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FUZZY DISCRIMINANT ANALYSIS OF MEDICINAL PLANT EXTRACTS ACCORDING TO THEIR TOTAL CONTENT OF PHYTOCHEMICALS AND THE ANTIOXIDANT CAPACITY

Augustin Cătalin MOŢ[a], Cezara ZĂGREAN-TUZA[a], Costel SÂRBU[a,*]

ABSTRACT. Fuzzy linear discriminant analysis, a robust supervised method, has been successfully applied for characterization and classification of 42 Romanian medicinal plant extracts according to the total content of eight phytochemical compounds (alkaloids, polyphenols, coumarins, o-diphenols, flavonoids, anthocyanins, flavonols, flavanols) estimated by dedicated molecular absorption spectrophotometry-based methods, and their antioxidant capacity determined by DPPH* method. The obtained results (fuzzy partitions) and parameters of the class centers (robust fuzzy means) clearly demonstrated the efficiency and information power of the advanced fuzzy method in plants characterization and classification, and allow a rationale choice of a medicinal plant extract with a specified phytochemical composition and/or antioxidant activity. Previous studies have investigated the association of certain classes of phytochemicals with the antioxidant activity in plant extracts. However, most of them are limited either in the number of the plants extracts they have analyzed or in the number of the employed phytochemical classes. The distinctness of this work is the application of a fuzzy multivariate analysis on data obtained for high number of plant extracts—42 widespread medicinal plants form various plant taxa—and a high number of ubiquitous phytochemical classes in plants. The methodology developed in this paper might be also extended in the authenticity and origin control of other fruits, herbs or derived products.

Keywords: *Fuzzy discriminant analysis, chemometrics, medicinal plants, phytochemical composition*

[a] *Faculty of Chemistry and Chemical Engineering, Department of Chemistry, Babeş-Bolyai University, 400028, Cluj-Napoca, Romania*
* *Corresponding author: costel.sarbu@ubbcluj.ro*

INTRODUCTION

Human beings, over the ages, used herbal and mineral drugs provided by nature for their ailments and treatments of disease. Being a rich source of natural antioxidants, medicinal plants—Romania is among the top three countries in Europe for the number of natural medicinal plants—fruits and vegetables have always been considered as part of a healthy and balanced diet, which more and more people are trying to achieve. According to World Health Organization (WHO) in many developing countries the main system used by the people to treat or to prevent diseases is traditional medicine, based on medicinal herbs, even though the modern medicine is available [1,2]. The complex chemical composition proved to be responsible for their curative properties [3-9] and for this reason they have begun to be used also in the food, beverage and cosmetics industry and more important to prevent lipid oxidation and food preservation.

Because of their complex composition, the development of a suitable analytical procedure to separate and evaluate all the constituents of herbal medicines is difficult, impractical and not to mention, time consuming. Therefore, the global evaluation of these samples seems more suitable, instead of focusing on individual compounds and fingerprinting methods fit this challenge by emphasizing and comprehensively characterizing the analyzed samples [10]. The Food and Drug Administration [11] and the European Medicines Agency [12] recommend that the chromatographic and spectroscopic techniques are the most appropriate for analytical procedures. Thus, in the last decades, many methods have been developed for analyzing different plant samples, including thin layer chromatography/high performance thin layer chromatography [10, 13, 14], high performance liquid chromatography and gas chromatography [15, 16], highly speed counter current chromatography [17], capillary zone electrophoresis [10-12]. These techniques are successfully completed by spectroscopic techniques, such as: nuclear magnetic resonance or mass spectrometry and representative results, comparable to those obtained by chromatography, were also obtained using also IR or UV-Vis spectroscopy [18]. The huge amount of spectrophotometric and chromatographic data can be efficiently processed and realistic interpreted using multivariate analysis methods as Cluster Analysis (CA), Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) [3-5, 13-15].

At a first sight it seems like these methods are able to solve all problems, but often they may lead to confusing results, because traditional chemometric methods have, indeed, difficulty in identifying outliers in large datasets, and in

finding real patterns. In this case these methodologies have to be improved, by hyphenation or by using more performant methods of discrimination such as fuzzy methods [19]. The applications of fuzzy techniques for characterization and classification of the analytical results are much broader and have significant potential in the authenticity and origin control of fruits, herbs or derived products [20].

The goal of the present study is to propose a new fuzzy classification method [21-23] for medicinal plants according to the total concentration of some classes of phytochemical compounds and their total radical scavenging capacity (RSC %) estimated by DPPH* method [20].

RESULTS AND DISCUSSION

The data used in this study are summarized in **Table 1** and **Figure 1 (a, b)**. It can be easily observed that in all cases, outliers and extremes values are highlighted and the distributions seem to be quite asymmetric. The mean concentration ($\mu g.mL^{-1}$) of anthocyanins and flavanols are the highest and the mean concentration of flavonols and coumarins are the lowest. The mean concentrations of alkaloids, polyphenols, o-diphenols, flavonoids, flavanols are more or less similar. These assertions are also supported by the matrix of correlation depicted in **Table 2**. The results of the correlation analysis also reveal that the phytochemical class most responsible of the antioxidant activity is by far the polyphenols and more specifically, its subclasses, flavonoids, o-diphenols and flavonols. Coumarins and alkaloids contribute to a much less extent to the antioxidant activity, at least to this antiradical ability that was tested via the DPPH bleaching assay in this current study. However, these compounds do have important contribution to other activities such as anti-inflammatory or other pharmacological activities.

According to the profile of antioxidant capacity of plant extract samples, the number of classes for FLDA was chosen to be 3. FLDA produced three fuzzy partitions, which were well represented by a prototype (a cluster center with the parameters corresponding to the fuzzy robust means [24] of the original phytochemical concentrations ($\mu g.mL^{-1}$) for the 42 samples weighted by DOMs corresponding to each partition) depicted in **Table 3**.

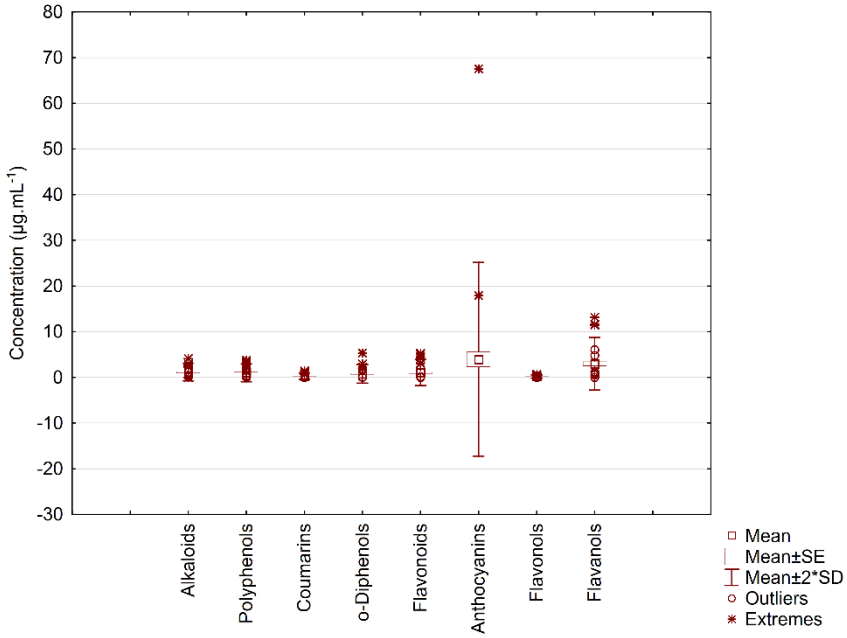
Table 1. The statistics of data discussed in this study (μg.mL^{-1})

Variable	Valid N	Mean	Minimum	Maximum	Range	Quartile Range	SD
DPPH (RSC* %)	42	11.18	0.220	46.71	46.49	17.04	12.15
Alkaloids	42	1.16	0.000	4.14	4.14	1.43	0.97
Polyphenols	42	1.03	0.008	3.78	3.77	1.13	0.98
Coumarins	42	0.25	0.000	1.45	1.45	0.25	0.34
o-Diphenols	42	0.78	0.000	5.35	5.35	0.96	1.02
Flavonoids	42	1.08	0.004	5.28	5.28	1.36	1.44
Anthocyanins	42	3.97	0.000	67.56	67.56	2.94	10.61
Flavonols	42	0.19	0.002	0.69	0.69	0.25	0.19
Flavanols	42	3.00	0.000	13.22	13.22	1.97	2.87

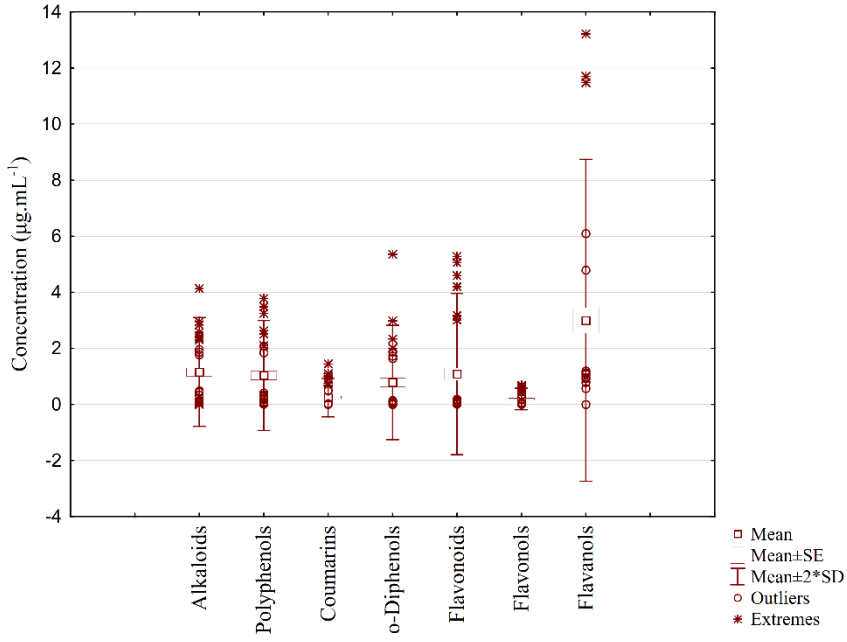
*relative total radical scavenging capacity

Table 2. Matrix of correlation concerning the concentration of phytochemicals

Variable	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	DPPH
Alkaloids (1)	1.000	0.577	0.278	0.582	0.591	0.119	0.533	0.598	0.468
Polyphenols (2)		1.000	0.544	0.871	0.912	0.143	0.878	0.735	0.936
Coumarins (3)			1.000	0.713	0.723	-0.030	0.489	0.357	0.529
o-Diphenols (4)				1.000	0.896	0.141	0.773	0.634	0.759
Flavonoids (5)					1.000	0.047	0.834	0.771	0.879
Anthocyanins (6)						1.000	0.167	-0.193	0.005
Flavonols (7)							1.000	0.655	0.824
Flavanols (8)								1.000	0.691
DPPH									1.000



(a)



(b)

Figure 1. Box and whiskers plot of all data (**a**) and data without anthocyanins (**b**)

Table 3. The results obtained applying fuzzy linear discriminant analysis

Fuzzy partition (class)	Parameters of partition centers (fuzzy mean) ($\mu g.mL^{-1}$)	Plant extract	Name of medicinal plant	RSC* %	DOM
A1	(1) 1.81 (2) 2.52 (3) 0.58 (4) 2.05 (5) 3.09 (6) 3.18 (7) 0.41 (8) 5.70	1-9	1.Blueberry 2.Lingon berry 3.Rosemary 4.Hoary willowherb 5.Lady's mantel 6.Quaking aspen 7.Lemon balm 8.Sage 9.Silver birch	46.71 36.58 31.01 29.82 29.61 27.97 27.36 27.36 26.27	0.934 0.821 0.921 0.999 0.874 0.168 0.976 0.842 0.623
A2	(1) 1.03 (2) 1.05 (3) 0.32 (4) 0.89 (5) 1.13 (6) 1.99 (7) 0.25 (8) 3.04	10-20	10.Saint John's wort 11.Hawthorn 12.Breckland thyme 13.Burdock 14.Great celandine 15.Lady's bedstraw 16.Common juniper 17.Yarrow 18.Spinycockle-bur 19.Lavender 20.Artichoke	21.12 18.74 15.48 13.98 12.86 11.16 10.13 9.45 9.44 8.93 7.42	0.848 0.718 0.796 0.880 0.916 0.662 0.784 0.712 0.168 0.342 0.261
A3	(1) 0.87 (2) 0.36 (3) 0.09 (4) 0.16 (5) 0.20 (6) 1.79 (7) 0.07 (8) 1.95	21-42	21.Liquorice 22.Gentian 23.Echinacea 24.Comfrey 25.Milk thistle 26.Nettle 27.Heart's ease 28.Motherwort 29.Ginger 30.Valerian 31.Shepherd's purse 32.Horsetail 33.Dill 34.Garlic 35.Mistletoe 36.Elder 37.Chili pepper 38.Sweet flag 39.Hogweed 40.Wolf's-foot clubmoss 41.Celery 42.Ramson	4.93 4.46 4.38 4.32 3.75 3.69 3.06 2.78 2.26 2.09 1.78 1.70 1.62 1.45 1.20 1.19 1.05 1.00 0.68 0.37 0.25 0.22	0.090 0.356 0.491 0.944 0.827 0.983 0.915 0.834 0.163 0.906 0.928 0.812 0.242 0.626 0.726 0.768 0.937 0.951 0.974 0.978 0.993 0.989

(1) alkaloids, (2) polyphenols, (3) coumarins, (4) ortho-diphenols,
(5) flavonoids, (6) anthocyanins, (7) flavonols, (8) flavanols
*relative total radical scavenging capacity [13]

To compare the fuzzy partitions (fuzzy classes) and the similarity and differences of the investigated medicinal plant extracts, we have to analyze both the characteristics of the prototypes (centers) corresponding to the three fuzzy partitions (**A1**-**A3**) obtained by applying FLDA and DOMs of samples corresponding to all fuzzy partitions, including also the canonical scores used usually in classical linear discriminant analysis [25].

The results presented in **Table 3** clearly illustrate the most specific characteristics of each fuzzy partition and their similarity and differences.

The mean values of prototype corresponding to the first partition (**A1**), including the medicinal plant extracts with the highest RSC (46.71-26.27%), are the highest for all phytochemicals. The blueberry extract (*a supper fruit*) has the strongest antioxidant capacity (46.71%). In addition, all the samples assigned to this group have a high DOM (0.821-0.999) except Silver birch (0.623) and Quaking aspen sample (0.168). This situation is well illustrated in **Figure 2**; Quaking aspen has a relatively high RSC, but in accordance with the total concentration of all phytochemical compounds is closer to the center of partition **A2**.

The partition **A2** contains plant extracts samples with moderate RSC (21.12-7.42%) and also quite different DOMs (0.916-0.168). All the concentrations obtained for the center of this partition are between the values corresponding to partition **A1** and **A3 (Figure 2)**.

The partition **A3** includes the medicinal plant extracts with the smallest RSC (4.93-0.22%) which is in good agreement with the concentration of phytochemical compounds (see center concentrations in **Table 3** and **Figure 2**). The 2D scatterplot of DOMs corresponding to the three partitions confirmed in a good way the results discussed above (**Figure 3a**).

The efficiency of the robust Fuzzy Linear Discriminant Analysis (FLDA) applied was measured by the correct classification rate of original data and by the values of quality performance features obtained applying leave-one-out (LOO) cross-validation approach including also relevant graphs obtained by scatterplot of degrees of freedom (DOMs) and fuzzy canonical scores on the plan defined by Root1-Root2 (**Figure 3b**) and the values of quality performance features obtained for the correct classification rate of the original data and by applying the leave-one-out (LOO) cross-validation approach (**Table 4**).

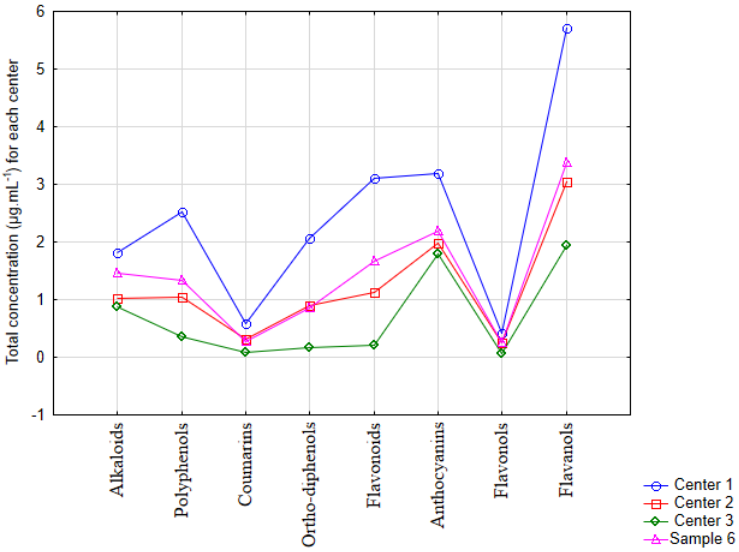
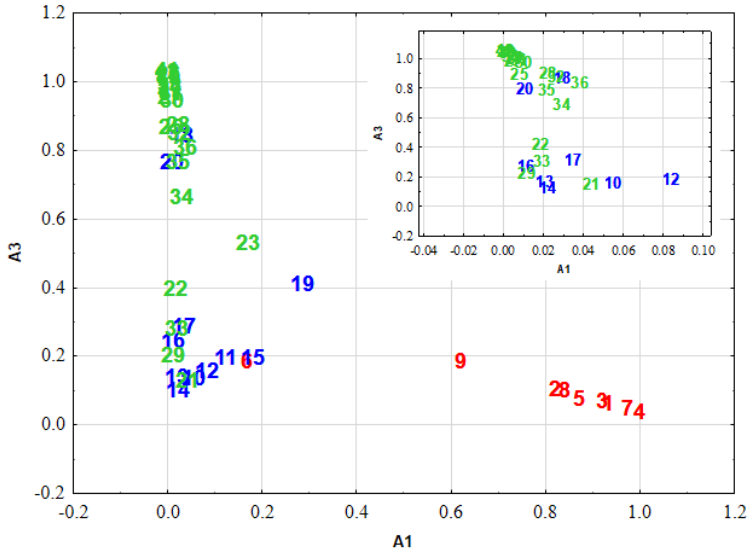


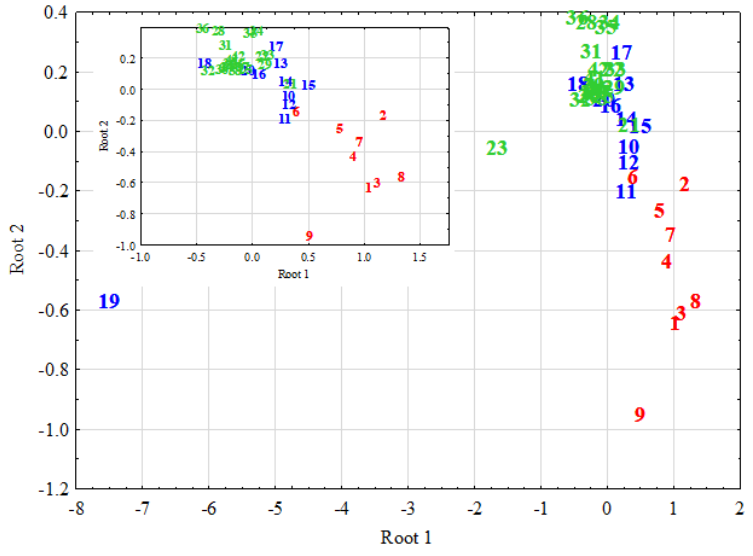
Figure 2. The concentration profile corresponding to all centers and the sample 6

Table 4. Matrix classification of medicinal plants

Class	Total	Classification matrix					
		Samples			%		
		A1	A2	A3	A1	A2	A3
A1	**9**	**8**	1	0	**88.89**	11.11	0.00
A2	**11**	0	**8**	3	0.00	**72.73**	27.27
A3	**22**	0	4	**18**	0.00	18.18	**81.82**
Leave-one-out cross-validation							
A1	**9**	**7**	1	1	**77.78**	11.11	11.11
A2	**11**	0	**8**	3	0.00	**72.73**	27.27
A3	**22**	0	4	**18**	0.00	18.18	**81.82**



(**a**)



(**b**)

Figure 3. Scatterplot of DOMs corresponding to partition **A1** and **A3** (**a**) and scatterplot of fuzzy canonical scores on the plan defined by root 1 and root 2. Inserts contain zoomed in regions, for heavily packed intervals, for better clarity.

Whereas the multivariate approach applied in this study allows for a fuzzy classification of the plant extracts in three main groups, mostly related to the antioxidant activity level, it also supports the expected finding that only to a limited extent their content and the diversity of the phytochemical classes are responsible for this activity but rather the chemical structure of the compounds (Figure 1, Table 3, Figure 2). For example, despite their small content compared to the other classes, flavonols do exhibit a significant positive correlation with the determined antioxidant activity ($r = 0.824$, $p < 0.05$). On the other hand, one might be aware that the determined phytochemical classes are chemically hierarchized, as indicated in Figure 4.

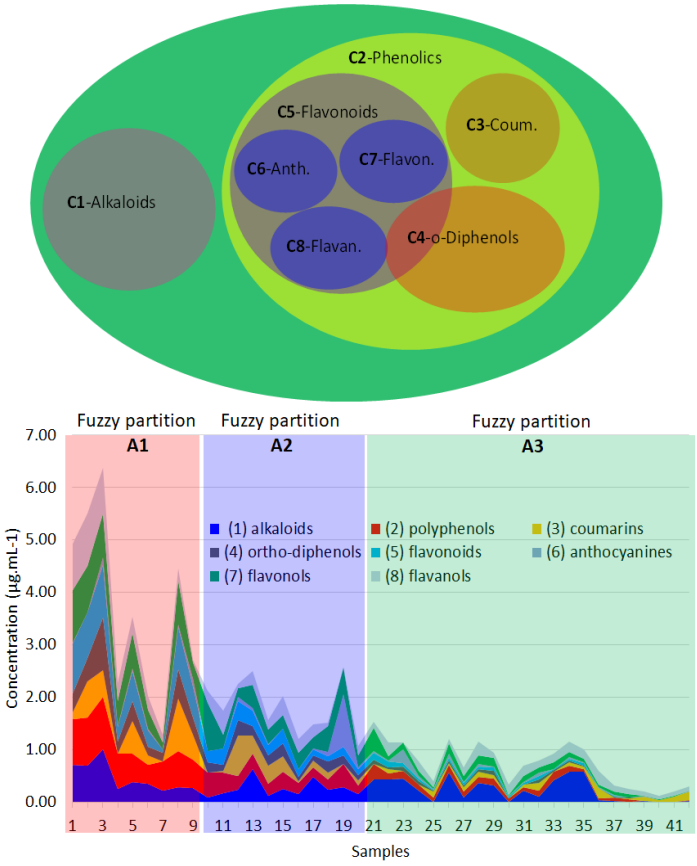


Figure 4. **Top**: Ven diagram of the analyzed phytochemical classes, according to their chemical relationships. **Bottom**: Concentration profile for the determined phytochemical classes for all the 42 plant extract samples.

Following the identification of the three fuzzy partitions by the FLDA, one might observe not only the strong association of the concentration profiles for certain phytochemical classes—polyphenols, o-diphenols, flavonoids, flavonols—with the antioxidant activity, but also the phytochemical groups responsible for the generation of the outliers/extremes and thus production of the significant variation within/between classes (Figure 4). Such systems could only be efficiently investigated using fuzzy analysis. One inconvenience of this work is that FLDA was applied only on DPPH antioxidant activity which is based mainly on electron transfer mechanism but possible more or less different plant classification might be observed if other types of antioxidant assays would be investigated.

CONCLUSIONS

In this study, the advantages of the fuzzy linear discriminant analysis for the characterization and classification of various medicinal plant extracts on the basis of their phytochemical composition and antioxidant capacity have been explored. The informative potential of this robust fuzzy method is clearly demonstrated. The new classification approach allows more relevant conclusions to be drawn, finding more specific groups. The parameters of the prototype (class center) illustrate much better than, for example, arithmetic mean the specific characteristics of each class. In addition, the 2D scatterplot of DOMs and fuzzy canonical scores allow a rationale comparison of the similarities and differences of medicinal plant extract samples investigated. This procedure can be successfully extended to other similar studies in different scientific and technical fields.

EXPERIMENTAL SECTION

Medicinal plant samples and analytical methods

The plant samples consisted of 42 hydroalcoholic extracts commercially available (**Table 2**), distributed by Dacia Plant manufacturer (Brasov, Romania).

Analytical methods

Total alkaloids content. This assay is a modified version of the analysis with the Dragendorff reagent [26]. Basically, the plant extract is treated with a mixture of bismuth nitrate and potassium iodide when a yellowish bismuth-nitrogen complex forms. Instead of separating and resuspending this precipitate, the turbidity of the reaction mixture was measured which was then correlated to the berberine equivalent from the calibration function. A 0.035 mM solution of $Bi(NO_3)_3$ was prepared, the solvent being mixture of water: acetic acid ratio of 4:1 v/v. The other prepared solution was one of KI of 50% (w/v). Vigorous shaking is required for dissolving both salts. The reagent was prepared by mixing 5 ml of $Bi(NO_3)_3$ with 2 ml of KI. A volume of 20 µl of extract were mixed with 200 µl of this solution along with 80 µl 50% ethanol in a 96 wells plate. The turbidity was measured using a Tecan spectrophotometer at 530 nm. The calibration function was done by using berberine as standard in concentrations ranging from 0.11 to 32.2 $\mu g.mL^{-1}$. The final values were expressed as berberine equivalents and were calculated by using the calibration function. All samples were analysed in duplicates.

Total polyphenolics content (*Folin-Ciocâlteu reducing capacity assay*). The basis of this method is the capacity of some phenolate anions to be oxidized by a molybdenum-based complex which changes color from yellow to blue. The polyphenols are exposed to a basic medium ensured by a saturated carbonate solution which deprotonates the compounds; as a result, phenolate ions are obtained. They are capable to react with the so-called Folin-Ciocâlteu reagent which contains a combination of phospho- tungsten and molybdenum compounds along with lithium sulfate as bromine. As a result of the chemical reaction, the polyphenols are oxidized, whereas the molybdenum is reduced, obtaining the metallic complex $(PMoW_{11}O_4)^{4-}$ which has a bluish shade. The main disadvantage of this assay is that not only polyphenols react with this reagent, but also other compounds with reducing abilities. In order to measure the antioxidant activity using this method, 2 µL of each extract was mixed with 236 µL water and 17 µL Folin-Ciocâlteu reagent in a 96 wells Tecan microplate. This solution was incubated in the dark for about 5 minutes. Then 45 µL of 20% sodium carbonate solution was added and the whole mixture was incubated for 30 minutes. After the time passed, the microplate was placed in a Tecan spectrophotometer and the absorbance was measured at 725 nm. All extracts were analysed in duplicates. In the meantime, a signal on concentration dependence was all performed, with values of concentration

varying from 0 to 40 $\mu g.mL^{-1}$ Gallic acid. The Gallic acid equivalents (GAE) of each extract were determined by calculating the concentration value from the calibration function by means of absorbance [27].

Total coumarins content. When exposed to pH variations coumarin phenolic hydroxyl moieties can suffer ionization which appears as a bathochromic effect in UV-vis spectrophotometry. A volume of 5 µL of extract were mixed with 295 µL 50% ethanol in ultrapure water along with 2 µL of 20 mM sodium carbonate solution in a 96 wells plate [28]. The absorbance was measured before and after the addition of the carbonate solution: first at 320 nm and then at 370 nm. The final absorbance value was calculated by adding up both values. A calibration function was built using coumarin as standard by plotting the absorbance values against the concentrations ranging from 1.65 to 33.11 $\mu g.mL^{-1}$. The final results were expressed as coumarin equivalents. All experiments have been performed in duplicates.

Total ortho-diphenols content. This method is based on the fact that the hydroxyl group of ortho-dipehnols can react with sodium molybdate thus producing a yellowish metallic complex in an aqueous medium [29]. In order to perform this analysis, 3 µL of extract were mixed with 97 µL 50% ethanol in ultrapure water, then 200 µL of 5 g/100 mL sodium molybdate was added in a 96 wells plate. The samples were incubated for 25 minutes and the absorbance was measured at 370 nm with the help of a Tecan spectrophotometer. A calibration function was built by using caffeic acid as standard in concentrations from 0.91 to 91.6 $\mu g.mL^{-1}$ by plotting the measured absorbance against the values of concentration. The final results were given as caffeic acid equivalents and they were obtained by applying the equation of the calibration function to each measurement. Each extract was analysed in duplicate.

Total flavonoids content. This method is based on the fact that flavonoids react with sodium nitrite, a potent nitration reagent, and aluminum chloride to form an adduct that in a strong basic media turns a bright red [30]. Basically, the aluminum ion is chelated toward a catechol moiety by reacting with its hydroxyls, fact that makes this analysis highly specific. A volume of 120 µL of ultrapure water were mixed with 5 µL extract and 80 µL of 5% $NaNO_2$ solution in a 96 wells plate and allowed to react for 5 minutes. Afterward 50 µL of 2% AlCl3 were added. After another 5 minutes 50 µL of 1M NaOH to increase the pH. Finally, after further 5 minutes while the compounds were left to react, the absorbance was measured at 510 nm using a Tecan spectrophotometer. The experiments were performed in duplicates and the calibration function was built by use of rutin solution varying from 1.1 to

110 μg.mL^{-1} by plotting the values of absorbance against the quantities of standard. The final results were showed as rutin equivalents and they were calculated by applying the equation of the calibration function.

Total anthocyanins content. A pH differential method is used for quantifying anthocyanins. This assay is based on the fact that these compounds exhibit different species at various pHs: at a low pH the flavylium cation is dominant, thus the anthocyanins having a bright powerful color [26]. If the pH is increased, a conversion to a pseudo base carbinol takes place with loss of color. Reading the absorbance at 700 nm is essential in order to correct the possible haze. In order to make this assay, 2 buffering solutions were prepared: one of pH=1 of 0.025 M KCl and another of pH=4.5 of 0.4 M sodium acetate. A volume of 270 μL of each buffer was separately mixed with 30 μL extract and the absorbance was read after 5 minutes at 520 nm. The absorbance values were obtained by subtracting the value of pH=4.5 from the value of pH=1 and then it was corrected with the 700 nm absorbance. The experiments were performed in duplicates for each pH value. The final cyaniding-3-glucoside-equivalents expressed results were calculated using the following formula:

Total anthocyanins (TA) = (absorbance at 520 * dilution factor)/ 98.2
where 98.2 is the extinction coefficient of the standard.

Total flavonols content. The basis of this method is the complexation reaction between aluminium and the hydroxyl groups of flavonols in slightly acidic pH, ensured by the presence of sodium acetate buffer [26]. For this purpose, 75 μL of 50% ethanol in ultrapure water was mixed with 5 μL extract and incubated for 5 minutes in the presence of 80 μL of 2% $AlCl_3$ solution in a 96 wells plate. Afterwards, 120 μL of 1M sodium acetate was added and the absorbance was recorded at 430 nm using a Tecan spectrophotometer in duplicates for each sample. The standard used to build the calibration function was quercetin in concentration range between 0.55 to 26.57 μg.mL^{-1}. The absorbance read for standard containing samples was then plotted against the values and concentration and the resulting equation was used to calculate the quercetin equivalents for each extract.

Total flavanols content. At low pH vanillin is protonated, one of its carbons becoming an electrophilic carbocation that can react with a flavonoid ring at the 6th or 8th carbon atom, giving rise to a reddish complex [26]. Even though, chemically speaking, the reaction is very selective towards flavanols, interferences with anthocyanins might occur when measuring the absorbance. In order to make this assay, 30 μL of extract were diluted in 70 μL 50% ethanol in ultrapure water in a 96 wells plate. A volume of 150 μL of 4% vanillin

solution was added, along with 75 µL of 35% H_2SO_4 and the mixture was left to react for 15 minutes at room temperature. Afterwards, the absorbance was recorded at 520 nm on a Tecan spectrophotometer for each extract in duplicates. Meanwhile, a calibration function was built by plotting the read absorbance against various concentrations of catechin (from 0.71 to 1146 $\mu g.mL^{-1}$). The final results were presented as equivalents of catechin obtained by applying the curve equation for each extract.

Fuzzy Linear Discriminant Analysis

Linear discriminant analysis (LDA) is a supervised classification technique based on the linear discriminant functions, which maximizes the ratio of between-class variance and minimizes the ratio of within-class variance. LDA selects directions, which accomplish maximum separation among the given classes. The Euclidean distance is used in the LDA algorithms in order to classify unknown samples and the stepwise algorithm to extract the most important variables. It is also possible to visualize how the functions discriminate between groups by plotting the individual scores for the discriminant functions. In addition, FLDA may offer some remarkable information for classification and discrimination of the considered samples according to DOMs and fuzzy canonical scores. The robust FLDA has been clearly described and efficiently applied in some interesting works [21-23].

All the graphs and some statistics were performed using Statistica 8.0 (StatSoft, Inc. 1984–2007, Tulsa, USA) software. All the other results were obtained using our own fuzzy software package.

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