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Phenolics content, *in vitro* antioxidant and anticholinesterase activities of combined berry extracts

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Abstract

Plant extracts are often incorporated into food products, mainly due to their protective effects such as antioxidant or antimicrobial. Usually, individual extracts were used for such purposes.

The aim of the present study was to investigate different combinations of bilberries-blackcurrants extracts as synergistic formulations in terms of phenolic compounds content, antioxidant and anticholinesterase activities.

Different behaviours such as additive, synergistic and antagonistic effects were noticed in combined extracts. With respect to antioxidant activity as measured by phenolics and FRAP assays, synergistic effects were observed in all tested combinations. The in vitro screening for acetylcholinesterase inhibition indicated inhibitory activity of combined extracts with different efficacies. The most promising formulation intended for antioxidant potential and pharmaceutical interest as well, was found to be 33% bilberry and 67% blackcurrant: 100 g of this freeze-dried formulation contains 2792 mg phenolics (1985 mg flavonoids, 1309 mg anthocyanins) and 3072 mg ascorbic acid equivalents (total antioxidant activity by FRAP). It can be considered a valuable starting point for the development of nutritional supplements or ingredients for food industry.

Keywords: bilberry, blackcurrant, antioxidant, anticholinesterase, synergistic effect

1. Introduction

Fresh high-anthocyanins fruits are excellent sources of phytochemicals with strong antioxidant power. Well-documented human health benefits of such fruits and their products are often related to the cooperative and synergistic effects of the large range of chemical structures highly correlated with each other (LIU [1]).

Bilberry (*Vaccinium myrtillus* L., family Ericaceae) and blackcurrant (*Ribes nigrum* L., family Grossulariaceae) are important wild fruit crops found in significant amounts in the spontaneous flora of Northern and Eastern European countries. Despite that they are mostly known for culinary properties, they found likewise numerous applications as phytotherapeuticals mainly in the form of extracts, and dietary supplements.

The key antioxidant compounds in bilberries and blackcurrants are of polyphenolic structure (phenolic acids, flavonoids, anthocyanins, tannins). Anthocyanins are hydrosoluble pigments located in plant cell vacuoles in the form of glycosylated derivatives of anthocyanidins, in particular cyanidin, pelargonidin, delphinidin, peonidin and malvidin. These compounds retain health-promoting properties principally based on their strong antioxidant capacity revealing anti-inflammatory, antiulcer, antiaging, antimicrobial, antimutagenic and neuroprotective effects (HE & al. [2], PAREDES-LÓPEZ & al. [3]).

The antioxidant activity of plant compounds of phenolic structure is attributed to the glycosylation pattern and to the number and position of substituted hydroxyl and methoxyl groups (MONTORO & al. [4]). Scientific literature describes various mechanism by which polyphenols behave as antioxidants, such as direct action through interaction with reactive oxygen species and free radicals, chelation of metal ions (Fenton chemistry), inhibition of oxidant enzymes, activation of endogenous antioxidant enzymes, and prevention of cellular lipids oxidation (GOSZCZ & al. [5]). Arising theories from recent research show that polyphenols molecular mechanism may be linked to the modulation of expression of genes connected to the *in vivo* inflammation pathways (RAY & al. [6]).

In spite of the large number of studies describing the phenolic profile and pharmacological properties of various fruit extracts, fewer investigations on the synergistic, antagonistic or additive interactions of combined extracts have been performed. Using a model system of different combinations of two phenolics (chlorogenic acid combined with others) or three phenolics (chlorogenic acid, cyanidin 3-rutinoside and quercetin 3-rutinoside) at 100 mg/L, some authors observed rather additive effects of antioxidant activity by ABTS than synergistic ones (HEO & al. [7]). Therefore, it can be concluded that the entire complex composition of a plant in particular polymerized phenolics might be more significant than individual or monomeric compounds.

In the present work, we prepared extracts from combinations of two wild berry fruits, which are known for their higher bioactive content than that of cultivated crops – bilberry (*Vaccinium myrtillus* L.) and blackcurrant (*Ribes nigrum* L.). Anthocyanin-rich extracts from mixed fruit powders were prepared from freeze-dried samples using an efficient short ultrasonic pre-treatment previously reported by us (OANCEA & al. [10]). The objective was to investigate the total content of important biochemical constituents (anthocyanins, phenolics, flavonoids, condensed tannins) and *in vitro* pharmacological effects (antioxidant, anticholinesterase) of individual *vs.* combined extracts. The total antioxidant activity was performed using three different test systems (total phenolics, FRAP and DPPH).

Although neuroprotective properties of several berry extracts has been reported (SUBASH & al. [8]), there is still a reduced number of such studies. The drugs used in the treatment of neurodegenerative diseases act as acetylcholinesterase inhibitors (ČOLOVIĆ & al. [9]). Considering this, our paper was brought about to explore the potential of bilberries, blackcurrants and their combination extracts for the *in vitro* inhibition of acetylcholinesterase.

2. Materials and methods

2.1. Plant material and reagents. Commercial fresh samples of wild fruits of bilberry (*Vaccinium myrtillus* L.) and blackcurrant (*Ribes nigrum* L.) were purchased from the local market of Sibiu, Romania. Samples were freeze-dried using a lyophilizer (Alpha 1 – 4 LD plus, Christ). The freeze-dried fruits were powdered using the knife mill (Grindomix GM 200, Retsch).

The powders of the two samples plus three combinations of them were further used for the chemical and biological analyses. Their moisture content was determined at 105 ºC using the moisture analyser (MAC 210/NP Radwag, Poland).

Chemical reagents of analytical grade without further purification were used.

2.2. Extraction procedures. Extraction of bioactive compounds (anthocyanins, phenolics, flavonoids, condensed tannins) from lyophilized fruits was conducted in 70% (v/v) ethanol in water. The experimental design for the extractive technology is shown in Table 1 based on the results of our previous work (OANCEA & al. [10]). The obtained mixtures were centrifuged at 8000 rpm for 10 minutes at 4 ºC. The refrigerated centrifuge (Hettich Universal 320R, Germany) was used.

Table 1. Experimental data for ultrasonic pre-treatment extraction of bioactives from the selected samples (total extraction time = 60 min)

Sample	bilberry/blackcurrant *ratio*	Solvent/solid *ratio*	Ultrasonic extraction		Conventional extraction
			time (min)	amplitude (%)	time at 4 ºC (min)
1	1/0	15	10	70	50
2	0/1	15	10	70	50
3	1/1	15	10	70	50
4	2/1	15	10	70	50
5	1/2	15	10	70	50

2.3. Total anthocyanins. The content of total anthocyanins in the crude extracts was determined spectrophotometrically by the pH differential method (SCHWARTZ [11]). The Specord 200Plus UV–Vis spectrophotometer (Analytik Jena, Germany) was used. The results were expressed as milligram cyanidin-3-O-glucoside (Cyn–3–O–G) equivalents per 100 g dry mass (mg $100g^{-1}$ DM).

2.4. Total flavonoids. The content of total flavonoids was determined spectrophotometrically using the aluminium chloride method (KUMAR & al. [12]). Quercetin was used as standard for the calibration curve. The results were expressed as milligram quercetin equivalents per 100 g dry mass (mg $100g^{-1}$ DM).

2.5. Total condensed tannins. The content of total tannins was determined spectrophotometrically using the vanillin assay (PRICE & al. [13]). Catechin was used as standard for the calibration curve. The results were expressed as milligram catechin equivalents per 100 g dry mass (mg $100g^{-1}$ DM).

2.6. Total antioxidant activity assays

2.6.1. Determination of total phenolics. The content of total phenolics was determined spectrophotometrically according to the Folin-Ciocalteu method (SINGLETON & al. [14]). The Specord 200Plus UV–Vis spectrophotometer (Analytik Jena, Germany) was used. Gallic acid was used as standard for the calibration curve. The results were expressed in milligram of gallic acid equivalents per 100 g dry mass (mg GAE $100g^{-1}$ DM).

2.6.2. Antioxidant assay using Ferric Reducing Antioxidant Power (FRAP). The total antioxidant activity of crude extracts was determined by the ferric reducing ability assay described by BENZIE & al. [15]. The results were expressed as milligram ascorbic acid per 100 g dry mass (mg $100g^{-1}$ DM).

2.6.3. Radical scavenging activity (RSA) using 1,1-Diphenyl-2-picryl-hydrazyl (DPPH). The RSA activity of crude extracts was determined by DPPH assay described by BRAND-WILLIAMS & al. [16]. The results were expressed as inhibition percentage calculated according to formula: RSA (%) = 100 x $(A_{control}-A_{sample})/A_{control}$

2.7. Acetylcholinesterase activity. The enzymatic activity was measured using purified extracts according to the Ellman's spectrophotometric method described by ATTA-UR-RAHMAN & al. [17]. Initially, the concentrated crude ethanol extracts were purified using solid-phase extraction SPE according to current protocol for anthocyanins (SCHWARTZ [11]). Sep-Pak C_{18} cartridges with 2 g sorbent (Waters Chromatography) were used. Acetylcholinesterase (AChE) from *Electrophorus electricus* Type VI-S 217 units/mg solid (Sigma) was used. The results were expressed as inhibition percentage calculated according to formula: AChE inhibition (%) = 100 x $(A_{control}-A_{sample})/A_{control}$

2.8. Statistical analysis. Data presented are the average of two replicates, expressed as mean ± standard deviation. Data processing was performed using mathematical and statistical methods with "IBM SPSS 21.0" software, following hypothesis testing. The correlation between variables was done by calculating the Pearson's correlation coefficient (r), at the significance level of risk $\alpha \leq 5\%$ and probability $P \geq 95\%$.

3. Results and discussion

Phytochemicals present in vegetables, whole grain diets, fruit, spices and various herbal teas was shown harbor many effects on health (NICU A& al [18, 19], GINGHINA& al [20]). Wildly grown berry fruits are highly appreciated by consumers for their beneficial effects, gaining a superfruit status and an increased interest for the market based on the large number of scientific studies behind them.

Freeze-dried bilberries (*Vaccinium myrtillus* L.), blackcurrants (*Ribes nigrum* L.) and their particular combinations were subjected to efficient extraction for phytochemical screening, antioxidant and anticholinesterase activities of five resulted crude or purified extracts. The anthocyanin-rich phenolic extracts were prepared using a combined ultrasounds-assisted/conventional extraction in hydroethanolic solution at low temperature in order to preserve most bioactive compounds. Optimized extraction and solvent/sample ratio favour an increased yield, and a high therapeutic effect of the final extract.

3.1. Comparative studies of antioxidant compounds content of individual and combined crude extracts

The concentration of the main phytochemicals with antioxidant properties, extracted from selected berry fruits – individual or combined, with the aid of the environmentally friendly aqueous ethanol, was calculated on the dry mass basis for homogeneity of the results to be compared. The following phytochemicals were quantified: anthocyanins, phenolics, flavonoids and tannins.

The obtained results regarding the concentration of phytochemicals in crude extracts are shown in Figure 1. The significance of the samples 1 – 5 is indicated in Table 1.

A high positive correlation between the variables and the average content of investigated bioactive compounds was found, with the Pearson correlation coefficient of $0.949 < r < 0.998$ depending on the sample. The highest r values were found for sample 2 and 5.

According to statistical analysis, the total phenolics content of sample 5 (extract from combined bilberries and blackcurrants in 1/2 *ratio*) was significantly higher than that of sample 1 (100% bilberry extract) and higher than sample 2 (100% blackcurrant extract) but the latter not statistically significant.

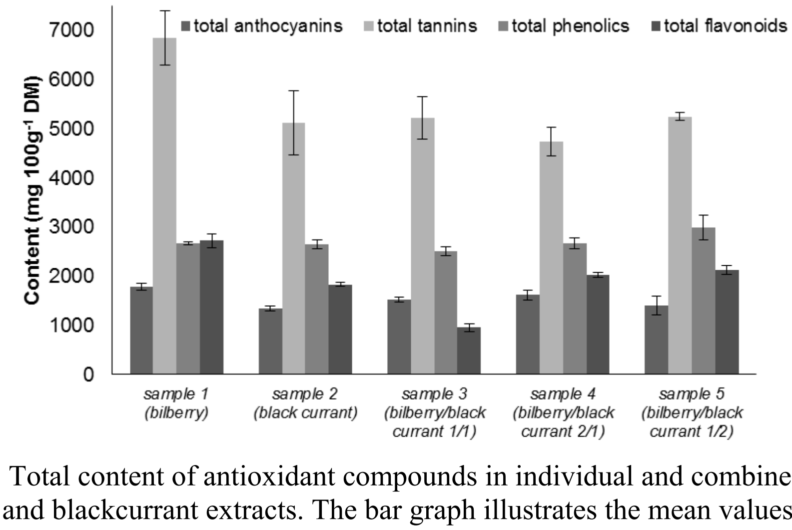

Figure 1. Total content of antioxidant compounds in individual and combined bilberry and blackcurrant extracts. The bar graph illustrates the mean values with error bars indicating ± SD of two independent extractions.

Among phenolic compounds, anthocyanins represent the main antioxidant compounds in wild berries conferring them valuable properties for human health. *Vaccinium myrtillus* L. wild fruits, in particular from Romanian regions, contain anthocyanins mainly in the form of glucosides, galactosides and arabinosides of cyanidin, delphinidin, malvidin, petunidin and peonidin (OANCEA & al. [21]). The anthocyanins in *Ribes nigrum* L. fruits consist mainly in glucosides and rutinosides of delphinidin and cyanidin (RUBINSKIENE & al. [22]).

According to statistical analysis, no significant difference was found between the content of total anthocyanins in individual and combined extracts.

In our investigation, bilberries showed significantly higher content of flavonoids than blackcurrants. Combination samples 4 and 5 showed significantly higher content of flavonoids than combination sample 3 and sample 2 (100% blackcurrant extract). All combinations showed significantly lower flavonoids content than bilberries.

The *ratio* of flavonoids to phenolics showed the highest value for bilberries (1.00) compared to blackcurrants (0.69), explained by the increased anthocyanins content in *Vaccinium myrtillus* L. fruits. This extract also showed the highest antioxidant activity by FRAP assay. Similarly, larger amounts of total phenolics and flavonoids were found in bilberries by other authors, with the flavonoids to phenolics *ratio* of 0.95 in 70% ethanolic extracts (POP & al. [23]).

Regarding the condensed tannins level, interestingly our results indicate that their content decreased in combinations compared to extracts from individual fruits, suggesting an antagonistic interaction. According to our results, fruits of bilberry contain larger amounts of tannins (6.8%), which is in accordance with other investigations which reported even higher amounts of catechin-type tannins (12%) (ROSLON & al. [24]).

Among the tested combinations, it seems that 33% bilberry / 67% blackcurrant blend has been the synergistic formula. This finding confirms that synergistic effects depend on the type of antioxidant compounds and their extent in the mixture.

Except tannins, the other bioactive compounds seem to develop a synergistic effect (phenolics, flavonoids) or an additive effect (anthocyanins) in specific combined extracts. Possible explanation for these effects could be the synergistic interactions between phenolic-based compounds and other chemical compounds in fruits samples, *e.g.* ascorbic acid and minerals are usually found in large amounts in blackcurrants. Change of the chemical profile of combinations, for example through esterification of anthocyanidin phenolic rings, polymerization, might also occur. It has been considered that pharmacological effects of plant extracts may modify in terms of additive or synergistic enhancement due to endo- and exo-interactions between phytochemicals, in particular phenolic compounds (LILA & al. [25]). Most of the published results come from experiments on individual antioxidant compounds and mixtures of particular chemical compounds. Such studies showed that some antioxidants when being in combination act in a regenerating way - either the stronger or the weaker antioxidant regenerating the other (PEYRAT-MAILLARD & al. [26]). This interaction leads to a synergistic effect if the weaker antioxidant regenerates the stronger one, or an antagonistic effect if the reverse is appearing. In addition to this explanation, other studies indicate as possible mechanism the reaction rates of the antioxidants, the polarity of the molecules in the combination or the real concentration of the antioxidants at the oxidation site (FRANKEL & al. [27], KOGA & al. [28], CUVELIER & al. [29]).

3.2. The *in vitro* antioxidant activity of individual and combined crude extracts

The total antioxidant activity of individual and combined crude extracts of bilberry and blackcurrant fruits was determined by Folin-Ciocalteu (total phenolics), ferric-reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assays. The obtained values were expressed on percentage and dry mass (DM) basis. The results on total phenolics are shown in Figure 1, while the results on FRAP and DPPH assays are presented in Figure 2.

As already shown, the total antioxidant activity in terms of total phenolics as measured by Folin-Ciocalteu assay, of sample 5 (extract from combined bilberries and blackcurrants) was significantly higher than that of sample 1 (100% bilberry extract), as resulted from statistical analysis.

The total antioxidant activity of bilberry extract is higher than of blackcurrant as measured by FRAP, while opposite occurs when determined by DPPH assay, but of no statistically significant difference. Regarding the total antioxidant activity as measured by FRAP and DPPH assays, no statistically significant difference was found between individual and combined crude extracts.

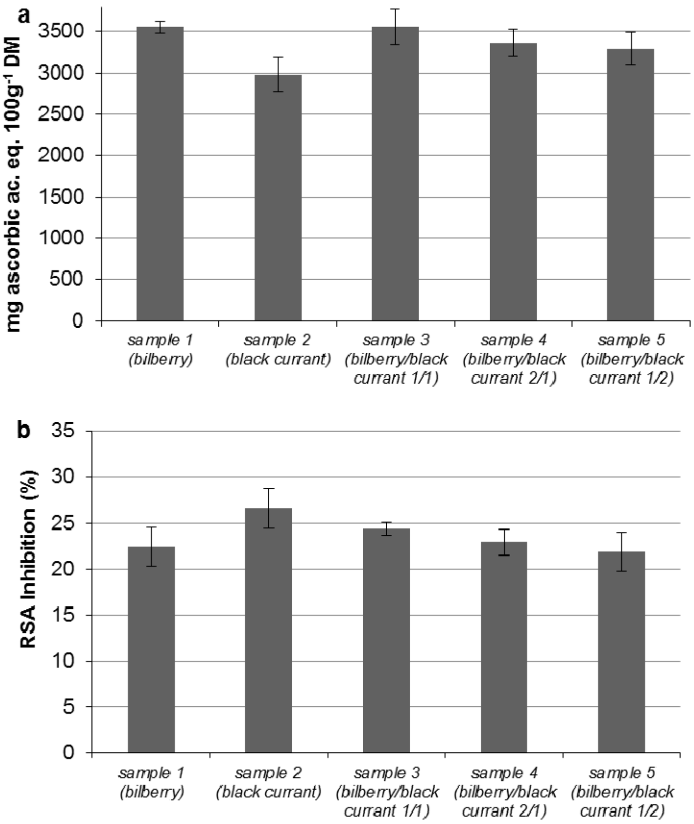

Figure 2. Total antioxidant activity of individual and combined bilberry and blackcurrant extracts, as measured by FRAP assay (a) and radical scavenging (RSA) by DPPH assay (b).The bar graph illustrates the mean values with error bars indicating ± SD of two independent extractions.

In order to compare such results, the synergistic effect regarding antioxidant activity of individual *vs*. combined extracts was calculated from the *ratio* of experimental value (as practically determined for combined samples) and theoretical value (calculated proportionally from the individually observed values for each of the three combinations). The *ratio* value > 1 indicates a synergistic effect, = 1 an additive effect, and < 1 an indifferent/antagonistic effect (FUHRMAN & al. [30]). All of the three prepared combinations showed a synergistic effect regarding the total antioxidant activity as measured by FRAP (the most effective one being sample 3), while two combinations (samples 3 and 4) work synergistically as determined by DPPH radical scavenging activity. Sample 5 was considered to determine an indifferent effect by DPPH as the obtained value was 0.92 very close to 1.

The results of statistical correlation by regression analysis indicate a good positive correlation between FRAP and total anthocyanins (r = 0.791, $p < 0.01$) in accordance to other studies which reported that anthocyanins are among the most effective predictors for the antioxidant activity as measured by FRAP (KAULMANN & al. [31]).

These results may be considered of practical importance, as particular combinations of bilberries and blackcurrants may be used as dietary supplement delivering a significant amount of antioxidant activity or as natural additive for oxidative stabilization of highly susceptible foods. 100 g of sample 5 (combined bilberries and blackcurrants) contains 2792 mg phenolics (1985 mg flavonoids, 1309 mg anthocyanins) and 3072 mg ascorbic acid equivalents (total antioxidant activity by FRAP).

3.3. The *in vitro* screening for acetylcholinesterase inhibition of individual and combined purified extracts

The cholinergic hypothesis suggests that the deficit of the neurotransmitter acetylcholine in the brain is linked to different brain pathologies. Because synthetic acetylcholinesterase (AChE) inhibitors as drugs used clinically for several neurological disorders including Alzheimer's disease may show several side effects (CHOPRA & al. [32]), new safe alternatives are required, including screening of natural products. Considering this approach and the already reported efficient anticholinesterase activity of several pure anthocyanidins (pelargonidin, delphinidin, cyanidin) (SZWAJGIER [33]), we aimed in the present work to test the AChE activity of the prepared extracts.

The five purified extracts were subjected to the *in vitro* screening for inhibition of electric eel AChE. Such anthocyanin-rich extracts were recovered from the crude extracts through purification on C_{18} cartridges by solid-phase extraction (SPE). Elution was conducted such as to remove only additional interfering compounds, such as acids and sugars, and keep the major phenolic-type compounds. The results are presented in Table 2.

Table 2. Anticholinesterase activity of the purified individual and combined bilberry and blackcurrant extracts (% of inhibition)

Sample	Inhibition of AChE (%)
1	23.4 ± 7.7
2	11.3 ± 4.9
3	6.1 ± 0.71
4	4.2 ± 1.4
5	10.4 ± 3.5

With the exception of sample 1 (100% bilberry extract) which gave moderate AChE inhibition, the other investigated extracts exhibited low inhibitory activity (below 20%) at the concentration of 1 mg ml^{-1} in PBS (each hydroethanolic extract of 10 mg ml^{-1} was diluted in PBS buffer such as to give 1 mg ml^{-1}). The difference between inhibition values of individual and combined extracts was not statistically significant. Among the three purified extracts containing combinations of bilberries and blackcurrants, sample 5 showed the most effective inhibition of AChE (similar to 100% blackcurrants) compared to other combinations. Regarding the AChE inhibition of bilberry extract, other authors reported similar moderate *in vitro* activity (<30%) of an extract from wild blueberries (fresh frozen from Wyman's) but a significantly decreased brain AChE activity as measured by *ex vivo* AChE assay (PAPANDREOU & al. [34]) suggesting the neuroprotective high potential of blueberry polyphenols.

A strong positive correlation was found between anticholinesterase activity and total condensed tannins (r = 0.853, p < 0.01) and a weak positive correlation between anticholinesterase activity and total flavonoids (r = 0.558, p < 0.10). There are studies demonstrating that dietary supplementation of mice with green tea known for its high content in tannins greatly inhibited the AChE activity (KIM & al. [35]), which indicate a possible link between tannins and inhibitory activity. Similar to other authors (KULIŠIĆ-BILUŠIĆ & al. [36]) we report that anticholinesterase activity does not correlate with the total phenolics content, despite that most reported studies on inhibitors of AChE correlated this inhibition potential of plant extracts to phenolics (ORHAN & al. [37], HLILA & al. [38], ZENGIN & al. [39]). This might be due to some phenolic acids removal through purification steps, compounds that might possess AChE inhibitory activity. The particular chemical composition of a sample, the extractive procedure for bioactives and the applied analytical methods for determination of bioactives composition, content and biological activities are influencing the results to a greater extent, so comparison to other results are difficult to perform.

To our knowledge, this is the first report on antioxidant compounds content and activities, synergistic or additive interactions, and acetylcholinesterase inhibitory activity of bilberries-blackcurrants combination extracts. The most promising combination seems to be 33% bilberry / 67% blackcurrant, which have potential to become a good antioxidant system due to the synergistic effects between the bioactive compounds responsible for the antioxidant activity (phenolics, FRAP). 100 g of this powder formulation contains 2792 mg phenolics (1985 mg flavonoids, 1309 mg anthocyanins) and 3072 mg ascorbic acid equivalents (total antioxidant activity by FRAP).

4. Conclusions

The content of biologically active compounds of phenolic structure, the total antioxidant activity and the acetylcholinesterase inhibition were determined for the first time in combined extracts of bilberries and blackcurrants, and compared to individual ones.

We reported different behaviours of the combined extracts *vs.* individual ones, as a result of their particular chemical structures and reactivity of their constituents. Therefore, additive effects were observed for anthocyanins, synergistic effects were found in specific combinations when measuring total flavonoids and total antioxidant activity (phenolics, FRAP), while antagonistic effects were registered for tannins.

Regarding the AChE inhibition, with the exception of 100% bilberry extract which gave moderate activity, the other investigated extracts exhibited low inhibitory activity. A strong positive correlation was found between anticholinesterase activity and total condensed tannins.

The most promising combination seems 33% bilberry / 67% blackcurrant showing potential for applications as new bioactive formulations, nutritional supplements or natural antioxidants as alternative to synthetic food additives, either in the form of powders or extracts highly concentrated in bioactive compounds. Likewise, this formulation may be of pharmaceutical interest due to the AChE inhibition.

Acknowledgements

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