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Bioactive and functional properties of cherry juice (Prunus cerasus)

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Complete List of Authors:	Casedas, Guillermo; Universidad San Jorge Les, Francisco; Universidad San Jorge Gomez-Serranillos, M. P.; University Complutense of Madrid, Pharmacology Smith, Carine; Stellenbosch University, Biochemistry López, Víctor; Universidad San Jorge,

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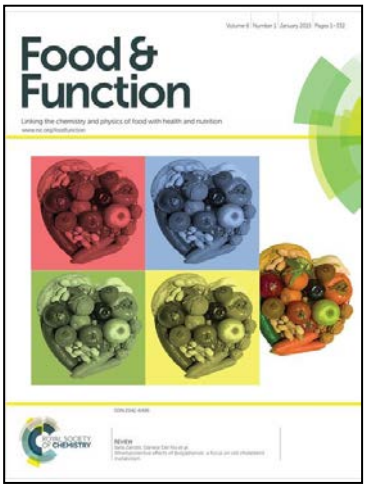

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Bioactive and functional properties of cherry juice (*Prunus cerasus*)

Guillermo Cásedas [a], Francisco Les [a], Maria Pilar Gómez-Serranillos [b], Carine Smith [c], Víctor López [d*]

[a] *Department of Pharmacy, Faculty of Health Sciences, Universidad San Jorge, 50.830 Villanueva de Gállego (Zaragoza), Spain*

[b] *Department of Pharmacology, Faculty of Pharmacy, University Complutense of Madrid, Plaza Ramón y Cajal s/n, 28040 Madrid, Spain*

[c] *Department of Physiological Sciences, Science Faculty, Stellenbosch University, Stellenbosch, South Africa*

*Corresponding author: Víctor López (ilopez@usj.es), Facultad de Ciencias de la Salud, Universidad San Jorge, Campus Universitario Villanueva de Gállego Autovía A-23 Zaragoza-Huesca Km. 299. 50.830 Villanueva de Gállego (Zaragoza)

ABSTRACT

Cherry juice is consumed as a nutritional supplement claiming health effects. The aim of the study was to evaluate different properties of cherry juice in terms of antioxidant activity and inhibition of target enzymes in the central nervous system and diabetes. The content of polyphenols and anthocyanidins was quantified. Different experiments were carried out to determine the radical scavenging properties of the juice. The activity of cherry juice was also tested in physiological relevant enzymes of the central nervous system (acetylcholinesterase, monoamine oxidase A, tyrosinase) and others involved in type 2 diabetes (α-glucosidase, dipeptidyl peptidase-4). Cherry juice showed significant antioxidant effects due to polyphenols but the activity was not superior to other common antioxidants such as ascorbic, gallic or chlorogenic acid. Furthermore, cherry juice and one of its main polyphenols known as chlorogenic acid were also able to inhibit monoamine oxidase A and tyrosinase as well as enzymes involved in diabetes. This is the first time that cherry juice is reported to inhibit monoamine oxidase A, α-glucosidase and dipeptidyl peptidase-4 in a dose dependent manner, which may be of interest for human health and the prevention of certain diseases.

KEYWORDS: natural products, antioxidant, anthocyanin, dietary polyphenols, chlorogenic acid, diabetes.

1. Introduction

Cherry belongs to the Rosaceae family, and specifically to the genus *Prunus*. The most common types of *Prunus* are *Prunus cerasus* and *Prunus avium*, the first one is known as sour cherry and the last is called sweet cherry. Both are considered nutrient dense food with a relatively low caloric content and a significant amount of important nutrients and bioactive food components [1]. Several studies have confirmed that eating a diet rich in fruits is in relation with a reduced risk of oxidative stress, cardiovascular disease, cancer, neurodegenerative disorders and diabetes [2-6]. This may be due to dietary polyphenols, which are formed by at least one aromatic ring with one or more hydroxyl groups attached [7].

Some of the most common dietary polyphenols presented in fruits and berries are anthocyanidins, which generate several anthocyanins. These anthocyanins are responsible for the red colour of fruits and the potential antioxidant activity. Although cherry is botanically classified as a stone fruit (drupe) due to the pit in the centre, it has the appearance of a berry. Several studies in animal models and in human subjects have demonstrated that dietary polyphenols are bioavailable and exert a protective role against oxidative stress and free radical damages [7]. Antioxidants have the ability to scavenge or to neutralize free radicals, or are necessary to enable other molecules to perform such a function [8].

There are strong evidences demonstrating that several ROS-mediated pathways may be involved in the neurodegenerative diseases, like Alzheimer's disease (AD) and Parkinson's disease (PD). It has been described that the accumulation of iron ion in the brain leads to higher ROS generation, involvement of mitochondrial pathways and to a decrease of endogenous antioxidants levels. Thus natural antioxidants may prevent neurodegenerative disorders [9].

Although mechanisms remain unclear, a body of evidence links type-2 diabetes with dementia and neurodegenerative diseases [10]. One therapeutic approach to treat diabetes is to retard the absorption of glucose via inhibition of enzymes, such as α-glucosidase, in the digestive organs. It has been confirmed that α-glucosidase activity *in vitro* can be inhibited by berry extracts, i.e. blueberry, blackcurrant, strawberry, and raspberry rich in polyphenols [11]. In recent years, there has also been an increasing interest in the ability of dietary factors to treat diabetes via modulating GLP-1 levels. GLP-1 is secreted from

enteroendocrine L cells, which are present in the lower small intestine and large intestine, and stimulates insulin secretion in a blood glucose concentration dependent manner. GLP-1 is inactivated by dipeptidyl peptidase-4 (DPP-4), a circulating catabolic enzyme, resulting in a rather short half-life of about two minutes in the blood. There are reports that non-nutrient dietary factors such as polyphenols can affect GLP-1 levels [12].

The aim of this study is to evaluate the bioactive properties of pure cherry juice in terms of antioxidant potential as well as activity in pharmacological targets of neurological diseases and diabetes. Antioxidant and protective effects of the juice have been studied in cellular and cell free systems. Potential inhibition of enzymes with relevant biological properties such as acetylcholinesterase, monoamine oxidase-A, tyrosinase, α-glucosidase and dipeptidyl peptidase 4 has also been carried out.

2. Materials and methods

2.1. Reagents and chemicals

Chemical reagents were acquired through Sigma-Aldrich, Cayman Chemical, Cymit química and Panreac (Spain). Cherry juice (Rabenhorst®) was kindly supplied by Natur Import. The juice is 100% organic, contains no additives and was obtained by pasteurization and expression and bottled into amber bottles, according to the manufacturer.

2.2 Cherry juice lyophilization

330 ml of Rabenhorst® cherry juice were lyophilized using Genesis VirTis 25 EL lyophilizer (Wizard 2.0 control system) during 7 days. The liquid sample was frozen at -80 ºC during 2 h while the lyophilizer was freezing at -80 ºC. After that, the temperature was modified at -30 ºC for a couple of hours and during 96 h at -60 ºC. Next transition was at -40 ºC again (4 h) and 24 h at -60 ºC. Finally, temperature grew up until -15 ºC (7 h) and dried 22 h at 20 ºC. Last 2 h temperature was established at 40 ^{O}C. A dried red powder was obtained and kept at -20 ^{O}C in a freezer until experiments were done.

2.3. Phytochemical analyses of lyophilized cherry juice

2.3.1. Total Polyphenols quantification

The Folin-Ciocalteu assay was used to quantify total phenolic compounds in cherry juice. 9 µl of the sample was mixed with 201 µl of Folin-Ciocalteu reagent. The sample was incubated 5 min at room temperature and preserved for the light; 90 µl of Na_2CO_3 (10%) was added to the mixture and incubated in the dark at room temperature for 40 min. Absorbance was measured at 752 nm. The standard curve was performed with different concentrations of gallic acid: 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625 and 0.0078125 mg ml^{-1}. Total polyphenol content is expressed as mg GAE (Gallic Acid Equivalents) per mg cherry juice lyophized [13].

2.3.2. HPLC-DAD analysis and anthocyanins quantification

Phytochemical screening of the cherry juice was performed by HPLC using an Agilent 1260 Infinity LC (column Eclipse plus C18 4.6 x 100 mm, 5 µm) equipped with a photodiode array detector. 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 [14]. 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}. The injection volume was 10 µl and the concentration of the sample was 40 mg ml^{-1}. Gallic acid, ellagic acid, chlorogenic acid, catechin and cyanidin 3-glucoside were used as standards with a concentration of 1 mg ml^{-1} in order to detect and compare peaks in cherry juice. Standards were dissolved in methanol. For detection of compounds, the chromatograms were recorded at 260, 280, 320, 360 and 520 nm. Polyphenols were identified according to retention times of standard pure compounds, elution order and comparing with a bibliographic revision of main phenolic compounds. Total anthocyanins were also quantified by HPLC at 520 nm using cyanidin 3-glucoside (kuromanin chloride) as standard for calibration curve.

2.4. Cytotoxicity screening in HeLa cells

HeLa cells were used to perform a cell viability test (MTT assay) [15]. HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine. Cultures were incubated in the presence of 5% CO2 at 37 ºC under a 100% relative humidified atmosphere. Cells were seeded in 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 cherry juice (0.001-1 mg ml-1) for 72 h and a MTT solution was added and incubated for 3 h at 37 ^oC. Cell survival was measured as the reduction of MTT into formazan at 550 nm in a microplate reader. Experiments were performed twice.

2.5. Antioxidant activity assays

2.5.1. Protective effects of cherry juice against hydrogen peroxide induced toxicity in *Artemia salina*

Dried cysts of *Artemia salina* were hatched in seawater with aeration for a whole week. Lyophilized cherry juice was dissolved in seawater and transferred to a 6-well plates at different concentrations (250, 500 and 1000 µg ml^{-1}) in 5 ml seawater with 10 nauplii in each well. Control wells were filled with 5 ml seawater and 10 nauplii also. After 24 h incubation at room temperature, survival viability was calculated.

As the viability of *Artemia salina* nauplii was not affected by different concentrations of cherry juice, the experiment was performed adding hydrogen peroxide to the wells at a concentration of 0.4 g L^{-1}. Two different control wells without cherry juice were also set, one with hydrogen peroxide and another with seawater. The viability of the nauplii study was measured every 24 h for 72 h [12].

2.5.2. Superoxide radical scavenging activity

Cherry juice was tested in the xanthine/xanthine oxidase assay in order to measure the capacity to scavenge superoxide radicals [16]. 90 µM xanthine, 16 mM Na_2CO_3, 22.8 µM NBT were dissolved in phosphate buffer pH=7 to reach a volume of 240 µl. Then, 30 µl of sample and 30 µl of xanthine oxidase (168 U L^{-1}) were added to start the reaction. The mixture was incubated for 2 min at 37 ^oC. Absorbance was measured at 560 nm and the activity of cherry juice was determined by checking the trasformation of NBT to the blue chromogen dye by the superoxide radical (O_2^-). Decreased absorbance of the

reaction mixture indicated increased superoxide anion scavenging activity. Gallic acid was used as reference compound.

2.5.3. Antiradical activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals

This assay is based on the measurement of the scavenging capacity of antioxidants [17]. The odd electron of the nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine. 150 µl of a DPPH methanolic solution were added to 150 µl of different cherry juice concentrations dissolved in MeOH. Absorbance was measured at 517 nm after 30 min of reaction at in a microplate reader. Controls contained a DPPH solution and the solvent. Radical Scavenging capacity was calculated by the formula: RSC (%) = $[(Abs_{control} - Abs_{sample})/Abs_{control}]$ x 100. Ascorbic, gallic and chlorogenic acids were also measured to compare with cherry juice antioxidant activity.

2.6. Bioassays regarding CNS enzymes

2.6.1. Acetylcholinesterase (AChE) inhibition

The Ellman's method was selected to perform the experiment using a 96-microplate reader [18]. Each well contained 25 µl of 15 mM ATCI in Milipore 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), 25 µl juice in buffer A (50 mM Tris-HCl, pH=8). Finally, 25 µl of the enzyme (0.22 U L^{-1}) was added to start the reaction. Absorbance was read 13 times times every 13 s at 405 nm. Galantamine was used as reference substance.

2.6.2. Monoamine oxidase A (MAO-A) inhibition

The activity was measured in a 96-well microplate using a described procedure [19]. Each well contained 50 µl of cherry 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 cherry juice. The absorbance was read at 490 nm every 5 min during 30 min. Clorgyline was used as reference substance.

2.6.3. Tyrosinase (TYR) inhibition

The assay was conducted in 96-well microplates using a microplate reader to measure absorbance at 475 nm [20]. 10 µl of cherry juice 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 cherry juice. α-Kojic acid was used as reference substance.

2.7. Bioassays regarding enzymes involved in type 2 diabetes

2.7.1. Inhibition of α-glucosidase (α-GLU)

The capacity of cherry juice to inhibit α-glucosidase was measured in a 96-well microplate reader at 405 nm [21]. 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 solvent (milipore water) instead of cherry juice. Acarbose was used as reference compound.

2.7.2. Inhibition of dipeptidyl peptidase-4 (DPP-4)

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

2.8. Statistical analysis

Results were expressed as the mean ± standard error of experiments performed in triplicates. GraphPad Prism v.5 was required to perform data analyses, nonlinear regressions and statistics.

3. Results

3.1. Phytochemical analysis of lyophilized cherry juice

Polyphenol content was measured by the Folin-Ciocalteu method and expressed as gallic acid equivalents (GAE). Cherry juice contained 9.835 ± 1.092 μg GAE mg^{-1} of lyophilized cherry juice. Only one (chlorogenic acid) out of five monitored polyphenolic compounds (gallic acid, ellagic acid, chlorogenic acid, catechin and cyanidin 3-glucoside) was detected and confirmed comparing retention times and UV spectra with standards (Fig. 1A). Anthocyanins were quantified by HPLC at 520 nm using cyanidin-3-glucoside chloride as external standard following the literature. Anthocyanins were found to be 0.301 ± 0.1735 μg cyanidin-3-glucoside equivalents mg^{-1}. However, other anthocyanins different from cyanidin 3-glucoside (also known as kuromanin) might be responsible for the red colour (Fig. 1B).

3.2. Cytotoxicity screening in HeLa cells

Cherry juice showed very mild antiproliferative 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 cherry components. Cell viability was approximately 60% at the highest tested concentration (1 mg/ml), which means that the juice is not considered cytotoxic in this type of cervix cancer cells (Fig. 2).

3.3. Antioxidant activity assays

Fig. 3 indicates that cherry juice increased the survival of *Artemia salina* nauplii compared to 0.4 g L^{-1} hydrogen peroxide at 24, 48 and 72 h. Different concentrations of cherry juice enhanced survival for nauplii exposed to hydrogen peroxide, reaching more than 90% at 24 h. At 48 h, survival of nauplii was around 30-50% and finally, at 72 h between 10-20% of the nauplii survived compared to 0 % of survival of nauplii exposed to hydrogen peroxide. Significant differences were only obtained at doses of 1000 and 500 μg/ml at 24h and 72h.

Fig. 4 shows the antioxidant effect of cherry juice compared to a reference standard such as gallic acid on superoxide radicals generated by the xanthine/xanthine oxidase system. IC_{50} values in this case were 54 μg ml^{-1} for cherry juice and 0.044 μg ml^{-1} for gallic acid.

DPPH radical scavenging effects of cherry juice are shown in Fig.5. The antiradical activity of cherry juice is this time compared with gallic, ascorbic and chlorogenic acid. IC_{50} values were also calculated by nonlinear regression. IC_{50} values were 236 μg ml^{-1} for cherry juice, 10 μg ml^{-1} for chlorogenic acid, 3 μg ml^{-1} for ascorbic acid and 1 μg ml^{-1} for gallic acid.

All this data indicate that the antioxidant and potential protective effects of cherry juice may be due to radical scavenging properties as it has been demonstrated through the DPPH and superoxide radical assays. In addition, the presence of polyphenols such as chlorogenic acid and anthocyanins in the juice, which was confirmed in the phytochemical analyses, seems to be crucial for the antioxidant and antiradical activities.

3.4. Bioassays regarding CNS enzymes

Cherry juice did not show activity in the AChE assay; however, it showed a clear dose dependent MAO-A inhibitory activity. Cherry and clorgyline inhibitions are shown in Fig. 6. IC_{50} values were calculated by nonlinear regression (0.02 μg ml^{-1} for clorgyline, 246.19 μg ml^{-1} for cherry juice). TYR inhibitory activity was not so clear as for MAO-A. Cherry juice produced a very mild inhibition (28% at 1mg/ml) like Fig. 6 shows. In both cases for MAO-A and TYR, chlorogenic acid showed a higher inhibition than cherry juice.

3.5. Bioassays regarding type 2 diabetes enzymes

Cherry juice exhibited *in vitro* an inhibition of α-GLU, but this activity was moderate compared to chlorogenic acid and acarbose, which is a reference inhibitor of this enzyme. As represented in Fig. 7, IC_{50} values were calculated by nonlinear regression (2783 μg ml^{-1} for cherry juice, 996 μg ml^{-1} for chlorogenic acid and 380 μg ml^{-1} for acarbose).

Sitagliptin, an antidiabetic drug, showed a clear dose dependent DPP-4 inhibition. In addition, the effects of cherry juice and chlorogenic acid in this enzyme are shown in Fig. 8. IC_{50} values were calculated by nonlinear regression (0.1 μg ml^{-1} for sitagliptin and 1003.41 μg ml^{-1} for cherry juice).

4. Discussion

Cherry juice is a good source of phytochemicals, specifically polyphenols and anthocyanins, which are the polyphenols responsible of the red skin and flesh colour [22].

The concentration of total phenolics (TP) was 9.835 ± 1.092 μg GAE mg^{-1} of lyophilized cherry juice (approx.. 100 mg/100 g), which is a lower concentration compared to other juices such as pomegranate [13] but can still be of significant importance to produce health benefits. HPLC-DAD analysis showed a peak of chlorogenic acid and previous works reveal that hydroxicinnamates such as caffeoylquinic acids are the main polyphenols in sweet and sour cherries [22]. Anthocyanins were also quantified by HPLC-DAD, obtaining approximately 30 mg /100 g. Other authors such as Wojdyło et al. compared 33 types of sour cherry in terms of polyphenol content and antioxidan activity [23]; our results are within the range of anthocyanins calculated for different cherry cultivars (7.56−94.20 mg/100 g) although the authors studied fruit content instead of juices as in our case.

Cherry juice is used in sport medicine to prevent muscle damage as some studies have shown that sour cherry is able to prevent these symptoms through anti-inflammatory and antioxidant properties [24-29]. Our results confirm the antioxidant potential of sour cherry juice. The protective effects against toxicity induced by hydrogen peroxide were measured using living organisms (*Artemia salina*). This experiment was performed by the authors using cherry juice as a co-treatment with hydrogen peroxide and the study demonstrated significant differences at 1000 and 500 μg/ml at 24h and 1000 μg/ml at 72h. Cherry juice was also able to scavenge both DPPH and superoxide radicals. It can be deduced that the antioxidant activity is mainly provided by polyphenols such as chlorogenic acid and anthocyanins. Other studies quantified *Prunus cerasus* antioxidant capacity using trolox as standard, which makes it difficult to compare with our results [30].

In addition, the study of the activity of cherry juice on enzymes was divided in two main groups, one related to central nervous system and another to glucose metabolism. We found for the first time that cherry juice was able to inhibit MAO-A and TYR. MAO-A is involved in deamination of catecholamines and serotonin and certain polyphenols such as anthocyanins are involved in this inhibition, which can drive to an antidepressant and anxiolytic effect [31]. Tyrosinase is a copper-containing enzyme

essential for tyrosine-melanin pigmentation and the role of toxic quinones in dopamine-induced neuronal damage catalyzed by TYR has been cleared in a number of studies [32]. According to our data, cherry juice may have potential as a neuroprotective agent via MAO-A or TYR inhibition; in fact, a recent interventional human study showed that consumption of anthocyanin-rich cherry juice for 12 weeks improves memory and cognition in older adults with mild-to-moderate dementia [33].

Finally, the inhibition of enzymes involved in glucose metabolism and type 2 diabetes was studied; this is the first time that cherry juice is reported to inhibit α-glucosidase and DPP-4 in a dose dependent manner. Anthocyanin content in fruits is also related with α-glucosidase inhibition [34]. According to our results, chlorogenic acid is also responsible for the activity. Polyphenols have also shown to facilitate 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 represent 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, cherry juice might be an interesting antioxidant nutritional tool to prevent these disorders.

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Conflicts of interest

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

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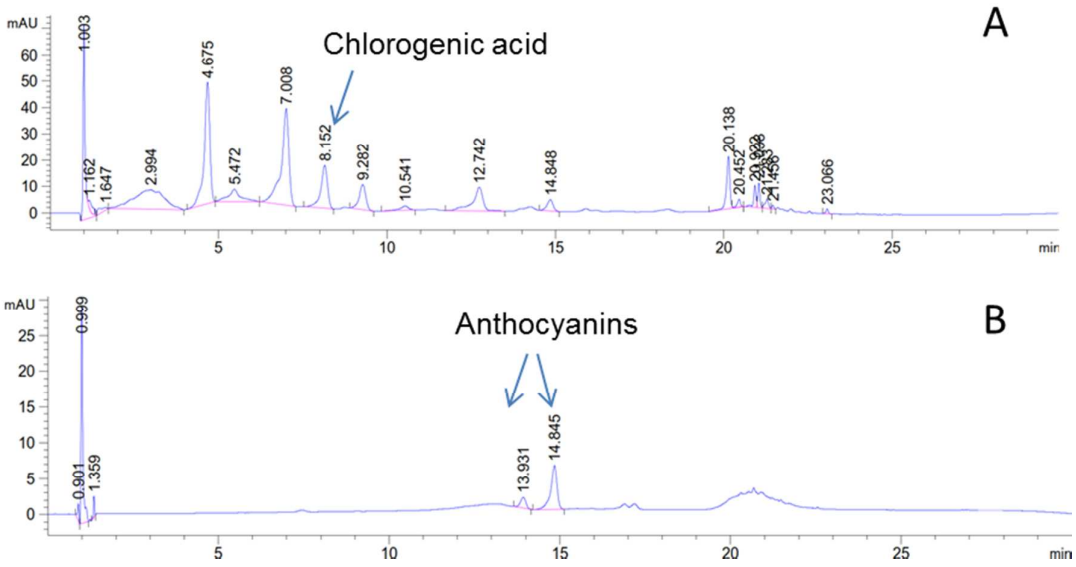


Figure 1. HPLC chromatogram at 320 nm (A) and 520 nm (B) of lyophilized cherry juice. Chlorogenic acid was detected at 320 nm (retention time = 8.152 min) and anthocyanins at 520 nm (retention time = 14.845 min)

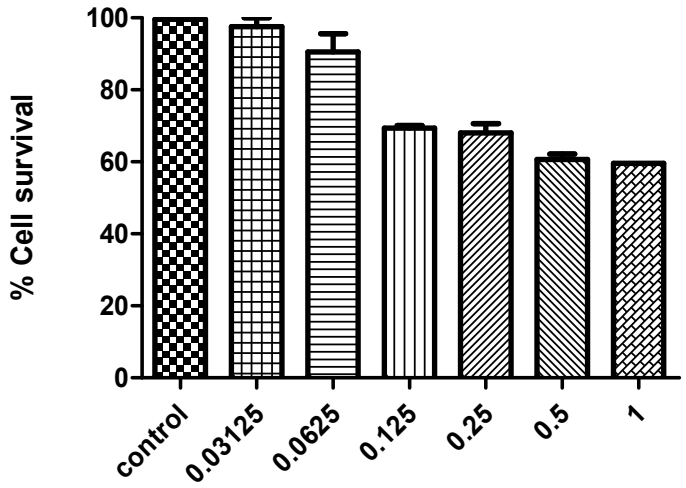


Figure 2. Viability of HeLa cells exposed to different concentration of cherry juice for 72 hours in the MTT assay. IC_{50} was not calculated as percentages of viability were more than 50 %

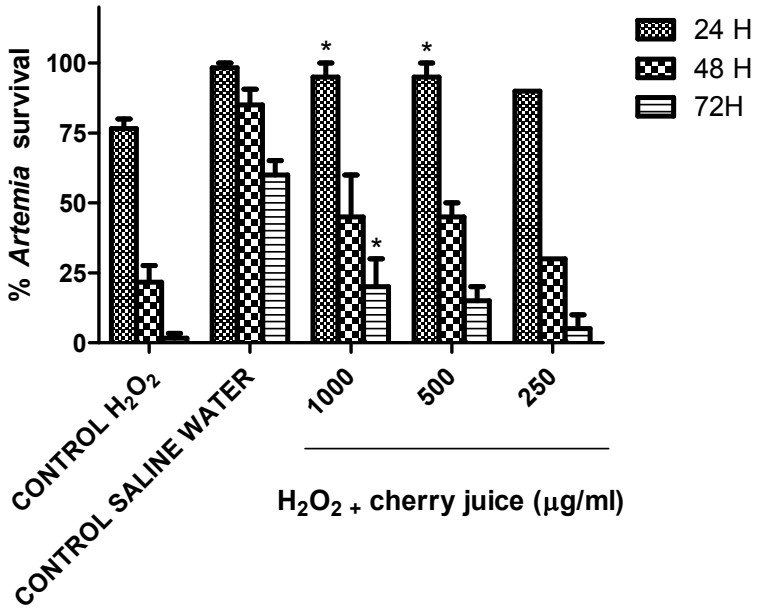


Figure 3. Effects of cherry juice in *Artemia salina* nauplii exposed to hydrogen peroxide (0.4 g L^{-1}). * Significant differences ($p < 0.05$) were detected between nauplii exposed to hydrogen peroxide (Control H_2O_2) and nauplii exposed to hydrogen peroxide + 1000 µg/ml cherry juice at 24h and 72 h. A lower dose of cherry juice (500 µg/ml) also showed a protective effect at 72 h. Differences were calculated using Student t tests.

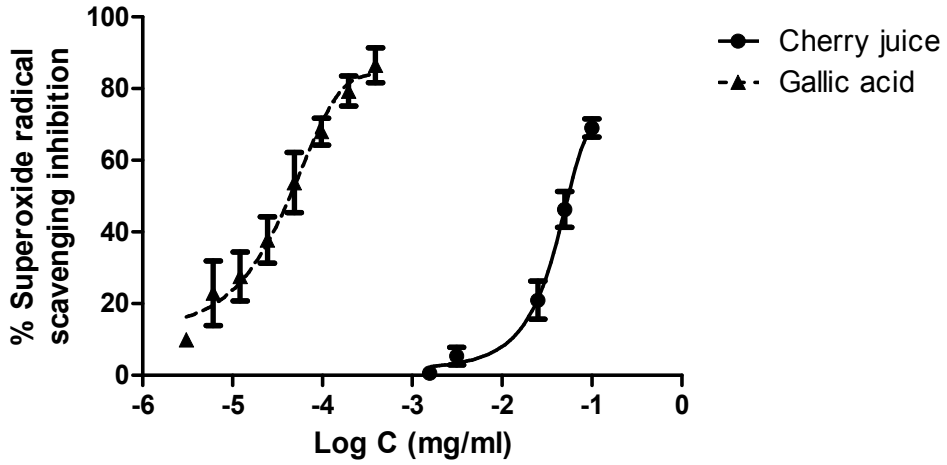
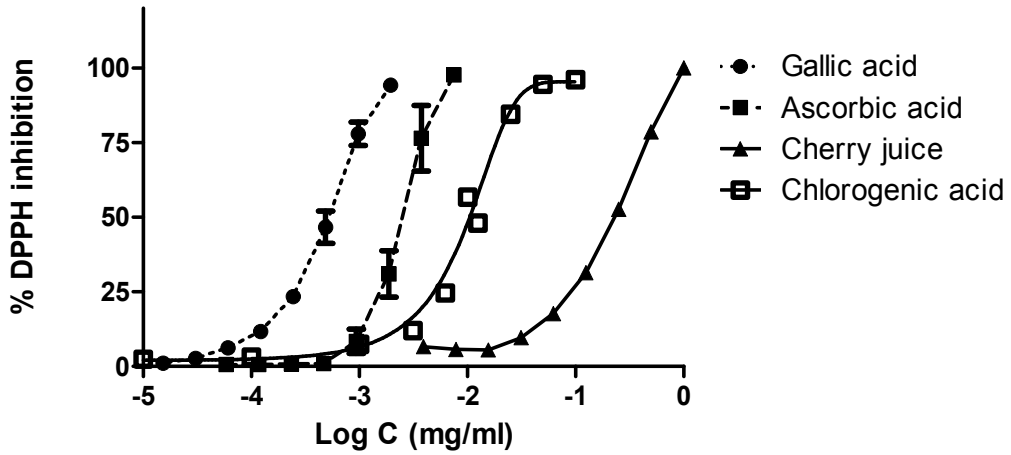
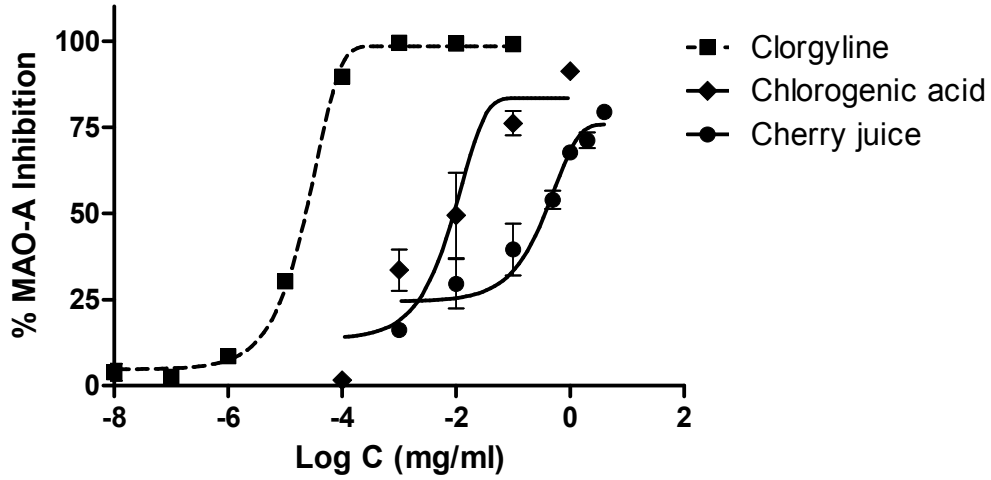


Figure 4. Antioxidant activity of cherry juice and gallic acid against superoxide radicals generated by the xanthine/xanthine oxidase system.



452

453 Figure 5. Antiradical activity of cherry juice, ascorbic acid, gallic acid and chlorogenic
454 acid against DPPH.



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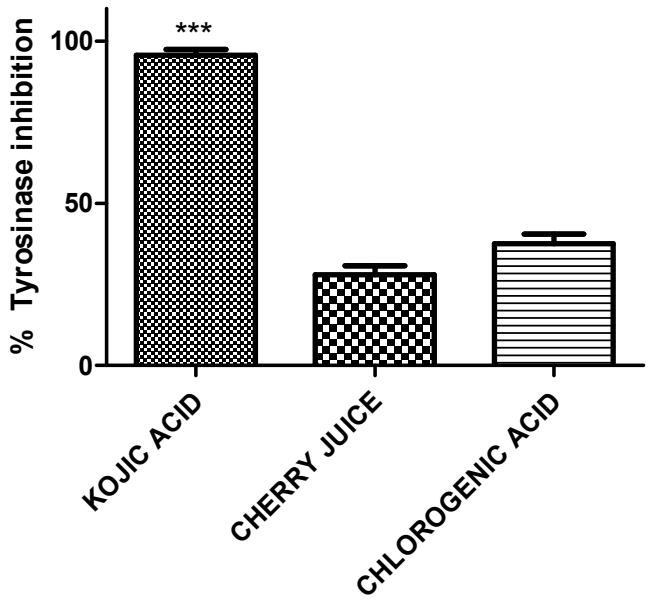
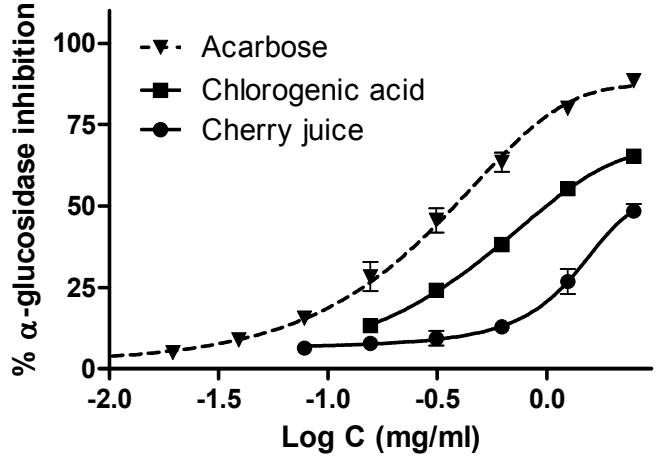
457 Figure 6. MAO-A inhibition performed by cherry juice, chlorogenic acid and clorgylyne



458

459 Figure 7. Tyrosinase inhibition performed by cherry juice (1mg/ml), chlorogenic acid (1
460 mg/ml) and kojic acid (0.2 mg/ml) as standard. *** $p < 0.0001$ *versus* cherry juice and
461 chlorogenic acid (One way ANOVA with post-hocTukey test).

462



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464 Figure 8. α-Glucosidase inhibition performed by cherry juice, chlorogenic acid and
465 acarbose as standard.

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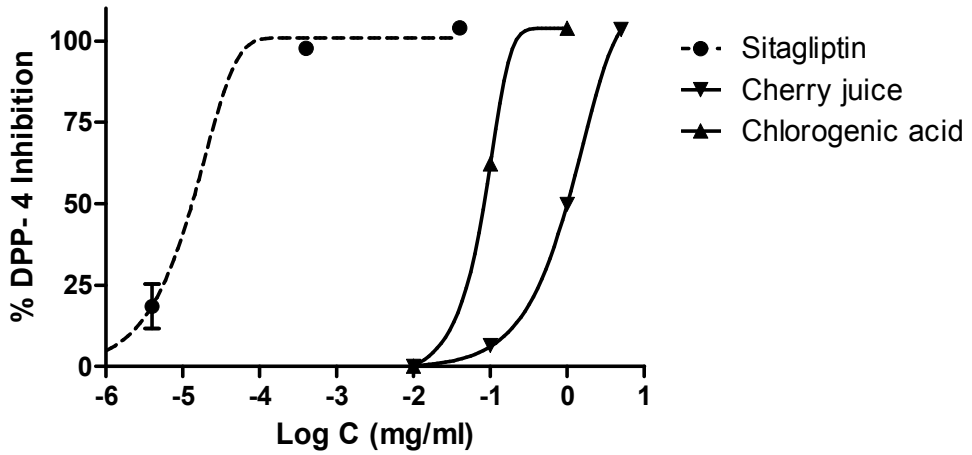

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468 Figure 9. Dipeptidil peptidase-4 inhibition performed by cherry juice, chlorogenic acid
469 and sitagliptin as standard.

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