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Anthocyanin profile, antioxidant activity and enzyme inhibiting properties of blueberry and cranberry juices: a comparative study

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ABSTRACT

Cranberry (*Vaccinium macrocarpon*) and blueberry (*Vaccinium myrtillus*) juices are commonly consumed as a source of antioxidants. The aim of this manuscript was to compare bioactivities as well as the differences in polyphenol content and anthocyanin profile of both juices. Polyphenol and anthocyanin content was quantified using spectrophotometric and chromatographic methods. Bioassays were carried out in terms of antioxidant properties in cell and cell free systems as well as inhibition of physiological enzymes that are targets involved in the prevention of chronic diseases (monoamine oxidase A, tyrosinase, acetylcholinesterase, α-glucosidase and dipeptidyl peptidase-4). Both juices contained a significant amount of anthocyanins (3.909 mg anthocyanins mg-1 extract for blueberry juice and 0.398 for cranberry juice) and also exhibited antioxidant properties against DPPH, superoxide radical and hydrogen peroxide. The juices showed inhibitory effects on the enzymes, showing substantial potential as antioxidant, neuroprotective and anti-hyperglycaemic agents. The total anthocyanin and polyphenol content was superior in blueberry juice, which is indicative of a higher antioxidant activity. Both juices were also able to inhibit monoamine oxidase A, tyrosinase, α-glucosidase and dipeptidyl peptidase-4 in a dose-dependent manner. However, cranberry juice had a greater capacity than blueberry juice as α-glucosidase inhibitor, revealing a similar activity to acarbose.

KEYWORDS: blueberry; cranberry; Ericaceae; anthocyanin; diabetes; neuroprotection.

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1. Introduction

Blueberries (*Vaccinium myrtillus*) and cranberries (*Vaccinium macrocarpon*) are drupe, berry-like fruits belonging to the Ericaceae family. In North America, blueberries are also known as bilberries, but in Europe the preferred denomination is blueberries. *Vaccinium macrocarpon* are sometimes referred to as "American cranberry" because it originally comes from North America. Certain studies have reported bioactive compounds such as vitamins, organic acids, dietary fibres and phenolic compounds (proanthocyanidins) in berry-like fruits.(1–3) Furthermore, cranberry products are medicinally used for the prevention of urinary tract infections caused by *Escherichia coli*(4) and is commonly recognized as a preventive natural product against urinary tract infections,(5) owing to its phenolic compounds; these polyphenols are also associated with therapeutic outcomes in diabetic retinopathy or fibrocystic disease. Antimicrobial properties of blueberries have been studied in the context of diarrhoea(6) and, as recent research has revealed anti-inflammatory capacity in HCECs cells,(7) the World Health Organization also approved its use in ophthalmologic disorders.

Colourful berry-like fruits are known to contain polyphenols of the anthocyanin type. Several studies have suggested that the intake of coloured fruits may reduce risk of cancer and that a diet rich in blueberries may support arterial structure, by contributing to healthy blood flow via LDL oxidation, normal platelet aggregation and improvement of endothelial function.(1–3) Berries also exert a protective role against oxidative stress and free radical damage.(8) There is strong evidence linking berry consumption to lower incidence of age-related neurodegenerative disease.(9)

Although it has not been directly proven, there is a connection between type-2 diabetes, dementia and neurodegenerative disease.(10) Certain papers confirm that berry

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components can inhibit α-glucosidase and α-amylase because of their polyphenolic composition.(11) However, the activity of blueberry and cranberry extracts on central nervous system enzymes has not been sufficiently investigated.

The main objective of the study was to screen the bioactivity of the juices and their capacity to modulate enzymes involved in the neurotransmission and glucose metabolism such as monoamine oxidase A, tyrosinase, acetylcholinesterase, α-glucosidase and dipeptidyl peptidase-4.

2. Materials and methods

2.1. Reagents and chemicals

Chemicals were supplied by Cymit química, Cayman Chemical, Sigma-Aldrich and Panreac (Spain). Blueberry and cranberry juices were kindly donated by Natur Import. Pure juices were industrially obtained, pasteurised and bottled in amber glass bottles by the manufacturer (Rabenhorst®).

2.2 Juice lyophilization

750 ml of Rabenhorst® cranberry juice and 330 ml of Rabenhorst® blueberry juice were lyophilized using Genesis VirTis 25 EL lyophilizer over 7 days. For each, the liquid sample was frozen for 2 hr at -80 ºC while the lyophilizer was freezing at the same temperature. After that, the temperature was modified (-30 ºC) and then maintained at -60 ºC for 96 hr. The next step was at -40 ºC (4 hr) and then 24 hr at -60 ºC. Temperature was increased to -15 ºC over 7 hours and the juice dried for 22 hr at 20 ºC. for the final 2 hr temperature was maintained at 40 ºC. A dried red powder was obtained for cranberry juice and blue for blueberry juice, and stored in a freezer at -20 ºC until experiments were carried out.

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2.3. Polyphenol content and anthocyanin profile

2.3.1. Folin-Ciocalteu method

This assay was performed to quantify total phenolic content in blueberry and cranberry juices measuring absorbances at 752 nm. 201 µl of Folin-Ciocalteu reagent were mixed with 9 µl of the samples. The samples were incubated at room temperature for 5 minutes, protected from light exposure. 90 µl of Na_2CO_3 (10%) was added to the mixture and incubated in the dark at room temperature for 40 min. Gallic acid was used as standard. A standard curve was constructed using different concentrations of gallic acid: 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625 and 0.0078125 mg ml^{-1}. Polyphenols were quantified as mg GAE (Gallic Acid Equivalents) per mg lyophilized juice.(12)

2.3.2. Anthocyanin profile

Anthocyanin profile was determined by HPLC using an Agilent 1260 Infinity LC (column Eclipse plus C18 4.6 x 100 mm, 5 µm) equipped with a DAD. A two-phase gradient system of trifluoroacetic acid/water (0.5/99.5, v/v) as mobile phase A, and trifluoroacetic acid/acetonitrile/water (0.5/50/49.5, v/v) as mobile phase B was used.(13) The gradient started at 92% of mobile phase A and 8% of phase B, reaching 18% mobile phase B at 1.2 min, 32% at 14 min, 60% of mobile phase B at 28 min and 100% at 34 min, at isocratic elution until 38.8 min. The gradient reached the initial conditions at 39.2 min and was maintained at isocratic elution for 0.8 min. Elution was carried out at a flow rate of 1 ml min^{-1}. 10 µl of sample were injected (concentration: 40 mg ml^{-1}). Kuromanin, keracyanin and peonidin were used as standards in order to detect and compare peaks in blueberry and cranberry juices. Standards were dissolved in methanol. Detection was made at 520 nm. Compounds were identified according to

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retention times of standard pure compounds, elution order and comparison with a bibliographic database (phenol-explorer) of main phenolic compounds. Keracyanin (cyanidin 3-O-rutinoside) was used as standard for calibration curve. The quantification was performed using conversion factors with the molecular weight of each anthocyanin.

2.4. HeLa cells viability

The MTT assay was carried out to study the survival of cells exposed to different concentration of the juices.(14) DMEM supplemented with 10% FBS and 1% penicillin-streptomycin-glutamine was used as cell culture medium. Cells were seeded into 96-well microplates at a density of $7x10^3$ cells/well and grown for 24 h at 37 ^oC. Cells were then treated with various concentrations of lyophilized blueberry and cranberry juice (0.001-1 mg ml-1) for 72 h, after which MTT solution was added and incubated for 3 h at 37 ^oC. Survival was measured at 550 nm in a microplate reader.

2.5. Antiradical and antioxidant activity

2.5.1. Antioxidant activity of blueberry and cranberry juices in *Artemia salina*

Artemia salina cysts were hatched in seawater. Lyophilized juices were transferred to 6-well plates at different concentrations in seawater (blueberry concentrations: 250, 500 and 1000 μg ml^{-1}; cranberry concentrations: 125, 250 and 500 μg ml^{-1}) with 10 nauplii and 5 ml seawater in each well. Control wells with 10 nauplii in seawater were also considered. Nauplii were incubated for 24 h at room temperature and survival was calculated.

Artemia salina nauplii were not affected by different concentrations of both juices and the experiment was again performed using hydrogen peroxide to the wells at a concentration of 0.4 g L^{-1} in order to induce mortality. Two different control wells

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without each juice were also set up, one with hydrogen peroxide and another with seawater. The protective effects of the juices in the nauplii were evaluated every 24 h during 3 consecutive days in order to (15).

2.5.2. Xanthine/xanthine oxidase system

The xanthine/xanthine oxidase assay was performed in order to measure the capacity to scavenge superoxide radicals.(16) 16 mM Na_2CO_3, 22.8 µM NBT and 90 µM xanthine were dissolved in phosphate buffer pH=7 to reach a volume of 240 µl. Then, 30 µl of each sample and 30 µl of xanthine oxidase (168 U L^{-1}) were added to start the reaction. After an incubation time of 2 min at 37 ^{o}C, the plate was measured at 560 nm. Gallic acid was used as reference compound.

2.5.3. DPPH scavenging activity

150 µl of a DPPH methanolic solution were added to 150 µl of different blueberry and cranberry concentrations dissolved in MeOH.(17) The plate was measured at 517 nm after an incubation time of 30 min under dark conditions. Control wells were also run. Radical Scavenging capacity was calculated by the formula: RSC (%) = [($Abs_{control}$ - Abs_{sample})/$Abs_{control}$] x 100. Different substances such as ascorbic, gallic and chlorogenic acids were also measured as antioxidant standards.

2.6. Neuroprotective potential

2.6.1. Inhibition of the acetylcholinesterase enzyme (AChE)

A modification of the Ellman's method was run using a 96-microplate reader. Each well contained 25 µl of 15 mM ATCI in Millipore water, 125 µl of 3 mM DTNB in buffer C (50 mM Tris-HCl, pH=8, 0.1 M NaCl, 0.02 M $MgCl_2 \cdot 6H_2O$), 50 µl buffer B (50 mM Tris-HCl, pH=8, 0.1% bovine serum) and 25 µl juice in buffer A (50 mM Tris-HCl,

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pH=8). Finally, 25 µl of the enzyme (0.22 U L^{-1}) was added to start the reaction. Absorbance was read 13 times, every 13 s at 405 nm. Galantamine was used as reference substance.

2.6.2. Inhibition of the monoamine oxidase A enzyme (MAO-A)

A described procedure was used to measure the inhibition of MAO-A.(18) Each well contained 50 µl of blueberry (or cranberry) juice in MilliQ water, 50 µl chromogenic solution (0.8 mM vanillic acid, 417 mM 4-aminoantipyrine and 4 U ml^{-1} horseradish peroxidase in potassium phosphate buffer pH=7.6.), 100 µl of 3 mM tyramine and 50 µl of 8 U ml-1 MAO-A. Control wells contained 50 µl of solvent instead of berry juice. The absorbance was read at 490 nm every 5 min for 30 min. Clorgyline was used as reference substance.

2.6.3. Inhibition of the tyrosinase enzyme (TYR)

A described procedure was conducted in 96-well microplates measuring absorbance at 475 nm.(19) 10 µl of blueberry and cranberry juices in MiliQ water, 40 µl of L-DOPA, 80 µl phosphate buffer, pH=6.8 and 40 µl of tyrosinase were mixed in each well. Controls contained 50 µl of solvent instead of berry juice. α-Kojic acid was used as reference substance.

2.7. Antidiabetic potential

2.7.1. α-Glucosidase (α-GLU) inhibition

The capacity of blueberry and cranberry juices to inhibit α-glucosidase was measured at 405 nm.(20) Each well contained 50 µl sample and 100 µl enzyme. After 10 min, 50 µl pNPG were added and incubated at 37 ^{o}C for 20 min. Control wells contained 50 µl of

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solvent (Millipore water) instead of berry juice. Acarbose was used as reference compound.

2.7.2. Dipeptidyl peptidase-4 (DPP-4) inhibition

The capacity of blueberry and cranberry juices and their reference compounds to inhibit the DPP-4 enzyme was measured using the fluorogenic substrate Gly-Pro-Aminomethylcoumarin (AMC) with a commercial kit (Cayman, item no. 700210). The authors followed the kit instructions. Blueberry and cranberry juices were tested at four different concentrations (5, 1, 0.1 and 0.01 mg/ml in the reaction mixture), using sitagliptin as a reference inhibitor of the enzyme. The percentages of inhibition of berry juice and other compounds were determined with the following formula: % Inhibition = [(Initial Activity - Inhibitor) / Initial Activity] x 100.

2.8. Statistical analysis

All experiments were performed in triplicates in different days. Results are expressed as mean ± standard error (±SEM). Data analyses were run with GraphPad Prism v.6 by nonlinear regressions using Student t-test or ANOVA followed by Tukey's test or Bonferroni's test for multiple comparisons with a confidence interval of 95% ($p < 0.05$).

3. Results

3.1. Polyphenol content and anthocyanin profile

Blueberry juice contained 27.44 ± 4.892 µg GAE mg^{-1} of lyophilized blueberry juice. On the other hand, cranberry juice contained 23.76 ± 1.407 µg GAE mg^{-1} of lyophilized cranberry juice (Table 1). Individual and total anthocyanins were also detected and quantified by HPLC at 520 nm using keracyanin (cyanidin-3-O-rutinoside) chloride as external standard, as prescribed in the literature (Table 1,Figure 1).

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3.2. HeLa cells viability

Cranberry juice showed very mild anti-proliferative effects in HeLa cells. Significant differences were detected at concentrations over 0.125 mg ml^{-1}, which indicates that this cell line seems to be partially sensitive to cranberry components. Cell viability was more than 50% at the highest tested concentration (1 mg/ml), which means that the juice is not considered cytotoxic to this type of cervical cancer cells (Figure 2A). Blueberry juice showed even less anti-proliferative effects in HeLa cells than cranberry juice.

3.3. Antiradical and antioxidant activity

Blueberry and cranberry juices increased *Artemia salina* survival when treated with 0.4 g L^{-1} hydrogen peroxide at 24, 48 and 72 h. Different concentrations of blueberry juice ameliorate nauplii survival with significant differences (Figure 2B).

In the case of cranberry juice, survival of nauplii was not as clear as for the case of blueberry as significant differences were only obtained at doses of 500 µg/ml at 48 h (Figure 2B).

Fig. 3A shows the capacity of both berry juices and other antioxidants to scavenge superoxide radicals generated by the xanthine/xanthine oxidase system. IC_{50} values in this case were 27 µg ml^{-1} for cranberry juice, 7 µg ml^{-1} for blueberry juice and 0.044 µg ml^{-1} for gallic acid (Table 2).

Blueberry and cranberry juices were also able to scavenge DPPH radicals as shown in Fig.3B. The antiradical activity of berry juices was compared to that of gallic, ascorbic and chlorogenic acid. IC_{50} values were also calculated by nonlinear regression. IC_{50} values were 177 µg ml^{-1} for cranberry juice, 99 µg ml^{-1} for blueberry juice, 10 µg ml^{-1} for chlorogenic acid, 3 µg ml^{-1} for ascorbic acid and 1 µg ml^{-1} for gallic acid (Table 2).

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Together, this data indicate that the potential protective effects of blueberry and cranberry juices may be due to radical scavenging properties, as demonstrated through the DPPH and superoxide radical assays. The presence of anthocyanins in these juices may explain the radical scavenging properties as well as the antioxidant capacity.

3.4. Neuroprotective potential

Neither blueberry juice, nor cranberry juice showed activity in the AChE assay. However, both showed a clear dose-dependent MAO-A inhibitory activity. Berries and clorgyline inhibition of enzymes are shown in Fig. 4A. IC_{50} values (Table 2) were calculated by nonlinear regression (0.02 μg ml^{-1} for clorgyline, 336.28 μg ml^{-1} for blueberry juice and 223.96 μg ml^{-1} for cranberry juice). TYR inhibition was less pronounced than that of MAO-A. For the latter, blueberry juice produced a significant inhibition (78% at 1mg/ml), while cranberry juice exhibited less pronounced inhibition (58% at 1mg/ml) than blueberry and kojic acid, as presented in Fig. 4B. For both MAO-A and TYR, blueberry juice showed a higher inhibition than cranberry juice.

3.5. Antidiabetic potential

The inhibition of α-GLU was moderate compared to acarbose, which is a reference inhibitor of this enzyme. As represented in Fig. 5A, IC_{50} values were calculated by nonlinear regression (1319 μg ml^{-1} for blueberry juice, 470 μg ml^{-1} for cranberry juice and 380 μg ml^{-1} for acarbose).

The effects on the DPP-4 enzyme are shown in Fig. 5B. IC_{50} values were calculated by nonlinear regression (0.1 μg ml^{-1} for sitagliptin, 82.8 μg ml^{-1} for chlorogenic acid, 552.4 μg ml^{-1} for blueberry juice and 868.20 μg ml^{-1} for cranberry juice).

4. Discussion

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Anthocyanins are available in human beings at least in part as a result of their consumption of berries and/or berry juices as a source of polyphenols. These anthocyanins are some of the main polyphenols in cranberry and blueberry juices. Being part of flavonoids, anthocyanins are the greatest natural pigments, producing the blue and red colour in blueberries and cranberries and showing antioxidant potential.(21,22)

According to polyphenols, blueberry juice revealed higher concentration of polyphenols than cranberry juice with no statistical differences between samples. Both contained higher proportion of these compounds than others fruit juices like sour cherry.(12,23) HPLC-DAD analysis showed a peak of cyanidin-3-O-glucoside and previous reviews have shown that cyanidin-derived compounds are the main anthocyanins in blueberry juice.(24) Blueberry juice also contained a greater variety of anthocyanins than cranberry juice.

These data confirm the antioxidant potential of blueberry and cranberry juices, with the results from blueberry juice being more promising than that for cranberry juice. The juices also acted as antioxidants in the context of protection against H_2O_2-induced toxicity in the *Artemia salina* model. Juices from both berries were able to scavenge DPPH and superoxide radicals. According to the literature, antioxidant activity in *Vaccinium* species is mainly provided by polyphenols such as anthocyanins (25). Many studies have quantified the antioxidant capacity of blueberry and cranberry juices using the ORAC method, with trolox as standard, which makes data difficult to compare with our results.(24) Although both berries exert interesting antiradical properties, it is confirmed that blueberry juice is a better source of antioxidants than cranberry juice, being anthocyanins key compounds for this activity.

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In addition, the juices revealed inhibitory activities of two main group of enzymes; one group in relation with neurotransmitters metabolism (MAO-A, TYR, AChE) whereas other tested enzymes are current targets for antidiabetic drugs (α-GLU and DPP-4). Blueberry and cranberry juices were able to inhibit MAO-A and TYR. MAO-A is involved in deamination of catecholamines and serotonin and certain polyphenols such as anthocyanins have been described to be involved in this inhibition, which may result in an antidepressant and anxiolytic effect.(26) Tyrosinase is a copper-containing enzyme essential for tyrosine-melanin pigmentation. The role of toxic quinones in dopamine-induced neuronal damage and the catalysing role of TYR in this process has been illustrated in several studies.(27) Our data reveal that these berry juices may have potential as neuroprotective agents via inhibition of MAO-A and/or TYR.(12,23) In fact, a human intervention study by American researchers showed that consumption of anthocyanin-rich wild blueberry juice for 12 weeks improves memory and cognition in older adults with mild to moderate dementia.(28)

Finally, enzymes involved in glucose metabolism and type 2 diabetes were also inhibited by the juices; this is the first time that blueberry and cranberry juices are reported to inhibit α-glucosidase and DPP-4 in a dose-dependent manner. Anthocyanin content in fruits is also related with α-glucosidase inhibition in previous studies (11) and other authors have shown that polyphenols can enhance the insulin response and attenuate secretion of glucose-dependent insulinotropic polypeptide and GLP-1. The DPP-4 enzyme also regulates glycaemia and its inhibitors such as sitagliptin represents some of the new treatments for type 2 diabetes. Taking in consideration that type 2 diabetes is linked to neurodegenerative diseases due to production of superoxide radicals, blueberry and cranberry juices might be a worthwhile nutritional antioxidant tool to prevent these disorders.

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In conclusion, this work reveals that blueberry and cranberry juices are potentially useful natural products to employ in the prevention of certain diseases in which oxidative stress may be a major aetiological role player. Both juices are a source of anthocyanins with potential effects as neuroprotective or antihyperglycemic agents but these effects should be verified in human and animal studies.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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413 Table 1. Polyphenol content and anthocyanins for blueberry and cranberry juice.
414

Phenolic compounds	Blueberry juice	Cranberry juice
Polyphenol content (Folin)	27.44 ± 4.892 μg GAE mg^{-1}	23.76 ± 1.407 μg GAE mg^{-1}
Anthocyanins (HPLC)	3.909 mg anthocyanins mg^{-1} extract	0.398 mg anthocyanins mg^{-1} extract
Delphinidin 3-O-galactoside	0.4322 ± 0.0302	
Delphinidin 3-O-glucoside	0.6832 ± 0.0559	
Cyanidin 3-O-galactoside	0.3655 ± 0.0211	
Delphinidin 3-O-arabinoside	0.1693 ± 0.0125	
Cyanidin 3-O-glucoside	0.8089 ± 0.0419	0.1381 ± 0.0293
Petunidin 3-O-glucoside	0.4669 ± 0.0402	
Cyanidin 3-O-arabinoside	0.1141 ± 0.0001	0.0169 ± 0.0194
Petunidin 3-O-arabinoside	0.0106 ± 0.0049	
Peonidin 3-O-glucoside	0.2287 ± 0.1044	0.1792 ± 0.0276
Malvidin 3-O-glucoside	0.4044 ± 0.0418	
Peonidin 3-O-arabinoside		0.0013 ± 0.0125

415 GAE: Gallic Acid Equivalents; HPLC: High performance Liquid Chromatography;

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418 Table 2. IC_{50} values (μg ml^{-1}) for different tested bioassays

Methods	Blueberry juice	Cranberry juice
Antiradical activity (DPPH)	99 ± 5.28 μg ml^{-1} ****	177 ± 7.96 μg ml^{-1}
Antioxidant (O_2^-)	7.55 ± 0.5 μg ml^{-1} **	27 ± 1.6 μg ml^{-1}
MAO-A	0.3336 ± 0.1219 μg mg-1	0.1850 ± 0.04471 μg mg-1
Tyrosinase	0.1064 ± 0.03630 μg mg-1*	0.4814 ± 0.09839 μg mg-1
Glucosidase	1.26 ± 0.109 μg mg-1	0.48 ± 0.43 μg mg-1**
DPP-4	0.453 ± 0.07156 μg mg-1	0.8821 ± 0.1828 μg mg-1

419 *$p < 0.05$; DPPH: 2,2-diphenyl-1-picrylhydrazyl; DPP-4: dipeptidyl peptidase 4;

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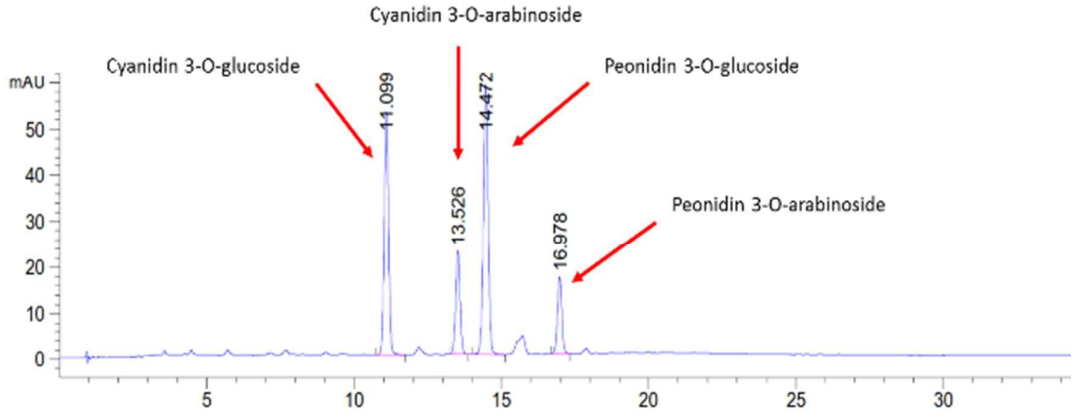
A



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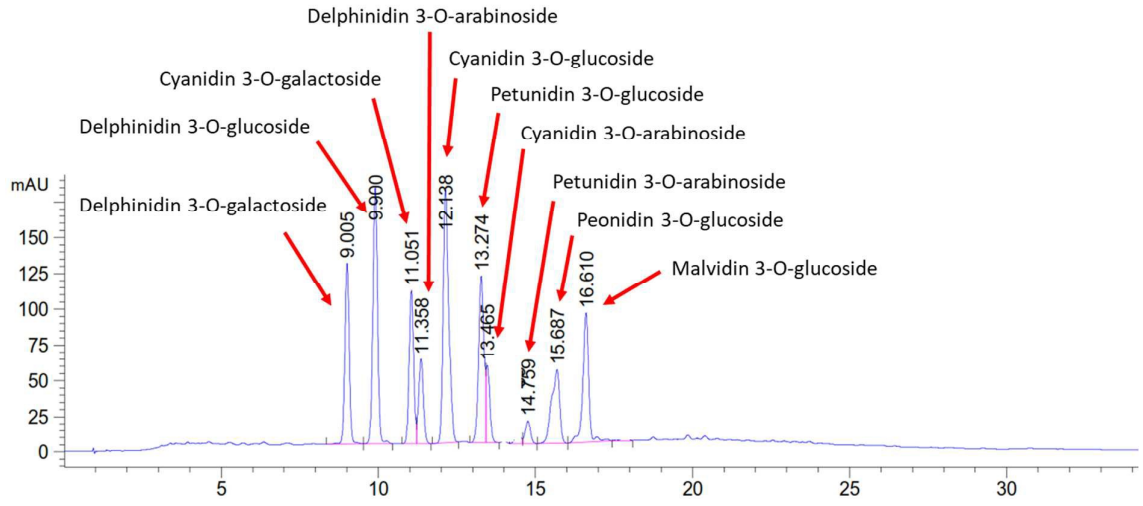

Figure 1. HPLC chromatogram at 520 nm of lyophilized cranberry juice (A) and lyophilized blueberry juice (B). Different anthocyanins were determined by their retention time.

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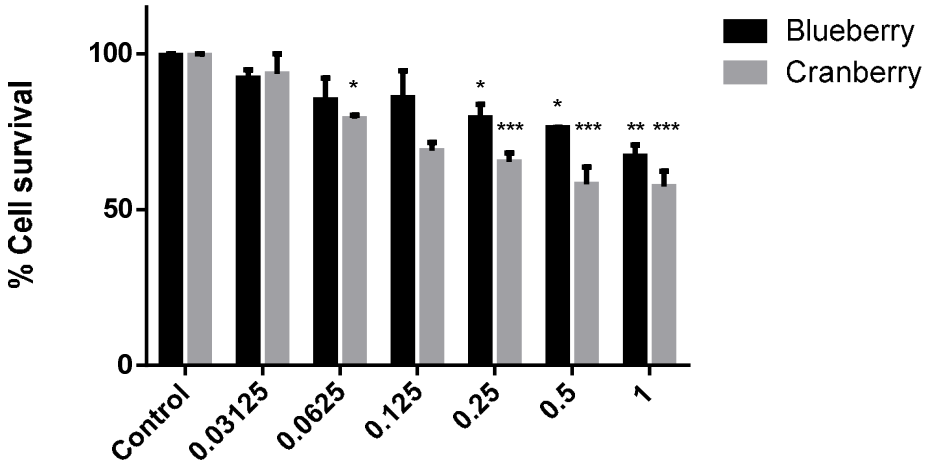
433 A



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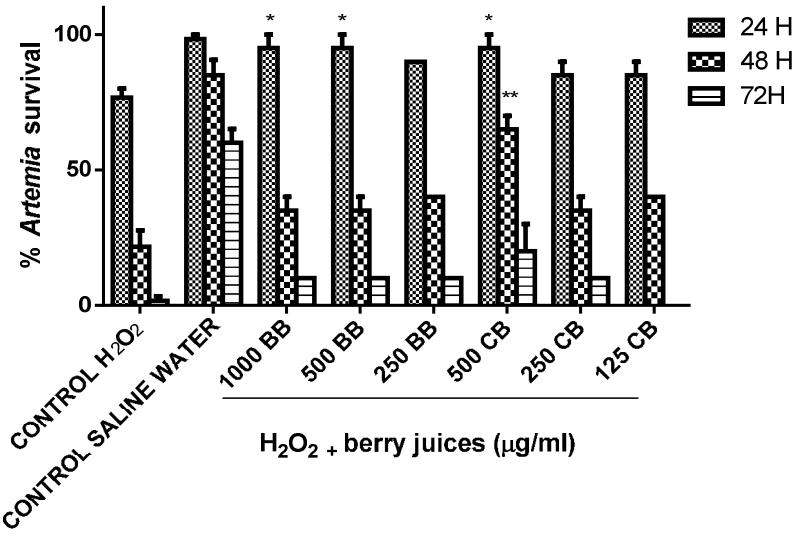

436

437 Figure 2. Viability of HeLa cells exposed to different concentration of berry juices for
438 72 hours in the MTT assay (A). Effects of berry juices in *Artemia salina* nauplii
439 exposed to hydrogen peroxide (0.4 g L^{-1}) (B). * Significant differences ($p < 0.05$) were
440 detected between nauplii exposed to hydrogen peroxide (Control H_2O_2) and nauplii
441 exposed to hydrogen peroxide + 500 μg/ml cranberry juice at 48 h.

442 BB: Blueberry; CB: Cranberry.

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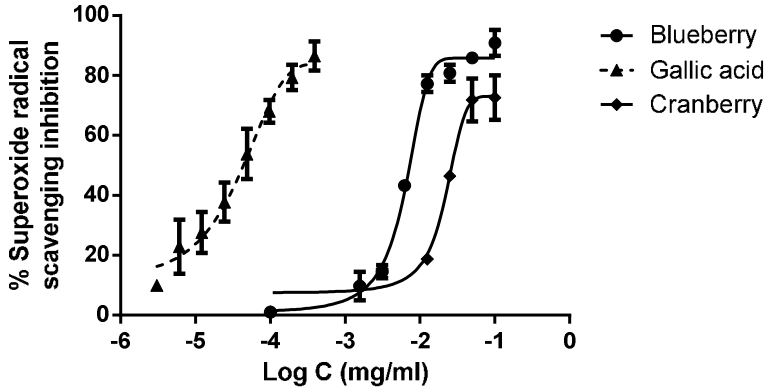
444 A



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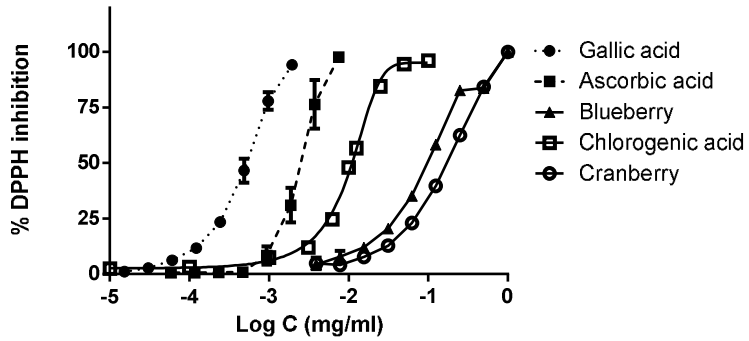
446 B



447

448 Figure 3. Antiradical activity of blueberry juice, cranberry juice, and standards against
449 superoxide radicals generated by the xanthine/xanthine oxidase system (A) and DPPH
450 radicals (B).

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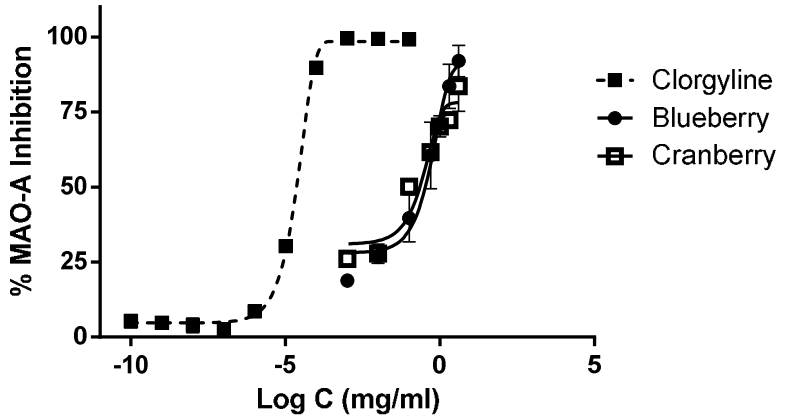
452 A



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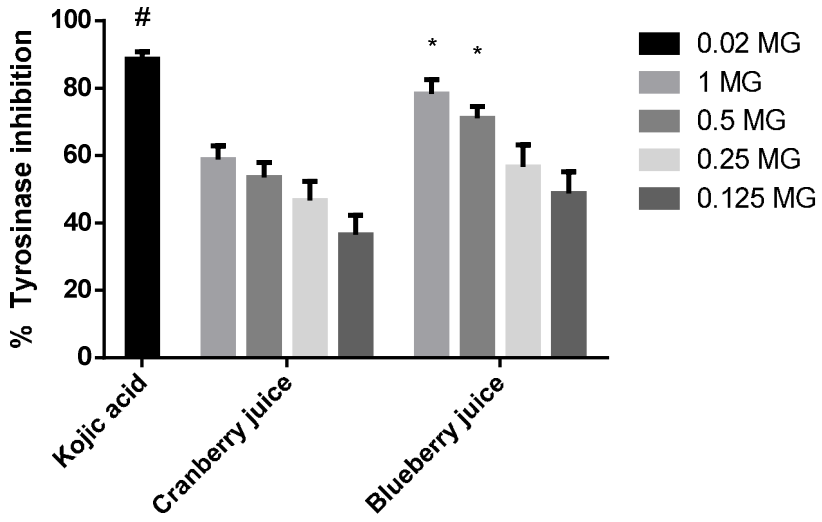
454 B



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456

457 Figure 4. Neuroprotective potential of the juices. MAO-A inhibition performed by
458 blueberry juice, cranberry juice and clorgyline (A). Tyrosinase inhibition performed by
459 different concentrations of cranberry juice, blueberry juice and kojic acid (0.2 mg/ml) as
460 standard (B). *$p < 0.05$ versus cranberry juice; # $p < 0.05$ versus berry juices.

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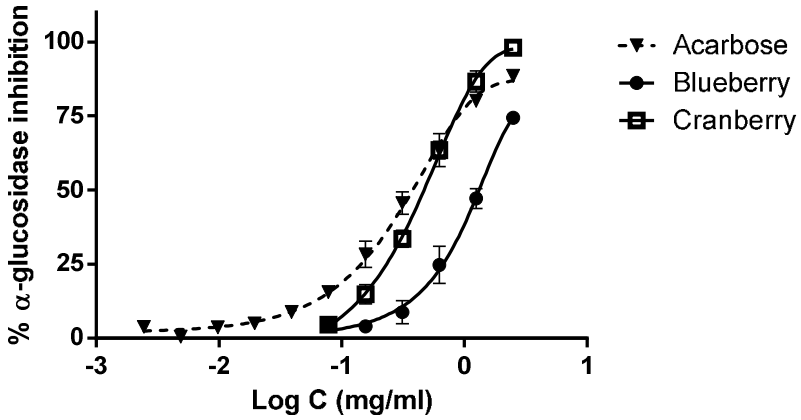
462 A



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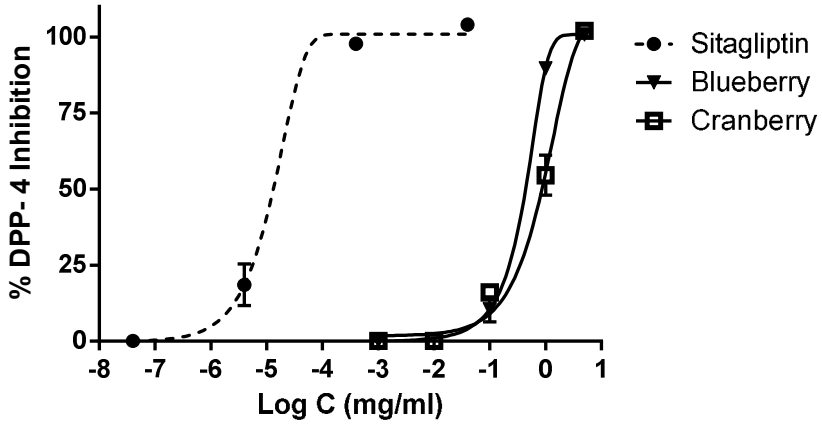
464 B



465

466 Figure 5. Antidiabetic potential of the juices. α-Glucosidase inhibition (A) and
467 dipeptidil peptidase-4 inhibition (B) performed by blueberry juice, cranberry juice and
468 acarbose or sitagliptin as standards.

GRAPHICAL ABSTRACT