

ASSESSMENT OF ANTIOXIDANT CAPACITY OF SOME EXTRACTS FOR FURTHER USE IN THERAPY

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Abstract

Salviae and *Plantago* species are plants known as having antioxidant effects. The aim of the study was to compare *in vitro* the antioxidant capacity of these medicinal plants from two different areas of Romania (Bihor and Arad counties) in order to use it in the future in the field of pharmacology and medicine. An UV-Vis spectra fingerprint (250-800 nm) of ethanol extracts from *Salviae* and *Plantago* species was recorded in order to establish the maximum absorption, specifically bioactive compounds. The bioactive compounds from those two extracts, polyphenols types were determined by the Folin-Ciocalteu method, and the total flavonoids have been determined through a spectrophotometric method.

Antioxidant capacity of the extracts was evaluated by the following methods: DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) and FRAP (the Ferric reducing antioxidant power). The results shown, that *Salviae* species are rich in polyphenols, while *Plantago* is rich in flavonoids being a good reason for their association.

Rezumat

Speciile de *Salviae* și *Plantago* sunt plante cunoscute ca având efecte antioxidante. Scopul lucrării a fost acela de a evalua capacitatea antioxidantă a acestora prin studiul comparativ a două surse vegetale din două zone diferite din România. Prin asocierea lor se poate realiza o formulă de suplimente nutritive cu rol protector celular. Extractelor vegetale li s-a trasat un spectru UV-VIS pentru a evidenția domeniile în care absorb diverșii compuși bioactivi. Speciile bioactive din cele două extracte au fost evidențiate, cele de tipul polifenolilor totali au fost determinate prin metoda Folin-Ciocalteu, iar flavonoidele totale au fost determinate printr-o metodă spectrofotometrică. Capacitatea antioxidantă a extractelor a fost evaluată prin următoarele metode: DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) și FRAP (the Ferric reducing antioxidant power). Rezultatele obținute au demonstrat că speciile de *Salviae* sunt mai bogate în polifenoli, iar cele de *Plantago* sunt bogate în flavonoide, fiind un bun argument pentru asocierea lor.

Keywords: antioxidant, polyphenols, *Plantago* sp., *Salviae* sp.

Introduction

Some food products contain compounds with antioxidant activity. In recent years the studies have shown that the widespread use of synthetic antioxidants in food can damage the health status of people.

Searching for compounds that can be used as natural additives and antioxidant, researchers' attention turned to plant foods.

Many sources of antioxidants of plant origin have been studied in recent years and identified as potential antioxidants.

Their effect is due to the presence of hydroxyl groups in their chemical structure [7]. Considering the antioxidant capacity of *Salviae* and *Plantago* species, we have aimed the evaluation of their activity comparatively, using two different plant sources, from two different areas of Romania, with

the possibility of associating them in nutritional supplements formulas with cell protecting role.

For this purpose, we have applied the method for determining the scavenger action of diphenylpicrylhydrazyl radicals after establishing the total polyphenols content for two extracts obtained from two species of *Salviae* and *Plantago*.

Materials and Methods

Preparing the herbal extracts

Two medicinal species were studied, each having their antioxidant efficiency demonstrated after their extracts were prepared. The studied species were *Salviae* sp. and *Plantago* sp. from different and unpopulated geographical areas of Bihor and Arad counties. Thus, two samples were obtained for each species, noted S1 (*Salviae* sp. – Bihor), S2 (*Salviae*

sp. – Arad), P1 (*Plantago* sp. – Bihor) and P2 (*Plantago* sp. – Arad), respectively [11, 15].

The fluid extract of *Salviae folium* was obtained by Squibb repercolation, having 70° alcohol as solvent [5]. The extract was standardized at a total polyphenols content of 0.25%, expressed in chlorogenic acid. The phenolic quantitative analysis was performed through thin layer chromatography and the quantitative determination through the official titrimetric method from the 10th edition of the Romanian Pharmacopoeia [14]. *Extractum Salviae fluidum* was produced in the Pharmaceutical Technology Laboratory of the Faculty of Medicine and Pharmacy Oradea [10].

The fluid extract of *Plantago folium* was prepared from *Plantago* sp. by Squibb repercolation, using 50° alcohol as a solvent [5, 14]. The extract was standardized at a total polyphenols content of 0.25% expressed in chlorogenic acid. The phenolic quantitative analysis was performed through thin layer chromatography and the quantitative determination through the official titrimetric method from the Romanian Pharmacopoeia X [14]. *Extractum Plantago folium* was produced in the Pharmaceutical Technology Laboratory of the Faculty of Medicine and Pharmacy Oradea [7].

UV-Vis spectrophotometric fingerprint of medicinal plant extracts

An UV-VIS spectrum was obtained for the 4 extracts (250-800 nm), after a prior dilution (1:500 v/v) using a Shimadzu UV-VIS 1700 PharmaSpec, Shimadzu Corp. Kyoto, Japan spectrophotometer.

Quantitative determination of bioactive compounds

Total polyphenols determination (Folin-Ciocalteu method)

The total polyphenols content was determined by the Folin-Ciocalteu method using the method proposed by Vicaș et al., with some modification. Briefly, the sample (*Salviae fluidum* extracts (S1 and S2) and *Plantago fluidum* (P1 and P2) extracts) (100 µL) was mixed with 1750 µL distilled water, 200 µL Folin-Ciocalteu reagent (dilution 1:10, v/v) and 1000 µL of 15% Na₂CO₃ solution, and the mixture was incubated at room temperature, in the dark, for 2 hours. The absorbance was measured at 765 nm using a spectrophotometer Shimadzu mini UV-Vis [12]. The calibration curve was linear for the range of concentrations between 0.1-0.5 mg/mL gallic acid. Total polyphenols content of extracts was expressed as mg gallic acid equivalents (GAE)/mL extract, using the following equation based on the calibration curve:

$$y = 1.9735x + 0.0261 \quad (R^2 = 0.9928),$$

where x was the absorbance recorded at 765 nm and y was the gallic acid equivalent (mg/mL) [9, 13].

Determination of total flavonoids

The total flavonoids content was determined using a colorimetric method [6]. Firstly, 1 mL of the extract (containing 0.1 mg/mL dry matter) was mixed with 4 mL water and accurately introduced in a volumetric flask (10 mL). Then, 3 mL of NaNO₂ (5%) solution were added, shaken up and allowed standing for 5 minutes. Secondly, 0.3 mL of the AlCl₃ (10%) solution was added to the volumetric flask, shaken, and was left to stand for 6 minutes. Finally, 2 mL of the NaOH (1M) solution was added to the volumetric flask, followed by addition of water to the scale, shaken, and left to stand for 15 minutes before determination. The absorbance was recorded at 510 nm, using a spectrophotometer Shimadzu mini UV-Vis and the results were expressed as mg quercetin equivalent (QE)/mL. The calibration curve was linear for the range of quercetin concentrations between 0.1-0.5 mg/mL. The equation based on the calibration curve was:

$$y = 56.818571x - 0.066498 \quad (R^2 = 0.9983),$$

where x was the absorbance and y was the mg quercetin equivalent/mL.

Assessing the antioxidant capacity of the extracts

DPPH (2,2-diphenyl-1-picrylhydrazyl) method

The DPPH radical-scavenging activity was determined using the method proposed by Brand-Williams *et al.*, [3]. Briefly, a volume of 100 µL plant extract at different concentrations and 2900 µL DPPH (80 µM) methanol solution were added and the decrease in the absorbance of the resulting solution was monitored at 515 nm after 5 min [3, 8]. The percentage of scavenging effect of extract against DPPH radicals was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_s) \times 100] / A_0$$

where,

A₀ - absorbance of the blank;

A_s - absorbance of the samples at 515 nm.

ABTS assay

Trolox Equivalents Antioxidant Capacity is a spectrophotometric method, widely used for the assessment of antioxidant activity of various extracts and performed using the method of Arnao *et al.* [1]. This method measures the ability of compounds to scavenge the ABTS⁺ cation radical in relation to Trolox. Shortly, the ABTS⁺ cation radical was produced by reacting the ABTS⁺ (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) solution (7 mM) with potassium persulfate (2.45 mM) solution, keeping the mixture in dark at room temperature for 16 h. ABTS stock solution was diluted in order to obtain an absorbance of 0.70 ± 0.02 at 734 nm. After addition of 25 µL extract to 2.5 mL of diluted ABTS⁺, the

mixture was mixed very well (using vortex) for 30 seconds and the interaction between the antioxidants and the $\text{ABTS}^{\cdot+}$ was monitored spectrophotometrically at 734 nm, exactly at 1 minute. The calibration curve was linear for the range of Trolox concentrations between 0.125 and 2 mmol/L, and the results were expressed in μmol Trolox equivalent/mL extract. The ABTS value was obtained using the following equation based on the calibration curve:

$$y = 1629x + 98.94 \quad (R^2 = 0.998),$$

where x was the absorbance and y was the μmol Trolox equivalent.

FRAP (Ferric Reducing Antioxidant Power) assay

The FRAP assay was applied according to the method of Benzie and Strain [2] with some modifications. The FRAP assay consists in the ferric tripyridyltriazine (Fe(III)-TPTZ) complex reduction to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by an antioxidant at low pH [2]. The stock solutions included: 300 mM acetate buffer; 270 mg $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ dissolved in 50 mL distilled water; 150 mg TPTZ and 150 μL HCl, dissolved in 50 mL distilled water. The working FRAP solution was freshly prepared by mixing 50 mL acetate buffer, 5 mL $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ solution and 5 mL TPTZ solution. Plant extracts (100 μL) were allowed to react with 500 μL FRAP solution and 2 mL distilled water, for 1 h, in dark. Absorbance was measured at 595 nm using the spectrophotometer Shimadzu mini UV-Vis. The calibration curve was linear for the range of Trolox concentrations between 50 and 500 $\mu\text{mol/L}$, and the results were expressed in μmol Trolox equivalent/mL extract. The FRAP value was obtained using the following equation based on the calibration curve:

$$y = 0.0157x + 0.0549 \quad (R^2 = 0.9981),$$

where x was the absorbance and y was the μmol Trolox equivalent/mL [4].

Statistical analysis

The antioxidant capacities comparison between crop locations of *Salviae* sp. and *Plantago* sp., was done by one-way analysis of variance, ANOVA ($n = 4$, $p = 0.05$), with GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, www.graphpad.com).

The multivariate ordination performed methods were principal component analysis, PCA, and hierarchical cluster analysis, HCA. The software used for the multivariate analysis was P.A.S.T. version 3.04 statistical software, (Palaeontology Statistics, Copyright Øyvind Hammer and D.A.T. Harper).

Results and Discussion

UV-VIS spectrophotometric fingerprint of medicinal plant extracts

UV-VIS spectrophotometric fingerprint of both medicinal plant extracts (*Salviae* sp. and *Plantago* sp.) were presented in Figure 1. The qualitative spectrophotometric analysis is a simple, very economic and easy to use technique to identify the main bioactive compounds. The maximum wavelengths specific for phenolic acids derivatives is 280, for flavonoids 280 and 340 nm, for carotenoids between 410-470 nm, for anthocyanins is between 520-535 and/or chlorophylls is 647 and 663 nm. In this case, both medicinal plants showed the maximum wavelengths in the domain between 280-375 nm (Table I), these means that these plant extracts are rich in bioactive compounds from phenolic acid and flavonoid classes.

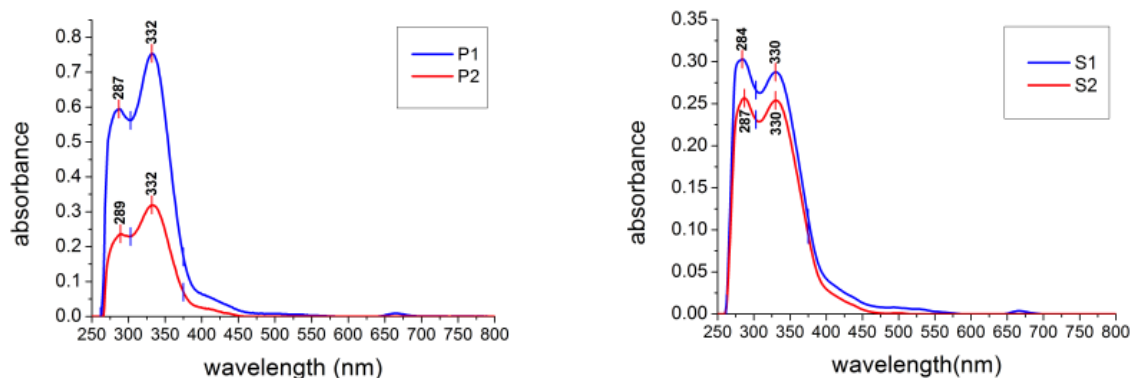


Figure 1.

UV-VIS spectrophotometric fingerprint of *Plantago* (P1 and P2) and *Salviae* (S1 and S2) plant extracts.

Table I

The absorption peaks of each plant extract from UV-VIS spectra (data presented as values from one sample, for exemplification)

Plant Extract	Peak Index	Peak Area	Area IntgP (%)	Row Index	Beginning λ (nm)	Ending λ (nm)	FWHM* (nm)	Center (nm)	Height
P1	1	19.941	30.19	87	262	303	34.10	287	0.595
	2	40.358	61.09	132	303	375	57.71	332	0.754
P2	1	7.389	28.37	89	262	303	32.52	289	0.237
	2	16.978	65.20	132	303	375	57.71	332	0.320
S1	1	10.230	32.57	84	262	303	33.89	284	0.303
	2	17.251	54.92	130	303	375	66.16	330	0.288
S2	1	8.446	32.79	87	262	303	33.02	287	0.257
	2	15.055	58.45	130	303	375	65.03	330	0.254

* FWHM = full width at half maximum

Quantitative analysis of total polyphenols and flavonoids

The results of quantitative analysis regarding the content of total polyphenols and flavonoids are shown in Table II. The highest content of polyphenols was recorded both in the *Salviae fluidum* (S1) and *Plantago fluidum* (P1) extracts

harvest from the same location Bihor county, (67.125 mg GAE/mL, 45.34 mg GAE/mL, respectively). Regarding the flavonoids content the highest values were obtained in the case of *Plantago fluidum* (P1) extract harvest from Bihor County (26.487 mg QE/mL).

Table II

Mean values ($\pm$ standard deviation) of total polyphenols and flavonoids of *Salviae fluidum* extracts (S1 and S2) and *Plantago fluidum* (P1 and P2) extracts from two different crop locations

	Total polyphenols (mg GAE/mL)	Rsqr; Rsqr. adj	p	Flavonoids (mg QE/mL)	Rsqr; Rsqr. adj	p
S1	67.125 ^a $\pm$ 1.995			11.289 ^d $\pm$ 0.112		
S2	38.973 ^c $\pm$ 1.088	0.991;	< 0.0001	11.759 ^c $\pm$ 0.085	0.999;	< 0.0001
P1	45.348 ^b $\pm$ 0.220	0.990		26.487 ^a $\pm$ 0.353	0.999	
P2	40.205 ^c $\pm$ 0.704			17.430 ^b $\pm$ 0.126		

Different letters across columns for each variable denotes statistical significant differences between the samples means. Results are expressed as mean $\pm$ standard deviation (n = 4).

Antioxidant capacity of medicinal plant extracts

Antioxidant properties of medicinal plants (*Salviae folium* and *Plantago folium*) were determined by three methods, DPPH, ABTS and FRAP. The results are shown in Table III. The scavenging effect of plant extracts, as determined by DPPH method, is measured as percentage of inhibition (%) of DPPH radical, and the antioxidant capacity of extracts from ABTS and FRAP methods is measured as Trolox equivalents (TE).

One-way analysis of variance of antioxidant capacities of *Salviae fluidum* and *Plantago fluidum* extracts revealed that there are significant differences ($p < 0.0001$) between the cropping locations (Bihor vs. Arad) with high level of variance explanation (i.e. high Rsqr/Rsqr. adj. values) (Table III).

In order to highlight which of the bioactive compounds content/antioxidant capacity are more affected by the crop locations, a relative ratio was calculated for *Salviae fluidum* (S1 vs. S2) and *Plantago fluidum* (P1 vs. P2) extracts (Figure 2, a-e).

Table III

The antioxidant capacities for *Salviae fluidum* (S1 and S2) and *Plantago fluidum* extracts harvested from two different crop locations

	FRAP (mmolTE/mL)	Rsqr; Rsqr. adj	p
S1	33.148 ^b $\pm$ 0.088		
S2	34.319 ^a $\pm$ 0.139	0.993; 0.991	< 0.0001
P1	29.771 ^c $\pm$ 0.490		
P2	25.639 ^d $\pm$ 0.399		
	DPPH (%)	Rsqr; Rsqr. adj	p
S1	59.173 ^a $\pm$ 0.071		
S2	52.957 ^b $\pm$ 0.682	0.986; 0.982	< 0.0001
P1	59.006 ^a $\pm$ 0.222		
P2	59.555 ^a $\pm$ 0.238		
	ABTS (umolTE/mL)	Rsqr; Rsqr. adj	p
S1	17.873 ^a $\pm$ 0.151		
S2	9.826 ^c $\pm$ 0.142	0.999; 0.998	< 0.0001
P1	15.262 ^b $\pm$ 0.077		
P2	15.337 ^b $\pm$ 0.101		

Different letters across column for each variable denote statistical significant differences between the samples means. Results are expressed as mean $\pm$ standard deviation (n = 4).

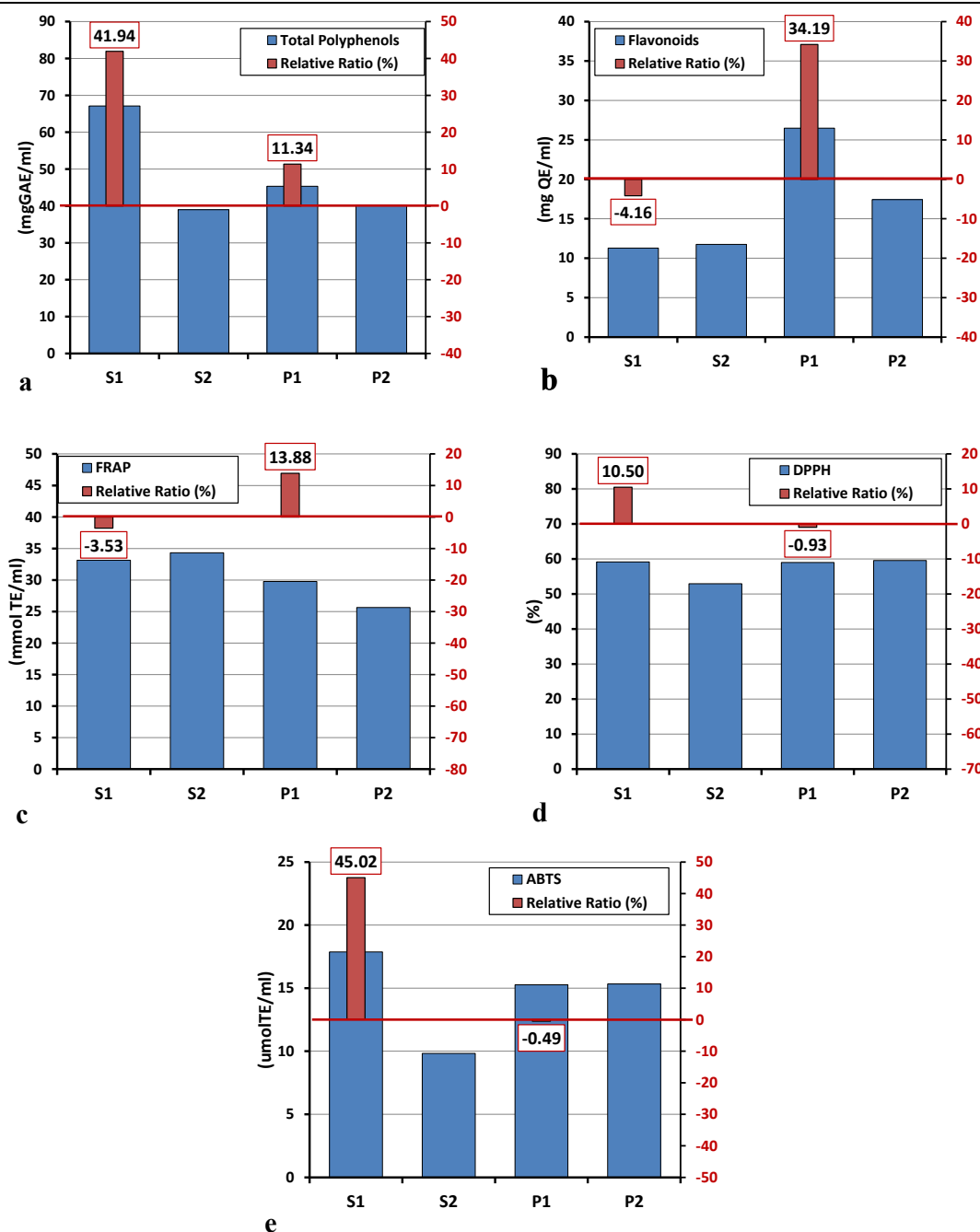


Figure 2.

The relative ratios between crop location (Bihor county –indexed with 1 and Arad county –indexed with 2) in the case of bioactive compounds, total polyphenols (a) and flavonoids (b) and antioxidant capacities, determined by three different methods: FRAP (c), DPPH (d), ABTS (e) of *Salviae fluidum* and *Plantago fluidum* extracts

Multivariate analysis

Multivariate analysis was performed in order to test the presence of relationships between variables and samples groups and to establish the differences, similarities or samples patterns of analysed data. Principal component analysis (PCA) considered quadruple samples (n=4) for each cropping location group. For PCA model input was considered the correlation matrix of data, due the fact that the variables present significant different variances; also,

in order to extract the sample grouping information, only the “between groups” analysis was performed. The considered variables were five antioxidant results. First two principal components (PC1 and PC2) explain 90.215% of total variance, thus the discrimination between cropping locations of *Salviae sp.* and *Plantago sp.* is statistical significant. First principal component (PC1) basically is a linear combination of the variables and has the main property to explain the most of the variable values variability. This means that PC1 will be strong by

correlated with the variable next to its axes and can “measure” the differences between the samples through the corresponding variables. The second principal component (PC2) is orthogonal on the first one denoting that it explains mostly the rest of unexplained variability by the PC1.

Eigen values of the first two principal components are high above the unit values showing that these components must be taken into account to analyse data in multivariate way (Table IV).

Antioxidant capacities such as ABTS and DPPH variables are positive by loaded against the first principal component, PC1 (Figure 3a). The FRAP variable is negative by loaded against the PC1 axis. These variables have the highest PC1 contributions and perform the discrimination between the PC1 positively loaded S1, P1, P2 and S2 which is negatively loaded against PC1.

Table IV

Principal components properties drawn out from principal component analysis (PCA) of antioxidant capacity for *Salviae fluidum* and *Plantago fluidum* extracts

PC	Eigenvalue	% variance	Cumulative % variance
1	2.67423	53.485	53.485
2	1.83651	36.730	90.215
3	0.489266	9.785	100.000

Total polyphenols variable is positive by loaded and flavonoids variable is negatively loaded against the second principal component, PC2 (Figure 3a). These variables have the highest PC2 contributions and perform the discrimination between S1 which is PC2 positively loaded, P1, P2 which are negatively loaded against PC2 and S2 which is null PC2 loaded. The total polyphenols content and antioxidant capacity have registered the highest levels in S1 sample group. In P1 and P2 sample groups, flavonoids content was more abundant than in S1 and S2 samples. Samples S1 and S2 presented high reducing potential of the extract reacting with a ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex (i.e. FRAP method). All these facts are valid due to high positive values of the mentioned samples PCA scores projections on variables vectors.

According with Figure 3a, P1 and P2 samples groups overlap so it appears that there are no differences between antioxidant capacities determined by ABTS and DPPH methods. The rest of the variables, FRAP method, flavonoids and total polyphenols are discriminators for these two samples groups (Figure 3b and Figure 4). However, only 9.785% of total variance is explained by the third principal component (PC3), thus this is not an accurate hypothesis (Table IV).

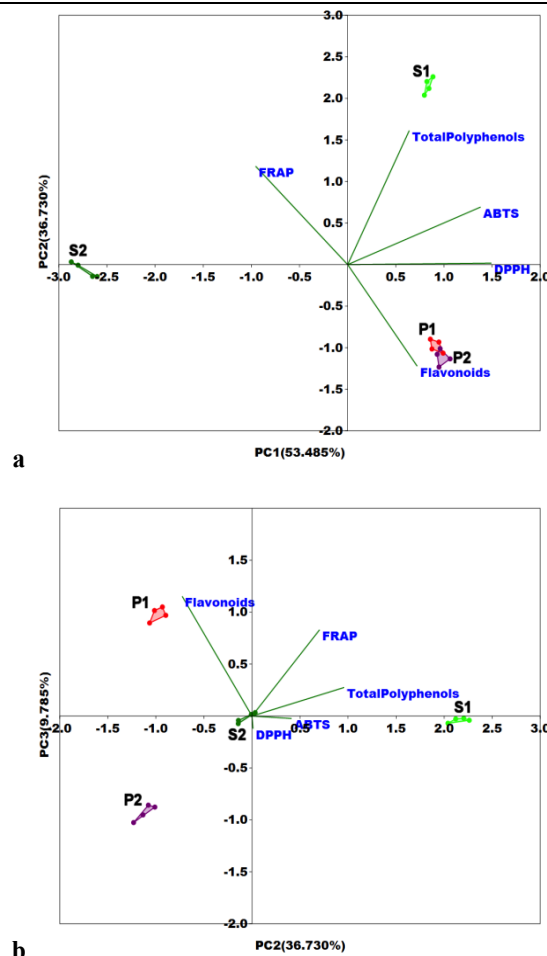


Figure 3.

Principal component biplot of antioxidant capacity from *Extractum Salviae fluidum* and *Extractum Plantago fluidum*: (a) representation for principal components PC1 and PC2 components; (b) representation for principal components PC2 and PC3 components

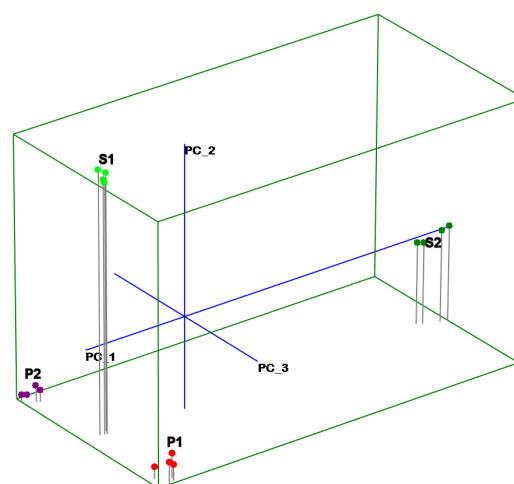


Figure 4.

Graphical 3D representation of factor scores of antioxidant capacity PCA from *Salviae fluidum* and *Plantago fluidum* extracts

This is the reason that in the hierarchical cluster analysis (HCA) of samples groups, the PCA factor scores for all three principal components (PC1, PC2 and PC3) were considered as input. In this way, HCA has successfully done the clusters validation at 1.9 cut-off similarity distance value (Figure 5) and furthermore, the motivation of using all three principal components in the multivariate analysis (Figure 5).

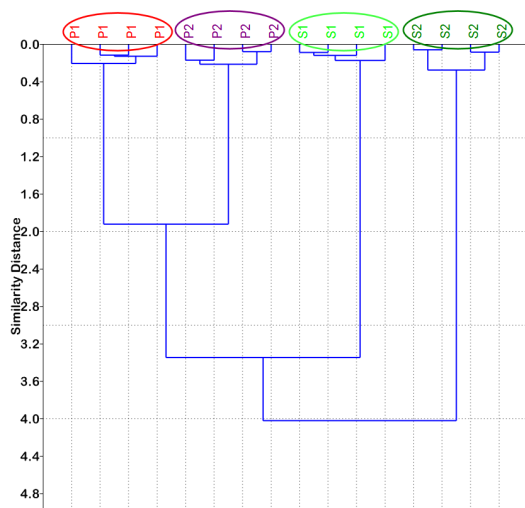


Figure 5.

Dendrogram from Hierarchical Cluster Analysis (HCA) of antioxidant capacity of *Salviae fluidum* and *Plantago fluidum* extracts

The success of the HCA samples clustering was proven by the fact that the group samples: P1, P2, S1 and S2, are not overlapping (or mixing one with other) after 1.9 cut-off similarity distance value (Figure 5). Furthermore, in order to finalize the HCA, the clusters were emphasized by non-overlapping ellipses.

Conclusions

The phytochemicals identified in *Salviae* and *Plantago* species made them a potential source of natural antioxidants that could have a great importance in medicine and pharmacology. Knowing the bioactive compounds from plant extracts (*Salviae fluidum* and *Plantago fluidum*) and their biological activity, these can be used in the desired direction with maximum efficiency, to improve the patient quality of life.

The method provided by the 10th edition of the Romanian Pharmacopoeia was performed in order to obtain the herbal extracts. As herbal species, *Salviae* sp. and *Plantago* sp. were studied, originating from the spontaneous flora of Bihor and Arad counties, each carefully selected as to have been found in unpolluted areas. The results of the analysis were demonstrated by determining the

antioxidant capacity, which may vary from one species to another, but closely connected to the polyphenols content that appears to vary depending on climatic conditions.

Multivariate analysis (PCA) revealed that the considered variables perform a clustering (HCA) of the analysed samples groups. The variables ABTS, DPPH and FRAP have the highest PC1 contributions and perform the discrimination between S1, P1, P2 clusters, located on the positive part of PC1 axis, and S2 cluster located on the negative part of the PC1 axis (Figure 3a). Furthermore, the variables total polyphenols, flavonoids and FRAP perform a discrimination between the samples species S1, S2 and P1, P2 (Figure 3a). The variables total polyphenols, ABTS and DPPH can discriminate in the same time between the samples species and the cropping location, but this is relevant only considering the third principal component, PC3 (Figure 3b and Figure 4). Hierarchical cluster analysis validated these conclusions (Figure 5) and emphasized the hypothesis that the analysed variables can be considered as markers for these two plant species extracts and their plant cropping locations.

We can affirm that both *Salviae* and *Plantago* extracts may be included in dietary supplements having an antioxidant effect, helpful in preventing different diseases.

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