of sterile stems [cm]									
	O1	O2	B1	B2	C1	C2	M1	M2	SA1	SA2
May	17.5	21.0	16.3	18.2	20.5	22.3	12.6	14.2	11.7	16.2
June	22.5	22.7	19.8	22.5	23.2	26.7	19.9	20.2	20.3	21.2
July	25.6	26.3	25.5	26.4	27.7	28.9	25.4	23.0	24.0	27.7
August	27.2	26.7	30.2	30.9	35.2	37.5	32.6	30.0	36.0	35.0
September	24.8	26.2	31.2	32.0	35.0	37.2	33.0	31.5	36.2	35.4

Table 2
EQUISETUM ARVENSE STERILE STEMS
LENGTH AVERAGE VALUE (IN 0.25 m²)

Period 2014	[Chl a] µg/mL									
	O1	O2	B1	B2	C1	C2	M1	M2	SA1	SA2
May	17.19	18.06	19.90	18.37	9.55	11.64	17.56	16.84	11.01	11.68
June	17.50	18.64	20.24	18.85	10.24	11.75	17.50	17.36	11.67	11.72
July	18.26	18.73	20.35	18.87	10.57	11.62	17.89	17.41	11.90	12.24
August	18.17	19.08	20.32	18.50	10.80	11.45	17.41	17.57	11.94	11.85
September	17.64	19.17	20.05	18.44	10.75	11.20	17.72	17.32	11.83	11.85

Table 3
EQUISETUM ARVENSE
CHLOROPHYLL A QUANTITYS IN
STUDIED POPULATIONS

Period 2014	[Chl b] µg/mL									
	O1	O2	B1	B2	C1	C2	M1	M2	SA1	SA2
May	36.30	35.73	35.92	33.90	33.88	32.62	30.58	31.20	32.41	31.34
June	36.45	35.67	36.32	34.23	33.93	32.75	31.35	31.65	32.78	31.62
July	36.78	35.60	36.37	34.62	33.54	32.50	31.67	31.63	32.81	31.50
August	36.40	35.82	36.45	34.27	33.20	32.14	31.48	31.44	32.46	31.71
September	36.56	35.53	36.52	34.34	33.43	32.36	31.52	31.28	32.70	31.56

Table 4
EQUISETUM ARVENSE CHLOROPHYLL
B QUANTITYS IN STUDIED
POPULATIONS

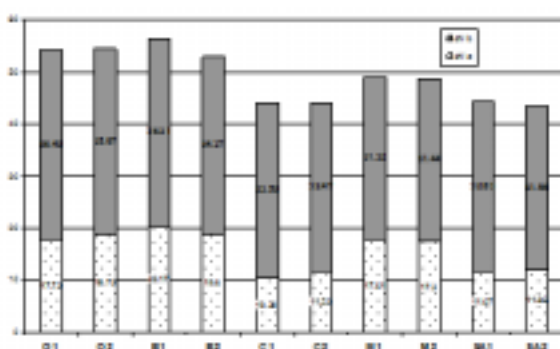


Fig. 3. *Equisetum arvense* chlorophyll A and chlorophyll B average values

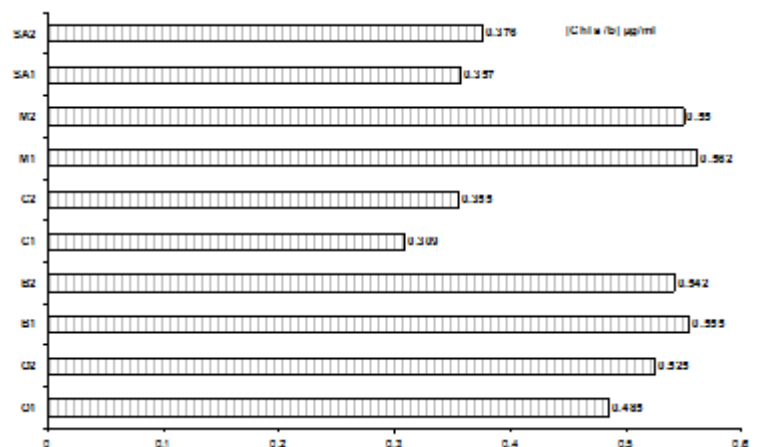


Fig. 4. *Equisetum arvense* chlorophyll A and chlorophyll B ratio

It is observed a big difference between the regions with wet soil, sandy soil and compact soil from the hill regions. At the beginning of the vegetation period, May, June, even July the quantities of polyphenols are higher than at the end of the period.

Total flavonoids contents

The amounts of total flavonoids found in the ethanolic extract of sterile stems are shown in figure 6.

Flavonoids are found in smaller amounts in *Equisetum* herba than polyphenols. The amount of flavonoids varies greatly depending on pedoclimatic conditions but also on harvesting period. Therefore it is observed that at the beginning of the vegetation period, in May and June, the quantities of flavonoids are much higher than in August and September. Greater amounts of flavonoids were

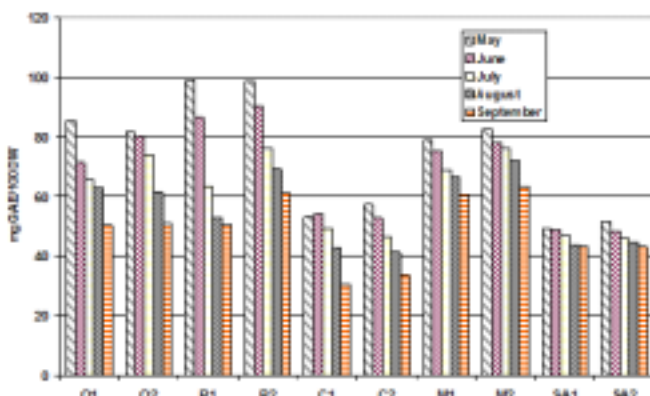


Fig. 5. *Equisetum arvense* total phenols amounts (mgGAE/100DW)

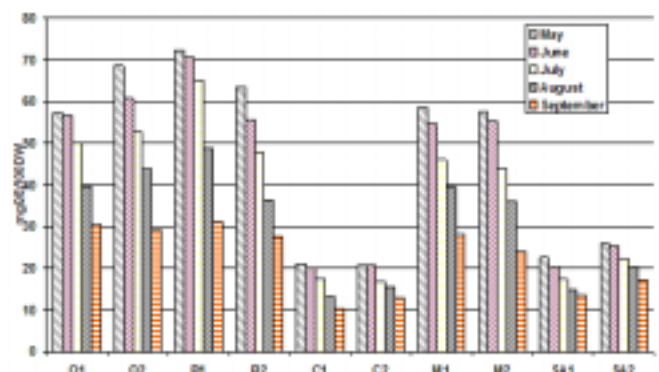


Fig. 6. *Equisetum arvense* total flavonoids amounts (mgQE/100DW)

Period 2014	Total flavonoids in dried drugs (%)									
	O1	O2	B1	B2	C1	C2	M1	M2	SA1	SA2
May	0.57	0.68	0.72	0.63	0.21	0.20	0.58	0.57	0.22	0.25
June	0.56	0.60	0.70	0.55	0.20	0.20	0.54	0.55	0.20	0.25
July	0.50	0.52	0.64	0.47	0.17	0.16	0.46	0.43	0.17	0.22
August	0.39	0.43	0.48	0.36	0.13	0.15	0.39	0.36	0.14	0.20
September	0.30	0.29	0.31	0.27	0.10	0.13	0.28	0.24	0.13	0.17

Table 5
EQUISETUM ARVENSE TOTAL FLAVONOIDS IN
DRIED DRUGS (%)

obtained in other studies [1, 6, 7] but the material used came from not spontaneous flora cultures. The European Pharmacopoeia and the Romanian Pharmacopoeia, require a minimum of 0.3% flavonoids in *Equiseti herba*. By transforming the obtained results in percentages (table 5), it is observed that in some areas (C1, C2, M1, M2, SA1, SA2) in August and September, which represent the traditional harvest months for the sterile strains (sterile stems), the quantities of flavonoids are well below the values provided. In May and June the quantities of flavonoids are high. Analyzing the results obtained, it can be observed a clear correlation between the pedoclimatic conditions, plant size and density, content of chlorophyll, polyphenols and flavonoids.

The chlorophyll level is directly linked to the compounds with antioxidant effect [26], which in our case are polyphenols and flavonoids. In the areas O1, O2, B1, B2, M1, M2, where harvesting was done from forested areas with clay soil, the stems density is high and the amount of chlorophyll is higher. Polyphenols and flavonoids with antioxidant activity are synthesized in large quantities, especially in May and June. In the areas C1, C2, SA1, SA2, where the soil is wet, the stems reach longer lengths, but have low density. Lower quantities of chlorophyll are correlated with lower quantities of polyphenols and flavonoids.

Conclusions

Equisetum arvense L. is a medicinal species used for a long time. As in the recent years the number of studies conducted on this species focusing on the antioxidant action has increased, based on the ability of neutralizing free radicals. The studied areas from the spontaneous flora of Romania, showed a clear correlation between the climatic conditions and the amount of substances with antioxidant capacity. These substances accumulate in large quantities in the stems from forested areas, especially in May and June. We recommend the therapeutic use of the sterile stems from these areas harvested in May and June, compared to traditional harvesting period between July and September.

Introduction into culture of this species should be taken in consideration; this will ensure the quantity and quality of the medicinal product, used in phytotherapy.

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