Universitatea Babeș Bolyai
Facultatea de Chimie și
Inginerie Chimică

Ileana Maria SIMION

Thesis

Characterization and fingerprinting of medicinal plant extracts (food suplements) found on the Romanian theritory

Summary

Conducător științific
Prof. Emerit Costel SÂRBU

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Introduction

Nature has always been involved in human lifestile, providing the necessary means to live a healty and careless life trough the presence of natural resources such as fruits, vegetables or even plants. Herbal medicine, as the name sugests, uses the products offerd by nature (plants) to prevent or even cure different disease.

The medicial plants are used as treatemets due to the fact that they are cheap, less toxic and lack of side effects compared to the chemically synthesized drugs. The terapeutic effect of every single plant is caused by the bioactive compounds produced as a result of scondary methabolism. The so called secondary metabolites represented by alkaloids, sterols, terpenes, flavonoids, tannins, glycosides, resins, volatile oils, etc., the alternative medicine started to play an important role in the treatment of diseases all over the world, mostly because the medicinal system in many underdeveloped countries is still inexistent.

Due to the above-mentioned considerations, the World Health Organization (WHO) has developed a strategic plan to promote alternative medicine by publishing four volumes containing 118 monographs regarding medicinal plants. The main purpose of WHO is to train people to develop their monographs due to the diversity of the flora that is characteristic from one territory to another.

The objective of the thesis entitled “Caracterizarea și amprentarea extractelor din plante medicinale (suplimentelor alimentare) de pe teritoriul României” is the usage of different chemometric methods on data obtained by various analytical methods (UV-Viz, thin layer chromatography, HPLC). The analyzed samples (42) are commercially available (hydroalcoholic extracts from medicinal plants), the main purpose of this study being the characterization and classification of those medicinal plants.

The thesis is structured in six chapters, the first four chapters contains introductive information’s about plants and the Romanian flora, as well as informations about the chemometric and analytical methods used in this study. Chapter five and six presents the experimental results and the conclusions of the study.

The results of this research aim to be a contribution to the development of the study of medicinal plant by using different analytical methods and the encouragement of the population to choose a healthier alternative as regards to the diet.

Chapter I – Medicinal plant universe

1.1. Romanian flora and medicinal plants

The World Health Organization refers to the medicinal plants as those plants that containes compounds that can be used as terapeutics [1]. The plants are divided in two categories: spices and herbs. The herba are the fresh green part of the plant while as the spices are represented by fruits flowers, seed and so on [2].

The first related data to the usage of herbs and spices dates back to the year 1550 befor Christ. In our days the usage of medicinal plants is restricted by the compilace with regulations established by authorised institutions such as WHO or Food and Drug Administration – FDA [2].

According to a study realised by Maarten și James in 2016 the were known a number of 374000 plants from which 308312 are superior plants and 295383 are plants with flowers. Although the herbal medicine is intensly promoted and studied, only a number of 28187 plant species are known to be medicinal [3].

On the Romanian theritory there are known a number of 3600 medicinal plants, aproximative 30% of the European flora [4]. Accordinfg to a study realised by the Ministerul Agriculturii și Dezvoltării Rurale (MADR), in 2002, a number of 29 plant species were registered as medicinal to which were added a number of 24 species from spontaneous flora and 10 species aclimatized to the Romanian environmental conditions. During 1980-1990 Romania was among top five exporting country, presently the things have changed, this sector beeing poorly exploited. Over the last few years the things have improoved many researches beeing made in the medicinal plants area [5,6].

1.2. Medicinal plants composition

Bioactive compounds are functional ingredients that occur in nature, they are part of the food chain and can help to improve the quality of food [7]. Among the valuable benefits, we mention the antioxidant activity, inhibition of enzymes, inhibition of activity receptors and inhibition of gene expression. Bioactive compounds are found in fruits, vegetables or medicinal herbs [8].

In the case of medicinal plants, different processes take place inside the plant cells resulting in two types of metabolites: primary and secondary. Primary metabolites are produced in the process of photosynthesis and are responsible for plant growth and metabolism. On the other hand, secondary metabolites are products of primary metabolites, which have no role in the metabolic processes. They are also known as bioactive compounds, and, among other things, are responsible for the beneficial effects felt by living organisms. The main classes of compounds found in medicinal plants are phenols, glucides, terpens, volatile oils etc [9,10].

1.3. Medicinal plants therapeutic effects

Pharmacognosy is the science that deals with the study of herbal products and of poisons and includes the analysis of all types of medicinal plants including mixtures complexes, raw plants or as extracts (phytotherapy), pure compounds (morphine), and foods with beneficial effects on health (nutraceuticals). Medicinal planets are considered the base point of traditional alternative medicine. Phytotherapy is the science that uses plants in the treatment and cure of various diseases. Ethnobotany and ethnopharmacology are fields that study exclusively the knowledge acquired by the natives related to the use of medicinal plants and the potential beneficial effects they have, but also the toxicity associated with the risk of using them as "drugs". In ethnopharmacology, an important aspect is the improvement of the obtained preparations from medicinal herbs. Therefore, it is essential to know the bioactive compounds in these plants and their therapeutic effects [11].

Depending on the chemical composition (bioactive compounds), medicinal plants have various therapeutic effects, from medicinal plants used to treat neurodegenerative diseases (Alzheimer's) [12], for example, to the treatment of various skin conditions (burns) [13]. That's why to promote and inform the population about the plant parts used, how to administer and use, today a large number of books / encyclopedias that describe these aspects are available the general public [14,15,16].

Chapter II – Methods of analysis used for the characterization and classification of medicinal plants

2.1. Spectroscopic methods

Spectroscopy deals with obtaining, measuring and interpreting the spectra obtained from the interaction between electromagnetic radiation with matter. Numerous spectroscopic methods are available for solving a wide range of analytical problems. These methods differ from each other depending on the species analyzed (atomic or molecular spectroscopy), the type of interaction of radiation with matter (absorption, emission or diffraction) and, last but not least, depending on the region of the electromagnetic spectrum used in the analysis. Spectroscopic methods are particularly informative and can be used for both quantitative and qualitative determinations. The spectroscopic methods, based on the absorption or emission of radiation in the fields of ultraviolet (UV), visible (Vis), infrared (IR) and radio (nuclear magnetic resonance - NMR) are among the most used methods in laboratories dedicated to the analysis of plants and herbal preparations/supplements [17-20].

2.2. Chromatographic methods

Chromatography is a general term applied to a wide variety of separation techniques based on the repeated distribution of the analyte (solute) between a mobile phase and a fixed or stationary phase. The relative interaction of a solute with these two phases is described by the partition (K) or distribution (D) coefficient (the ratio between the solute concentration in the stationary phase and the solute concentration in the mobile phase). The mobile phase can be either a gas (GC), a liquid (LC) or a supercritical fluid (SFC). The stationary phase can be a solid or usually a liquid. Among the most employed methods to carry out medicinal plants analysis are thin layer chromatography - TLC, high performance liquid chromatography - HPLC and gas chromatography – GC [17-21].

Chapter III – Methods employed for the antioxidant activity analysis

Reactive Oxygen Species (ROS), such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$), hydrogen peroxide (H_2O_2) and oxygen ($O_2{}^1\Delta g$) can attack biological macromolecules, giving rise to structural changes in proteins, lipids and DNA, cell aging, diseases caused by oxidative stress (eg, cardiovascular and neurodegenerative diseases), and cancer. Antioxidants eliminate or extinguish ROS and Reactive Nitrogen Species (RNS), produced as a result of respiration, including free radicals [22-25].

The terms "antioxidant activity" and "antioxidant capacity" have different meanings: antioxidant activity refers to the kinetics of the reaction between an antioxidant and a prooxidant, while antioxidant capacity measures the thermodynamic conversion efficiency of a reaction between an oxidant and an antioxidant. The measurement of the antioxidant capacity of food and biological fluids (eg human serum) is performed for the significant comparison of the antioxidant content of food and for the diagnosis and treatment of diseases associated with oxidative stress. ROS can lead to oxidation of amino acid side chains, cross-linking between proteins and oxidation of peptides. When different antioxidants work together, greater protection against ROS/RNS attack is provided than in the presence of a single antioxidant compound (which makes measuring the total antioxidant capacity even more important) [22].

An antioxidant can be defined as "any substance which, present in relatively low concentrations in comparison with those of the oxidizable substrate, significantly delays or inhibits the oxidation of the substrate". Antioxidant compounds act in the human body in two different stages: in the first phase they prevent oxidative damage by blocking the appearance of free radicals, and then they are positioned as a shield, protecting the remaining healthy cells. Antioxidants were divided into two classes: primary antioxidants and secondary antioxidants [22,26-28].

The mechanism of primary antioxidants follows the following reactions:

$$R^{\bullet} + AH \rightarrow RH + A^{\bullet}$$

$$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet}$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

As for secondary (preventive) antioxidants, they delay the rate of oxidation. The chemical diversity of antioxidants (relative abundance of glycosides and their isomers) makes it difficult to separate and quantify them from foods/biological matrices where their combined action may be more relevant. Therefore, it is desirable to measure the level of total antioxidant activity directly from plant extracts and biological fluids. Antioxidant methods can be classified according to the type of reaction into two groups [22]:

• Hydrogen transfer methods (HAT)

• Electron transfer methods (ET)

3.1. Hydrogen transfer methods (HAT)

Methods based on the HAT mechanism measure the ability of an antioxidant to quench free radicals by donating a hydrogen atom. The HAT mechanisms by which the hydrogen atom (H) of a phenol (Ar - OH) is transferred to a radical $ROO^{\bullet}$ is described by the reaction below:

$$ROO^{\bullet} + AH/ArOH \rightarrow ROOH + A^{\bullet}/ArO^{\bullet}$$

where the aryloxy radical ($ArO^{\bullet}$) formed as a result of the reaction between the antioxidant phenol and the peroxyl radical is stabilized by resonance. Effective phenolic antioxidants must react faster than free radical biomolecules to protect the latter from oxidation. Since in HAT-based antioxidant methods, both the sample and the antioxidants react with $ROO^{\bullet}$, the antioxidant activity can be determined by measuring the analytical signal reduction curve of the sample in the absence and presence of antioxidants, integrating the area under the curve and determining the difference between them.

Methods for determining the antioxidant capacity following a HAT mechanism include the ORAC (oxygen radical absorbance capacity), TRAP (total radical trapping antioxidant) and croton and β-carotene bleaching [22,29-31].

3.2. Electron transfer methods (ET)

In most electron transfer assays, the antioxidant action is simulated with a sample that has a suitable redox capacity, namely, antioxidants react with a fluorescent or colored sample (oxidizing agent) instead of peroxyl radicals. Electron transfer-based spectrophotometric tests measure the ability of an antioxidant to reduce an oxidant, which changes color when reduced. The degree of color change (either an increase or decrease in sample absorption at a given wavelength) is correlated with the concentration of antioxidants in the sample. The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), TEAC (trolox-equivalent antioxidant

capacity) and DPPH (2,2-Diphenyl-1-picrylhydrazyl) methods are discoloration assays, while in the Folin-Ciocâlteu method for determining the total phenol content, FRAP (ferric reduction antioxidant power) and CUPRAC (cupric reduction antioxidant capacity), there is an increase in absorbance at a specified wavelength, following the reaction of the antioxidant with the chromogenic reagent (i.e. in the last two methods, the lower valences of iron and copper, namely Fe (II) and Cu (I), form load transfer complexes with the corresponding ligands).

Electron transfer tests generally establish a fixed time for the redox reaction in question and measure the thermodynamic conversion (oxidation) during that period. ET-based tests include ABTS/TEAC, DPPH (although the first two methods are considered mixed methods based on HAT/ET mechanisms by some researchers), the Folin-Ciocalteu method (FCR), FRAP and CUPRAC using different chromogenic redox reagents with potentials. different standards[22,29-31.]

Chapter IV – Chemometric methods

4.1. Introduction – Multivariate Data Analysis

According to Massart's definition, chemometry is: "the chemical discipline that uses mathematical, statistical, and other methods using formal logic (a) to design or select optimal measurement procedures and experiments, and (b) to provide maximum relevant chemical information by analyzing chemical data" [18].

Multidimensional data analysis involves the use of data tables (numbers) and the visualization of the information they contain. The role of data tables is very important, being the most appropriate means to use in terms of communication, processing and storage of information. In a table there is a certain message that can be commented and supported by graphs. The data table consists of a set of numbers distributed in the form of horizontal lines and vertical columns. The tables can be symbolized by the group of letters XLC, each of the letters representing the lines (L) and the columns (C) respectively. Usually, in the case of tables the lines are represented by the objects or subjects (individuals) studied, and the columns represent the experimental values or observations of different characteristics (properties, attributes, properties) specific to the objects (subjects). There is a significant difference between experimental values and observations. Experimental values, resulting from measurement processes performed in physics and chemistry laboratories, are associated with a high degree of accuracy and precision. The observations are devoid of this high degree of precision and accuracy, and are at best orderly, as is often the case in psychological examinations.

An XLC table can be represented with the help of coordinate axes, each column element corresponds to a coordinate axis in the data space. Thus, each line element (LN) is identified as a point on such an axis, the position of each such point is determined by the numerical value corresponding to the respective column in the data table. All coordinate axes thus obtained are orthogonal to each other, together defining a coordinate system originating in 0. A two-dimensional coordinate axis system is obtained when the number of columns is equal to two, when the CN is equal to three, we have three-dimensional space, and multidimensional space occurs if the NC is greater than three (the graphical representation of this space is impossible).

In algebra, a table made up of numbers arranged in the form of horizontal lines and vertical columns is called a matrix. The lines of an array are composed of a set of numbers arranged in

a certain order (line-vector). Matrix columns are called vector-columns. The scalar represents an individual numerical value. Scalar quantities, vectors and matrices can be represented geometrically. If we talk about the scalar it is devoid of dimensional information, a vector can be considered either a line element or a column element in a metric, and a matrix can be constructed by overlapping several line vectors.

In statistics, when we refer to the word characteristic or attribute (variable), it means that it is a property that a certain object either possesses or not. The variable is of three types (numerical, nominal and ordinary and respectively binary variable), being a quantity that can take any numerical value from a certain domain of definition.

The most emploied chemometric methods for the data obtained from the analitical methods applied to the medicinal samples are: principal component analysis (PCA), cluster analysis (CA) and liniar discriminat analysis (LDA). Due to the fact that during the analysis a large number of variables are obtained, preprocessing (or data reduction) techniques have become essential techniques for reducing the number of data. The need for data processing arises for three main reasons: the data generated by the analysis methods may be incomplete (lack of values), the data set is affected by noise (may contain errors or extreme points) and last but not least the data set data may be inconsistent, containing discrepancies in names or codes. These preprocessing methods together with the chemometric ones are fairly discussed in this doctoral thesis [32].

Chapter V - Original results

5.1. Studied medicinal plants

This first subchapter (of the chapter V) describes in detail the medicinal plants characteristics. There were a number of 42 hydroalcoholic extracts, commercially available, obtained from 42 medicinal plants. 41 of the samples are provided by S.C. Dacia Plant S.R.L., Brașov, Romania and a sample from Fares Orăștie, Hunedoara, Romania. The plants are characteristic to the Romanian flora, and the manufacturing process is specified on the prospect that accompanies the product. Different ethanol ratios were used in the maceration process of the extracts: water (35-85% ethanol v/v). The distribution between the dry plant part (aerial parts, leaves, flowers, bulbs, seeds, fruits, germs and roots or mixtures depending on the plant species) and solvent was 1/(2–10) g/mL. The name and phylum of the 42 samples are presented in Table 5.1.

Table 5.1. The name, scientific name and branch of the medicinal plants studied.

Nr.	Name	Scientific name	Phylum
1	Horsetail	*Equisetum arvense*	Pteridophyte
2	Wolf's-food clubmoss	*Lycopodium clavatum*	Pteridophyte
3	Hoaru willowherb	*Epilobium parviflorum*	Magnoliophyte
4	Quaking aspen	*Plopus nigra*	Magnoliophyte
5	Lemon balm	*Melissa officinalis*	Magnoliophyte
6	Great celandine	*Chelidonium majus*	Magnoliophyte
7	Lady's bedstraw	*Galium verum*	Magnoliophyte
8	Echinacea	*Echinacea purpurea*	Magnoliophyte
9	Milck thistle	*Silybum marianum*	Magnoliophyte
10	Ginger	*Zingiber officinale*	Magnoliophyte
11	Hogweed	*Heracleum sphondylium*	Magnoliophyte
12	Ramson	*Allium ursinum*	Magnoliophyte
13	Blueberry	*Vaccinium myrtillus*	Spermatophyte
14	Lingonberry	*Vaccinium vitis-idaea*	Spermatophyte

15	Rosemay	*Rosmarinus officinalis*	Spermatophyte
16	Lady's mantle	*Alchemilla vulgaris*	Spermatophyte
17	Sage	*Salvia officinalis*	Spermatophyte
18	Silver brich	*Betula pendula*	Spermatophyte
19	Saint John's wort	*Hypericum perforatum*	Spermatophyte
20	Hawthorn	*Crataegus monogyma*	Spermatophyte
21	Breckland thyme	*Thymus serpyllum*	Spermatophyte
22	Burdock	*Arctium lappa*	Spermatophyte
23	Juniper	*Juniperus communis*	Spermatophyte
24	Yarrow	*Achillea millefolium*	Spermatophyte
25	Spinycockle-burr	*Xanthium spinosum*	Spermatophyte
26	Lavander	*Lavandula augustifolia*	Spermatophyte
27	Artichoke	*Cynara scolymus*	Spermatophyte
28	Liquorice	*Glycyrrhiza glabra*	Spermatophyte
29	Gentian	*Gentiana asclepiadea*	Spermatophyte
30	Comfrey	*Symphytum officinale*	Spermatophyte
31	Nettle	*Urtica dioica*	Spermatophyte
32	Heart's ease	*Viola tricolor*	Spermatophyte
33	Motherwort	*Leonurus cardiaca*	Spermatophyte
34	Valerian	*Valeriana officinalis*	Spermatophyte
35	Shepherd's purse	*Capsella bursa-pastoris*	Spermatophyte
36	Dill	*Anethum graveolens*	Spermatophyte
37	Garlic	*Allium sativum*	Spermatophyte
38	Mistletoe	*Viscum album*	Spermatophyte
39	Elder	*Sambuctus nigra*	Spermatophyte
40	Chili pepper	*Capsicum annuum*	Spermatophyte
41	Sweet flag	*Acorus calamus*	Spermatophyte
42	Celery	*Apium graveolens*	Spermatophyte

5.2. Medicinal plant extract classification according to the phylum

According to the Medicinal Flora of Romania and http://www.plante-medicinale.ro/pm/specii.php the plants described in Table 5.1. belong to the branches Spermatophyta, Magnoliophyte and Pteridophytes [33-37].

The Spermatophyta branch includes woody and herbaceous plants, their originated theoretically derived from the Pteridophyte branch. Depending on whether or not the seed is enclosed in the fruit, two new subcategories called Gymnosperms and Angiospermae are created [38].

The Pteridophyte phylum contains the first plants in which organs and tissues can be distinguished, with features such as lack of flowers and multiplication by spores, which are mainly ferns. The importance of plants belonging to this category is given by the presence of salicylic acid [4].

The Magnoliophyte cluster brings together in the same category the most evolved plants, characterized by the seed enclosed in the fruit and a complex arrangement of the petals. They are grouped into two subdivisions: monocotyledons and dicotyledons [4].

5.2.1. UV-Vis Spectroscopy

In this subchapter the UV-Viz spectra together with the first four derivatives and the chemometric methods were employed to classify the samples according to the phylum. The results obtained by using the cluster analysis demonstrates a weak classification of the samples into the given phylum, the best results being obtained for the first derivative. The second method was PCA, resulting as in the previous case a weak separation of the samples, except the first derivative. As for the third method employed PCA-LDA, completes the set of methods employed resulting in a good classification of the samples according to the phylum also for the first derivative (Figure 5.1.). Moreover, the preprocessing of the data did not bring any improvement to the classification.

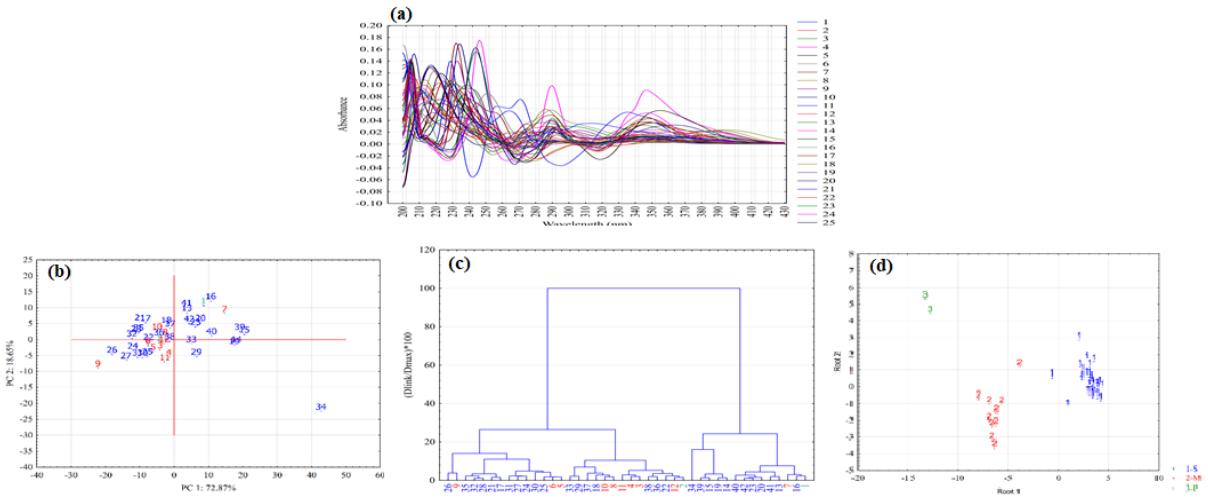

Figure 5.1. The (a) UV-Vis spectra, (b) dendrograms, (c) graphical representation of PC1-PC2 and (d) graphical representation of Root1-Root2, of the data obtained for the 42 medicinal plant extracts for the first derivative.

5.2.2. Thin layer chromatography

The images of the chromatographic plates used in this study (HPTLC Silica gel 60 și HPTLC Silica gel 60 F_{254}) were obtained at two different wavelength 254 and 365 nm. The data were processed further using TLC Analyzer software.

Evaluating of the plates under UV light reveals dark spots, blue/green fluorescence (254 nm) and light spots on a blue background (365 nm), highlighting different classes of compounds present in the analyzed samples. When the color channels related to images were investigated (obtained at 365 nm), the classes were identified as dark spots on a colored background. Different chromatograms (related to the number of compounds viewed / sample) were observed depending on the selected color (red, green, blue or gray channel). Also, the fingerprints on the red and green channels are similar highlighting the same classes of compounds, while the gray and blue channels bring additional information. Thus, the fingerprints on the gray channel better highlight certain compounds and on the blue channel some compounds are missing for samples such as 17-three spotted brothers, 18-fluffy with small flowers, 22-cress, 25-cranberry, 34-sage, 36-shepherd's purse (Figure 5.2.). As a general observation based on the results obtained from the use of the channels, the red one highlights the classes of yellow/orange fluorescence compounds (365 nm), while the blue channel highlights the blue fluorescence compounds (365 nm).

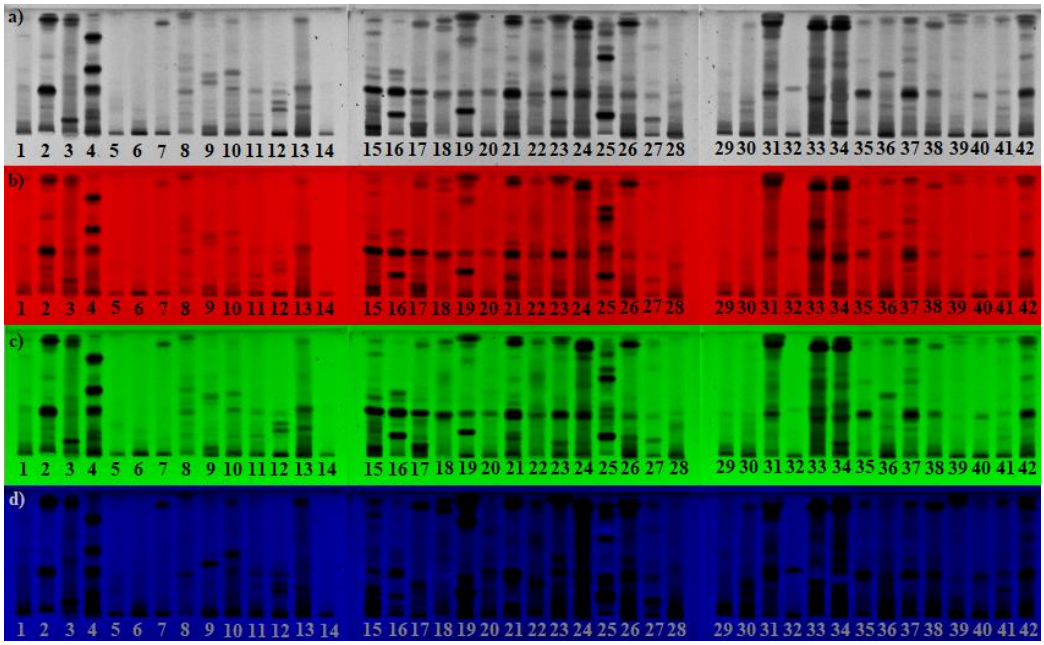

Figure 5.2. Image of HPTLC silica gel 60 F_{254} chromatographic plate sprayed with NTS solution (0.02%) in methanol: (a) gray channel; (b) red channel; (c) green canal; (d) blue channel for investigated medicinal plants.

All PCA models resulting from the 2D and 3D graphical reprezentation of the scores corresponding to different combinations between the main components, showed a weak and more or less similar separation of the samples, according to phylum.

The PCA method combined with LDA lead to the best discrimination of the samples according to the phylum, the best results are obtained for the HPTLC Silica gel 60 F_{254} gray channel (Figure 5.3).

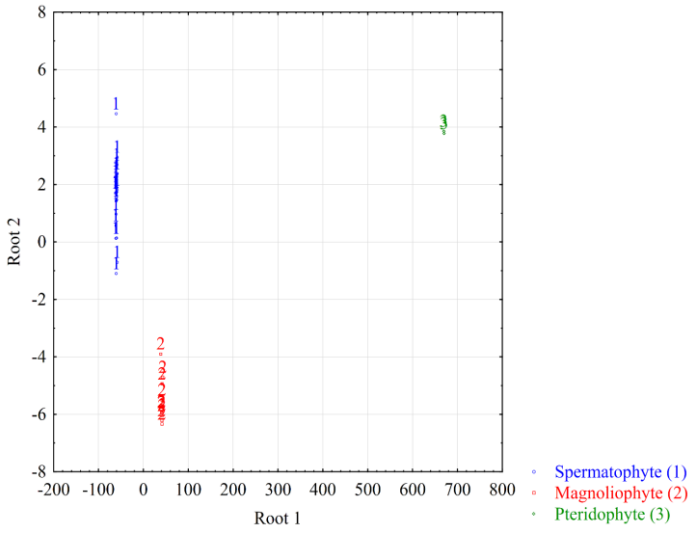

Figure 5.3. Graphical representation of Root1-Root2 of canonical scores: HPTLC Silica gel 60 F_{254}, gray channel 254 nm.

5.3. Medicinal plant classification according to the therapeutic effects

Most of the known medicinal plants are found in the Monographs of the World Health Organization (WHO) classified according to the different therapeutic effects [39] they manifest. They are used to treat different diseases such as neurodegenerative disease [40], digestive disease [41], asthma or even skin disease [42].

5.3.1. UV-Vis Spectroscopy

According to the PCA method a sepparation of the samples according to the therapeutic effecat occur. The graphical representation of the first five PCAs revealed a good separation of the samples according to this parameter, the samples number 26 an 31 corresponding to the liquorice and quaking aspen appear as extereme points. This separation is caused by the fact that this to samples are rich in salicin and respective in glicirhizin, according to the literature. As for the fuzzy PCA method there were separated 4 principal groups each one of them contaning samples that cure a certain disease. The exception in this case to is quaking aspen . When the F_OPCA was emploied there were noticed similar reults with F_1PCA the main differences beeing underlined by the FoPC2 direction. Quaking aspen is a extrem point in this case too (Figure 5.4).

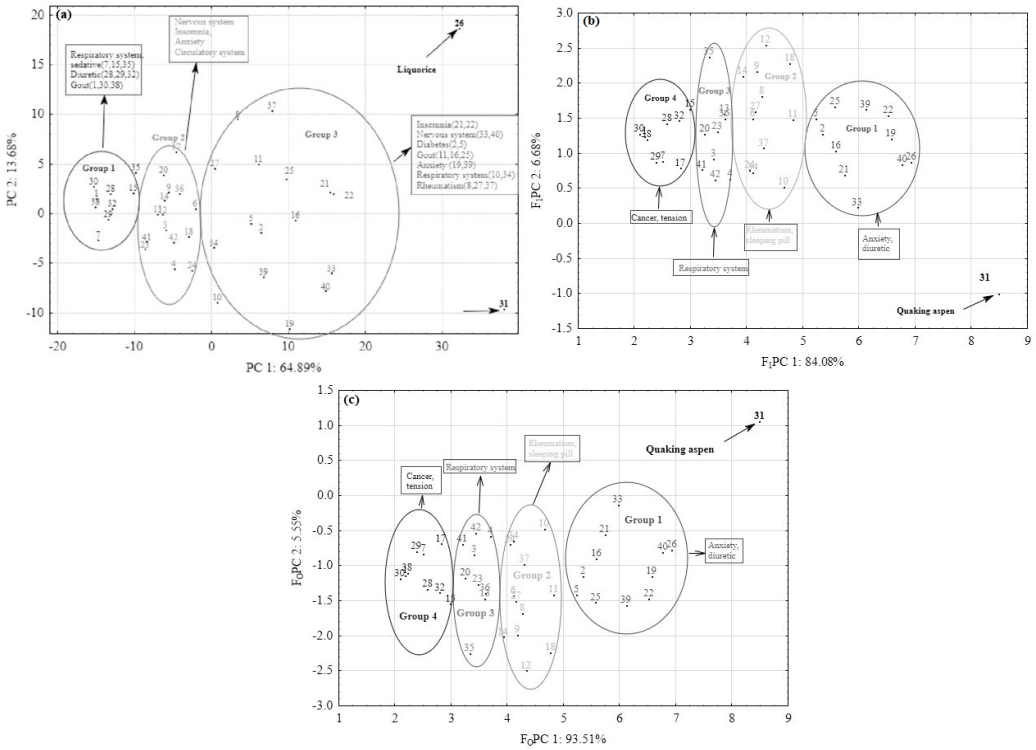

Figure 5.4. Graphical representation of (a) PC1-PC2, (b) F_1PC1-F_1PC2 (d) FoPC1-FoPC2 of the 42 investigated medicinal plant exctracts

5.3.2 Thin layer chromatography

The results of chemometric analysis revealed a good separation of the samples according to the medicinal effects for the image of the plates obtained at 254 nm. Thus the results obtained for this wavelenght will be presented further. The cluster analysis revealed a classification of the samples in four groups, according to the medicinal effects. A good separation of the samples in to the four groups it is observed on all channels when the PCA analysis was used. The best results were obtained for the HPTLC Silica gel 60 F_{254} plates on blue channel. The PCA-LDA method revealed a good separation of the samples according to this parameter on the HPTLC Silica gel 60 F_{254} blue and gray channel (Figure 5.5).

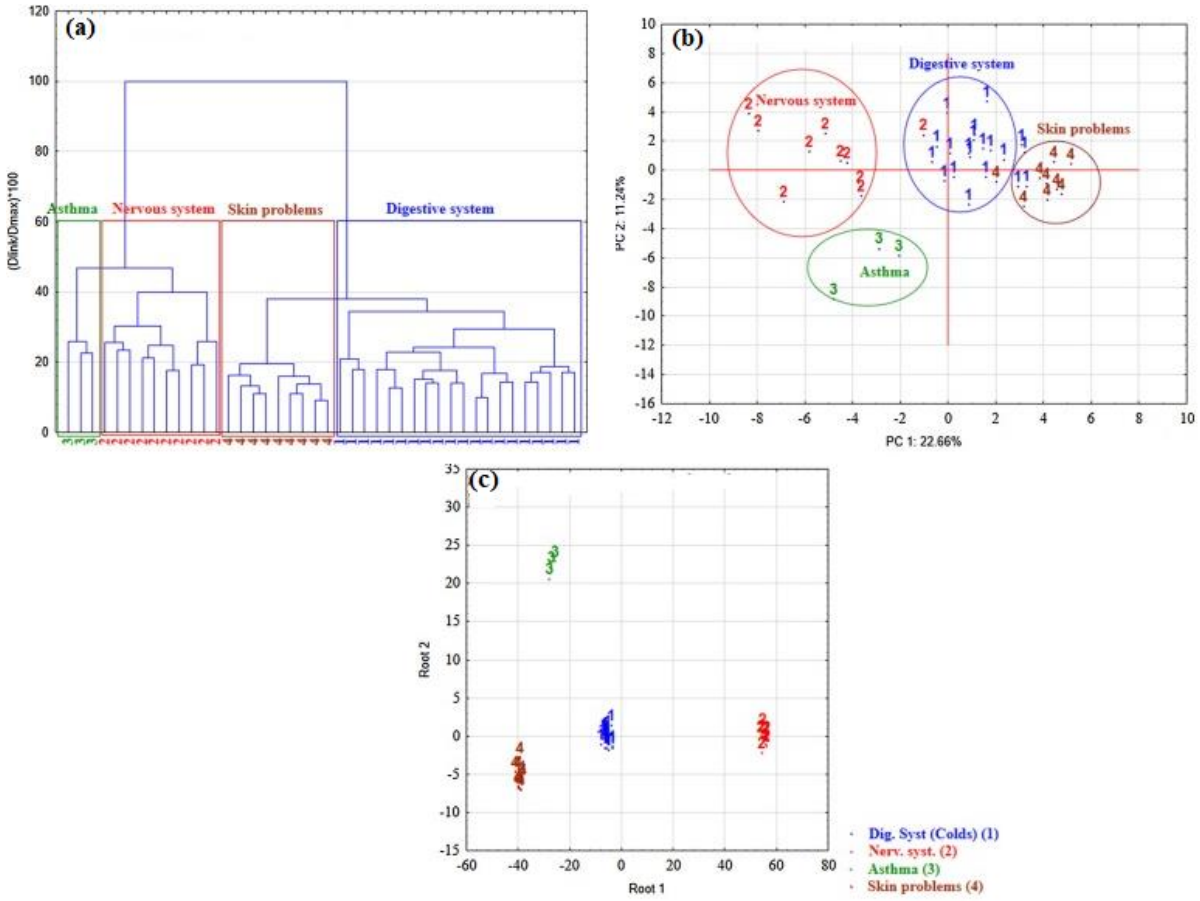

Figure 5.5. (a) Dendrograms, (b) Graphical representation of PC1-PC2, (c) graphical representation of Root1-Root2 of 42 medicinal plants extracts

5.4. Medicinal plant classification according to the antioxidant capacity

Antioxidants are substances which, present in small quantities, comparable to that of the oxidizable substrate, significantly delay or inhibit its oxidation. The interaction between free radicals and antioxidants is considered an important factor associated with the initiation and progression of various diseases and maintaining an optimal level of health. If the generation of free radicals exceeds the limits of the protective effects of antioxidants, oxidative stress occurs which has negative effects on the body, such as aging, cardiovascular disease, cancer, neurodegenerative disorders and other chronic diseases [43,44]. The best known antioxidants are found in fruits, vegetables, teas, wine, medicinal plants and spices.

5.4.1. Thin layer chromatography

The presence of antioxidant compounds (white bands on a purple background) with low or high reactivity, reported in DPPH•, was easily observed after a thorough investigation of the

image of chromatographic plates obtained 10, 20 and 30 minutes after immersion in DPPH solution (Figure 5.6).

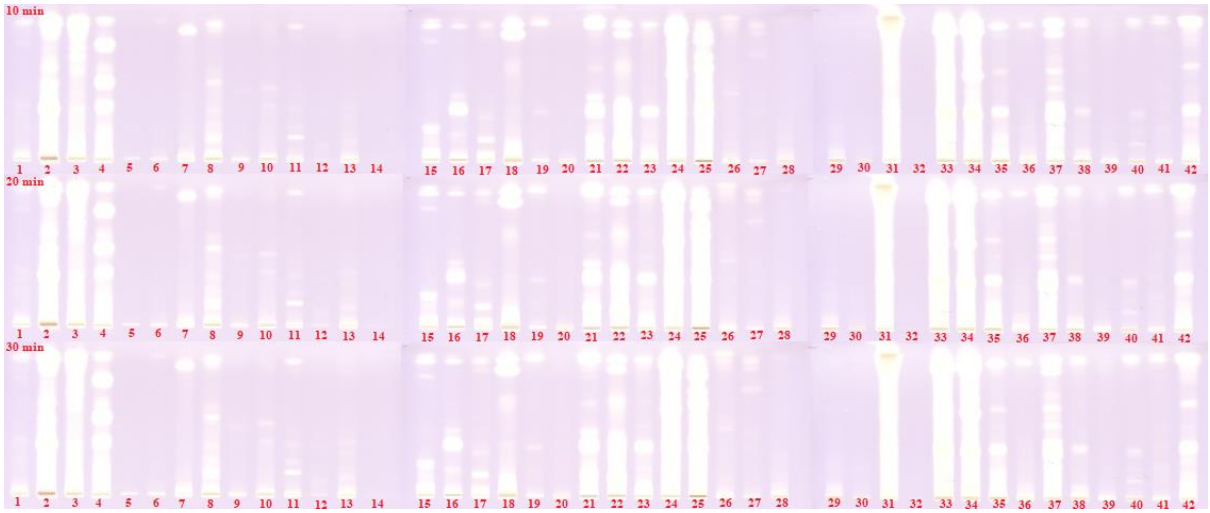

Figure 5.6. HPTLC chromatograms of hydroalcoholic extracts obtained after reaction with DPPH solution (0.02% in ethanol) for HPTLC Silica gel 60 F254 (10, 20 and 30 min). Mobile phase used in the separation process: ethyl acetate: toluene: formic acid: water (30: 1.5: 4: 3, v / v / v): (a) detection at λ = 365 n; (b) detection at λ = 254 nm.

The models obtained after the graphical representation of the scores related to PC1-PC2, indicate similar results for both types of plates. The samples are separated into two distinct groups: one large (more dissipated) which includes samples with high/medium antioxidant activity (positive scores) and a second (more compact, negative scores) consisting of samples with low/medium activity. Cluster analysis revealed a separation of the samples in to three main clusters according to the antioxidant activity. In LDA analysis HPTLC Silica gel F254 plates, indicate a correct classification of the samples (100%) according to the three groups while as the LDA results corresponding to HPTLC Silica gel 60 plates obtained under the same conditions (38 PCs) are significantly different, so the groups are more slightly compact and partially separated (Figure 5.7.)

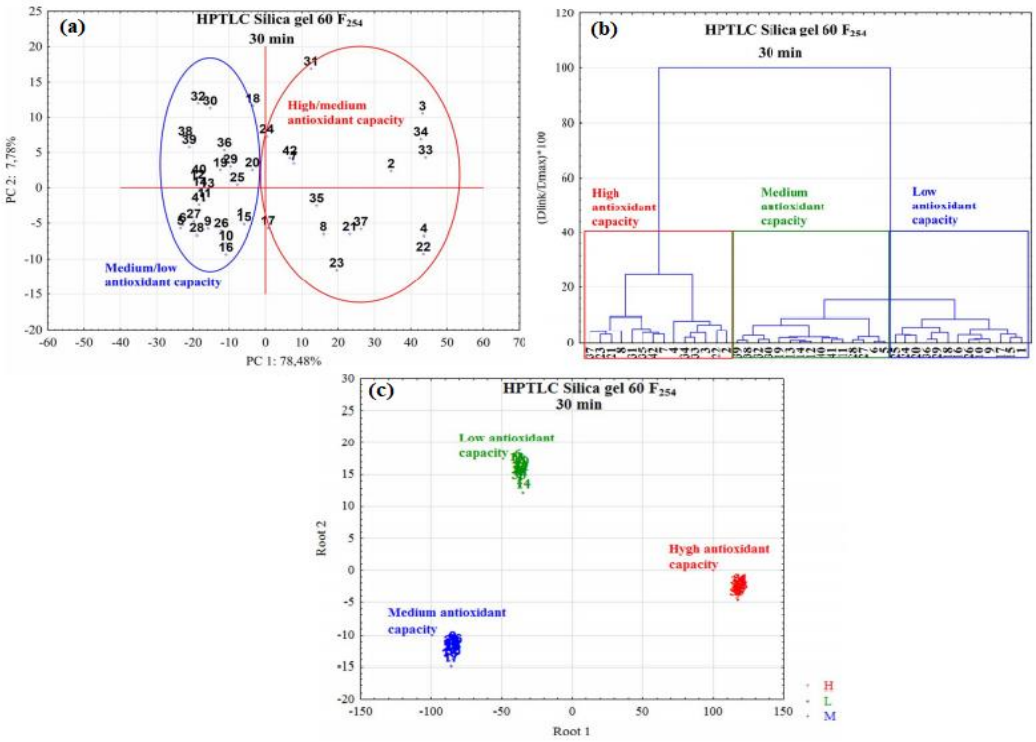

Figure 5.7. Graphical representation of (a) PC1-PC2, (b) dendrograms and (c) graphical representation of Root1-Root2 for the results obtained for HPTLC Silica gel 60 F_{254} nm.

5.4.2. High performance liquid chromatography

The results of horizontal clustering using c means and the data obtained at different wavelength (242, 260, 280, 320, 340, 380 nm) revealed a classification of the samples according to the antioxidant activity. The best results were obtained for 242 and 280 with few exceptions 21 and 22 were classified in the group of medium antioxidant activity and samples 14, 16, 18 and 20 were classified in the group of low antioxidant activity. However, regarding the DOMs of the samples assigned to the first partition (A1) in the group with moderate antioxidant activity and to the third partition (A3) from the marked group with high activity the differences are almost imperceptible.

Partitions obtained with FDHC, using the same chromatographic data (without preprocessing), seems to bring better results by offering two classes in most cases. Thus, the first includes plant extracts with high and moderate antioxidant activity and the second contains samples of plants with low antioxidant activity. The assignment of samples 21, 22 and 14, 16, 18 and 20 is the same.

The grouping of samples into two classes at the first partition level is similar to that previously obtained with FDHC in all cases (242, 260, 280, 320, 340 and 380 nm). The novelty consists in obtaining the partitions of the chromatographic retention time interval and the

association with different plant extracts. The main region (chromatographic fingerprint group) of all chromatographic retention time intervals (0.000-30,000 min) associated with the class of samples with high and moderate antioxidant activity (A1) including samples 21 and 22 and samples with low antioxidant activity (1-9, 10-20, 21, 22) is 7.83-13.35 min, and other shorter retention time intervals. In contrast, the most representative retention time regions for the group of plants with low antioxidant activity (A2), including samples 13, 14, 16, 18 and 20 with moderate antioxidant activity (13, 14, 16, 18, 20, 23-42), are 3,185-7,478 min, 16,732-24,138 min and 26,691-30,003 min, as well as other more or less restricted subintervals. Considering that the variables and samples are assigned to the fuzzy partition according to the highest value of the DOMs (defusification), it is also possible to build new DOM-based fingerprints. These are relevant in terms of sample similarities/differences, because they indicate both the position and the degree of association of the chromatographic peaks for the different classes of individual samples.

However, surprisingly, the graphical representation of the samples using the first two components corresponding to the data obtained at 242 nm indicates a good separation of the samples according to the antioxidant activity. These data are consistent with the results obtained from the application of the fuzzy cluster analysis method. In addition, all the results obtained after the use of PCA support the idea of using orthogonal and noise-cleaned scores for the first 41 PCs, in the HCA and LDA classification of extracts (Figure 5.8.).

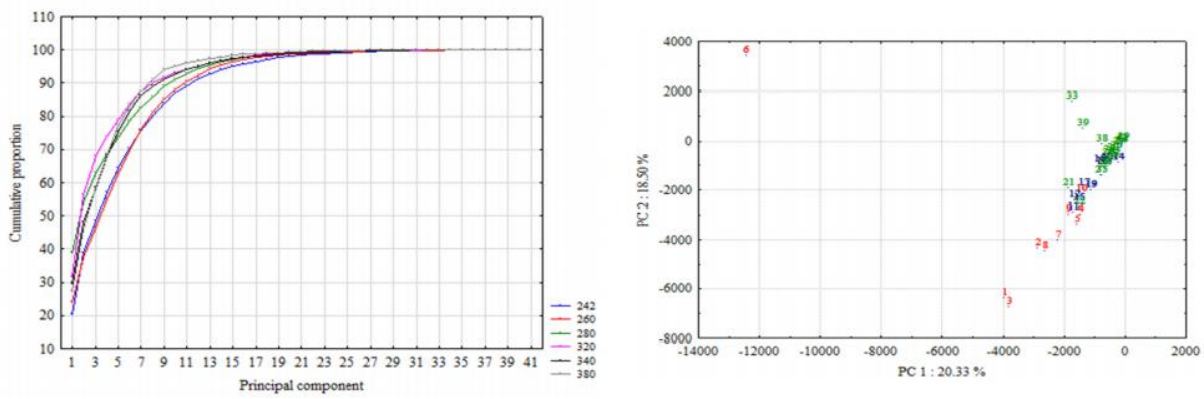

Figure 5.8. (a) Cumulative proportions profile and (b) graphical representation PC1-PC2.

The dendrogram provided by the cluster analysis using the Ward technique as a binding method and the Manhattan distance as a measure of similarity, for the data associated with the 41 PCs (242 nm), highlights well-defined groups of extracts, similar to those obtained. applying the methods of analysis of fuzzy clusters and PCA (Figure 5.9.).

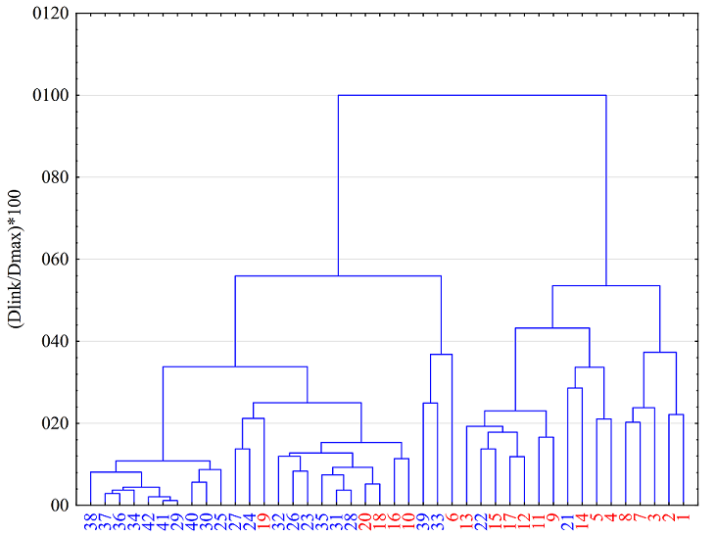

Figure 5.9. Dendrogram corresponding to herbal extracts.

The combination of the PCA method with LDA led to the best discrimination of the investigated samples, respectively, in two classes. The idea of the existence of three given classes was also considered. The minimum value of the correct classification supports the hypothesis of classifying medicinal plant extracts into two classes according to the criterion of antioxidant activity.

Chapter VI – Conclusions

The present research had as central theme the characterization and classification of medicinal plants characteristic of the Romanian flora, depending on different parameters/characteristics, by carrying out multiple types of analysis.

A first attempt was to use UV-Viz spectra as well as their derivatives (first to fourth order derivatives), to demonstrate their impact on the classification of extracts by phylum. The PCA methods combined with LDA, generated a high discrimination of the samples in the phylum to which they belong to. The first order derivative provided the best results, confirmed by the leaves one out validation 90.5%; 71.4% and 76.2%, respectively, attributed to unprocessed, standardized and standardized data. It is necessary to mention that the processing of data by normalization or standardization, or the use of various Savitzky-Golay parameters did not favor the improvement of the results.

The combination of thin layer chromatography with chemometric and image editing methods has led to a classification of medicinal plants in terms of phylum. It was concluded that the best results are obtained by using PCA-LDA, where the gray channel provides a more efficient separation (HPTLC Silica gel 60 F254 plates viewed at 254 nm) with a correct classification percentage of 90.5 %. The above observations are supported by the leave one out validation that confirms the effectiveness of the gray channel and the plates, with a correct classification percentage of 83.3%.

UV-Viz spectra together with the data obtained by combining thin layer chromatography with image processing methods, were used to classify the plants according to another criterion, namely the main therapeutic effect.

UV-Viz data demonstrated the feasibility of chemometric studies used on spectroscopic data to understand the relationship between medicinal plants and their healing properties. Robust fuzzy methods applied, provide an additional input of information, the groups obtained are better highlighted and defined, in accordance with the therapeutic effects. The total variance is described by a smaller number of main components and a more obvious delimitation of them. The results obtained should encourage the use of fuzzy methods for other types of data as well.

Thin layer chromatography has proven to be a suitable method for classifying medicinal plant samples according to their therapeutic effect. The contribution of PCs to the retention of compounds on the chromatographic plate was highlighted by the profile of PCA loadings. PC1 has a high contribution to the compounds retained on the HPTLC Silica gel 60 F254 plates, contrary to what is found on the HPTLC Silica gel 60, where the contribution is small and uniform, except for the compounds retained close to the front. In the LDA analyze, the

substitution of new, noise-free variables with PC scores, doubled by the use of different color channels, has proven to be an effective technique for increasing the selectivity of samples depending on the therapeutic effect. The highest percentage of correct classification was registered for the HPTLC Silica gel 60 F_{254} plates blue channel (92.9%) (PCA and FPCA methods) respectively the red channel on HPTLC Silica gel 60 (93.9% FPCA).

The classification of medicinal plants according to antioxidant activity was performed using HPLC chromatographic methods and thin layer chromatography.

Chromatographic data obtained by HPLC-DAD analysis at different wavelengths (242, 260, 280, 320, 340, 380), together with the methods of analysis of fuzzy clusters and classical methods, proved to be suitable in the classification of plants, according to this parameter. The best results were identified for all chemometric methods for wavelengths of 242 nm. The samples (which include relatively the same compounds) were grouped into two well-defined classes (high and low antioxidant activity, respectively) and three classes (high/medium/low antioxidant activity), respectively, supplemented by a few exceptions: *Glycyrriza glabra, Gentiana asclepiadea, Caelidonium majus, Juniperus communis, Xantium spinosum* and *Cynara scolymus* caused by differences in concentration.

The separation into two classes mentioned above is obtained in the case of the unsupervised methods PCA and HCA, and the supervised method PCA-LDA, results that overlap with those obtained by FDHC and FDHAC. Furthermore, FDHAC offers the opportunity to associate each fuzzy sample partition with a set of fuzzy features using characteristic regions of specific chromatographic fingerprints or peaks (chromatographic and fuzzy markers).

Cross-validation indicates the division of samples into two classes with a correct classification percentage of 100%, compared to 90.5% for the use of three classes. In addition, data do not require preprocessing, standardization and autoscaling do not improve results. Another important conclusion is that the initial variables (4501 variables) can be replaced by the scores corresponding to the main components totaling 100% of the variance (41 PCs), with the same results, but minimizing the speed and duration of the analysis.

The use of HPTLC together with chemometric and image processing methods, led to the characterization and classification of plants according to their antioxidant capacity, determined by immersing the plates in the free radical solution DPPH•. The results of applying the LDA method on the scores associated with the main components, demonstrate a complete separation of the samples (100%) into three compact groups and confirm the classification obtained by cluster analysis, the best results being obtained for HPTLC Silica gel 60 F_{254} plates.

The determination of the antioxidant activity of the sample set was performed, both separately, by using the DPPH method but also by comparing 17 more or less known techniques.

By running the SRD-CRRN program, and comparing the 17 methods, it was found that the best results are obtained for the methods: beta carotene, ABTS, FRAP, DPPH, CUPRAC, ORAC, CERAC.

The DPPH method is also suitable for other types of samples, among which we can mention the analysis of a set of 32 vegetable oils, the samples being successfully classified according to the antioxidant activity it manifests.

In addition to the 17 methods used to determine the antioxidant capacity of the 42 herbal extracts, the Micro TLC method was also used. By relating it to the other two methods, namely immersing the plates in DPPH and ABTS (results similar to those obtained by immersing the plates in DPPH) and the spectrophotometric method, the results are identical: compounds with antioxidant activity being highlighted by the three methods. Micro TLC analysis involves a number of advantages such as cheap equipment (no spectrophotometer is needed), short analysis time and a small amount of sample, being dedicated to application on complex samples.

To determine the stability of the samples, a set of five samples was chosen from the 42 medicinal plant extracts: blueberry, lady's bedstraw, licorice, sage and nettle, respectively. The samples were kept in a dry place, protected from light at a temperature of 21 ºC. According to the results obtained from thin layer chromatography, it can be seen that they have a rich composition (blueberry - 2, sandalwood - 21) in bioactive compounds and respectively low (sorrel - 7, licorice - 26, nettle - 30). The UV-Viz spectra of the five samples were measured over a period of six months (for one month the spectra were measured daily and for four months the spectra were taken once a week). Following this analysis, it can be seen that the UV-Viz spectra did not undergo significant changes, the small differences that appear being due to the way of working.

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Annex 1. List of original publications and participation in international conferences

Publications elaborated within the doctoral thesis:

Simion Maria, Cobzac Simona Codrtuţa, Casoni Dorina. Image analysis approaches to improve the thin layer chromatography-chemometric based investigations of natural extracts. STUDIA UBB CHEMIA, **2017**, *62*, 67–80. (**IF: 0,447; Q3; citation number: 3**)

Simion Ileana Maria, Casoni Dorina, Sârbu Costel. Characterization and classification of medicinal plants according to their antioxidant profile estimated by thin layer chromatography assisted by chemometric expertise. *J. Liq. Chrom. Relat. Tech*. **2018**, *41*(6), 342–348. (**IF: 1,312; Q3; citation number: 3**)

Simion Ileana Maria, Pop F. Horia, Sârbu Costel. Spectrophotometric characterization of Roumanian medicinal herbs assisted by robust chemometrics expertise. *Rev. Roum. Chim*. **2018**, *63(5-6),* 489–496. (**IF: 0,381; Q4; citation number: 0**)

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Simion Ileana Maria, Casoni Dorina, Sârbu Costel. Multivariate color scale image analysis–Thin layer chromatography for comprehensive evaluation of complex samples fingerprint. *J. Chromatogr. B Biomed. Appl.* **2021**, *1170*, 122590. (**IF: 3,205; Q1; citation number: 1**)

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Maria Simion, Costel Sârbu, Taxonomy of plants in phylum using UV fingerprints and robust chemometrics, Global Conference on Plant Science and Molecular Biology" 11-13 septembrie, 2017, Valencia, Spania.

Maria Ileana Simion, Augustin Cătălin Moț, Costel Sârbu, Classification of medicinal plants according to their antioxidant capacity using chromatographic profiles and chemometrics, Global Conference on Plant Science and Molecular Biology" 20-22 septembrie, 2018, Roma, Italia.