ORIGINAL ARTICLE

A NEW NATURAL ANTIOXIDANT SUPPLEMENT – DESIGN AND DEVELOPMENT

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Abstract

Various preparations based on herbs, when taken in sufficient doses and correct combinations, neutralize free radicals before they lead to the appearance of diseases in the body. In order to obtain a new nutritional supplement, we selected raw vegetal products: blueberry fruits (*Vaccinium fructus*), seabuckthorn fruits (*Hippophaë Rhamnoides fructus*), sage leaves (*Salviae folium*) and marigold flowers (*Calendulae flos*). The parts of plants originate from spontaneous flora. The pharmaceutical form of the final product is represented by antioxidant capsules. The antioxidant activity of the final product was demonstrated by the quantification of polyphenols (Folin-Ciocâlteu method), flavonoids (colorimetric method) of anthocyanins (spectrophotometric UV-VIS method). It has been also determined the content of heavy metals, elements that are good catalysts for biochemical processes. *In vitro* dissolution test is a quality parameter, in order to assess the bioavailability of the active ingredients in this pharmaceutical form achieved. The evaluation of the *in vitro* release of the active ingredients in the capsules under compendial conditions was performed on a number of 3 capsules, for 45 minutes according to the Romanian Pharmacopoeia 10th edition. The profile of the *in vitro* release of the active substances was carried out using 6 points, corresponding to the amount of active ingredients released at 5, 10, 15, 20, 30 and 45 minutes. The evaluation of antioxidant activity of the released active ingredients was performed by the α,α-diphenyl-β-picrylhydrazyl free radical scavenging method (DPPH). It has been also carried out the evaluation of microbial load of required parts of plants in order to reduce the risk of an onset of infections.

Rezumat

Diverse preparate pe bază de plante medicinale, atunci când sunt luate în doze suficiente şi în combinaţii corecte, neutralizează radicalii liberi înainte ca aceştia să ducă la apariţia unor afecţiuni în organism. Ca materii prime pentru realizarea unui nou supliment nutritiv am selectat fructe de afin (*Vaccinium fructus*), fructe de cătină *(Hippophaë rhamnoides fructus*), frunze de salvie (*Salviae folium*) şi flori de gălbenele (*Calendulae flos*). Produsele vegetale sunt provenite din flora spontană. Produsul finit este condiţionat sub formă de capsule cu proprietăţi antioxidante. Activitatea antioxidantă a produsului finit a fost demonstrată prin determinarea cantitativă a polifenolilor (metoda Folin-Ciocâlteu), a flavonoidelor (metodă colorimetrică), a antocianilor (metoda spectrofotometrică UV-VIS). De asemenea a fost determinat conţinutul în metale grele, elemente cu rol de catalizatori ai proceselor biochimice. Testul de dizolvare *in vitro* este un parametru de calitate, pentru evaluarea biodisponibilităţii principiilor active din forma farmaceutică realizată. Evaluarea cedării *in vitro* a principiilor active din capsule, în condiţii compendiale, s-a realizat pe un numar de 3 capsule, timp de 45 minute conform prevederilor Farmacopeei Române ediţia a X-a. Profilul cedării *in vitro* al substanţelor active s-a efectuat utilizând 6 puncte, corespunzătoare cantităţii de principii active eliberată la 5, 10, 15, 20, 30 şi 45 minute. Evaluarea activităţii antioxidante a principiilor active eliberate s-a realizat prin metoda DPPH. De asemenea s-a efectuat şi evaluarea încărcăturii microbiene a produselor vegetale necesară pentru a reduce riscul unor infecţii.

Keywords: natural antioxidant supplement, microbial test, *Calendulae flos, Hippophae rhamnoides fructus, Vaccinium fructus, Salviae folium*

Introduction

Capitalization of spontaneous flora is due to obtain nutritional supplement directions, to which the industry of pharmaceuticals is increasingly oriented. Sea buckthorn fruits, blueberry fruits, sage leaves and marigold flowers for their content rich in anthocyanins and flavonoids, can be used in the prevention of various diseases [5, 8, 14].

They are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium [11]. The differences between individual anthocyanins are related to the number of hydroxyl groups, the nature and the number of sugar attached to the molecule, the position of this attachment, and the nature and the number of aliphatic or aromatic acids attached to the sugar in molecule [23, 24].

Flavonoids are the most widespread group of natural compounds and probably the most important natural phenols. Total phenolic and flavonoids possess a broad spectrum of chemical and biological activities including radical scavenging properties. This property is especially distinct for flavonols. The medicinal effects of plants are often attributed to the antioxidant activity of phytochemical constituents mainly phenols, flavonoids and flavonols [16]. It is claimed that phenolic compounds are powerful chain breaking antioxidants [21]. The scavenging activity of the phenolic group is due to its hydroxyl group [9].

The studied species are rich in anthocyanins and flavonoids. Many reports have suggested that these compounds exhibit a wide range of biological activities, e.g., antioxidant, anti-inflammatory and anticancer effects [12, 22, 27]. Thus, they are assumed to promote health by protecting one from various degenerative diseases and diabetes as well as enhancing visual function and slowing the progress of neurological disorders. Consumption of flavonoid-rich plant foods has been claimed to protect against cardiovascular diseases and certain cancers, such as lung cancer [3]. It is known that the oxidation of low density lipoproteins (LDL) is associated with cardiovascular diseases, and thus flavonoids, compounds possessing antioxidant activity, are postulated to have potential benefits in the prevention of these diseases [2, 15, 17].

Materials and Methods

Reagents: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (Trolox), potassium persulfate, Folin-Ciocâlteu's reagent,1,1-diphenyl-2-pycrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (FRAP) were purchased from Sigma Aldrich (St. Louis, MO, USA). Gallic acid and sodium carbonate were purchased from Fluka (Switzerland). All the chemicals were of analytical grade.

Plant materials: Fruits from *Vaccinium myrtillus* and *Hippophaë rhamnoides*, leaves of *Salvia officinalis* and flowers from *Calendula officinalis* were collected from Bihor County in 2013. The parts of plants used in this study were dried, at an average temperature of 40°C for 96 h. The dried product it was macerated with ethanol (95%), for 72 h at 20°C. The ethanolic solution was concentrated under reduced pressure, at 50°C using BUCHI-Rotavapor (R-210/215), with control vacuum V850/855 and vacuum-pump V700/710. Each extract was immediately placed in a freezer (Freeze dryer Alpha 1–2 Christ - Martin Christ, Osterode am Harz, Germany) at -60^{o}C, where they remained for 48 h.

Instrumental and operating conditions in inductively coupled plasma atomic emission spectrometry (ICP-AES)

An acid-assisted microwave digestion procedure is optimized for the determination of metals in the vegetal products, analysed by using of Inductively Coupled Plasma Atomic Emission Spectrometry techniques (ICP-AES Agilent). After air drying, the vegetal products were pulverized with ceramic instruments, avoiding the contact with metals. Microwave Milestone MLS-1200 mega microwave digestion was used. The ICP-AES measurements were performed with a sequential ICP spectrometer IRIS Intrepid II. The parts of plants were subjected to digestion using an acid mixture (HNO_3 and H_2O_2). An amount of 0.1500-0.2500 g of dry sample was treated with 4.5 mL concentrated HNO_3 and 0.5 mL H_2O_2 were placed in the vessel. We closed the vessel with a valve and tightened with ECM-30, introduced in single safety shield and inserted in the microwave unit. After being cooled and reopened, the residue was diluted to 50 mL with ultrapure water in a plastic volumetric flask. The resulting solutions were clear, colourless and odourless, with no observed residue – complete dissolution in most cases was achieves.

The antioxidant capacity of herbs extracts

DPPH method

Radical scavenging activity of plant extracts against stable 2,2-diphenyl-2-picryl-hydrazyl-hydrate (DPPH) was determined by the slightly modified method of Brand-Williams *et al* 1995 [4]. DPPH reacts with an antioxidant compound, hydrogen donor, and reduce DPPH. Absorbance was measured at 517 nm by using UV visible light spectrophotometer. The solution of DPPH in methanol, of $6 \cdot 10^{-5}$ M, was prepared *in extempore* before UV measurements. The samples were kept at dark for 15 minutes at room temperature and the decrease in absorbance was measured. The experiment was carried out in triplicate. Radical scavenging activity was calculated using the following formula:

$$\% \text{ Inhibition} = [(AB - AA)/AB] \times 100,$$

where AB = absorption of blank sample (t = 0 min), AA = absorption of test extract solution (t =15 min) [27].

FRAP method (ferric reducing antioxidant power)

FRAP method is a simple spectrophotometric method that assesses the antioxidant power of the studied samples, based on the reduction of ferric tripyridyltriazine complex [Fe(III)-TPTZ] by a reductant, at an acid pH. The stock solutions included: 300 mM acetate buffer; 270 mg $FeCl_3 \cdot 6 H_2O$ dissolved in 50 mL distilled water; 150 mg TPTZ and 150 µL HCl, dissolved in 50 mL distilled water. The working FRAP solution was prepared *in extempore* by stirring 50 mL acetate buffer, 5 mL $FeCl_3 \cdot 6 H_2O$ solution and 5 mL TPTZ solution.

Trolox was used as a standard solution, the calibration curve was made for concentrations between 0 and 75 µg/mL, having a coefficient of determination $R^2 = 0.9956$ and the regression equation ($y = 0.0017x + 0.0848$), where *y* represents one absorbance detected at 595 nm. The results are expressed as µmol Trolox equivalents (TE)/100 µL extract [13].

2,2'- azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or TEAC method (Trolox equivalent antioxidant capacity)

This method is based on the ability of antioxidants to decrease the cation-radical life ($ABTS^+$), a blue-green chromophore that absorbs at 734 nm, compared to Trolox. $ABTS^+$ is produced in the reaction between the ABTS stock solution (7 mM) and the potassium persulfate (2.45 mM) for 12-16 hours. For the study of antioxidant activity, the $ABTS^+$ solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. After adding 100 µL sample to 2900 µL $ABTS^+$ solution, the mixture was spectrophotometrically monitored at 734 nm. The calibration curve was performed with Trolox standard, and the results were expressed as µmol Trolox Equivalents/100g dry weight.

The bioactive compounds of herbal extracts

Determination of polyphenols content

Total phenolic content was determined by the Folin Ciocâlteu method. Using Folin-Ciocâlteu method, the OH groups can be evaluated from the studied samples, in an alkaline medium (adjusted with sodium carbonate). The absorbance at the wavelength of 765 nm increases proportionally with the number of OH groups of the polyphenols. To 0.5 mL of herbal preparation made up with 0.5 mL of distilled water, 0.5 mL Folin Ciocâlteu reagent was added and gently mixed. After 2 minutes, 0.5 mL of 100 mg/mL sodium carbonate was added. The contents were mixed and allowed to stand for 2 hours. The optical density of the blue coloured samples was spectrophotometrically measured at 765 nm. Standard gallic acid at a concentration of 100 - 500 g/mL was used. The concentration of total phenolic is expressed as milligrams of gallic acid equivalents (GAE)/g of mixture.

Determination of total anthocyanin content

The total anthocyanin content was determined using a method based on their property of changing colour pH dependent [10, 21]. The basic principle of anthocyanin determination lies in the fact that these compounds present different absorption spectra. The coloured oxonium form predominates at pH 0.1, and the colourless cetallic form at pH 4.5. The spectrophotometric method based on pH difference enables total anthocyanin evaluation, even in the presence of degraded polymerized pigments or other interfering compounds.

Approximately 0.15 g of each sample were weighed, later blended using an *Ultra-Turex* homogenizer for 1 minute at 3000 rpm, in methanol acidulated with 0.3% HCl. The samples were then centrifuged at 5000 rpm, for 20 minutes. The supernatant was isolated and the residue was once more homogenized by centrifugation. The procedure was repeated 3 times. The supernatants were reunited and the total anthocyanin content was determined. Two dilutions of the samples were made, one in KCl buffer, pH 1.0 and the other in acetate buffer pH 4.5, so that the absorbance of the sample at λ_{max} would not surpass the value of 1.2. After a resting time of 15 minutes, each diluted sample had its absorbance measured at λ_{max} and 700 nm in regard to the solvent. The absorbance values for the two diluted solutions were calculated using the following formula:

$$A = (A\ \lambda_{max} - A_{700})_{pH\ 1.0} - (A\ \lambda_{max} - A_{700})_{pH\ 4.5}.$$

The total flavonoid content was determined using a previously described colorimetric method [13]. 1 mL sample (containing 0.1 mg/mL dry substance) was mixed with 4 mL water and placed in a 10 mL volumetric flask. Firstly, 3 mL 5% $NaNO_2$ solution where added, after 5 minutes 0.3 mL 10% $AlCl_3$ and after 6 minutes 2 mL NaOH 1M. The flask was filled up to its calibration mark with distilled water. The solution was mixed and its absorbance was detected at 510 nm.

Preparation of capsules with different proportions of herbs

In view of the properties of capsules, we have achieved their filling. In order to obtain the capsules, we have pursued that the lyophilized powder mixture match to the requirements of quality grading.

Dissolution time determination

The *in vitro* release of active ingredients from capsules in compendial conditions was assessed using 3 capsules, for a period of 45 minutes according to the guidelines of the 10th edition of the Romanian Pharmacopoeia. The dissolution time according to the 10th edition of the Romanian Pharmacopoeia is determined using an ELECTROLAB TDT-08L Dissolution Tester (USP) testing device. The number of cycles is 23 - 24/min and the amplitude is 50 - 60 mm. The device performs the cycles within the basket containing the liquid medium. The water bath thermostat in which the basket is introduced must ensure a temperature of 37°C for the liquid. The *in vitro* release profile of the active ingredients was determined using 6 samples, each corresponding to the released quantity of active ingredients at 5, 10, 15, 20, 30 and 45 minutes. The antioxidant activity of these active ingredients was determined by DPPH method as describe above.

Microbial test

The contamination of plant products with microorganisms can cause the release of alterative processes on raw vegetable products, thereby

reducing their shelf life. The most common plant pathogens in plant products are *Staphylococcus spp., Enterobacter spp., Ascomycota spp*. Based on these considerations, the aim of our research was the assessment of microbial load and configuration for the four plant products, on which our nutritional supplement was based. For the determination of the microbial load and configuration, four samples were taken.

Ascomycota germs were identified using the Sabourand selective media for 5 days at temperatures between 20ºC and 25ºC. *Enterobacter* germs were identified using the Con Mc Key selective media for 24 hours at 37ºC. *Staphylococcus* germs were identified using the Chapman selective media for 3 days at 37ºC. After processing the obtained data, it has been determined a low load plant products.

Statistical analysis

The data analysis was performed by one-way analysis of variance, ANOVA, with GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). The mean values were considered statistically significant when $p < 0.05$.

Results and Discussion

Determination of the polyphenolic and total flavonoid content

The Folin-Ciocâlteu method, generally used to assay the total phenolic content also measures the total reducing capacity of a sample. Total phenolic generally correlates with redox and antioxidant capacities as measured by the TEAC or DPPH methods [4, 19]

In this study, the aim was to analyse four herbals extracts from the point of view of bioactive compounds (like total polyphenols, anthocyanins and flavonoids) and antioxidant capacity. Also, the content in metal ions of herbs was determined. The results of total phenolic and flavonoid contents of studied lyophilized herbal parts and alcoholic extracts are shown in Table I.

Table I

Total polyphenolic and flavonoid content in lyophilized herbal parts and alcoholic extracts

No.	Sample	Total polyphenolic content (mg GAE/100 DW	Total flavonoid content mg QE/100 DW
1.	Lyophilized blueberry fruits	343.93 ± 0.01^{c}	72.80 ± 7.61^{e}
2.	Lyophilized sea-buckthorn fruits	251.47 ± 0.02^{e}	145.44 ± 8.72^{c}
3.	Lyophilized common marigold flowers	151.62 ± 0.03^{g}	266.62 ± 6.91^{b}
4.	Lyophilized sage leaves	258.87 ± 0.01^{d}	427.23 ± 9.42^{a}
5.	Alcoholic blueberry extract	812.13 ± 0.02^{b}	125.82 ± 8.91^{d}
6.	Alcoholic sea-buckthorn extract	211.91 ± 0.02^{f}	43.14 ± 2.42^{f}
7.	Alcoholic common marigold extract	112.42 ± 0.01^{h}	42.12 ± 2.41^{f}
8.	Alcoholic sage extract	963.01 ± 0.01^{a}	267.42 ± 8.21^{b}

*Data is expressed as mean ± standard deviation. Different letters in the same column indicate significant differences. The results are generated with one-way ANOVA and Tukey's Multiple Comparison Test ($p < 0.05$).

** DW – dry weight, GAE – gallic acid equivalent, QE – quercetin equivalent

The amount of total phenolic, assessed by Folin–Ciocâlteu method, varied widely in herb materials and ranged from 151.61 to 343.93 ± 0.01 mg GAE/100 g for lyophilized extract, common marigold and blueberries, respectivelly 112.42 to 963.01 ± 0.01 mg GAE/100 g DW for alcoholic extract, common marigold extract and sage (Table I). Analysing results, it can be observed that the alcoholic samples contained the highest quantity of polyphenols, increasing in the following order: common marigold < sea-Buckthorn < blueberry < sage. The obtained poliphenolic data are comparable to previous findings which reported values between 251 - 310 mg GAE/100 g for some cultivated blueberries and between 577 and 614 mg GAE/100 g for wild Italian blueberries [6]. The total flavonoid expressed in quercetin (QE) content in blueberry ranged from 125.82 ± 8.92 in alcoholic extracts to 72.80 ± 7.61 mg QE/100 g DW in lyophilized blueberries extract. The highest level of flavonoids was found in lyophilized sage extract, while the lowest concentration was found in alcoholic sea-buckthorn extract. Lyophilized common marigold extract (266.62 mg QE/100 g DW), lyophilized sea-buckthorn extract and alcoholic blueberry extract (145.48 mg GAE/100 g DW and 125.82 mg QE/ 100 g DW) also had very high levels of flavonoids.

Only blueberry samples contained the anthocyanin pigments and the results are displayed in Table II.

Table II

The anthocyanin content of blueberries samples

No.	Blueberry samples	Anthocyanin quantity mg cyanidin/100 g DW
1.	Alcoholic blueberries	284.8 ± 17.2

*DW – dry weight

Determination of antioxidant capacity

DPPH method (1,1-diphenyl-2-picrylhydrazyl) is a spectrophotometric method, widely used to test components' ability to remove neutralise free radicals scavenger or their hydrogen donating capacity. The antioxidant capacity of the samples, measured by different complementary assays, is shown in Table III.

Table III

Antioxidant capacity of samples

Sample	DPPH%	ABTS (mmol Trolox equivalent/g DW)	FRAP (mmol Trolox equivalent/g DW)
Lyophilized blueberries	$8.51 \pm 1.11^{c,d,e}$	326.95 ± 5.98^{a}	73.05 ± 0.00^{c}
Lyophilized sea-buckthorn	$4.12 \pm 0.11^{c,d,e}$	48.67 ± 31^{d}	44.41 ± 0.01^{f}
Lyophilized common marigold	$2.69 \pm 0.51^{d,e,f}$	3.45 ± 3.21^{e}	17.53 ± 0.02^{g}
Lyophilized sage	$5.61 \pm 1.83^{d,e}$	67.84 ± 11.78^{d}	61.07 ± 0.01^{d}
Alcoholic blueberry extract	34.02 ± 2.01^{b}	280.91 ± 1.12^{b}	201.91 ± 0.05^{a}
Alcoholic sea-buckthorn extract	47.41 ± 3.22^{a}	$56.85 \pm 3.25^{d,f}$	52.78 ± 0.01^{e}
Alcoholic common marigold extract	4.17 ± 1.31^{e}	6.68 ± 1.14^{e}	15.01 ± 0.03^{h}
Alcoholic sage extract	46.94 ± 3.56^{a}	244.12 ± 23.42^{c}	177.62 ± 0.11^{b}

*Data is expressed as mean ± standard deviation. Different letters in the same column indicate significant differences. The results are generated with one-way ANOVA and Tukey's Multiple Comparison Test ($p < 0.05$).

**ABTS - 2,2'- azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) method, DW – dry weight, FRAP - 2,4,6-tri-(2-pyridyl)-s-triazine method

The alcoholic extract of blueberry had the highest content of phenolic compounds (812.13 ± 0.00) mg GAE/100 g DW, as well as the best antioxidant activity in the DPPH assay (34.02 ± 2.01%). The highest scavenger capacity of DPPH radicals was recorded in the case of alcoholic compared with aqueous extracts. The highest DPPH inhibition was recorded in the case of alcoholic sea-buckthorn extract followed by alcoholic sage extract (47.41 ± 3.22% and 46.94 ± 3.56%, respectively). The lowest value of DPPH inhibition was recorded in the case of lyophilized common marigold (2.69 ± 0.51%). The present ABTS values are higher than the values obtained by Sellappan *et al.* (8.11 - 38.29 µmol/g) [20]. The high level of antioxidant activity obtained for alcoholic blueberry extract, by all used methods, could be due to its high level of total polyphenol content.

ICP-AES measurement

The mineral content found in the samples under investigation is shown in Figure 1.

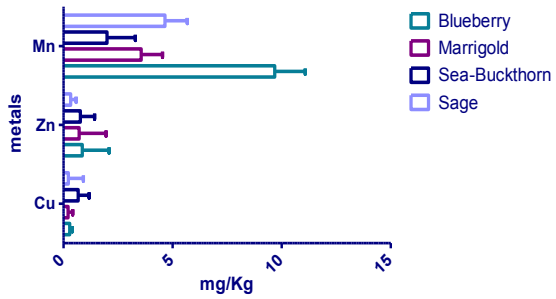

Figure 1.

The mineral content of analysed plants

The Zn content of the herbs is very low (0.3295 - 0.8608 mg/kg raw), when compared to the normative zinc requirement for humans (4.6 mg/day) [19]. The method is also used for the evaluation of the copper: the assessed samples contain 0.2109 - 0.2835 mg Cu/kg raw, the requirement being 0.75 mg/day [1, 18]. The highest concentrations of Mn were found in blueberry (9.68 mg/kg raw), followed by sage (4.6547 mg/kg raw). The manganese content of blueberry (9.6807 mg/kg raw) is not so high in comparative with the average Mn concentration of herbaceous species (54 mg Mn/kg), or compared to vegetables 5 - 34 mg/kg raw and fruits < 10 mg/kg raw. When assessing the content in cooper, zinc and manganese, manganese is best represented, being followed by zinc and copper.

The statistical analysis has shown that the insignificantly different content of Cu and Zn were recorded, instead significantly differences were obtained in the case on Mn, between the following samples: blueberry *vs.* marigold, blueberry vs. sea-buckthorn and blueberry *vs.* sage ($p < 0.05$).

As a result of the conducted studies, three combinations of powders were suggested, each combination containing blueberries, sea-buckthorn, sage and common marigolds in the following proportion: mixture 2:2:1:1 > mixture 2:2:2:1 > mixture 2:1:1:1 (g/g/g/g).

The prepared mixtures were extracted with 70% ethanol, each having its total polyphenol and flavonoid content determined and DPPH antioxidant capacity analysed by the previously described methods.

Considering of the obtained results, the series in decreasing order of total polyphenol content expressed in gallic acid per mixture was the following: mixture 2:2:2:1 > mixture 2:2:1:1 > mixture 2:1:1:1.

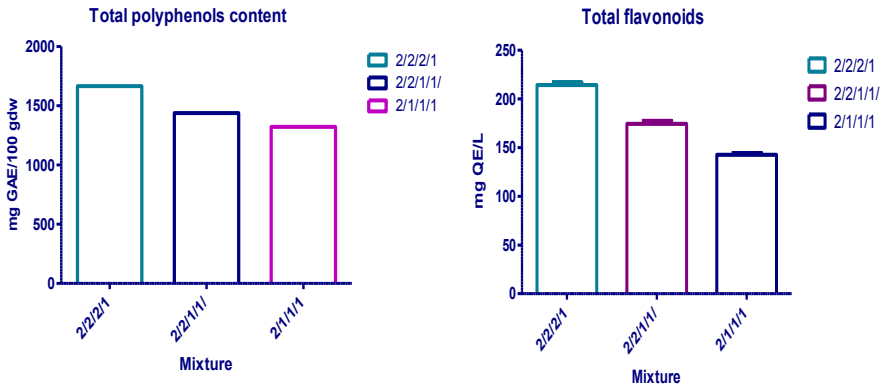

Figure 2.
Total polyphenol and flavonoid content of the mixtures

The determined total polyphenol content in primary herbal resources is responsible for the antioxidant action, by binding oxide and peroxide free radicals which cause aging and infectious diseases, these actions being mentioned in the scientific literature [23]. Also, the series in increasing order of flavonoid content expressed in quercetin per mixtures is the following: mixture 2:2:2:1 > mixture 2:2:1:1 > mixture 2:1:1:1. In addition, the series in decreasing order of antioxidant activity, determined by DPPH method, was the following: mixture 2:2:2:1 > mixture 2:2:1:1 > mixture 2:1:1:1.

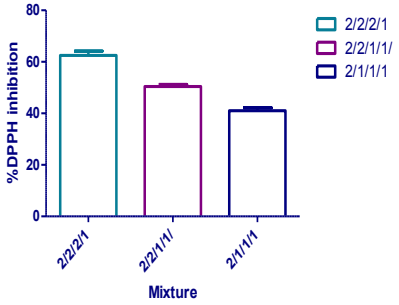

Figure 3.
Antioxidant capacity of the mixtures determined by the DPPH method

Regarding the statistical analysis, the content of bioactive compounds (total polyphenols and flavonoids) and antioxidant capacity of plants (determined by DPPH assay) significantly differ among the mixtures ($p < 0.05$).

Flavonoids can alter physiological effects of other dietary antioxidants, such as vitamins E and C, whereas it synergizes with them, favouring their regeneration. The high anticarcinogen effect of flavonoids must be remarked: the action of flavonoids pro-synthesizing enzymes of phase I (monooxygenase) and phase II (glucuronyl-transferases, sulfotransferases etc.) was demonstrated, flavonoids acting in the first stages of tumour initiation [25].

In conclusion, the combination blueberries, sea-buckthorn, sage and common marigolds in proportion of 2:2:2:1 proved to be the most promising for the intentions of the study.

Microbial test of the plants

The most common pathogenic species found within plant materials are: *Staphylococcus spp., Enterobacter spp., Ascomycota spp.* Thus, limiting contamination level with these agents may be done only by respecting certain hygiene measures, good manufacturing practice and standard procedures for hygienization. Based on these considerations, the purpose of this determination was to assess the pathogens loading and configuration for the plant materials (Table IV).

Table IV
Pathogen load of the raw vegetable products

Pathogenic species/group	*Vaccinium fructus*	*Hippophaë rhamnoides fructus*	*Salviae folium*	*Calendulae flos*
Staphylococcus spp.	Negative	Negative	Negative	Negative
Enterobacter spp.	Negative	Negative	Negative	Negative
Ascomycota spp.	Negative	Negative	Negative	Negative
Grounds	Negative	Negative	Positive	Negative
Molds	Negative	Negative	Positive	Negative
Yeasts	Negative	Negative	Negative	Negative

Capsule filling technology

The herbal products were brought in the following proportion: blueberries:sea-buckthorn:sage:common marigold = 2:2:2:1. Lactose was used as a powder diluent. To carry out the capsule filling in pharmacy,

the method chosen was manual filling by pouring and levelling, using ProFill 100 capsule filling system. The capsules used were Capsugel no.1 type containing 0.5 g herbal products and diluents. The filled capsules, closed and removed from the encapsulating system were stored in opaque plastomer pharmaceutical boxes. After closing the boxes, the labels were applied, describing information regarding the product, administration mode, therapeutic indications and precautions of use.

Determination of disintegration time

The disintegration time of the obtained capsules falls within the maximum limit of 15 minutes allowed by FR X [29]. The antioxidant activity of the active ingredients after disintegration determined by DPPH method was 48.5% and 62.5% before dissolution.

Conclusions

Methods of analysis carried out for the extracts of parts of plants, taken under study, have enabled us their physico-chemical characterization. On the basis of the bioactive ingredients, we were able to achieve the standardization of lyophilized extracts in gallic acid and quercetin.

The determination of bioactive compounds suggested that the lyophilized extract blueberry: buckthorn: sage: marigold 2:2:2:1 so that we have a new formula of nutritional supplement with antioxidant properties.

ICP-AES analysis of studied raw vegetable products shows that they may constitute a contribution of oligo-elements that are in the interests of biochemical processes of the body.

Following the performed determinations, the raw vegetal products do not had microbial charge, so they can be used for creating the new supplement.

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