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Cyanidin-3-O-glucoside inhibits different enzymes involved in central nervous system pathologies and type-2 diabetes

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ABSTRACT

Cyanidin-3-O-glucoside, also known as kuromanin, is one of the most important anthocyanins in nature. The scope of this paper is to discuss the potential role of this anthocyanin as therapeutic agent to prevent or treat chronic diseases in which oxidative stress may be involved through a modulation of certain enzymes. The inhibitory potential of cyanidin-3-O-glucoside against enzymatic targets of neurodegenerative or metabolic diseases was tested using monoamine oxidase A (MAO-A), acetylcholinesterase (AChE), tyrosinase (TYR), fatty acid amide hydrolase (FAAH), α-glucosidase (α-GLU) and dipeptidyl peptidase-4 (DPP-4). The antioxidant activity was evaluated through the xanthine/xanthine oxidase system. Cyanidin-3-O-glucoside inhibited MAO-A, TYR and FAAH enzymes whereas it could not inhibit AChE activity. IC_{50} values for these assays were 7.6 μM, 18.1 μM and 152.1 μM, respectively. Additionally, cyanidin-3-O-glucoside was able to inhibit α-GLU (IC_{50} = 479.8 μM) and DPP-4 (IC_{50} = 125.1 μM). Finally, the antioxidant activity of cyanidin-3-O-glucoside was confirmed by the xanthine/xanthine oxidase method, being more efficient than gallic acid as superoxide radical scavenger. In conclusion, cyanidin-3-O-glucoside has demonstrated to be a candidate as enzyme inhibitor with neuroprotective, antioxidant and antidiabetic potential.

1. Introduction

Cyanidin-3-O-glucoside, also known as asterin, chrysanthemin or kuromanin, belongs to the flavonoid family of plant secondary metabolites, and specifically to the subgroup of anthocyanins. It can be found in flowers and fruits of species such as *Elymus repens* (couch grass), *Morus alba* (mulberry), *Sambucus nigra* (elderberry), *Vitis vinifera* (grape), *Vaccinium myrtillus* (bilberry) and *Vaccinium corymbosum* (blueberry) (Brewer, 2011; Carradori et al., 2014; Chorfa et al., 2016; Koyu et al., 2018). More than 250 different anthocyanins have been discovered and they are considered to be responsible for the red, purple and blue colors in fruits, leaves and flowers (Strack and Wray, 1989; Francis and Markakis, 2009; Spínola et al., 2018).

Apart from acting as pigments in plants, they are considered as healthy bioactive agents in human diet (Wallace and Giusti, 2015). The daily intake and bioavailability of anthocyanins depend on the amount of fruits and vegetables consumed, as well as harvest region and season (Fang, 2014). Although there are no reference values for anthocyanins, Chinese Dietary Reference Intakes propose levels of 50 mg/day (Chinese Nutrition Society, 2013).

Pharmacokinetic studies reveal that anthocyanins could be absorbed both in the stomach and the intestine, which make them rather different from other flavonoids (Fang, 2014). Cyanidin-3-O-glucoside and pelargonidin-3-O-glucoside could be absorbed in their intact form into the gastrointestinal wall; however, metabolites have been found in the blood stream in much higher concentrations than their precursors (Bonarska-Kujawa et al., 2012; Fang, 2014).

Anthocyanins have extensively been described as antioxidant agents in different types of *in vitro*, *in vivo* and human studies (Tsuda, 2012; Del Rio et al., 2013). The accumulation of ROS induces oxidative damage of mitochondrial DNA, proteins, and lipids, and has been shown to contribute to the decline in physiological function of cells resulting in a variety of diseases and accelerated aging. Natural products

Abbreviations: CNS, central nervous system; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); DOPA, L-3,4-dihydroxyphenylalanine; pNPG, 4-nitrophenyl β-D-glucopyranoside; MAO-A, monoamine oxidase A; AChE, acetylcholinesterase; TYR, tyrosinase; FAAH, fatty acid amide hydrolase; α-GLU, α-glucosidase; DPP-4, dipeptidyl peptidase-4; XO, xanthine oxidase.

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might slow the progression of Alzheimer's disease (AD) by simultaneously protecting neurons from oxidative stress and acting as cholinesterase inhibitors (Pervin et al., 2014). Randomized trials propose that purified anthocyanins also exhibit beneficial significant effects on LDL cholesterol in patients with hyperlipidemia. On the other hand, less beneficial tendencies for total cholesterol and HDL cholesterol among those with hyperlipidemia have been reported (Wallace and Giusti, 2015). In parallel, anthocyanin-rich fruits such as blackcurrants, bilberries, and blueberries, have demonstrated a decrease in LDL- cholesterol levels and an increase of plasma antioxidant capacity (Erlund et al., 2008; Gupta et al., 2009). Anthocyanins, or their metabolites, could also exhibit cardiovascular protective effects through antiplatelet and anti-inflammatory activities (Erlund et al., 2008). Obese mice fed cyanidin-enriched diet (2 g/kg), showed that anthocyanins were able to significantly reduce body fat accumulation induced by high-fat meals (60% of energy), when compared with controls (Tsuda et al., 2003; Tsuda, 2008). Intake of high-purity cyanidin-3-O-glucoside inhibits elevation of blood glucose levels and improves insulin sensitivity in a type-2 diabetes model (Sasaki et al., 2007).

Type-2 diabetes and neurodegenerative diseases such as Alzheimer's disease (AD) are linked and connected from a physiopathological perspective due to oxidative stress and ROS (Fawole et al., 2012). AD, which is the most common form of dementia, is characterized by the accumulation of extracellular amyloid beta (Aβ) peptide and tau protein. These two proteins are supposed to drive and accelerate oxidative stress and inflammatory processes leading to neurodegeneration. It is not so clear that type 2 diabetes can exacerbate these neurodegenerative processes but animal and cell culture studies have indicated that insulin resistance and impaired insulin signaling in the brain could initiate other aspects of brain injury, including inflammatory and oxidative stress processes and the early accumulation of Aβ (Verdile et al., 2015).

Anthocyanins contain a pseudo aromatic ring C that increases their structural planarity and promotes amyloid fibril disruption due to effective incorporation of anthocyanins inside the amyloid beta fibril groove (Lakey-Beitia et al., 2015). Additionally, several studies have shown that some polyphenols, including anthocyanins, can cross the blood–brain barrier and exert neuroprotection (Xu et al., 2010; Ebrahimi and Schluesener, 2012; Carradori et al., 2014), which gives scientific evidence of the neuroprotective role of these substances.

Due to the fact that anthocyanins could be used to modulate chronic diseases in which oxidative stress is underlying, the potential of cyanidin-3-O-glucoside as neuroprotective and antidiabetic agent was evaluated through *in vitro* enzyme inhibition bioassays. CNS enzymes such monoamine oxidase A (MAO-A), acetylcholinesterase (AChE), tyrosinase (TYR), and fatty acid amide hydrolase (FAAH) were selected as targets for neuroprotection; other enzymes like α-glucosidase (α-GLU) and dipeptidyl peptidase-4 (DPP-4) were designated as current targets in type-2 diabetes.

2. Materials and methods

2.1. Reagents and chemicals

Cyanidin-3-O-glucoside, also referred as kuromanin throughout the manuscript, (Fig. 1) was acquired through Extrasynthese (France). DPP-4 and FAAH kits were purchased from Cayman Chemicals (Michigan, USA); α-GLU, pNPG MAO-A, clorgyline, DTNB, Tris, galantamine, L-DOPA, tyramine, horseradish peroxidase, bovine serum albumin, vanillic acid, 4-aminoantipyrine and tyrosinase were obtained from Sigma-Aldrich (Madrid, Spain), clorgyline, α-kojic, acarbose were from Cymit Química (Barcelona, Spain), Na_2CO_3, HCl, NaCl, MeOH, potassium phosphate were from Panreac (Barcelona, Spain).

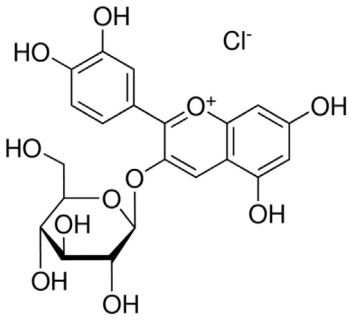

Fig. 1. Structure of cyanidin-3-O-glucoside. Synonyms: chrysanthemin, kuromanin, cyanidin 3-O-β-glucopyranoside.

2.2. Potential to inhibit CNS enzymes

2.2.1. Monoamine oxidase A (MAO-A) inhibition

MAO-A inhibition assay was carried out following the protocol of Olsen et al., 2008 in order to determine the potential activity of the anthocyanin. Each well contained 50 μL of different sample concentrations in MilliQ water, 50 μL chromogenic solution (800 μM vanillic acid, 417 mM 4-aminoantipyrine and 4 U/mL horseradish peroxidase in potassium phosphate buffer pH = 7.6.), 100 μL of tyramine (300 μM) and 50 μL of MAO-A (8 U/mL). Clorgyline was used as reference inhibitor. Control wells contained 50 μL of solvent in place of sample. The absorbance was read at 490 nm every 5 min during 30 min in a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The percentages of inhibition of cyanidin-3-O-glucoside and clorgyline were determined with the following equation: % Inhibition = [1 − (Inhibitor Slope / Control Slope)] × 100.

2.2.2. Tyrosinase (TYR) inhibition

The inhibition of tyrosinase was performed in a 96-well plate as previously described (Sezer Senol et al., 2015). The reaction mixture contained 10 μL of different sample concentrations in MiliQ water, 40 μL of L-DOPA (substrate), 80 μL phosphate buffer (pH = 6.8) and 40 μL of tyrosinase (TYR) in each well. α-Kojic acid was used as reference inhibitor. Controls wells contained 50 μL of solvent instead of sample. The absorbance was measured at 475 nm (endpoint) using a FLUOstar Omega microplate reader.

2.2.3. Acetylcholinesterase (AChE) inhibition

AChE inhibition was performed with Ellman's method in a 96-well microplate using a FLUOstar Omega microplate reader for measuring the absorbance at 405 nm during 13 times every 13 s (Rhee et al., 2001). Each well contained 25 μL of ATCI (15 mM) in ultrapure water, 125 μL of DTNB (3 mM) in buffer C (50 mM Tris–HCl, pH = 8, 100 mM NaCl, 20 mM $MgCl_2 \cdot 6H_2O$), 50 μL of buffer B (50 mM Tris–HCl, pH = 8, 0.1% bovine serum), and 25 μL of different sample concentrations in buffer A (50 mM Tris–HCl, pH = 8). Galantamine was used as reference inhibitor. To end up, 25 μL of the enzyme (0.22 U/L) was added to complete the reaction. Blanks contained 25 μL of buffer A instead of the enzyme.

2.2.4. Fatty acid amide hydrolase (FAAH) inhibition

The inhibition of FAAH enzyme was measured by a fluorescence procedure using a Synergy H1 hybrid multimode reader (Biotek) and following manufacturer's instructions of a commercial kit (Cayman, item no. 10005196). JZL 195 was use as reference inhibitor to inhibit FAAH enzyme.

2.3. Potential to inhibit enzymes involved in type 2-diabetes

2.3.1. α-Glucosidase (α-GLU) inhibition

The inhibition of the α-glucosidase enzyme, extracted from *Saccharomyces cerevisiae*, was assessed in 96-well microplate and read in a FLUOstar Omega microplate reader at 405 nm following the procedure of Kazeem et al., 2013. The mixture assay contained 50 μL of different sample concentrations, cyanidin-3-O-glucoside, acarbose (reference inhibitor) and 100 μL α-glucosidase enzyme (1 U/mL). After 10 min of pre-incubation, 50 μL of pNPG (3 mM) dissolved in phosphate buffer (20 mM, pH = 6.9) were added and incubated for 20 min at 37 °C. Blanks contained buffer instead of α-glucosidase.

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

The inhibition of DPP-4 enzyme was evaluated by a fluorometric procedure using a Synergy H1 hybrid multimode reader (Biotek) and following manufacturer's instructions of a commercial kit (Cayman, item no. 700210). A fluorogenic substrate Gly-Pro-Aminomethylcoumarin (AMC) was selected for measuring the capacity of cyanidin-3-O-glucoside and sitagliptin (reference inhibitor) to inhibit DPP-4 enzyme.

2.4. Antioxidant activity: superoxide radical generated by xanthine/xanthine oxidase

The antioxidant activity of cyanidin-3-O-glucoside was measured by the xanthine/xanthine oxidase method (Rodríguez-Chávez et al., 2015). 90 μM xanthine, 22.8 μM NBT and 16 mM Na_2CO_3 were mixed in phosphate buffer (pH = 7). 240 μL of this mix were added to the well. 30 μL of cyanidin-3-O-glucoside and 30 μL of xanthine oxidase (168 U/L) were added to start the reaction. Incubation of 2 min at 37 °C was required. The transformation of NBT to formazan by the superoxide radical (O_2^-) was measured at 560 nm. Gallic acid was used as a reference antioxidant compound. The formation of uric acid was evaluated to ensure if the compound does or does not affect xanthine oxidase activity at 295 nm.

2.5. Statistical analysis

Results were expressed as the mean ± standard error (SE) of different assays. GraphPad Prism v.6 was required to perform data analyses, nonlinear regressions and statistics. Statistical differences were detected comparing IC_{50} values of cyanidin-3-O-glucoside and reference compounds using Student's *t*-test.

3. Results & discussion

3.1. Bioassays regarding CNS enzymes

The inhibition of CNS enzymes performed by cyanidin-3-O-glucoside may increase the levels of certain neurotransmitters and neuromodulators leading to a neuroprotective activity.

Data revealed a very clear dose dependent MAO-A inhibition. Fig. 2A shows 100% of MAO-A inhibition at the highest tested dose. IC_{50} values were calculated by nonlinear regression for clorgyline, reference inhibitor, and cyanidin-3-O-glucoside, being 0.02 μg/mL (=0.07 μM) and 3.77 μg/mL (=7.6 μM), respectively. Dreiseitel et al., 2009 evaluated the inhibitory activity of different anthocyanins against MAO-A, obtaining IC_{50} values of 34.3 μM for cyanidin-3-O-glucoside. Another flavonoid, quercetin, extracted from *Hypericum hircinum*, showed a great response against MAO-A, 0.01 μM (Carradori et al., 2016; Gidaro et al., 2016). A recent study revealed that an enriched-flavonoid extract from the bark of *Trichilia catigua*, which is used in traditional Brazilian medicine as antidepressant, was able to inhibit MAO-A in a concentration-dependent manner (IC_{50} = 121.06 ± 2.13 μg/mL) (Bernardo et al., 2018). Different polyphenols as curcumin or ellagic acid were also tested as possible target inhibitors of MAO showing

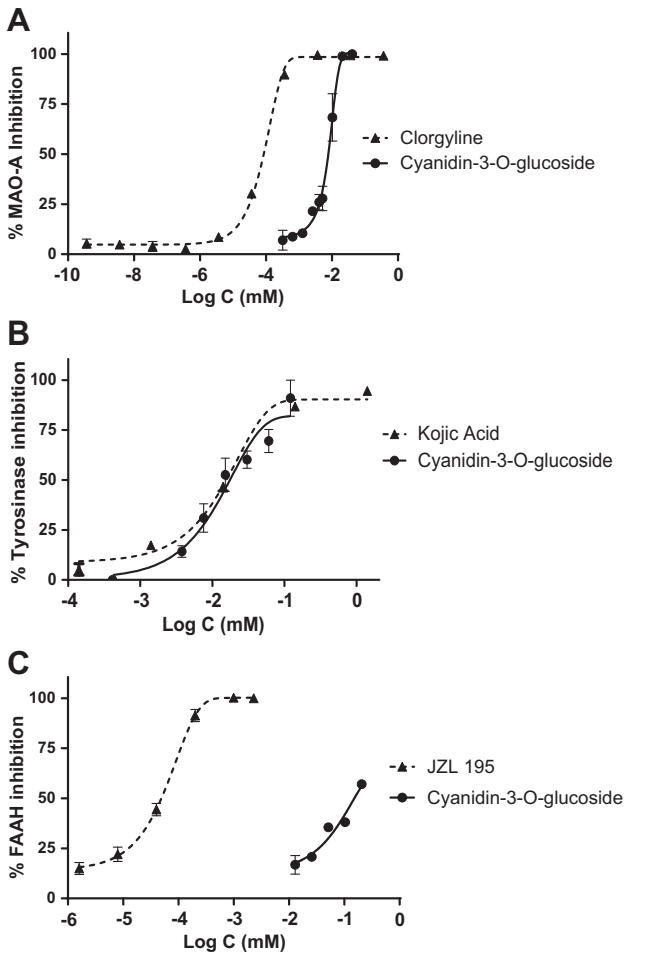

Fig. 2. Bioassays regarding CNS enzymes. A) MAO-A inhibition profiles of cyanidin-3-O-glucoside and clorgyline. B) Tyrosinase inhibition profiles of cyanidin-3-O-glucoside and kojic acid C) Fatty acid amide hydrolase inhibition profile of cyanidin-3-O-glucoside and JZL 195. Cyanidin-3-O-glucoside was not able to inhibit acetylcholinesterase.

0.50 μM and 0.41 μM IC_{50} values, respectively (Khatri and Juvekar, 2016). Finally, a study carried out in a mice model demonstrated that the uptake of proanthocyanidins from a grape seed inhibited MAO-A enzyme in mouse brain (Xu et al., 2010).

It seems that the catechol group of certain flavonoids and phenolics could be involved in the inhibition of the MAO-A enzyme. Due to their structures, the effect of flavonoids on MAO-A could be different, as we can observe between cyanidin-3-O-glucoside and quercetin. The reason could be that the sugar moiety of the anthocyanoside may interfere in the inhibition of MAO-A. These results indicate that cyanidin-3-O-glucoside could act as a neuroprotective and antidepressant agent due to potential effects on monoaminergic neurotransmitter systems.

The concentration of cyanidin-3-O-glucoside tested in tyrosinase inhibition assay was higher than the one in MAO-A assay, but also for kojic acid, which is a reference inhibitor. The inhibitory profile was similar to kojic acid (Fig. 2B); IC_{50} values were 18.1 μM for cyanidin-3-O-glucoside and 16.0 μM for kojic acid. A recent paper based on *Morus nigra*, established a positive response for tyrosinase inhibition (IC_{50} = 1.6 mg/mL) (Koyu et al., 2018). Another work focused on the tyrosinase inhibition activity of seven different pomegranate cultivars, showing that all these cultivars inhibited more than 50% of the enzyme activity being "Bhagwa" cultivar the most active with an IC_{50} value of 3.66 μg/mL (Fawole et al., 2012). Liposome-capsulated anthocyanin (LCA) from *Hibiscus sabdariffa* have also inhibitory activity against tyrosinase enzyme in a human A375 melanoma model decreasing in more than 50% its activity (Hwang et al., 2013).

As we can observe in the literature, peel from pomegranate ("Bhagwa" cultivar) has a better IC_{50} value than cyanidin-3-O-glucoside which could be attributed to the other compounds present in the extract. But comparing anthocyanins, cyanidin-3-O-glucoside equivalents from *Morus nigra* demonstrated lower inhibitory activity than cyanidin-3-O-glucoside.

On the other hand, different concentrations of cyanidin-3-O-glucoside were tested in the acetylcholinesterase (AChE) assay, but the compound did not display activity against this enzyme. *Trichilia catigua*, cited previously as MAO-A inhibitor, was able to reduce enzymatic activity by Ellman's method (Bernardo et al., 2018). Furthermore, anthocyanin-rich tea 'Sunrouge' was able to inhibit AChE in 52% at 410 μg/mL (which contained 0.6 μg/mL of anthocyanin) in a neuroblastoma model (Maeda-Yamamoto et al., 2012). Ethanolic extracts from pomegranate leaves exhibited AChE inhibitory activity with IC_{50} values between 2.65–14.83 mg/mL (Bekir et al., 2013). The inhibition of AChE by certain plant extracts may be in relation with polyphenols different from the anthocyanin type.

Finally, cyanidin-3-O-glucoside was also able to modulate the endocannabinoid system through inhibition of the FAAH enzyme, almost reaching 60% inhibition at the highest tested dose (Fig. 2C). The estimated IC_{50} values were 152.1 μM for cyanidin-3-O-glucoside and 0.05 μM for the reference inhibitor. It is the first time that this anthocyanin shows the capacity to inhibit this enzyme, which is involved in the endocannabinoids metabolism. The inhibition of this endocannabinoid hydrolase could offer beneficial properties in the treatment of pain, obesity, and various neurological diseases, where higher endocannabinoid activity would be beneficial (Lambert and Fowler, 2005).

3.2. Bioassays regarding type 2-diabetes enzymes

Acarbose, an antidiabetic oral drug, is able to inhibit α-GLU with an IC_{50} value of 379 μg/mL; interestingly, cyanidin-3-O-glucoside displayed a better IC_{50} value for α-GLU inhibition, showing 238 μg/mL (=479.8 μM) (Fig. 3A). Both IC_{50} values were calculated by nonlinear regression. In order to compare the IC_{50} values of the isolated anthocyanin with the reference inhibitor, Student's *t*-test was performed, and

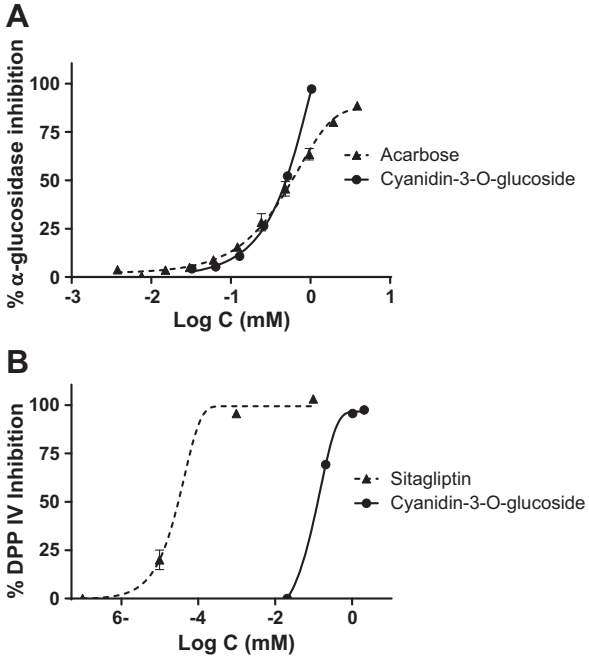

Fig. 3. Bioassays regarding antidiabetic potential. A) α-Glucosidase inhibition performed by cyanidin-3-O-glucoside and acarbose. B) Dipeptidyl peptidase-4 inhibition performed by cyanidin-3-O-glucoside and sitagliptin.

no significant differences were observed, confirming their similarity in this activity.

The α-glucosidase inhibitory activity of blueberry anthocyanins has already been evaluated in amberlite extracts (CAE) and rehydrated powders. These samples demonstrated a high content in anthocyanins such as cyanidin, peonidin, petunidin and malvidin. Rehydrated powder was able to inhibit this enzyme up to 91.49% and CAE in 92.83% (Flores et al., 2013). In addition, an anthocyanin fraction from black carrots (*Daucus carota* L.), showed inhibition on α-amylase and glucosidase in a dose-dependent manner reaching 100% of inhibition at 50 mg/mL (Esatbeyoglu et al., 2016). Other researchers have studied colored grains such as black rice or purple corn and its capacity of inhibiting the α-glucosidase enzyme; black rice had the highest anthocyanin content (3.83 mg anthocyanin/g of extract) and also the maximum inhibitory enzymatic activity (IC_{50} = 13.56 μg/mL) which suggested a link between anthocyanin and antidiabetic effects (Yao et al., 2010). Furthermore, literature supports that certain fruits with high anthocyanin content (blueberry and blackcurrant) have greater activity as α-glucosidase inhibitors than others (strawberry and raspberry) containing more soluble tannins (McDougall et al., 2005). Another report, based on cyanidin-3-O-galactoside, showed an IC_{50} value of 500 μM (Adisakwattana et al., 2009) while cyanidin-3-O-glucoside showed 491.78 μM confirming very similar effects in both anthocyanins. The same authors analyzed cyanidin (IC_{50} = 300 μM) and its glycosides finding differences in the 3-O-position of glucose and galactose for modulating the inhibition of glucosidase (Akkarachiyasit et al., 2010). Blend wines from blueberry and blackcurrant fruits also inhibited the glucosidase enzyme, which IC_{50} values lower than those herein reported (27.6–36.2 μM). This is an interesting point because fermented fruits could increase this inhibition (Johnson et al., 2013). Furthermore, anthocyanins of two different *Vaccinium* berries were the main inhibitory agents towards α-glucosidase (Spínola et al., 2018).

Cyanidin-3-O-glucoside and sitagliptin (reference drug), revealed a clear dose dependent DPP-4 inhibition. Results are presented in Fig. 3B and IC_{50} values were calculated by nonlinear regression (62.05 μg/mL = 125.1 μM for cyanidin-3-O-glucoside and 0.013 μg/mL for the reference inhibitor). Black carrots (*Daucus carota* L.) were also tested as possible inhibitors of DPP-4. However, these fractions could not inhibit the enzyme (Esatbeyoglu et al., 2016). Cyanidin-3-O-glucoside was the main anthocyanin in blueberry wine, displaying DPP-4 inhibitory activity (Fan et al., 2013). This blueberry wine reached and IC50 value of 15.9 μM (Johnson et al., 2013) while cyanidin-3-O-glucoside 128.21 μM. A similar trend was observed with previous work. The main differences could be attributed mainly to differences in analytical procedures rather than the fermentation process. The capacity of different berry juices (blueberry, cranberry and cherry) with a relevant anthocyanin content has also been on the DPP-4 enzyme (Cásedas et al., 2016, 2017).

According to literature, polyphenol-rich foods may be beneficial for prevention of type-2 diabetes owing to their protective effect on pancreatic beta-cells against glucotoxicity, antioxidant and anti-inflammatory effects as well as inhibition of α-glucosidase, α-amylase and DPP-4 enzymes. Many researchers have suggested that anthocyanins may delay glucose absorption *via* inhibition of α-amylase and α-glucosidase (Xiao and Hogger, 2014) enabling also insulin response and decrease secretion of Glucagon-like peptide-1 (GLP-1) and Gastric inhibitory polypeptide (GIP) which are the two primary incretin hormones secreted from the intestine on ingestion of glucose or nutrients to stimulate insulin secretion from pancreatic β cells (Seino et al., 2010).

3.3. Inhibition of superoxide radical generated by xanthine/xanthine oxidase

The capacity to inhibit XO has previously been attributed to flavan-3-ols and phenolic acids (Aron and Kennedy, 2008); however, cyanidin-3-O-glucoside, which is an anthocyanin, is not able to inhibit

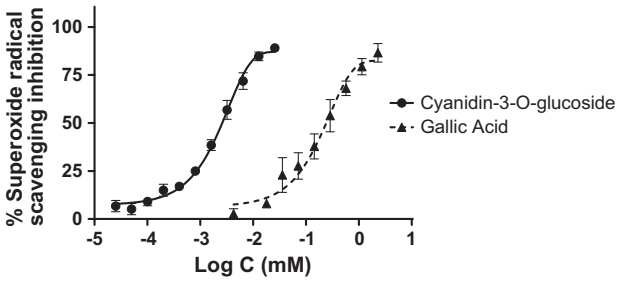

Fig. 4. Cyanidin-3-O-glucoside scavenges superoxide radicals generated by the xanthine/xanthine oxidase system. Gallic acid was used as reference.

XO. Nevertheless, this anthocyanin has demonstrated antioxidant potential through superoxide radical scavenging resulting from xanthine oxidase (Fig. 4). Gallic acid was used as standard showing a concentration-dependent activity (IC_{50} = 44 μg/mL). In contrast, cyanidin-3-O-glucoside reached the same inhibition at lower concentration (IC_{50} = 1 μg/mL, 2.01 μM). Polyphenols are considered exogenous antioxidants since they counteract the oxidative stress through direct radical scavenging and enzymatic inhibition, or indirectly by interaction/modulation of signal pathways (Brewer, 2011; Bernardo et al., 2018). In this sense, anthocyanins might be used as exogenous antioxidant to prevent oxidative stress. Other papers have also confirmed the potential of polyphenolic antioxidants on neurodegenerative diseases and type-2 diabetes (Les et al., 2015).

4. Conclusion

Cyanidin-3-O-glucoside has revealed inhibitory properties of key enzymes such as MAO-A, TYR, FAAH, α-GLU and DPP-4. The inhibition of these enzymes together with its antioxidant activity makes this anthocyanin an interesting starting point to develop therapeutic agents in the areas of neuroprotection and type 2-diabetes.

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Conflicts of interest

The authors declare that they do not have any conflicts of interest.

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