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Advances on the Antioxidant Activity of a Phytocomplex Product Containing Berry Extracts from Romanian Spontaneous Flora

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Abstract: The present study aimed to evaluate the antioxidant properties of a phytocomplex product obtained using 10% hydroalcoholic extractive solutions, in equal proportions, from *Ribes nigrum*, *Rubus idaeus*, *Rubus fruticosus* and *Fragaria moschata* fresh fruits harvested from the spontaneous flora of Romania. These plant products were recognized for their rich antioxidant content. The phytochemical profile was assessed using HPLC chromatography and UV-Vis spectrometry. The obtained results highlighted the presence of complex bioactive compounds with antioxidant actions, namely anthocyanins, proanthocyanins and vitamin C. The antioxidant actions of the hydroalcoholic extractive solutions and the phytocomplex product were evaluated using chemiluminescence, electrochemical and superoxide dismutase (SOD) methods. The experimental results showed evident antioxidant activity in both the hydroalcoholic extracts and the phytocomplex product.

Keywords: hydroalcoholic plant extracts; antioxidant activity; oxidative stress; Romanian berry fruits; vitamin C

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1. Introduction

Recently, numerous studies have been conducted on free radicals formed in the human body as a result of endogenous metabolic activity and the influence of various agents with higher or lower degrees of toxicity on the cells and tissues of living organisms [1–4]. The attack produced by free radicals is manifested either locally on the component cells of an organ or simultaneously on several tissues, gradually leading to serious local or general disorders. In the case of analyses performed on the potency of different free radical types, it was established that, in most cases, the evolution of biochemical processes and reactions triggers (simultaneously or at different times) some factors which favor the effects of oxidative stress [5–8].

Oxidative stress represents the aggression produced at the molecular level by an imbalance between pro-oxidants and antioxidants—in favor of the first—that is manifested as negative effects at the tissue and organ level. Reactive oxygen species (ROS) are produced in aerobic organisms under physiological conditions. These intermediate metabolic products induce pathological changes only when they can no longer be neutralized by endogenous

antioxidants [9–15]. Numerous recent studies have highlighted the main mechanisms occurring at a cellular level through which reactive oxygen species start to show harmful and toxic effects: (i) the peroxidation of membrane lipids, which causes changes in the functionality of cell membranes and cell organs; (ii) damage to DNA molecules, which induces mutagenic processes and cell death; (iii) the alteration of protein and polycarbohydrate structures (hyaluronic acid); and (iv) the disruption of calcium homeostasis in the vascular wall [16–19].

In cases where oxidative stress leads to pathological conditions, therapeutic intervention with exogenous, synthetic or natural antioxidants is necessary. Current trends in medicine focus on the use of natural products from a plant or animal origin [20–26].

The most important mechanisms through which oxidative stress occurs are: (i) an increase in the formation of free oxygen and nitrogen radicals following exposure to large amounts of oxygen in the presence of toxins; (ii) a decrease in endogenous, physiological antioxidants, and especially of some enzymes, such as glutathione-peroxidase (GSH), manganese or copper-zinc superoxide-dismutase, with a role in the protection of organisms; and (iii) a decrease in antioxidants in the human diet.

Oxidative stress induces apoptosis. Antioxidants may reduce the ability of ROS to participate in processes that lead to apoptosis, such as the infiltration and adhesion of neutrophils, by activating myocyte receptors or altering cell membranes; by altering the homeostasis of calcium ions, which affects the sarcoplasmic reticulum membrane; and by the oxidation of nucleic acids [27,28].

During the processes by which the formation of free radicals takes place, an increase in vascular permeability and the extravasation of macromolecules, manifested as edema, inflammation, etc., has been observed. The considerable formation of oxygen free radicals during the inflammatory process, as a result of oxidative toxins, produces increases in vascular permeability [29–31].

Oxidative stress can also occur intracellularly (i) after intoxication with certain chemicals, such as ethanol, paracetamol, acetanilide derivatives and adriamycin, or (ii) after the reperfusion of ischemic tissues and organs [25,32]. In this case, the antioxidants used must be able to cross the cell membrane. For this purpose, the antioxidants must be lipophilic or amphipathic, such as vitamin E or β-carotene [16,33,34].

The use of hydrophilic antioxidant compounds leads to a longer time requirement for the restoration of intracellular antioxidant capacity [35,36].

Since the compounds that are richest in vitamin C, an especially effective antioxidant, are fruits, it is a challenge to obtain natural pharmaceutical products with improved therapeutic qualities that contain the whole range of active properties. Phytocomplex technology represents a promising alternative; it incorporates phytoconstituents that are soluble in water (terpenoids or flavonoids) or standardized plant and fruit extracts. In this context, we obtained a phytocomplex with antioxidant activity by combining 10% hydroalcoholic extractive solutions obtained from the fresh fruits of *Ribes nigrum*, *Rubus idaeus*, *Rubus fruticosus* and *Fragaria moschata* in equal proportions. In general, berry fruits are one natural resource of antioxidants, and contain a high concentration of phenolic compounds (benzoic acid, cinnamic acid, tannins, stilbenes and flavonoids, such as anthocyanins, flavanols and flavonols), vitamin C and other antioxidant compounds [37–39]. However, the amount of bioactive compounds is determined by diverse factors, such as the variety, species, region, cultivation, ripeness, harvesting time, weather conditions and storage time [40–45]. Therefore, this study aimed to evaluate each hydroalcoholic extractive solution component from a qualitative and quantitative chemical point of view, as well as investigate its antioxidant activity.

Numerous studies have shown the benefits of phytocomplexes in increasing vitamin C bioavailability due to the presence of other essential components, such as bioflavonoids, in the structure of the extract. It has been demonstrated that these ingredients act as antioxidants, thus considerably reducing vitamin C oxidation [46–48].

2. Materials and Methods

2.1. Materials

Fresh fruits, including *Ribes nigri* (blackcurrants), *Rubi idaei* (raspberries), *Rubi fruticosi* (blackberries) and *Fragariae moschatae* (strawberries), were collected from spontaneous flora in the forest zone of Arges County, Romania. All reagents and reference substances used were chromatographically pure (manufacturer: Merck, Fluka). Ultra-pure water obtained by ultra-purification with a Millipore system was used.

All reagents used (chloroform, Na_2HPO_4, KH_2PO_4, nitrotetrazolium blue (NBT), methyl blue, tetramethylene diamine (TMED) and 0.05 M phosphate buffer, pH 7.8) for the determination of the antioxidant action of the phytocomplex by the superoxide dismutase method were of analytical reagent grade (from Merck).

2.2. Methods

Preparation of the phytocomplex

The extracts of *Ribes nigri* fructus, *Rubi idaei* fructus, *Rubi fruticosi* fructus and *Fragariae moschatae* fructus were obtained according to a working protocol previously developed in the literature [36,49,50].

10 g of vegetable product was brought into contact with 100 mL of a 70% hydroalcoholic mixture (1/10 ratio).

10 g of vegetable product and 100 mL of 70% hydroalcohol (1/10 ratio) were mixed in a glass vial, wrapped in paper, covered and left to soak for 7 days in a cool place (15–25 °C) protected from direct sunlight. The mixture was shaken 3–4 times/day.

After 7 days, the hydroalcoholic extracts were filtered and the hydroalcoholic extractive solutions were placed into hermetically sealed vessels and kept in the refrigerator. Similarly, 10 g of partially solubilized vegetable products were subjected to a new solubilization process with a 70% hydroalcoholic mixture for another 4 days in similar conditions. After that, the hydroalcoholic extracts were filtered and mixed with the previously obtained hydroalcoholic solutions. Finally, hydroalcoholic extractive solutions at a concentration of 10% in 70% ethyl alcohol were obtained. In these conditions, the phytocomplex consisted of equal parts of the 10% hydroalcoholic solutions obtained from *Ribes nigri*, *Rubi idaei*, *Rubi fruticosi* and *Fragariae moschatae* fructus.

Qualitative chemical analysis of hydroalcoholic extractive solutions

The obtained hydroalcoholic solutions were chemically analyzed, both qualitatively and quantitatively. The identification of compounds with antioxidant action (flavones, anthocyanins, proanthocyanins and tannins) was performed using specific reactions [51–55].

Varian-ProStar equipment with a quaternary pump and UV-Vis detector with a diode array was used to perform the UV-Vis determinations. A C18 type stationary phase column (Discovery C18 250 × 4.6 mm, 5 µm particles) was used. The mobile phase consisted of two solvents: Solvent A was an aqueous phosphoric acid solution (3 mL of 85% phosphoric acid diluted to 1000 mL with water) and Solvent B was acetonitrile.

The composition of the mobile phase was determined with a linear gradient from 0 to 100%. The measured time was from 0 min to 70 min (0, 20, 25, 65 and 70 min). The composition mobile phase was chosen as follows: Solvent A/Solvent B = 90/10; 70/30; 60/40 and 90/10.

The mobile phase flow rate was 1 mL/min, and the detection was performed at 335 nm. The spectra were recorded in the range of 200–400 nm. The injection volume was 20 µL. All determinations were performed at room temperature.

The peaks were determined by comparing the retention times of the analyzed substances with the retention times of the reference substances.

Quantitative analysis of the extractive solutions from the phytocomplex

The determination of anthocyanosides and proanthocyanosides was made using a spectrophotometric method with a Carry 50 UV-Vis spectrophotometer, equipped with a quartz cuvette (optical path = 1.0 cm). Dilutions (1/10 (*v/v*)) of hydroalcoholic extractive solutions

in 95% ethanol were prepared. The quantitative determination of anthocyanosides and proanthocyanosides was made using the method described in our previous research [36].

Quantitative determination of ascorbic acid

Ascorbic acid was determined using the HPLC method. Considering its antioxidant role, as well as the fact that it is unstable, the samples were analyzed immediately after preparation and also after 60 days.

The HPLC methods used in the literature [56,57] propose determination by reversed-phase liquid chromatography. The determinations were performed with a Varian-ProStar chromatograph with a quaternary pump and a PDA (photo-diode assay) detector. The chromatographic system was previously described in another study [36], together with the accuracy, precision (repeatability and reproducibility) and linearity of the method. The HPLC method was validated and previously published [36].

The sample solutions were obtained by diluting 0.10 mL of antioxidant phytocomplex to 25.0 mL with 0.1% H_3PO_4 in water with addition of 1% CH_3OH.

Accuracy and repeatability were verified by injecting standard solutions in triplicate on the same day, and reproducibility was verified by repeating the determinations for three consecutive days.

The specificity was demonstrated by the chromatogram obtained by analyzing a prepared standard matrix, using the standards of flavones and polyphenolic derivatives of ODP (*ortho*-dihydroxy phenols), which usually appear with ascorbic acid in vegetable hydroalcoholic extractive solutions.

The linearity and concentration range were established by applying the regression analysis of the experimental data obtained by analyzing three sets of standard solutions prepared as described in our previous study [36]. The results are presented in Figures S1 and S2 in the Supplementary Material.

Analysis of the antioxidant action of the phytocomplex

Determination of antioxidant action by chemiluminescence [36]

To determine the antioxidant activity, a Turner Designs TD 20/20 chemiluminometer was used. The chemiluminometer was coupled with a computer equipped with 1.5 mL encapsulated glass cuvettes, a luminol chemiluminescence generating system, and hydrogen peroxide in TRIS-HCl buffer at pH = 8.6, with a 1 mL volume.

All measurements were made in triplicate.

Determination of the antioxidant action of the phytocomplex by electrochemical methods **[36]**

The cyclic voltammetry (CV), linear potential sweep voltammetry (LSV) and square wave voltammetry (SWV) techniques were used. The inert electrode was 0.12 mol L^{-1} H_2SO_4 in an alcoholic solution (methanol). The reagents (methyl alcohol, sulfuric acid, Trolox (6-hydroxy-2,5,7,8 -tetramethyl chroman-2-carboxylic acid) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH)) were of analytical purity (from Sigma). Measurements were made at a temperature of 25 °C under an inert atmosphere, after a de-aeration of the used solutions.

For stock alcoholic solutions, 10^{-3} mol L^{-1} Trolox, freshly prepared and maintained overnight at 4 °C, and 3×10^{-3} mol L^{-1} DPPH were used.

UV-Vis spectrometric determinations were performed using a JASCO V-530 spectrometer.

Determination of the antioxidant action of the phytocomplex using the superoxide dismutase (SOD) method **[36]**

The hydroalcoholic extractive solutions from *Ribes nigri* fructus, *Rubi idaei* fructus, *Fragariae moschatae* fructus and *Rubi fruticosi* fructus, and the phytocomplex solution were tested for antioxidant action. The presence of oxygen in the reaction mixture was ensured by purging air for 20 min. The sample and standard were placed under a 17 W fluorescent lamp for 15 min. A wavelength of 560 nm was used. The quantities used are listed in Table 1.

Table 1. Working technique.

Sample	Dry Residue/g%	SOD Like/(U/mL)
Ribes nigri fructus	17.5	1000
Rubi idaei fructus	2.2	1000
Rubi fruticosi fructus	2.44	120
Fragariae moschatae fructus	3.3	1000
Phytocomplex	5	120
Reagents	**Sample/mL**	**Blank/mL**
Phosphate buffer 0.05 M pH 7.8	2.6 mL	2.7 mL
TMED 0.78 M	0.05 mL	0.05 mL
AM 2×10^{-4} M	0.05 mL	0.05 mL
NBT 3×10^{-3} M	0.2 mL	0.2 mL
Vegetable extract	0.1 mL	-

An SOD unit was expressed as the number of enzymes (unit/mg protein) that reduced 50% of formed formazan [58–60].

3. Results

Qualitative chemical composition of hydroalcoholic extractive solutions

In the hydroalcoholic extractive solutions obtained from *Ribes nigri* fructus, *Rubi idaei* fructus, *Rubi fruticosi* fructus and *Fragariae moschatae* fructus was detected the presence ofanthocyanins, proanthocyanins and tannin, and flavone only in *Ribes nigri* fructus and *Rubi fruticosi* fructus (Table 2).

Table 2. The results of the qualitative chemical analysis.

Extractive Solution	Flavone	Anthocyanins	Proanthocyanins	Tannin
Ribes nigri fructus	+	++	++	++
Rubi fruticosi fructus	+	+	++	+
Rubi idaei fructus	–	++	+	+
Fragariae moschatae fructus	–	+	+	+

"–"—negative reaction; "+"—positive reaction; "++"—intensely positive reaction.

The UV-Vis absorption spectra for the hydroalcoholic extractive solutions of *Ribes nigri* fructus, *Rubi idaei* fructus, *Rubi fruticosi* fructus and *Fragariae moschatae* fructus are shown in Figures S3–S5 in the Supplementary Materials. The obtained UV-Vis absorption spectra for the hydroalcoholic extractive solutions of *Ribes nigri* fructus, *Rubi idaei* fructus, *Rubi fruticosi* fructus and *Fragariae moschatae* fructus demonstrated that compounds with antioxidant action from the flavonoid class, such as naringin and naringenin, were present in all the extractive solutions (Figures S6–S9 in the Supplementary Materials) [61,62].

Quantitative chemical composition of hydroalcoholic extractive solutions

The results of the quantitative chemical determinations are presented in Table 3.

Table 3. The chemical composition of the analyzed hydroalcoholic extracts.

Extractive Solution	Anthocyanins/(mg%)	Proanthocyanins/(LA‰/100 mL)
Ribes nigri fructus	0.1760 ± 0.0031	0.0150 ± 0.0018
Rubi fruticosi fructus	0.1040 ± 0.0028	0.0720 ± 0.0023
Rubi idaei fructus	0.0733 ± 0.0015	0.0092 ± 0.0009
Fragariae moschatae fructus	0.0244 ± 0.0011	0.0100 ± 0.0012

In the UV-Vis spectra of all the hydroalcoholic extracts, compounds with a maximum absorption between 270–290 nm were detected, which corresponds with the UV-Vis spectra of naringenin and naringin compounds; this indicates that this type of derivative is also present in the studied solutions.

It is also well known in the literature that flavanols have two major UV-Vis absorption bands at 250–285 nm and 320–385 nm [63].

Determination of vitamin C content

The calculation of the ascorbic acid concentration in the phytocomplex was performed via the external calibration method, using a regression curve linearity analysis for 10 analyses.

The ascorbic acid in the sample was determined using the retention time and the HPLC spectrum of the eluate (ascorbic acid in aqueous solution presents an absorption band at 243 nm). The obtained content of ascorbic acid was 14.14 ± 1.10 mg/mL.

During the analysis, a low stability of ascorbic acid in the solution was observed. Because of this, the determination of ascorbic acid in the phytocomplex was also performed after 60 days.

The two freshly prepared phytocomplex samples were kept for 2 months in parallel (i) at room temperature and (ii) in the refrigerator (4–8 °C). The ascorbic acid content was determined every two weeks by the HPLC method. During this time, the ascorbic acid content of the antioxidant phytocomplex kept in the refrigerator decreased by about 5.5%. The results are presented in Table 4.

Table 4. Variation of ascorbic acid content in the antioxidant phytocomplex after 60 days.

Sample	Number of Analyses	Ascorbic Acid after 60 Days (at Room Temperature)		RSD % (Relative Standard Deviation)	Ascorbic Acid after 60 Days (In Refrigerator)	
		mg/mL	%		mg/mL	%
Antioxidant phytocomplex	10	13.66	96.60	1.18	13.85	95.47

The variation of the ascorbic acid content of the antioxidant phytocomplex is shown in Figure S10 in the Supplementary Materials.

Evaluation of the antioxidant action of the phytocomplex by chemiluminescence

In Table 5, the values of the chemiluminescent signal and antioxidant activity of the hydroalcoholic solutions are shown.

Table 5. Values of the chemiluminescent signal as a function of time and antioxidant activity in the presence of hydroalcoholic solutions.

Sample	I_p	AA%	v (s^{-1})
Ribes nigri fructus	396.2	79.50	79.2
Rubi fruticosi fructus	1084	51.04	216.8
Rubi idaei fructus	287.7	83.8	57.6
Fragariae moschatae fructus	1272	40.54	254.4
Phytocomplex	2820	68.54	564.0

Ip—chemiluminescent signal intensity in the presence of the studied solutions at t = 5 s; AA%—antioxidant activity; V—chemiluminescent signal speed after the first 5 s.

The variation in the antioxidant activity of the studied solutions is presented in Figure 1.

The antioxidant activity is explained by the presence of active compounds with antioxidant actions (polyphenolic derivatives), including flavones, catechols and ODPs that were present in the extractive solutions from which the mixtures were formed. Additionally, this category of active compounds included the proanthocyanosides and anthocyanosides identified in the extractive solutions [64–66].

Determination of the antioxidant capacity of the phytocomplex by the electrochemical method

In Figures 2–6, the behaviors of the hydroalcoholic extracts and phytocomplex with concern to the long-lived radical DPPH are shown at the optimal times established for the measurements.

As shown in Figures 2–6, the presence of some antioxidant properties was observed in all investigated samples. Considering that all extracts contained polyphenolic derivatives, including flavones, catechols, ODPs, proanthocyanosides and anthocyanosides, the antioxidant effect was demonstrated [26].

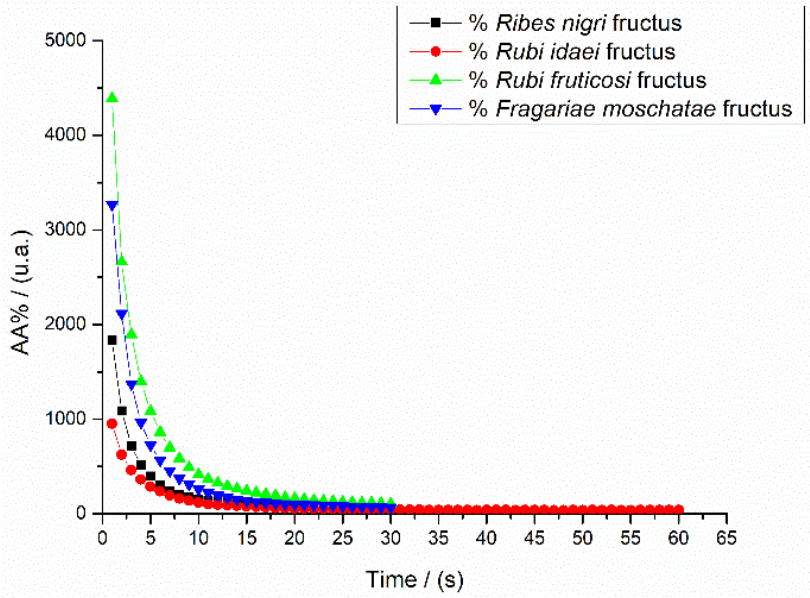

Figure 1. Variation of antioxidant activity values in the studied solutions as a function of time.

Using this method, the total antioxidant capacity of the extracts was determined, and no fractional separation of the components from the mixture was performed.

The peak current intensities corresponding to each sample were determined, and the differences at 0 and 2 min from the peak current intensity belonging to DPPH in the absence of any antioxidant were also determined. The obtained values are shown in Table 6.

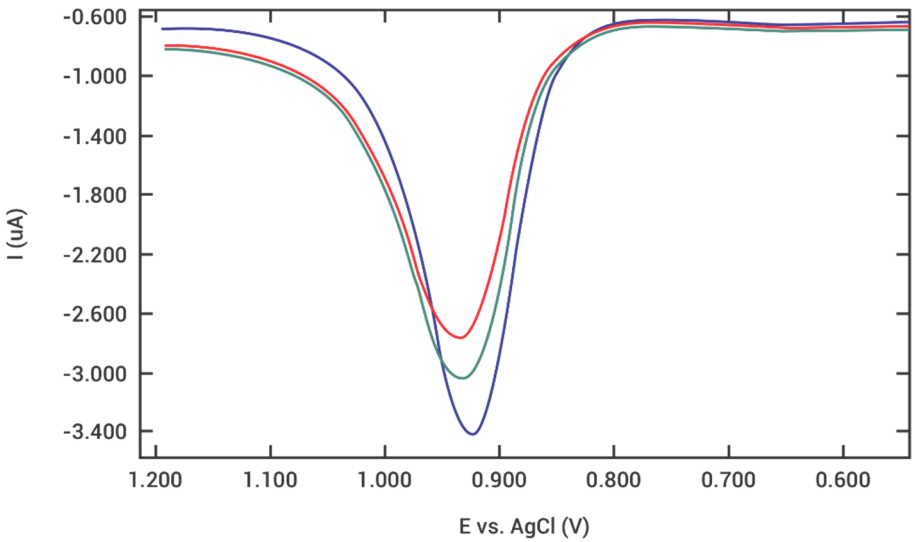

Figure 2. Overlapping voltammograms corresponding to DPPH and the addition of 10 µL of the hydroalcoholic solution of *Ribes nigri* fructus at 0 and 2 min, respectively.

The antioxidant capacity of the samples was determined using Equation (1) as follows:

$$AC_{\text{sample}} = V_{\text{sample}} \times K \times \frac{I_{\text{sample}} - I_{\text{blank}}}{I_{\text{Trolox}} - I_{\text{blank}}} \quad (1)$$

where AC_{sample} is the antioxidant capacity of the sample, V is the sample volume, K is the sample dilution factor, I_{sample} is the intensity of the anodic peak current of DPPH in the presence of the sample, I_{blank} is the intensity of the peak current of DPPH in the absence of any antioxidant and I_{Trolox} is the peak current intensity for DPPH in the presence of Trolox. The obtained values are presented in Figure 7.

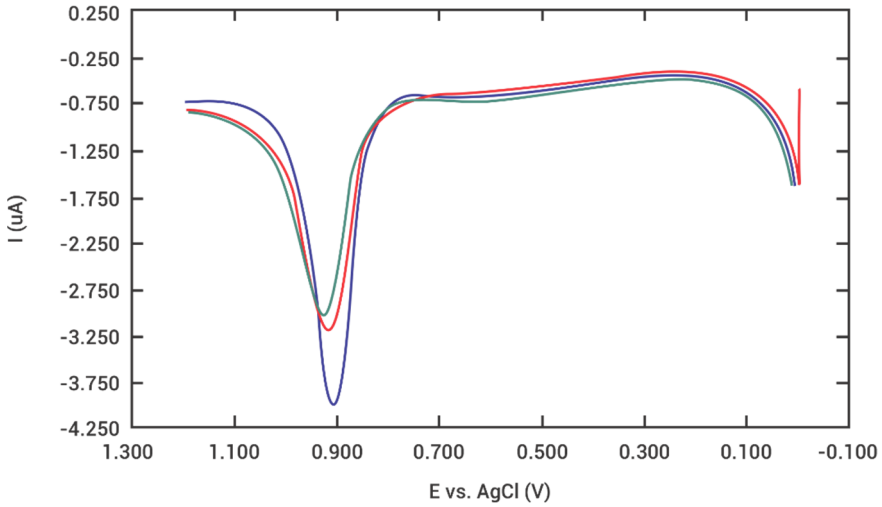

Figure 3. Overlapping voltammograms corresponding to DPPH and the addition of 10 µL of the hydroalcoholic solution of *Rubi fruticosi* fructus at 0 and 2 min, respectively.

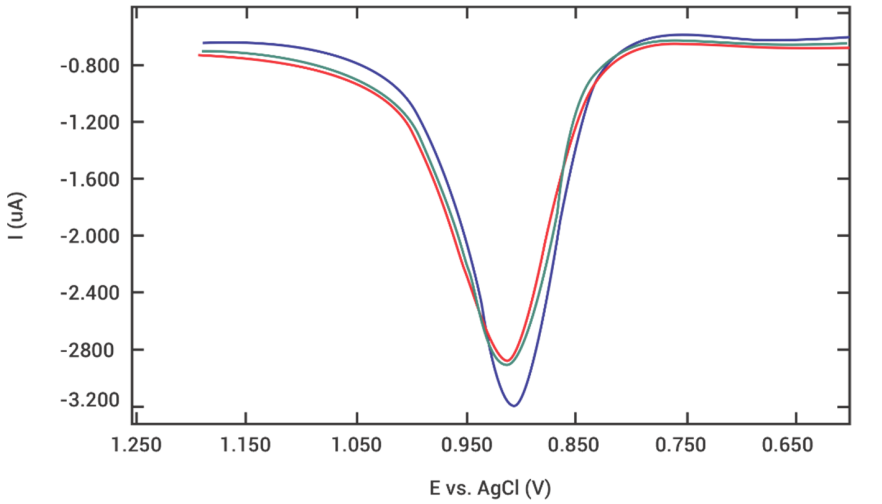

Figure 4. Overlapping voltammograms corresponding to DPPH and the addition of 10 µL of *Rubi idaei* fructus hydroalcoholic solution at 0 and 2 min, respectively.

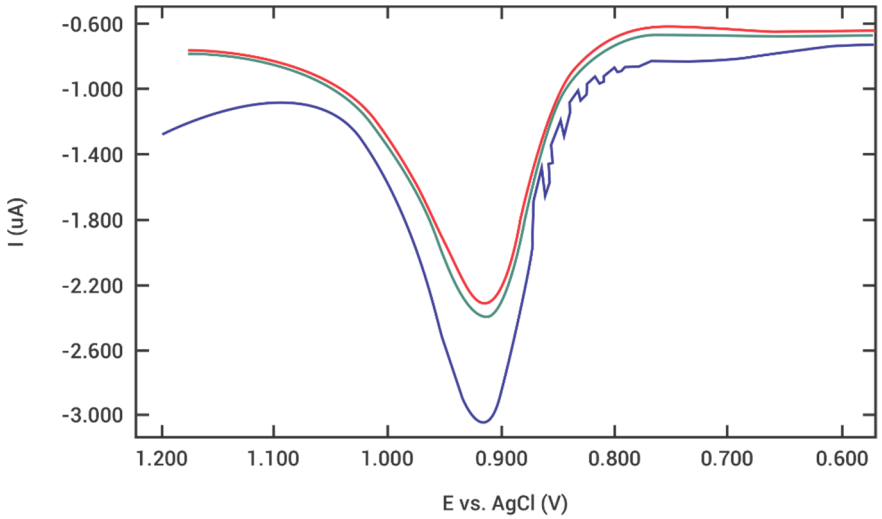

Figure 5. Overlapping voltammograms corresponding to DPPH and the addition of 10 µL of the hydroalcoholic solution of *Fragariae moschatae* fructus at 0 and 2 min, respectively.

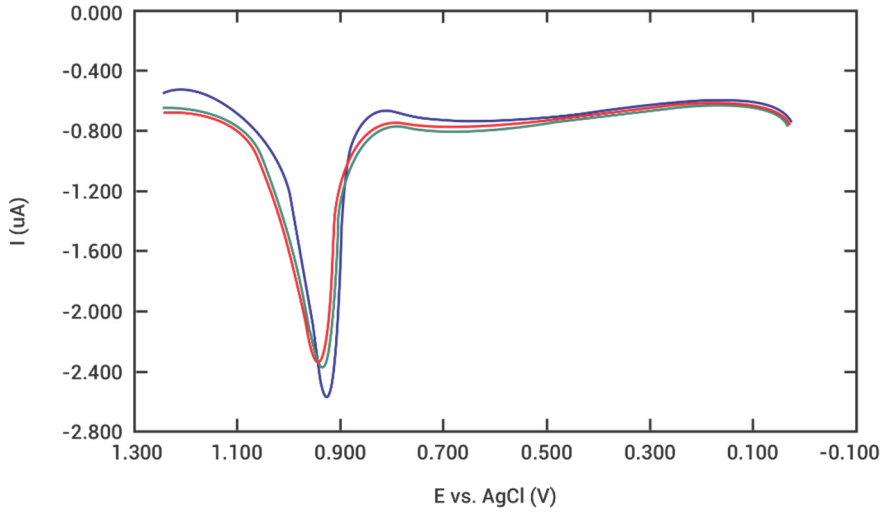

Figure 6. Overlapping voltammograms corresponding to DPPH and the addition of 10 µL of the hydroalcoholic solution of the phytocomplex at 0 and 2 min, respectively.

Table 6. Values of anodic peak currents of DPPH in the presence of vegetable hydroalcoholic solutions and the phytocomplex.

Sample	I_{DPPH} (mA)	Time (min)	Intensity
Ribes nigri fructus	3.444 3.208	t = 0 t = 2	0.628 0.864
Rubi fruticosi fructus	2.261 2.24	t = 0 t = 2	0.317 0.338
Rubi idaei fructus	3.445 3.205	t = 0 t = 2	0.620 0.856
Fragariae moschatae fructus	2.801 2.743	t = 0 t = 2	0.096 0.154
Phytocomplex	2.338 2.064	t = 0 t = 2	0.437 0.711

The obtained data were verified by using UV-Vis spectrometry to determine the observable changes in the absorption maxima of DPPH at 519 nm. However, the spectrometric determinations for *Ribes nigri* fructus, *Rubi idaei* fructus, *Rubi fruticosi* fructus, *Fragariae moschatae* fructus and the phytocomplex were inconclusive since the extracts had absorption maxima in the 500–520 nm region, which made it difficult to observe possible changes in the maximum of DPPH.

The electrochemical method has the advantage of avoiding the interference encountered in the spectrometric method; therefore, it is widely applicable.

Determination of the antioxidant capacity of the phytocomplex by the superoxide dismutase (SOD) method

From the analysis of the data presented in Table 7, it was observed that all the solutions used at 1/10 dilutions showed antioxidant activity that inhibited the amount of enzyme by more than 50%. Among the studied solutions, the most accentuated antioxidant action was found in the extract solution from *Fragariae moschatae* fructus (100%). The antioxidant phytocomplex inhibited the activity of the enzyme by 81%.

The antioxidant activity of the phytocomplex was due to the presence of polyphenolic derivatives, represented by flavones, catechols and ODPs.

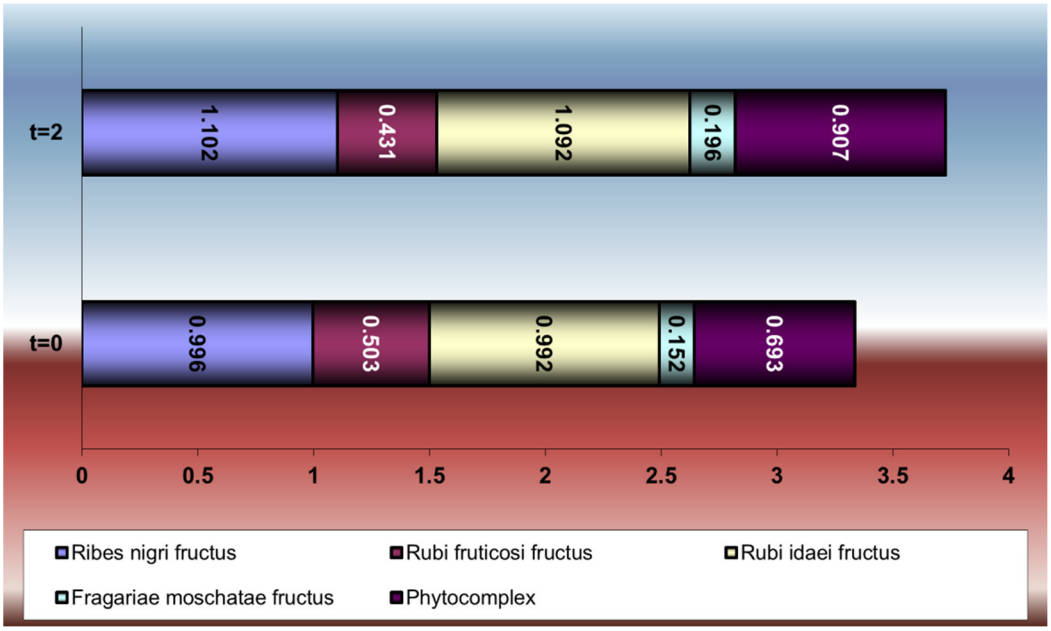

Figure 7. Graphical representation of the antioxidant capacity of the hydroalcoholic solutions of *Ribes nigri* fructus, *Rubi fruticosi* fructus, *Rubi idaei* fructus, *Fragariae moschatae* fructus and the phytocomplex.

Table 7. Percentage inhibitions (%) of lipid peroxidation.

Sample	Dilutions	Dilutions	Dilutions
	1/10	1/100	1/1000
Ribes nigri fructus	75	49	37
Rubi fruticosi fructus	91	48	37
Rubi idaei fructus	51	37	37
Fragariae moschatae fructus	100	49	43
Antioxidant phytocomplex	81	43	11

4. Discussion

All the hydroalcoholic extractive solutions from *Ribes nigri* fructus, *Rubi idaei* fructus, *Rubi fruticosi* fructus and *Fragariae moschatae* fructus contained anthocyanins, proanthocyanins and tannins. Flavones were identified only in *Ribes nigri* fructus and *Rubi fruticosi* fructus. In the final mixture, obtained by mixing equal proportions of the extractive solutions of *Ribes nigri* fructus, *Rubi idaei* fructus, *Rubi fruticosi* fructus and *Fragariae moschatae* fructus, the same chemical constituents were identified. The obtained results agree with the data reported in the literature [42,56,67–70]. The variation of the total anthocyanin and proanthocyanin content is due to environmental factors.

Several authors have reported a total polyphenol amount of 267.5 mg/100 g [67], 720.0 mg/100 g [56] and 799.0 mg/100 g [70] for *Ribes nigri*; 320.0 mg/100 g [67], 260.0 mg/100 g [56] and 264.0 mg/100 g [70] for *Rubi idaei*; and 507.5 mg/100 g [67], 320.0 mg/100 g [56] and 460.0 mg/100 g [70] for *Rubi fruticose*. The total anthocyanins reported by the same authors are as follows: 235.0 mg/100 g [67], 260.0 mg/100 g [56] and 229.0 mg/100 g [70] for *Ribes nigri*; 37.5 mg/100 g [67], 40.0 mg/100 g [56] and 141.0 mg/100 g [70] for *Rubi idaei*; and 115.0 mg/100 g [67], 80.0 mg/100 g [56] and 141.0 mg/100 g [70] for *Rubi fruticose*. The ascorbic acid content was between 6.0 and 20.4 mg/100 g for *Rubi fruticose*, 18.3 and 30.17 mg/100 g for *Rubi idaei*, and 44.5 and 167.8 mg/100 g for *Ribes nigri* [56,67,70]. Light, temperature, soil conditions, rainfall and agronomic practices are factors that may contribute to the differences observed in total polyphenol, anthocyanin and ascorbic acid contents between various studies.

From a chemical quality point of view, the phytocomplex had a content of 0.105 mg of anthocyanosides/100 mL solution and proanthocyanins of 0.041 LA ‰/100 mL solution. In the literature, a strong correlation between phenol and anthocyanin content and antioxidant activity has been observed [71]. Our results confirmed that antioxidant activity is related to the total anthocyanins. Wu et al. determined the total content of anthocyanins in redcurrant compared to blackcurrant. The authors concluded that the blackcurrant, which had a higher concentration of anthocyanins, possessed the highest antioxidant activity [72].

The same range of chemical constituents was identified in the final preparation product, obtained by mixing the extractive solutions of *Ribes nigri* fructus, *Rubi idaei* fructus, *Rubi fruticosi* fructus and *Fragariae moschatae* fructus in equal proportions. Based on the spectrophotometric results of studies performed on the hydroalcoholic extractive solutions from the extracts of Romanian spontaneous flora berries, it can be concluded that they contain bioactive compounds with antioxidant action. This is confirmed by the presence of active molecules with antioxidant properties (polyphenolic derivatives), including flavones, anthocyanins, proanthocyanins and tannin.

The results also confirmed the presence of ascorbic acid after 60 days (at room temperature) in the antioxidant phytocomplex. Ascorbic acid also acts as an antioxidant compound by preventing free radicals from inducing DNA damage [73].

In the present study, the obtained values of antioxidant activity (AA%) proved that the hydroalcoholic extractive solution of *Rubi idaei* fructus had the most pronounced antioxidant activity (AA% = 83.80), followed by the hydroalcoholic extractive solution of *Ribes nigri* fructus (AA% = 79.50), *Rubi fructicosi* fructus (AA% = 51.04) and that of *Fragariae moschatae* fructus (AA% = 40.54). The phytocomplex had a lower antioxidant activity (AA% = 68.54) compared with that of the hydroalcoholic extractive solutions of *Rubi idaei* fructus and *Ribes nigri* fructus, and a more pronounced activity compared to that of the extractive solutions of *Rubi fructicosi* fructus and *Fragariae moschatae* fructus.

Angela and Meireles reported a high antioxidant potential for ethanol samples due to the effect of ethanol in increasing the extraction of bioactive compounds, mostly anthocyanins [74]. The same results were obtained by Peschel et al. with ethanol extract of blackcurrant residue, which had a higher antioxidant potential (20.08%) compared to aqueous extract residue (9.95%) [75].

Our results showed that the berry fruits (dark or red colored fruits) are an abundant source of active substances, and their mixed product showed a significant antioxidant potential due to the synergetic effect of the individual compounds. Subsequently, important steps to improve the final antioxidant properties include the extraction method, processing and storage of the extracts.

5. Conclusions

Based on the studies performed on the hydroalcoholic extractive solutions from *Ribes nigri* fructus, *Rubi idaei* fructus, *Rubi fruticosi* fructus and *Fragariae moschatae* fructus using specific analytical methods, it can be concluded that bioactive compounds with antioxidant action, such as anthocyanins, proanthocyanins, tannins and vitamin C, are present in their composition. By the determination of ascorbic acid dosage using the HPLC method, it was found that the antioxidant phytocomplex contained 14.14 mg/mL of ascorbic acid.

The methods used to determine the antioxidant activity of the prepared phytocomplex indicated a significant antioxidant activity of both the extractive plant solutions that were part of the phytocomplex and the mixture obtained in the form of a phytocomplex-type nutritional supplement. The higher antioxidant activity of the phytocomplex is due to the presence of several compounds, such as anthocyanins, proanthocyanins, tannins and flavones, with antioxidant actions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10040646/s1, Figure S1. Calibration curve for ascorbic acid dosage in the antioxidant phytocomplex; Figure S2. Response factor to ascorbic acid determinations; Figure S3. Chromatogram of the hydroalcoholic extractive solution from Ribes nigri fructus; Figure S4. Chromatogram of the hydroalcoholic extractive solution from Rubi fruticosi fructus; Figure S5. Chromatogram of the hydroalcoholic extractive solution from Fragariae moschatae fructus; Figure S6. UV-Vis spectrum of the hydroalcoholic extractive solution from Ribes nigri fructus; Figure S7. UV-Vis spectrum of the hydroalcoholic extractive solution from Rubi fruticosi fructus; Figure S8. UV-Vis spectrum of the hydroalcoholic extractive solution from Rubi idaei fructus; Figure S9. UV-Vis spectrum of the hydroalcoholic extractive solution from Fragariae moschatae fructus; Figure S10. Chromatogram with time variation of ascorbic acid concentration in the antioxidant phytocomplex.

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