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Sweet cherries from Fundão as health promotors:
chemical characterization and biological potential

Ana Carolina Almeida Gonçalves

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Orientador: Prof. Doutor Luís Rodrigues da Silva
Co-orientador: Prof.ª Doutora Branca Monteiro da Silva
Co-orientador: Prof. Doutor Marco Alves

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Dedication

To the most important persons of my life, my parents, my little brother, my grandmother, and my boyfriend, for everything they do for me. A *thank you* will never be enough!

"Sou feliz, muito feliz.

Querem saber a razão?

Tenho paz e amor, e agradeço tudo à minha família do fundo do coração."

Obrigada!

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Step-by-step, dreams come true!

Resumo

As cerejas são um dos frutos de verão mais apreciados pelos consumidores devido à sua cor, sabor doce, elevado conteúdo em água, baixo teor em calorias e por possuírem numerosos compostos bioativos na sua constituição, que produzem efeitos metabólicos e fisiológicos importantes para a prevenção de algumas patologias, tais como diabetes, doenças cardiovasculares e cancro. Portugal produz cerca de 15 mil toneladas de cerejas por ano, sendo a maioria destas cultivadas no nordeste do País, nomeadamente na região da Beira Interior (Fundão, Covilhã e Belmonte). Neste trabalho, procedeu-se à determinação do perfil fenólico e do potencial biológico de cinco variedades de cereja do Fundão (*Saco, Sweetheart, Satin, Maring* e *Hedelfinger*). A avaliação por cromatografia líquida com deteção por arranjo de díodos permitiu a identificação de um total de vinte e três compostos fenólicos, incluindo um ácido hidroxibenzóico, oito ácidos hidroxicinâmicos, cinco flavonóis, três flavan-3-óis e seis antocianinas, comprovando-se que as cerejas são uma excelente fonte natural de compostos bioativos, principalmente ácidos hidroxicinâmicos e antocianinas. O potencial antioxidante foi avaliado através do FRAP e contra os radicais DPPH e óxido nítrico. Após a análise dos resultados obtidos, constatou-se que a variedade *Sweetheart* foi a que apresentou maior potencial antioxidante no FRAP (IC_{50} = 26,7 μM Fe^{2+}). As variedades *Hedelfinger* e *Maring* foram as mais ativas contra os radicais DPPH e óxido nítrico (IC_{50} = 12,1 μg/mL e IC_{50} = 140,9 μg/mL, respetivamente). Seguidamente, avaliou-se o potencial antidiabético dos extratos das cerejas através da determinação da capacidade inibitória da *α*-glucosidase, destacando-se a variedade *Hedelfinger* como a mais ativa (IC_{50} = 10,3 μg/mL). Posteriormente, analisou-se a capacidade dos extratos da variedade *Saco* para inibir a oxidação da hemoglobina e da hemólise dos eritrócitos humanos. Ambos os ensaios revelaram um efeito inibitório dependente da concentração (IC_{50} = 38,57 μg/mL e IC_{50} = 73,03 μg/mL, respetivamente). Em suma, verificou-se que a *Saco* e a *Hedelfinger* foram as variedades mais ativas, tendo-se verificado uma correlação entre o conteúdo fenólico das diferentes variedades de cereja e o potencial biológico apresentado. Os resultados obtidos neste estudo permitiram concluir que as cerejas possuem um grande potencial biológico, sendo ainda necessária a realização de mais estudos para incentivar a utilização dos extratos de cereja em suplementos alimentares e em novas aplicações terapêuticas, farmacológicas e nutracêuticas.

Palavras-chave

Cereja, Compostos fenólicos, Benefícios para a saúde, Potencial biológico.

Resumo alargado

Os frutos e vegetais são importantes objetos de estudo científico pela importância que possuem numa alimentação saudável, a nível medicinal e terapêutico. Esta relevância prende-se com os inúmeros compostos bioativos que fazem parte das suas composições, conferindo-lhes propriedades antioxidantes, antimicrobianas, anti-inflamatórias, entre outras.

As cerejas (*Prunus avium* Linnaeus) são dos frutos de verão mais apreciados pelos consumidores devido à sua cor, sabor doce, elevado conteúdo em água, baixo teor em calorias e por possuírem numerosos compostos bioativos na sua constituição, que produzem efeitos metabólicos e fisiológicos importantes na prevenção de algumas patologias, tais como diabetes, doenças cardiovasculares e cancro, sendo, atualmente um dos frutos mais consumidos e com maior importância económica em Portugal.

Neste trabalho realizou-se a determinação do perfil fenólico (fenóis não corados e antocianinas) e avaliou-se o potencial biológico de cinco variedades de cereja provenientes do Fundão (*Saco*, *Sweetheart, Satin, Maring* e *Hedelfinger*).

As determinações por deteção através do arranjo de díodos permitiram a identificação e quantificação de um total de vinte e três compostos fenólicos, incluindo um ácido hidroxibenzóico, oito ácidos hidroxicinâmicos, cinco flavonóis, três flavan-3-óis e seis antocianinas.

Relativamente aos compostos fenólicos corados, o seu conteúdo variou entre 1077,0 e 2183,6 μg/g, sendo a variedade *Maring* a mais rica em antocianinas, seguida pelas variedades *Hedelfinger* e *Saco*. Em contrapartida, a *Satin* mostrou ser a variedade mais pobre neste tipo de compostos. A cianidina-3-*O*-rutinósido foi quantificada como sendo o composto maioritário, variando entre 87,7% e 91,9% relativamente ao conteúdo total dos compostos corados e o segundo mais comum nas cerejas, representando 42,5% a 68,7% relativamente ao teor total dos compostos fenólicos.

Em relação aos compostos fenólicos não corados, o seu conteúdo total variou entre 389,1 e 2024,4 μg/g. A variedade *Hedelfinger* revelou ser a mais rica neste tipo de compostos, seguida pelas variedades *Saco* e *Satin,* enquanto que a *Sweetheart* foi a que exibiu o conteúdo mais baixo. Os ácidos fenólicos são o constituinte maioritário das cerejas (variando entre 86,3% e 95,2% relativamente ao teor total dos compostos fenólicos não corados), sendo o ácido 3-*O*-caffeolquínico o composto maioritário em todas as variedades, excepto na *Satin*, onde o ácido *p*-cumaroilquínico foi encontrado em maiores quantidades. Em relação aos

flavonóides não corados (flavonóis e flavan-3-óis), a epicatequina (flavan-3-ol) foi o flavonóide não corado presente em maior quantidade na variedade *Saco* (correspondendo a 3,2% do total dos flavonóides não corados). Por outro lado, os flavonóis mostraram ser os mais abundantes nas outras variedades, sendo a quercetina-3-*O*-rutinósido o composto maioritário nas variedades *Sweetheart*, *Maring* e *Hedelfinger* (6,3%; 2,8% e 2,2 % em relação ao teor total dos compostos fenólicos não corados, respetivamente). A quercetina-3-*O*-glucósido foi o flavonóide não corado mais abundante na variedade *Satin* (1,6% do teor total dos compostos fenólicos não corados).

O metabolismo celular gera radicais livres de azoto e de oxigénio, necessários para a sobrevivência e atividade das células que, quando presentes em excesso, danificam os lípidos, as proteínas e os ácidos nucleicos, aumentando o risco de ocorrência de diversas patologias, tais como cancro, diabetes, gota e doenças cardiovasculares. O organismo humano produz antioxidantes durante o seu metabolismo normal, mas estes também podem ser obtidos por via exógena a partir dos alimentos, em especial a partir do consumo de frutos e vegetais, que são considerados como boas fontes de antioxidantes naturais, promovendo a proteção contra os radicais livres. A atividade antioxidante dos extratos das cerejas foi avaliada através de ensaios *in vitro* do FRAP e contra os radicais DPPH e óxido nítrico. A variedade *Satin* revelou ser a mais ativa para o FRAP (26,7 μM Fe^{2+}) e a variedade *Maring* foi a mais ativa contra o radical de óxido nítrico (IC_{50} = 140,9 ± 1,9 μg/mL). A variedade *Hedelfinger* foi a que obteve melhores resultados contra o $DPPH^{\bullet}$ (IC_{50} =12,1 ± 0,4 μg/mL). De uma forma geral, as variedades mais ativas foram as que apresentaram um maior conteúdo em compostos fenólicos. Esta atividade deve-se ao conteúdo e à estrutura destes compostos, onde a presença de um grupo catecol e de grupos hidroxilo aumentam a capacidade para capturar espécies reativas e de doação de hidrogénios.

A diabetes *mellitus* é das doenças inflamatórias mais prevalentes e sem cura conhecida. Esta doença metabólica é caracterizada por hiperglicemia resultante de defeitos na secreção e/ ou na ação da insulina. Uma das terapias adotadas para o seu tratamento é inibir a ação de enzimas-chave, como a *a*-glucosidase, que convertem os hidratos de carbono em monossacarídeos para serem absorvidos. Inibidores desta enzima, como os compostos fenólicos, retardam a absorção da glucose, normalizando os níveis de açúcar e aumentando a sensibilidade à insulina. A avaliação do potencial antidiabético dos extratos das cerejas foi realizada pela primeira vez neste trabalho através da análise da capacidade inibitória da *a*-glucosidase. As variedades *Hedelfinger* (IC_{50} = 10,2 ± 0,5 μg/mL), *Saco* (IC_{50} = 10,7 ± 0,4 μg/mL) e *Maring* (IC_{50} = 11,4 ± 0,5 μg/mL) foram as mais ativas. Todos os extratos de cereja analisados revelaram maior atividade inibitória em relação à *a*-glucosidase quando comparados com o controlo acarbose, que é um dos fármacos mais comercializados na inibição de enzimas envolvidas na diabetes m*ellitus*. Para além dos compostos fenólicos conseguirem captar os radicais de óxido nítrico, largamente aumentados em doenças

inflamatórias como a diabetes *mellitus*, também são capazes de competir com o substrato desta enzima, diminuindo os níveis de absorção da glucose.

Os eritrócitos são outras das células facilmente danificadas pelas espécies reativas, devido ao facto de terem na sua constituição um grande número de ácidos gordos e por serem um local de transporte de oxigénio. Os radicais livres, quando atacam os eritrócitos, comprometem o transporte deste gás, causando fenómenos de hipoxia e aumentando os casos de anemia hemolítica. Nesta dissertação, avaliou-se pela primeira vez o potencial dos extratos da variedade *Saco* para protegerem os eritrócitos humanos contra $ROO^{\bullet}$ a nível da oxidação da hemoglobina e da hemólise. Os resultados obtidos comprovaram a existência de um efeito inibitório dependente da concentração em ambos os ensaios (IC_{50} = 38,6 ± 1,0 µg/mL e IC_{50} = 73,0 ± 1,5 µg/mL, respetivamente). A proteção conferida aos eritrócitos deve-se à capacidade que os compostos fenólicos apresentam para se ligarem à membrana, aumentando a rigidez e resistência desta contra os radicais livres.

Os dados obtidos através deste estudo comprovam que as cerejas apresentam grande potencial biológico, principalmente devido à sua capacidade antioxidante, protegendo as células dos danos causados pelos radicais livres. Para além disso, apresentam propriedades antidiabéticas e de proteção dos eritrócitos. Estas descobertas são relevantes para o futuro desenvolvimento de novas abordagens terapêuticas, farmacológicas e nutracêuticas, e para a incorporação dos seus extratos em suplementos alimentares.

Abstract

Cherries are one of the most appreciated summer fruits by consumers because of their attractive color, sweet taste, high water content, low level of calories and due to the presence of numerous bioactive compounds in their constitution, which produce metabolic and physiological effects, that are important to prevent some pathologies, such as diabetes, cardiovascular diseases and cancer. Portugal produces about 15 thousand tons of cherries per year, most of them cultivated in the northeast of the country, more specifically in the regions of Beira Interior (Fundão, Covilhã and Belmonte). In this work, we evaluated the phenolic profile and biological potential of five varieties of sweet cherries from Fundão region (*Saco*, *Sweetheart, Satin, Maring* e *Hedelfinger*). The analysis performed by liquid chromatography with diode-array detection allowed to identify a total of twenty-three phenolic compounds, including one hydroxybenzoic acid, eight hydroxycinnamic acids, five flavonols, three flavan-3-ols and six anthocyanins, confirming that cherries are an excellent natural source of bioactive compounds, mainly hydroxycinnamic acids and anthocyanins. The antioxidant potential was assessed by FRAP, and against DPPH and nitric oxide radicals. After a detailed analysis of the obtained results, it was possible to infer that *Sweetheart* cultivar showed the highest antioxidant potential in FRAP (26.7 μM Fe^{2+}), while *Hedelfinger* and *Maring* were the most active against DPPH and nitric oxide radicals (IC_{50} = 12.1 μg/mL e IC_{50} = 140.9 μg/mL, respectively). Thereafter, it was studied the antidiabetic potential of cherries extracts by determination of the inhibitory capacity of α-glucosidase, highlighting *Hedelfinger* cultivar as the most active (IC_{50} = 10.3 μg/mL). Subsequently, it was assessed the capacity of the extracts from *Saco* to inhibit the oxidation of hemoglobin and the hemolysis of human erythrocytes. Both assays revealed an inhibitory effect dependent on the concentration (IC_{50} = 38.6 μg/mL e IC_{50} = 73.0 μg/mL, respectively). In a general way, it was found that *Saco* and *Hedelfinger* were the most active, having been found a correlation between the phenolic content of the different varieties of cherries and the biological potential displayed by extracts. The results obtained with this study allowed us to conclude that sweet cherries possess a great biological potential, being necessary to carry out further investigations in order to encourage the use of cherries extracts in food supplements and in new pharmaceutical and nutraceutical applications.

Keywords

Cherry, Phenolic compounds, Health benefits, Biological potential.

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COX	Cyclooxygenase
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DW	Dry weight
ESBL	Extended spectrum β-lactamase
FRAP	Ferric Reducing Antioxidant Power
FW	Fresh weight
GLUT	Glucose transporter
H_2O_2	Hydrogen peroxide
IL	Interleukin
iNOS	Nitric oxide synthase
IFN-γ	Interferon gamma
LC-DAD	Liquid chromatography with diode-array detection
LDL	Low density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
MAPK	Mitogen-activated protein kinases
$^{\bullet}NO$	Nitric oxide radical
OH	Hydroxyl
$^{\bullet}OH$	Hydroxyl radical
PKC	Protein-kinase C

RNS	Reactive nitrogenous species
ROO•	Peroxyl radical
ROS	Reactive oxygen species
SGLUT1	Sodium-dependent transporter 1
TNF-α	Tumor necrosis factor-alpha
TPTZ	2,4,6-Tripyridyl-s-triazine

I. Introduction

In the last years, it became very evident the linkage between dietary food choices and health risks. Several epidemiological studies have shown the benefits of a non-diet fat (rich in vegetables, fruits and plant infusions) in cardiovascular, neurological and cancer pathologies (Kim et al. 2005; Ferretti et al. 2010; Duarte & Silva 2014; Nunes et al. 2015). Besides, this diet provides a high amount of bioactive compounds (as phenolics), improving the action and benefits of pharmaceutical drugs (Wallace 2011).

Sweet cherries (*Prunus avium* Linnaeus (L.)) are an example of phenolic-fruits sources which have been gaining great scientific interest concerning their beneficial effects. In addition, they also possess few calories and low glycaemic response (McCune et al. 2010). Their chemical constitution offers resistance to oxidative stress (Seeram et al. 2001; Usenik et al. 2008), anti-inflammatory (Seeram et al. 2001; Delgado et al. 2012), antibacterial (Hanbali et al. 2012) and anticancer (Kang et al. 2003; Serra et al. 2011) properties, as well as neurological (Kim et al. 2005) and cardiovascular (Snyder et al. 2011; Bueno et al. 2012) protections and also helps retarding the aging process (Serra et al. 2011).

1. *Prunus avium* L.

Prunus avium L., known as sweet cherries are native from the area between the Black and Caspian seas of Asia Minor. Therefore, they came from regions with a temperate climate, mainly in Mediterranean and Central Europe, North Africa, Near and Far East, South Australia and New Zealand, United States of America and Canada, Argentina and Chile (Bastos et al. 2015), and have their harvest period is between May and July (González-Gómez et al. 2009). Domestic cultivars began with Greeks, followed by Romans (Lim 2012). Sweet cherries are diploid fruits (2n=16) (Sermonti 2009) and also the smallest one that belongs to the Rosaceae family, *Prunoideae* subfamily, *Prunus* genus, and *Cerasus* and *Padus* subgenera (Figure 1) (Ferretti et al. 2010). They are composed by an edible and thin protective red, maroon or purplish black skin (exocarp), an edible red and sometimes white succulent flesh (mesocarp) and an inedible seed (endocarp) (Lim 2012).

Most of commercialized sweet cherries have a red flesh, juice and skin (e.g.: *Benton*, *Blackgold*, *Glacier*, *Hedelfinger*, *Kiona*, *Kordia*, *Kristin*, *Regina*, *Selah* and *Skeena*), but others are constituted by a yellow flesh, clear juice and skin (e.g.: *Gold* and *Nugent*), or by a yellow flesh and skin, that turns to red colours depending to light exposures, and a clear juice (e.g.: *Emperor Francis*, *Napoleon* and *Rainier*) (Mulabagal et al. 2009). Other cultivars also consumed are *0-900 Ziraat*, *Bigarreau*, *Big Burlat*, *Bing*, *Edirne*, *Jerte Valley, Karabodur*, *Lambert*, *Noir de Guben*, *Stella*, *Turfanda* and *Van* (Vursavuş et al. 2006; Beyhan & Karakaş

2009). *Morangão* and *Saco* (or *Saco da Cova da Beira*) are Portuguese sweet cherries cultivars very appreciated around the world. *Saco* possess more antioxidant capacity than *Morangão* due to its phenolic-rich composition in flavan-3-ols (catechin and epicatechin), hydroxycinnamic acids (3-*O*-caffeolquinic, 5-*O*-caffeoylquinic and *p*-coumaroylquinic acids), flavan-3-ols (quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside) and anthocyanins (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, pelargonidin-3-*O*-rutinoside, peonidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside) (Serra et al. 2011).

Figure 1. *Prunus avium* L. flower (A) and fruit (B).

Several factors, such as cultivars, weather conditions, ripeness time of harvest and storage conditions, alter sweet cherries weight, firmness, sweetness and skin colours, influencing consumers cherry choice (Faniadis et al. 2010). The most appreciated cherry it has dark-red colour, sweet taste, large diameter (Kappel et al. 1996), between 28-30 mm (Schmitz-Eiberger & Blanke 2012), is free of cracks and with a perfect round shape (Basanta et al. 2014).

According to Food and Agriculture Organization of the United Nations (FAO 2015), Turkey is the world biggest producer of cherries (480 748 tones), followed by United States of America (384 646 tones), Iran (200 000 tones) and Italy (104 766 tones). It was reported that, in 2015, Portugal produced approximately 10 500 tons of this fruit (Bickford & Valverde 2015).

Most of sweet cherries are produced to be consumed as fresh fruits, but as they are seasonal fruits, they are not available during all year in the supermarket, so they are frozen, brined, canned, dried and processed into jams and juices (McCune et al. 2010).

1.1. Chemical composition of *Prunus avium* L.

Sweet cherries are extremely nutritious and a great source of many bioactive compounds, which work together to increase health-promoting effects. Primary metabolites (sugars and fibers, minerals, vitamins, amino and fatty acids) are used to vital functions, as growth, development (photosynthesis and respiration) and reproduction of the plants; while the secondary metabolites, such as carotenoids (lipophilic), phenolics and ascorbic acid (hydrophilic molecules) act to protect the plants against pathogenic agents, to attract

pollinators and seed-dispersing animals or to suppress the growth of surrounding plants. They are also responsible for the organoleptic properties, of the fruit, in addition with carotenoids and vitamin E, as the smell, taste and colour (Fürstenberg-Hägg et al. 2013).

Sweet cherries are composed by around 80% of water, low caloric content with approximately 60 kcal per 100 g of fresh edible portion (Figure 2 and Table 1). The main nutrients and bioactive compounds found are carbohydrates (sugars and fibers), proteins, fatty acids, organic acids, minerals, amino acids, vitamins , carotenoids, volatile compounds, serotonin, melatonin and phenolic compounds (Table 1 and Figure 2) (Duarte & Silva 2014).

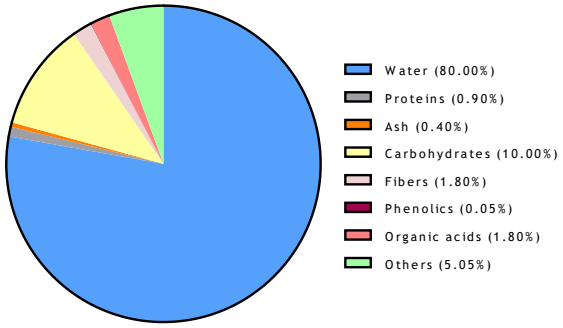


Figure 2. Nutritional composition of sweet cherries.

Moreover, their brix varies between 19.1º and 22.7º (Long 2000) and have a pH between 3.81 and 3.96 (Vavoura et al. 2015).

1.1.1. Macronutrients

Nutrients are environmental substances used to obtain energy for growth and body functions by organisms. Depending on the type of nutrients, these are needed in small amounts (micronutrients) or larger amounts (macronutrients). There are four macronutrients required by human body: carbohydrates (sugars, starches and fibers), proteins, fatty acids and organic acids.

1.1.1.1. Carbohydrates

Carbohydrates (sugars, starches and fibers) are the most common biomolecules in the world and have the empirical formula (CH_2O). Sugars can be divided into monosaccharides, oligosaccharides or polysaccharides. The first two classes have sweet flavor and are the responsible for fruits' taste due to sugars/ acid ratio (Crisosto et al. 2003). Most of fruits contain between 10-25% of sugars, however sweet cherries have lower levels of sugars (13.30%, mostly in edible portion) (Belitz et al. 2009). Glucose and fructose account more than 80% of the total sweet cherries content (6.59 and 5.37 g per 100 g of fresh weight, respectively) (Papp et al. 2010), followed by sorbitol, which ranges between 0.93 to 6.77 g per 100 g of dresh weight (fw) (Ballistreri et al. 2013). Sucrose, maltose and galactose are

also found in sweet cherries, but in small amounts (0.15; 0.12 and 0.59 g per 100 g of fw, respectively) (USDA 2015).

In regards to fibers, sweet cherries are not a great source (their percentage in sweet cherries constitution is around 1.80%), but their presence contributes to increase this fruit benefits, as lipid metabolism, cholesterol and glucose levels, and water-holding capacity improvements, reducing constipation and decreasing cardiovascular and colon pathologies (Kader & Barrett 2005; Dembitsky et al. 2011).

1.1.1.2. Organic acids

Organic acids are important intermediary metabolism products, which interact with sugars, developing aroma, chemical and sensory properties (pH, total acidity, microbial stability and sweetness) of cherries (Mahmood et al. 2012). Sweet cherries are fruits high in several organic acids, representing 1.80% of the total fw. The organic acids previously reported in sweet cherries were ascorbic, citric, fumaric, malic, shikimic and succinic acids (Usenik et al. 2008; Serradilla et al. 2011; Demir 2013). Malic acid is the most common organic acid in this fruit (353 to 812 mg per 100 g of fw) accounting for more than 98% of the total content of organic acids found in sweet cherries, followed by citric (11 to 54 mg per 100 g of fw), shikimic (0.66 to 2.67 mg per kg of fw) and fumaric acids (0.10 to 0.76 mg per 100 g of fw) (Usenik et al. 2008).

The metabolization of these organic compounds inside cells by the Krebs cycle originates many constituents as amino-acids (which are the building blocks of proteins) and provides the energy required for maintenance of cell integrity (Kader & Barrett 2005). Furthermore, organic acids also have many physiological functions in plants protection, including the induction of systemic resistance against pathogens (bacteria, fungi and viruses) and prevention of degradation of anthocyanins, by conjugating with them, generally with a glucose at position 3, and esterification, mainly with citric, malic acids and phenolic acids (Manach et al. 2004).

Table 1. Nutritional composition of raw, frozen and juice of sweet cherries.

Nutrient (Unit)	Raw sweet cherry	Frozen sweet cherry	Juice of sweet cherry
Basic chemical composition			
Water (g/100g)	82.25	75.53	84.95
Energy (kcal/100g)	63.00	89.00	54.00
Macronutrients			
Total protein (g/100g)	1.06	1.15	0.91
Total lipids (g/100g)	0.20	0.13	0.02
Fatty acids, total saturated (g/100g)	0.038	0.030	0.004
Fatty acids, total monounsaturated (g/100g)	0.047	0.036	0.005
Fatty acids, total polyunsaturated (g/100g)	0.052	0.040	0.006
Carbohydrates (g/100g) (by difference)	16.01	22.36	13.81
Dietary fiber (g/100g)	2.10	2.10	1.50
Total sugars (g/100g)	12.82	20.26	12.31
Sucrose (g/100g)	0.15	Not available	Not available
Glucose (dextrose) (g/100g)	6.59	Not available	Not available
Fructose (g/100g)	5.37	Not available	Not available
Maltose (g/100g)	0.12	Not available	Not available
Galactose (g/100g)	0.59	Not available	Not available
Micronutrients			
Minerals			
Calcium, Ca (mg/100g)	13.00	12.00	14.00
Iron, Fe (mg/100g)	0.36	0.35	0.58
Magnesium, Mg (mg/100g)	11.00	10.00	12.00
Phosphorus, P (mg/100g)	21.00	16.00	22.00
Potassium, K (mg/100g)	222.00	199.00	131.00
Sodium, Na (mg/100g)	0.00	1.00	3.00
Zinc, Zn (mg/100g)	0.07	0.04	0.10
Cooper, Cu (mg/100g)	0.06	0.02	0.07
Manganese, Mn (mg/100g)	0.07	0.11	0.06
Fluoride, F (µg/100g)	2.00	Not available	Not available
Vitamins			
Vitamin C (mg/100g)	7.00	1.00	2.50
Thiamin (mg/100g)	0.03	0.03	0.018
Riboflavin (mg/100g)	0.03	0.05	0.02
Niacin (mg/100g)	0.15	0.18	0.41
Pantothenic acid (mg/100g)	0.20	0.13	0.13
Vitamin B-6 (mg/100g)	0.05	0.04	0.03
Folate, total (µg/100g)	4.00	4.00	4.00
Folate, DFE (µg/100g)	4.00	4.00	4.00
Folate, food (µg/100g)	4.00	4.00	4.00
Choline, total (mg/100g)	6.10	5.60	4.70
Vitamin A, RAE (µg/100g)	3.00	9.00	6.00
Carotene, beta (µg/100g)	38.00	113.00	75.00
Vitamin A, IU (IU/100g)	64.00	189.00	125.00
Lutein+zeaxanthin (µg/100g)	85.00	85.00	57.00

Table 1. Nutritional composition of raw, frozen and juice of sweet cherries (cont.).

Nutrient (Unit)	Raw sweet cherry	Frozen sweet cherry	Juice of sweet cherry
Vitamin E (mg/100g)	0.07	0.07	0.23
Tocopherol, beta (mg/100g)	0.01	Not available	Not available
Tocopherol, gamma (mg/100g)	0.04	Not available	Not available
Vitamin K (µg/100g)	2.10	2.10	1.40
Amino acids			
Trypthophan (g/100g)	0.009	Not available	Not available
Threonine (g/100g)	0.022	Not available	Not available
Isoleucine (g/100g)	0.020	Not available	Not available
Leucine (g/100g)	0.030	Not available	Not available
Lysine (g/100g)	0.032	Not available	Not available
Methionine (g/100g)	0.010	Not available	Not available
Cystine (g/100g)	0.010	Not available	Not available
Phenylalanine (g/100g)	0.024	Not available	Not available
Tyrosine (g/100g)	0.014	Not available	Not available
Valine (g/100g)	0.024	Not available	Not available
Arginine (g/100g)	0.018	Not available	Not available
Histidine (g/100g)	0.015	Not available	Not available
Alanine (g/100g)	0.026	Not available	Not available
Aspartic acid (g/100g)	0.569	Not available	Not available
Glutamic acid (g/100g)	0.083	Not available	Not available
Glycine (g/100g)	0.023	Not available	Not available
Proline (g/100g)	0.039	Not available	Not available
Serine (g/100g)	0.030	Not available	Not available
Flavonoids			
Anthocyanidins			
Cyanidin (mg/100g)	30.20	Not available	Not available
Pelargonidin (mg/100g)	0.27	Not available	Not available
Peonidin (mg/100g)	1.50	Not available	Not available
Flavan-3-ols			
(+)-Catechin (mg/100g)	4.35	Not available	Not available
(-)-Epigallocatechin (mg/100g)	0.34	Not available	Not available
(-)-Epicatechin (mg/100g)	5.00	Not available	Not available
(-)-Epicatechin-3-gallate (mg/100g)	0.05	Not available	Not available
Flavonols			
Isorhamnetin (mg/100g)	0.05	Not available	Not available
Kaempferol (mg/100g)	0.24	Not available	Not available
Myricetin (mg/100g)	0.05	Not available	Not available
Quercetin (mg/100g)	2.28	Not available	Not available
Vitamin E (mg/100g)	0.07	0.07	0.23
Tocopherol, beta (mg/100g)	0.01	Not available	Not available
Tocopherol, gamma (mg/100g)	0.04	Not available	Not available
Vitamin K (µg/100g)	2.10	2.10	1.40
Amino acids			
Trypthophan (g/100g)	0.009	Not available	Not available

1.1.1.3. Protein and amino acids

Protein content in sweet cherries has been reported to be 1.06 g per 100 g of fw (Table 1), which is considered a low value. Relatively to amino acids, the scarce number of reports available showed the presence of 18 essential and nonessential amino acids: aspartic acid, cystine, glycine, glutamic acid, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. Tryptophan is the main one (USDA 2015). *Navalinda* is the richest variety in tryptophan content (82.65 μg/ g of fw), followed by *Van* (68.85 μg/ g of fw), *Pico-Limón* (62.69 μg/ g of fw), *Burlat* (61.36 μg/ g of fw), *Ambrunés* (449.26 μg/ g of fw), *Pico-Negro* (37.76 μg/ g of fw) and *Pico-Colorado* (36.53 μg/ g of fw) (Cubero et al. 2010). Tryptophan can pass through the blood-brain barrier, being converted into serotonin, thus increasing the melatonin, and can also change the levels of tryptamine, quinolinic acid, kynurenic acid, and coenzymes NAD and NADP production (Sainio et al. 1996). Accordingly, sweet cherries consumption increases the availability of tryptophan in the brain, and consequently its metabolism rises brain and blood serotonin and melatonin levels (Delgado et al. 2012). Amino acids are essential to produce enzymes, hormones, antibodies, transporters and muscle fibers in human body (Nelson & Cox 2011).

1.1.1.4. Fatty acids

Fatty acids cannot be synthesised by humans due to the lack of desaturase enzymes, being obtained through the diet. After consumption, fatty acids originate the omega-3 and the omega-6 fats by Δ^5-desaturase enzyme action, which are biosynthetic precursors of eicosanoids, and thus, make part of diverse metabolic functions (WHO 2008).

The data collected reveals the existence of low levels of these compounds in sweet cherries. According to the current knowledge, sweet cherry fruits have around 0.38 mg of saturated fat, 0.47 mg of monounsaturated fat and 0.52 mg of polyunsaturated fat of dry weight (dw) (Table 1). The concentration of fatty acids in sweet cherries is shown in Table 1. They are mostly present in edible portion (USDA 2015). A total of nineteen fatty acids were reported in sweet cherries, linoleic (C18:2n6) (25.08%), oleic (C18:1n9) (23.95%), palmitic (C16:0) (22.27%) and *α*-linoleic acids (C18:3n3) (15.39%) are the main ones (Bastos et al. 2015).

Fatty acids are important in diverse metabolic functions and are able to decrease cholesterol levels, cardiovascular threat risks. They are also involved in volatile compounds formation by *β*-oxidation and oxidation via lipoxygenase enzymes (Vavoura et al. 2015).

1.1.2. Micronutrients

Unlike the macronutrients, micronutrients are needed in small amounts, being involved in many vital chemical reactions for human body (as regulatory and plastics functions). They

include minerals and vitamins. The sweet cherries micronutrients content is showed in Table 1.

1.1.2.1. Minerals

Fruits are a natural source of minerals, essential to vascular function, controlling high blood pressure and heart diseases risk (D'Elia et al. 2011). The percentage of sweet cherries minerals is around 0.23%.

Potassium is the most abundant in sweet cherries (222 mg per 100 g of fw) (Table 1) (Ferretti et al. 2010), usually occurring combined with organic acids, keeping cellular osmolarity and membrane potentials and being essential to vascular tone and cardiovascular health (Steinberg et al. 2003). Calcium, magnesium and phosphorus are also present in considerable amounts (Table 1) (Duarte & Silva 2014). Copper is another mineral present in sweet cherries but in small amounts, that together with calcium are involved in collagen and neurotransmitters synthesis, nerve impulses transmission, muscle relaxation, energy production and in bone and teeth adsorption (Steinberg et al. 2003). Sodium is absent in cherries, which reduces stroke risk (D'Elia et al. 2011).

1.1.2.2. Vitamins

Vitamins are minor constituents of fruits, they are essential to the normal growth, maintenance and functioning of human body (Belitz et al. 2009). Sweet cherries contain both fat-soluble (A, E and K) and water-soluble (B and C) vitamins (Duarte & Silva 2014).

Water-soluble vitamins are the most commons: vitamin C (ascorbic acid) is the most prevalent, ranging between 7 to 37 mg of fw, followed by B vitamin complex (choline, pantothenic acid and niacin) (6.1 mg; 199 µg and 154 µg per 100 g of fw, respectively) (Belitz et al. 2009; Ballistreri et al. 2013; Demir 2013).

As we can see in Table 1, sweet cherries are a great source of vitamin C, which is a great electron donor and the main human body antioxidant in plasma and cells (Kim et al. 2002). Vitamin C is also able to protect melatonin against oxidation (Garcia-Parrilla et al. 2009). Choline is the second vitamin most common in sweet cherries, and it is needed to acetylcholine and essential membranes compounds synthesis, to lipid transport, for homocysteine reduction and for inflammatory processes, breast cancer and heart attack risks. Furthermore, it also plays an important role in brain and memory development of fetuses and decreases the risk of formation of neural tubes with defects (Zeisel & Da Costa 2009). Pantothenic acid is part of coenzyme A, participating in the Krebs cycle, in the disposal of carbohydrates and ketogenic amino acids, in acylation of proteins and in the lipids synthesis

(e.g.: fatty acids, glycerides, cholesterol, ketone bodies, sphingosine…), while niacin regulates cholesterol levels and reduces the risk of cardiovascular pathologies (Hankey 2003).

On the other hand, fat-soluble vitamins are present in trace amounts: vitamin K_1 (2.1 µg per 100 g of fw), vitamin A (64 IU per 100 g of fw) and vitamin E (70 µg per 100 g of fw) (USDA 2015). Vitamin K_1 is associated to bleeding disorders, mainly in obstetric and pediatric patients (Hankey 2003). Vitamin A (retinol) is linked to normal growth, vision, epithelial differentiation, immunological tolerance and adaptive immune responses (Hall et al. 2011), reducing T-cells production of pro-inflammatory mediators interleukin (IL)-6 and interferon gamma (IL-6 and IFN-*γ*, respectively) and promoting differentiation of regulatory T-cells in anti-inflammatory mediator Il-4 (Røsjø et al. 2014). Vitamin E is involved in protein-kinase C (PKC) inhibition in different types of cells, by activating at cellular level the protein phosphatase 2A, causing the dephosphorylation of PKC and consequently the inhibition of platelet aggregation. It also helps keeping vascular integrity, endothelial cells, nitric oxide (•NO) and glial cells superoxide production in neutrophils and macrophages. Furthermore, vitamin E also captures CD36 and SR-A receptors (related to arteriosclerosis), diminishes molecular adhesion, blocks IL-6, IFN-*γ* and reactive oxygen species (ROS) production, by interfering with monocytes activity, and increases connective tissue growth factor expression (Azzi et al. 2002; Mora et al. 2008).

1.1.3. Phytochemicals

Phytochemicals are natural chemical compounds found in plants and the major responsible for protecting the plants against diseases and damages. They contribute to colour, aroma, flavour and also to the antioxidant, anti-inflammatory and anti-mutagenic properties displayed by fruits (Batta 2016), providing healthier benefits for human body than those attributed to macro and micronutrients (Kurmukov 2013). They are more concentrated in exocarp than in the flesh of sweet cherries (Contreras-Calderón et al. 2011) and include carotenoids, volatile compounds, serotonin, melatonin and phenolics.

1.1.3.1. Carotenoids

Carotenoids (precursors of vitamin A) confer to fruits colours from yellow to red. They derive from a 40-carbon structure and their double bonds determine their antioxidant capacity (Stahl & Sies 2003). Based on their composition, they are divided in two classes: carotenoid-hydrocarbons, containing only carbons and hydrogens (*β*-carotene and lycopene) and oxygenated carotenoids (xanthophylls, as astaxanthin, canthaxanthin, fucoxanthin, lutein, spirilloxanthin and zeaxanthin) (Arathi et al. 2015). More than 700 different carotenoids have been characterized so far in fruits and vegetables, but only six were described in sweet cherries. Lutein (100-130 µg per 100 g of fw), *β*-carotene (78-87 µg per 100 g of fw), *α*-carotene (27-37 µg per 100 g of fw), *β*-cryptoxanthin (18-23 µg per 100 g of fw) and

zeaxanthin (16-33 μg per 100 g of fw) were reported in *Saco* (Dias et al. 2009). Furthermore, the *β*-Apo-8-carotenal were found in other sweet cherries cultivars: *Lambert* (302 μg per 100 g of fw), *Churchill (*111 μg per 100 g of fw), *Hasan Kazak* and *Karakiraz* (103 μg per 100 g of fw), *Napoleon* and *Z0900-1* (45 μg per 100 g of fw) and *Z0900-2* (32 μg per 100 g of fw) (Demir 2013).

As they quench singlet oxygen, there is an increase in protection of cells against oxidative damages, and in cardiovascular and ophthalmologic diseases (Cooper 2004; von Lintig 2010). In addition, lutein and zeaxanthin can absorb damaging blue-light that enters into the eyes, preventing damages, as macular degeneration and cataracts (Krinsky & Johnson 2005).

1.1.3.2. Volatile compounds

Volatiles are the responsible for fruits aroma. Sweet cherries possess different varieties of volatiles (about 97), although they are present in trace amounts (<100 mg per g of fw) (Kader & Barrett 2005). The most common subclass of volatiles found in sweet cherries are the C6 aromatic compounds, mainly (E)-hexen-1-ol and (E)-2-hexanal (Serradilla et al. 2011). Benzaldehyde, a primary contributor to the flavour of sweet cherries originated from the enzymatic hydrolysis of the amygdalin, is a volatile also found in this fruit (Vavoura et al. 2015). Terpenoids, namely geraniol, linalool, nerol, terpineol, *β*-citronellol, geranial and neral are another volatile compounds that constitute sweet cherries. These glycosylated compounds interact in a synergistic way with polyphenols, offering fruits an antibacterial activity against pathogens. In addition, acetic acid is the volatile acid most found in sweet cherries, followed by hexanoic, tetradecanoic, 9-hexadecenoic and hexadecanoic acids (Serradilla et al. 2012).

1.1.3.3. Serotonin and melatonin

Serotonin is a monoamine neurotransmitter which is involved in many neuronal functions, such as sleep-wake cycle regulation and in some behaviours control, like aggression, impulsivity, compulsion of food and alcohol, arousal, sexual behaviour, pain reaction, among others, as well as in melatonin biosynthesis pathway (Sainio et al. 1996). It is also found in sweet cherries, where its content varies between the different cultivars: *Ambrunés* (37,6 ng), *Pico-Colorado* (36.6 ng), *Navalinda* (30.7 ng), *Pico Limón Negro* (27.1 ng), Van (19.2 ng), *Burlat* (12.6 ng), *Sweetheart* (10.6 ng) and *Pico-Negro* (2.8 ng) per 100 g of fw (González-Gómez et al. 2009).

In respect to melatonin (an indolamine), it is distributed along the skin, flesh and seed of sweet cherries (Vitalini et al. 2011), ranging from 0.01 and 0.22 ng per g of fw: *Van* (0.01 ng), *Navalinda* (0.03 ng), *Pico-Colorado* (0.05 ng), *Sweetheart* (0.06 ng), *Pico-Negro* (0.12 ng) and *Burlat* (0.22 ng) per g of fw (González-Gómez et al. 2009; Kirakosyan et al. 2009). This

secondary metabolite is involved in antioxidant activities by scavenging toxic hydroxyl radicals (•OH) and stimulating antioxidant enzymes, diminishing free radical formation in mitochondria and stopping the leakage of electrons in electron transport chain, and also in immune responses, inhibiting cyclooxygenase (COX) II, and in aging processes and sleep-cycles, too (Hardeland et al. 1993; Wang et al. 1999; Zhdanova et al. 2001; Paredes et al. 2007).

1.1.3.4. Phenolic compounds

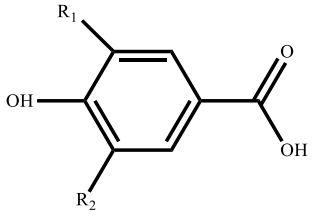
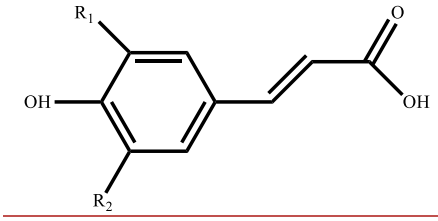
According to the literature data, exist over 10 000 phenolics identified in plants (Fürstenberg-Hägg et al. 2013). They all possess at least one benzene ring, containing at least one free hydroxyl group (OH). Despite having alcohol groups, they don't show properties of an alcohol, displaying a reactivity associated with the acid character of phenolic nucleophilic function and the benzene ring. In addition, their structures may range from a simple phenolic molecule to a complex high-molecular weight polymer (Bravo 1998). Most phenolics found in fruits, vegetables and other foods possess two or more OH groups and they are divided according to their chemical structure, as non-flavonoids (phenolic acids, stilbenes and lignans) or as flavonoids (flavonols, flavan-3-ols conferring health benefits, as a normal cellular metabolism and a reduction of free radical species, flavones, flavanones and anthocyanins) (Ross & Kasum 2002). In addition to protecting vegetables and fruits against ultraviolet radiation, pathogens, poor-soil fertility and climate variations, they are also the major responsible for the antioxidant action, conferring health benefits, such as a normal cellular metabolism and a reduction of free radical species (Figure 3) (Thi et al. 2016).

1.1.3.4.1. Phenolic acids

Phenolic acids are divided in hydroxybenzoic and hydroxycinnamic acids, and occur in fruits as esters of organic acids, glycosides or bound to protein or other cell wall polymers (Chanet et al. 2012). They all have one functional carboxylic acid. Furthermore, they differ in the number of carbons: hydroxycinnamic acids have nine carbons, while hydroxybenzoic acids (Table 2) have seven carbons (Robbins 2003). Both interact with anthocyanins, increasing colours diversity and altering stability, flavour, nutritional values, antioxidant activities and other food properties (Dixon & Steele 1999).

Hydroxycinnamic acids derivatives (C6-C3) (Table 2) are the most common phenolic acids found in sweet cherries, namely 3-*O*-caffeolquinic acid, an ester of caffeic and (-)-quinic acids, caffeoyl tartaric acid, 5-*O*-caffeoylquinic acid and 3-*ρ*-coumaroylquinic acid (a derivate of *ρ*-coumaric acid) (Ballistreri et al. 2013). 3-*O*-Caffeoylquinic acid is the major one, representing 24-65% of the total phenolic content, ranging between 4.74 to 11.9 mg per 100 g of fw, followed by *ρ*-coumaroylquinic acid (0.77 to 7.20 mg per 100 g of fw) and 5-*O*-caffeoylquinic acid (0.60 to 2.61 mg per 100 g of fw) (Usenik et al. 2008).

Table 2. Hydroxycinnamic acid (A) and hydroxybenzoic acid (B) structures.

Structural formula	Representative Flavonoids	Substitutions	
		R_1	R_2
Hydroxybenzoic acids			
	*ρ*-Hydroxybenzoic acid	H	H
	Gallic acid	OH	OH
	Protocatechuic acid	H	OH
	Syringic acid	CH_3O	CH_3O
	Vanillic acid	CH_3O	H
Hydroxycinnamic acids			
	*ρ*-Coumaric acid	H	H
	Caffeic acid	H	OH
	Ferulic acid	CH_3O	H
	Sinapic acid	CH_3O	CH_3O

Derivatives of hydroxybenzoic acids (C6-C1) (Table 2) are also, occasionally, found in sweet cherries, in trace glycosylated amounts. Their total contents range from 3.3 to 14.3 mg per 100 g of fw, being the gallic and the *ρ*-hydroxybenzoic acids the most commons, ranging between 0.10 to 1.40 mg per 100 g of fw, respectively (Mattila et al. 2006; Kelebek & Selli 2011; Serradilla et al. 2011; Ballistreri et al. 2013).

Phenolic acids have considerable antioxidant capacity, principally the hydroxycinnamic acids, being able to reduce oxidative stress, scavenge ROS and reduce •OH formation in cells, which contributes to reduce cancer and neurological and cardiovascular disorders (Laranjinha et al. 1994).

1.1.3.4.2. Flavonoids

Actually, more than 9 000 different flavonoids can be found in plants (Mouradov & Spangenberg 2014). They are present in most edible fruits, as glycosides forms, usually combined with sugars (D-glucose and less commonly with *D*-galactose, *L*-rhamnose, *L*-arabinose and *D*-xylose), or as aglycones (forms without sugar moieties) (Erlund 2004). They have low molecular weight and are structurally defined by 15 carbons with a common phenylbenzopyrone structure C6-C3-C6: two aromatic rings, A and B (where A ring derives from acetate/ malonate pathway and B ring derives from shikimate pathway) linked by a 3-carbon chain that forms an oxygenated heterocyclic ring (C ring) with one or more OH substituents, and linked to A ring by a connection and to ring B by an single bond (Setchell & Cassidy 1999). Substitutions on C ring results in different saturation levels, different degrees of hydroxylation and oxidation and opens the central ring into eight major flavonoid classes. Flavonoids containing an OH group in C3 of C ring are classified as 3-hydroxyflavonoids

(anthocyanidins, flavan-3-ols or catechins and flavonols), and those that lack an OH group as 3-desoxyflavonoids (flavanones and flavones) (Table 3). Particularly, anthocyanidins and flavan-3-ols do not have the carbonyl group in C4 (Erlund 2004). The substitution pattern on B ring is responsible for the differences within each group, differing in the number and location of the hydroxy and methoxy groups located in this ring. Typically, the substitution verified is a group hydroxy at position 4 with an additional substitution of an oxygen in position 3 and/ or 5 (which may be a hydroxy or a methoxy) (Wallace 2011).

Flavonoids not only induce the systemic acquired resistance against a wide range of pathogens and protect the plants against ultraviolet radiation, but also display antiallergic, anticancer, anti-inflammatory and anti-hemorrhagic properties (Wang et al. 1996), inducing or inhibiting enzymes involved in cellular proliferation and division, and in immune and inflammatory processes, and also promoting detoxification and platelet aggregation (Sandhar et al. 2011). These beneficial effects are due to the fact that flavonoids are electron donors, broking their phenolic bound between oxygen and hydrogen, in the presence of free radical species, producing the phenoxyl radical and H^+ ion. This last specie is able to scavenge and stabilize radical species (Wang et al. 1996; Rice-Evans et al. 1997).

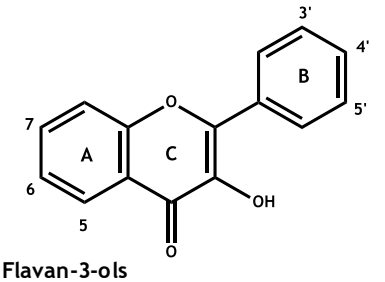
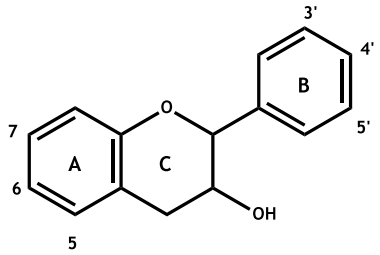
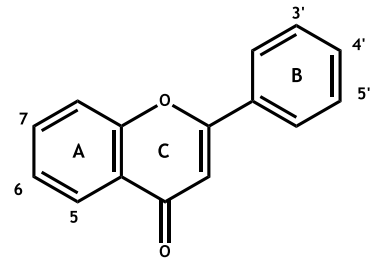
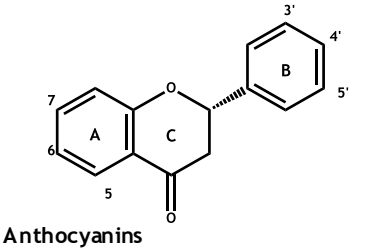
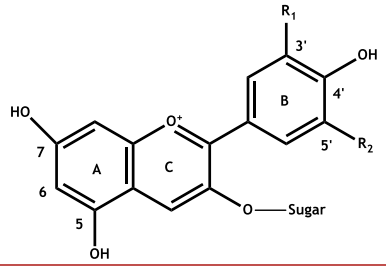
Sweet cherries are a great source of flavonols and anthocyanins, but also have in their constitution contents of flavanones, flavones and flavan-3-ols. All together contribute to biologic potential and health benefits of sweet cherries consumption.

1.1.3.4.2.1. Flavonols

Flavonols (Table 3) are widespread in fruits and vegetables as *O*-glycosides. Only four aglycones are common in fruits, including sweet cherries, namelly quercetin, kaempferol, myricetin and isorhamnetin (Casagrande & Darbon 2001; Mattila et al. 2006; Jakobek et al. 2007; Galluzzo et al. 2009). Quercetin is the major flavonol found in sweet cherries, being reported amounts ranging between 1.14 to 4.97 mg per 100 g of fw in the form of quercetin-3-*O*-rutinoside (rutin) and 0.16 to 0.79 mg per 100 g of fw as quercetin-3-*O*-glucoside (Kim et al. 2005).

Kaempferol is the second most found flavonol in sweet cherries, in the form of kaempferol-3-*O*-rutinoside (0.30 to 1.39 mg per 100 g of fw), followed by myricetin-3-*O*-rutinoside (0.02 mg per 100 g of fw). Isorharmnetin-3-*O*-rutinoside is also found in trace amounts (ranging between 0.08 to 0.13 mg per 100 g of fw) (Giménez et al. 2014).

Table 3. Subclasses of flavonoids present in sweet cherries based on variations in the heterocyclic C-ring.

Structural formula	Representative flavonoids	Substitutions 5	6	7	3'	4'	5'	Major food sources
Flavonols								
	Isorhamnetin	OH	H	OH	H	OH	OCH_3	onions, kale, broccoli, apples, berries, tea, red wine
	Kaempherol	OH	H	OH	H	OH	H	
	Myricetin	OH	H	OH	OH	OH	OH	
	Quercetin	OH	H	OH	OH	OH	H	
Flavan-3-ols								
	Catechin	OH	H	OH	OH	OH	H	cocoa, green tea, chocolate, red wine, hawthorn, bilberry, motherwort and other herbs
	Gallocatechin	OH	H	OH	OH	OH	OH	
Flavones								
	Apigenin	OH	H	OH	H	OH	H	parsley, thyme, celerey, honey, propolis, sweet red peppers
	Chrysin	H	H	OH	H	H	H	
	Luteolin	OH	H	OH	OH	OH	H	
Flavanones								
	Eriodictyol	OH	H	OH	OH	OH	H	citrus
	Hesperetin	OH	H	OH	OH	OMe	H	
	Naringenin	OH	H	OH	H	OH	H	
Anthocyanins								
	Cyanidin	OH	OH	OH	OH	OH	H	cherries, grapes, berries, red cabbage
	Malvidin	OH	OH	OH	OCH_3	OH	OCH_3	
	Petunidin	OH	OH	OH	OCH_3	OH	OH	

1.1.3.4.2.2. Flavan-3-ols

Flavan-3-ols (catechins, flavanols or proanthocyanidins, the last one also known as condensed tannins) (Table 3) are another class of phenolic compounds present in sweet cherries. They are based on the skeleton of 2-phenylchromen-4-one (Dajas et al. 2013) and the substitution pattern in B ring varies between monomeric units, with (+)-catechin and (-)-epicatechin ortho-hydroxylated at C3 and C4, while (+)-gallocatechin and (-)-epigallocatechin have a third hydroxy group on C5. In addition, the two chiral centres at C2 and C3 of the monomeric flavanol produce four isomers, two of which, for each level of B ring hydroxylation (+)-epicatechin, (-)-epicatechin, (+)-catechin and (-)-catechin, which have great distribution in nature, while (-)-epiafzelechin is another flavonol but with more limited distribution (Del Rio et al. 2013; Silva & Costa 2014).

About sweet cherries flavan-3-ol content, it ranges between 0.43 to 13.38 mg per 100 g of fw, being reported the presence of (+)-catechin, (-)-epigallocatechin, (-)-epicatechin, (-)-epicatechin-gallate, (-)-epigallocatechin-3-gallate and (+)-gallocatechin (Casagrande & Darbon 2001; Mattila et al. 2006; Galluzzo et al. 2009; Usenik et al. 2008; González-Gómez et al. 2010; Kelebek & Selli 2011). Epicatechin is the most dominant, ranging from 6.33 to 14.84 mg per 100 g of fw (Kelebek & Selli 2011).

1.1.3.4.2.3. Flavones

Flavones are also based on the skeleton of 2-phenylchromen-4-one (Table 3) (Dajas et al. 2013), but the lack of oxygenation at C3 makes possible several substitutions, including hydroxylation, methylation, *O*- and C-glycosylation and alkylation (Del Rio et al. 2013). The most commons are apigenin, luteolin, diosmetin and chrysoeriol and they occur as 3-glycosides forms, and less frequently, as the 7-glycosides (Belitz et al. 2009). In sweet cherries are reported apigenin and luteolin in trace amounts (Table 3) (Marchand 2002; USDA 2015).

1.1.3.4.2.4. Flavanones

The most flavanones found in fruits are narigenin-7-rutinoside (narirutin), narigenin, hesperidin and hesperetin (Table 3) (Erlund 2004). They are mostly present in citrus fruits and their derived products (Garrido et al. 2013), but can also be found in sweet cherries. Flavanones are characterized by the absence of $\Delta^{2,3}$ double bond and by the presence of a chiral centre at C2, that attributes a saturated heterocyclic C ring. They occur as OH, glycosylated and *O*-methylated derivatives and they are the unique class of phenolic compounds that contains various glycosides of three main aglycones: hesperetin (4'-methoxy-3',5,7-trihydroxyflavanone), naringenin (5,7,4'-trihydroxyflavanone) and eriodictyol (5,7,3',4'-tetrahydroxyflavanone) (Manach et al. 2003).

Hesperetin and naringenin are the only flavanones reported in sweet cherries, both with contents around 4 mg per 100 g of fw (Harnly et al. 2006). They increase the biologic potential of sweet cherries, being associated to lipid metabolism regulation (for instance, apolipoprotein B secretion by HepG2 cells), cholesterol ester synthesis, HMG-CoA reductase and acyl CoA:cholesterol-*O*-acyltransferance reducing plasma and hepatic cholesterol inhibition, supressing oxidative stress, preventing cancer and cardiovascular diseases (Borradaile et al. 1999).

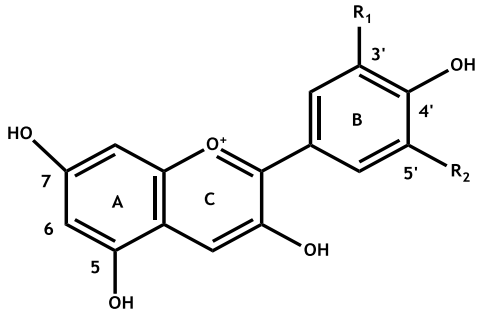
1.1.3.4.2.5. Anthocyanins

Anthocyanins possess the best antioxidant capacity and are the most abundant flavonoid found in sweet cherries, comprising more than 70% of total phenolic content (Kim et al. 2005; Patras et al. 2010). Actually, 23 anthocyanidins and more than 500 anthocyanins (glycosides of aglycones anthocyanidins and more stables than them) are known, and all possess a C6-C3-C6 carbon structure (Wallace 2011). The common anthocyanidin skeletons in fruits and vegetables are cyanidin, delphinidin, pelargonidin, malvidin, petunidin and peonidin (Figure 3). Their presence is associated with the colour, attraction and flavour of fruits, being the only phenolic water-soluble pigment responsible for the orange, red and blue colours of foods, depending on the pH (Hassimotto et al. 2008). The substitution pattern on B ring offers different colours, being blue colour more associated with OH groups and the red colour to methoxy groups (Miguel 2011).

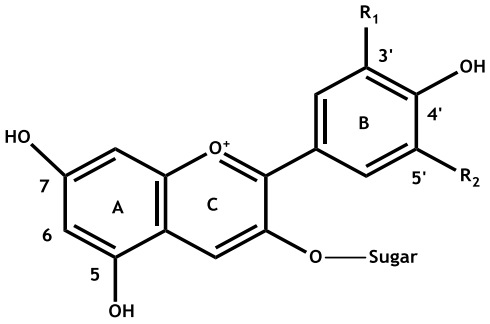
The anthocyanins reported in sweet cherries are cyanindin-3-*O*-rutinoside. Which is the main one, ranging between <1 mg to 30 mg per 100 g of fw (Schüller et al. 2015), followed by cyaniding-3-O-glucoside (<1 mg to 70,1 mg per 100 g of dw), and small traces of peonidin-3-*O*-rutinoside (<1 mg to 3.6 mg per 100 g of dw), peonidin-3-*O*-glucoside (1.23 mg per 100 g of dw) and pelargonidin-3-*O*-rutinoside (0.01 to 0.88 mg per 100 g of dw) (Gonçalves, Landbo, Knudsen, et al. 2004; Usenik et al. 2008; Kelebek & Selli 2011; Serradilla et al. 2011; Serra et al. 2011; Grigoras et al. 2012). Some aglycones, as petunidin (4 mg per 100 g of fw) and delphinidin (4 mg per 100 g of fw), malvidin (ranging from 0.08 to 0.64 mg per 100 g of fw), pelargonidin (0.27 mg per 100 g of fw) and peonidin (1.50 mg per 100 g of fw) also exist in sweet cherries composition, constituting only <5% of the total anthocyanidin content in this fruit.

Protective effects showed by anthocyanins are associated with the number of free OH groups around the B ring (Miguel 2011). They are responsible to define the scavenging capacity of ROS species and chelate metals of these compounds, reducing oxidative stress involved in cancer, neurological and cardiovascular disorders (Kim et al. 2005).

In addition, OH groups also give to anthocyanins antimicrobial and anti-inflammatory properties (Cisowska et al. 2011; Hanbali et al. 2012).



Anthocyanidin



Anthocyanin

Figure 3. More common anthocyanins and anthocyanidins in sweet cherries.

Name	R_1	R_2	R_3
Cyanidin	OH	H	H
Cyanidin-3-*O*-glucoside	OH	H	Glucose
Cyanidin-3-*O*-rutinoside	OH	H	Rutinoside
Delphinidin	OH	OH	H
Malvidin	OCH_3	OCH	H
Pelargonidin	H	H	H
Pelargonidin-3-*O*-glucoside	H	H	Glucose
Pelargonidin-3-*O*-rutinoside	H	H	Rutinoside
Peonidin	OCH_3	H	H
Peonidin-3-*O*-rutinoside	OCH_3	H	Rutinoside
Peonidin-3-*O*-glucoside	OCH_3	H	Glucose
Petunidin	OCH_3	H	H

1.2. Biologic potential and health benefits of *Prunus avium* L.

It exists several epidemiological studies based on bioactive compounds present in sweet cherries. Phenolic compounds have been reported by their medicinal and therapeutic properties, namely antioxidant, anti-mutagenic, anti-allergenic and antimicrobial effects (Figure 4) (Rice-Evans et al. 1997; Seymour et al. 2009; Duarte & Silva 2014). Furthermore, the sequencing of the human genome allowed a better knowledge about their action in various chronic pandemic disorders, as obesity, hypertension, diabetes, cancer, rheumatoid arthritis, cardiovascular and neurological pathologies, being able to reduce oxidative stress by scavenging free radical species, by strengthening immune, gonadotrophic, gastrointestinal, renal and hepatic systems (Figure 4) (Seymour et al. 2009; Ferretti et al. 2010; Shrinath et al.

2011). Sweet cherries use dates back to ancient times to threat ague, diarrhoea, tonsillitis, jaundice, urogenital disorders, sickness, urinary and intestinal inflammations and bellyache (Duarte & Silva 2014).

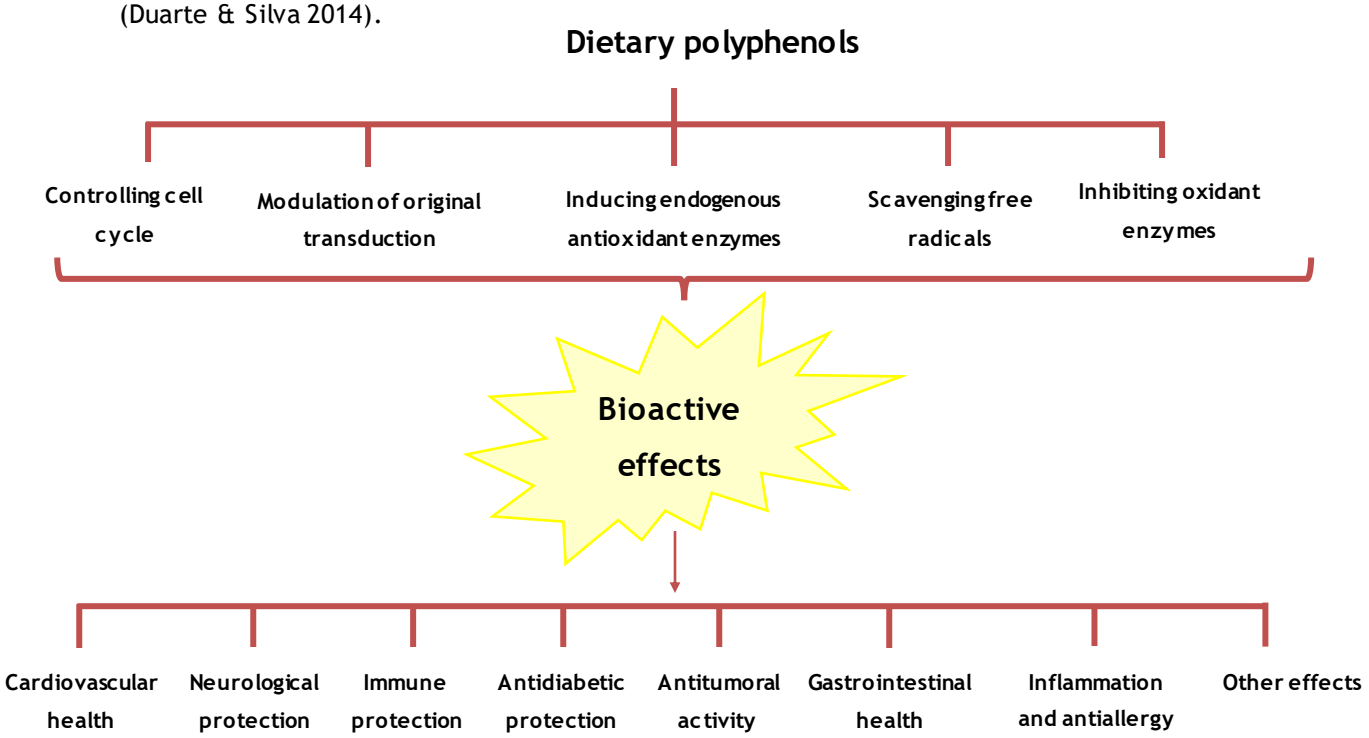


Figure 4. Bioactive effects of polyphenols daily intake.

Several biological studies have been associating the consumption benefits of sweet cherry fruit, teas, seeds and stems, to their phenolic constitution, which is related to the antioxidant activity. As mentioned above, antioxidant activity helps reducing oxidative-stress and consequently all the threats associated, such as cancer, neurological and cardiovascular disorders and inflammatory process, the major contributing factor in chronic diseases, as type 2 diabetes, arthritis and gout, cancer, neurological and cardiovascular pathologies (Duarte & Silva 2014). Moreover, antimicrobial activity is also reported (Ferretti et al. 2010; McCune et al. 2010).

Since sweet cherries possess various compounds with important antioxidant properties, particularly anthocyanins and hydroxycinnamic acids, it would be very useful to incorporate these fruits in dietetic additives, new foods, nutraceutical supplements, pharmaceutical and cosmetic products (Ayala-Zavala et al. 2011).

Next, it will be discussed some aspects related with the biological potential evaluated in this dissertation.

Cellular metabolism generates free radical species (atoms and molecules that have one or more unpaired electrons), as reactive oxygen and nitrogenous species (ROS and RNS, respectively), which are essential for the living state of cells and organisms. ROS include not

only oxygen radicals, but also •OH and superoxide radicals, and nonradical derivatives, as hydrogen peroxide (H_2O_2), hypochlorous acid, ozone and singlet oxygen (Wiseman & Halliwell 1996; Silva & Teixeira 2015). •NO is a reactive nitrogenous specie, which is involved in various physiological processes, such as in neurotransmission, blood pressure control and immune responses (Silva & Teixeira 2015). However, and knowing that they are pro-oxidant molecules, an abnormal accumulation of them causes oxidative stress, damaging lipids, proteins and nucleic acids in cells (Contreras-Calderón et al. 2011), increasing the risk of cancer, gout, neurodegeneration, cardiovascular pathologies, hypertension, type 2 diabetes, among others (Wang et al. 2011). They are typically detoxified by intracellular enzymes (glutathione, superoxide dismutase and catalase) (Batta 2016). Furthermore, diet also contributes to increase antioxidant molecules in the organism, not only phenolics but also vitamin E, *β*-carotene and ascorbic acid (Balasundram et al. 2006). In addition, H_2O_2 may be converted into •OH by Fenton and/ or Haber-Weiss reactions, resulting in the formation of peroxyl radicals (ROO•) that damage erythrocytes membrane, promoting autoxidation of oxyhemoglobin and generation of more superoxide radicals and H_2O_2, causing erythrocytes hemolysis and compromising oxygen transport around the body (Chisté et al. 2014a). Erythrocytes are considered as major targets for free radicals because they are constituted by a high concentration of polyunsaturated fatty acids and also transport oxygen, being very susceptible of oxidation (Carvalho et al. 2010).

Oxidative stress is also associated with type 2 diabetes. This type of diabetes is characterized by an increase of insulin requirement and in some inflammatory markers, as IL-1*β* and IL-6, that appeared reduced after cherries consumption (Donath & Shoelson 2011). These reductions in the levels of inflammatory markers caused by cherries ingestion are related to theirs anthocyanins' content, which are well-known free radical scavengers with antidiabetic activity (Ferretti et al. 2010; Delgado, del Pilar Terrón, et al. 2012; Garrido et al. 2013; Kirakosyan et al. 2015). Lachin and Reza (2012) and Lachin (2014) showed that hydroethanolic extracts of tart and sweet cherries reduced blood glucose levels and protected *β*-cells from oxidative damage, increasing insulin secretion (Lachin & Reza 2012; Lachin 2014). This was achieved because phenolics capacity to inhibit *α*-glucosidase activity, an enzyme responsible for the digestion of carbohydrates in absorbable monosaccharides (Silva & Costa 2014).

However, when present in high concentrations, these compounds can act as pro-oxidant molecules too, interacting with proteins and inhibiting several enzymes, affecting growth, digestibility, drug bioavailability and pharmacokinetics, as benzodiazepines, terfenadine and cyclosporine drugs (which have their bioavailability increased in three times due to polyphenol cytochrome P450 3A4 inhibition), and depleting iron (which is more prejudicial in individuals with marginal iron status) by enhancing iron absorption (Mennen et al. 2005). This fact can cause several health threats, as cancer and neurodegenerative and cardiovascular threats.

Cancer is a global and growing threat that affects a large portion of people worldwide, being a major cause of mortality and morbidity. The World Cancer Research Fund and the American Institute for Cancer Research release some recommendations, such as being physically active, eat vegetables, fruits, wholegrains, live with happiness and limit consumption of alcohol. These recommendations can reduce overall cancer risk (5%), with great reductions in colorectal (12%) and stomach (16%) cancers (Rodriguez-Romaguera et al. 2012).

Most of cancers are associated with mutations in key regulators of cell cycle proliferation-cyclin-dependent kinases gene or in their inhibitors. Sweet cherries are useful to fight against cancer (Serra et al. 2011; Pacifico et al. 2014). Phenolic compounds can stop or prevent mutagenesis because they are potent free radical scavengers (Ren et al. 2003). Extracts of sweet cherries can inhibit proliferation of colon cancer cells HT29 and breast cancer cells MCF-7, being this anti-proliferative activity related to the vitamin C, anthocyanins and ß-carotene contents (Olsson et al. 2004). *Saco* ethanolic extract acts against the colon cancer cells, inhibiting 50% of its viability (Serra et al. 2010). *Del Monte* and *Della Recca* sweet cherries cultivars showed efficiency against five cancer cell lines: HepG2 human hepatoblastoma, A549 lung epithelial cell line, HeLa cervical carcinoma, SK-B-NE(2)-C human bone marrow neuroblastoma and SH-SY5Y human neuroblastoma (Pacifico et al. 2014).

Oxidative stress is one of the major causes of neurodegenerative diseases (e.g. Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease and multiple sclerosis), characterized by the deterioration of nerve cells (Zhang et al. 2015) from brain and spinal cord that leads to functional loss (ataxia) or sensory function (dementia) (Uttara et al. 2009). However, the daily intake of antioxidants has an important role in neuronal and mental protections (Perrone et al. 2008). The changes in cognitive functions in 49 adults (more than 70 years) with dementia, after 6 and 12 weeks of daily consumption of anthocyanin-rich sweet cherry juice (200 mL/day) were also evaluated and it was verified improvements in verbal fluency, short and long term memories (Kent et al. 2015). In addition, dried ethanolic extracts from sweet cherries proved to have neuroprotective actions in 24 streptozotocin induced mice with cognitive decline (cholinergic deficiency, choline acetyltransferase activity reduced and acetylcholinesterase activity increased) (Vinitha et al. 2014).

Sweet cherries, not only contain high concentrations of anthocyanins and other phenolic compounds (González-Gómez et al. 2009), but also substantial amounts of melatonin, serotonin (González-Gómez et al. 2010) and tryptophan (Cubero et al. 2010). All these compounds mentioned before, interact each other, regulating sleep cycles and improving antioxidant defences (Paredes et al. 2007). Their consumption in our diet has beneficial effects on mood (Garrido et al. 2012), has antidepressant actions and are important for stress responses because they have an inter-relationship between the hypothalamus–pituitary-adrenal axis, brain serotonin and circulating levels of the cortisol (biological marker of stress,

anxiety, and depression) (Levine et al. 2007). Several authors reported that sweet cherries exhibit sleep promoting actions, and increase urinary 6-sulfatoxymelatonin, a metabolite that is considered to reflect the nocturnal melatonin concentration, as well as antioxidant status in young, middle-aged, and elderly subjects; and also improves sleep quality (Garrido et al. 2010; Delgado et al. 2012).

Cardiovascular diseases are a consequence of bad habits like smoking, fat diet and lack of physical activity, which result in abnormal vascular endothelium function, hypertension, high levels of LDL cholesterol in plasma, platelet aggregation and diabetes (Wallace 2011). The excessive production of free radical species activates pro-inflammatory molecules, leading to heart problems, as ischemic heart disease, cardiomyopathy and coronary heart problems. Antioxidants, vitamins and compounds like polyphenols, folate, fiber, potassium and magnesium prevent the oxidation of cholesterol and other lipids in the arteries, diminishing the risk of coronary events (Nyyssonen et al. 1997; Rissanen et al. 2003).

Although there is a lack of specific studies concerning the role of sweet cherries in cardiovascular protection, a study with 12 volunteers that consumed 45-90 frozen tart cherries per day, presented an increase in the antioxidant response detected in blood and urine samples, after 12h of cherries' ingestion, which is an evidence that antioxidant power positively affects endothelial function vascular reactivity and, consequently, blood pressure and tissue perfusion, offering cardiovascular protection, reducing low density lipoprotein (LDL) oxidation and preventing atherogenicity of blood vessels. Volunteers also showed a raise of cyanidin-3-*O*-rutinoside, the most antioxidant anthocyanin, and also, the anthocyanin most common in sweet cherries (Seymour et al. 2014).

Cherries are also gaining importance related to their anti-inflammatory actions. They can accelerate immune responses to finish inflammation and to diminishing the medication intake (McCune et al. 2010). Sweet cherries inhibit inflammatory pathways, after observed that the consumption of 280 g per day of Bing sweet cherries decreased plasma urate, C-reactive protein (CRP) and nitric oxide levels in 10 healthy women volunteers (Steenge et al. 2003). Another study supported by 18 healthy women and men that also ate 280g of *Bing* sweet cherries during 28 days related that, after 12h of ingestion was already observed in blood samples, a decrease of reactive plasma uric acid, LDL cholesterol (associated of heart attacks), tumor necrosis factor-alpha (TNF-α) and nitric oxide levels, a normal T-cell expressed and secreted, and an increase of high-density lipoproteins cholesterol. These samples analyzed also showed changes in another inflammation biomarkers, as ferritin, IL-18, IL-1Ra, ET-1, EN-RAGE, and PAI-1, as well as reduced risks for arthritis (CRP, TNF-α, IL-18, IL-1Ra), diabetes, cardiovascular diseases (CRP, ferritin, ET-1, EN-RAGE, PAI-1, IL-18), cancer (ET-1, EGF) and hypertension (ET-1) (Kelley et al. 2006).

Anti-inflammatory effects of sweet cherries are also related to flavonoids' content (Wang et al. 1996; Wang et al. 1999; Wang et al. 2000; Seeram et al. 2001). High levels of anthocyanins (40 mg/kg) were tested in rats with induced arthritis, and all showed an increase of the superoxide dismutase level and reduction of the level of TNF-α, IL-6 and malondialdehyde, prostaglandin-2 and COX enzymes in serum (He et al. 2006). Particularly, cyanidin can inhibit the activities of COXs I and II enzymes in vitro, reducing the COX II activity in 47.4%, which is similar to ibuprofen and naproxen actions (39.8% and 41.3%, respectively) (Bell, Mchugh, et al. 2014), and the COX I activity in 28%, by inhibiting mitogen activated protein kinases (Seeram et al. 2001). Melatonin present in sweet cherries contributes to reduce the inflammation, regulates cellular metabolism and reduces oxidative stress, acting in a synergistic and additive way with phenolic compounds, increasing benefits of this fruit (González-Gómez et al. 2009; Delgado et al. 2012).

Another health benefit associated with sweet cherries bioactive compounds is their antimicrobial action, which has been gaining interest, since pathogens are developing resistance against existing drugs (Abdallah et al. 2012). Although there are few studies about this potential, Ördogh et al. (2010) reported that sweet cherry juices can inhibit the growth of gram-positive bacteria involved in medical issues, like *Propionibacterium acnes* (cause acne), *Streptococcus pyogene* (involved in bacterial pharyngitis and scarlet fever), but not *Staphylococcus epidermidis* (responsible for hospital infections) (Ördögh et al. 2010). Ankolekar et al. (2011) observed that fermented extracts of sweet cherries are able to inhibit cultures of *Helicobacter plyori* (gram-negative bacteria involved in stomach diseases) due to their phenolic content, included caffeic acid and anthocyanins (Ankolekar et al. 2011). More recently, Hanbali et al. (2012) studied the potential of four different sweet cherry extracts (aqueous-extracted pomace, methanol extracted juice, methanol-extracted pomace and whole juice extract) to inhibit five gram-positive (*Enterococcus Group D*, *Baccilus subtilis*; *Staphylococcus aureus*, *Streptococcus Group A* and *Streptococcus Group B*) and seven gram-negative bacteria (*Escherichia coli*, *E. Coli* produced by extended spectrum *B*-lactamase (*E. coli ESBL*), *Proteus vulgaris*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Citrobacter koseri*), obtaining different results. The methanol extracted juice and methanol-extracted pomace inhibited *B. subtilis*, *Enterococcus Group D*, *C. koseri*, *E. coli*, E. *coli ESBL* and *P. vulgaris*, while aqueous-extracted pomace inhibited *Enterobacter cloacae*, *E. coli* and *E. coli ESBL* and whole juice extract only inhibited *E. coli*, *Enterococcus Group D* and *P. vulgaris*.

This potential is closely linked to phenolic constitution due to the presence of free OH groups (Hanbali et al. 2012; Strugala et al. 2015), which allow phenolics to alter the oxidant-responsive transcription factors, hypoxia-inducible factor-1*a* and nuclear factor kappa B on mitogen-activated protein kinases (MAPK) (Hanbali et al. 2012), disturbing the function of bacterial cell membranes by creating complexes with extracellular and soluble proteins

present in bacterial cell walls, slowing the growth and multiplication of bacteria, and also participate in adhesion, protein and cell wall binding, enzyme inactivation, and intercalation into the cell wall and/or DNA inactivating the pathogens (Cisowska et al. 2011). More specifically, anthocyanins and chlorogenic acid induce the release of lipopolysaccharides from the outer membrane of gram-negative bacteria, as *E. coli,* causing their disruption (He and Giusti 2010; Lee and Lee 2010) and quercetin is able to inhibit bacterial DNA gyrase (Cushnie and Lamb 2005). Recently Daglia (2012) reported that flavan-3-ols (mainly catechins) and flavonols (as quercetins) are the phenolic compounds with the most antimicrobial power, being able to supress microbial virulence factors, such as inhibiting biofilm formation, reducing host ligands adhesion, neutralizing bacterial toxins and acting in a synergistic form with antibiotics, enhancing their protective effects (Daglia 2012).

II. Aims of the study

The aim of this study was to improve the knowledge about coloured and non-coloured phenolic profile of sweet cherries from Fundão and evaluate their biological potential. To elaborate this work, several specific objectives were defined:

- Liquid chromatography with diode-array detection (LC-DAD) characterization of the non-coloured phenolics and anthocyanins profile of five sweet cherries cultivars from Fundão;
- Validation of a LC-DAD method for routine determination of non-coloured phenolic compounds in cherries;
- Validation of a LC-DAD method for routine determination of anthocyanins in cherries;
- Evaluation of the antioxidant capacity of sweet cherries hydroethanolic phenolic rich extracts against FRAP, DPPH and NO radicals;
- Determination of the inhibitory potential of sweet cherries hydroethanolic phenolic rich extracts against *α*-glucosidase enzyme; and
- Evaluation of the capacity of *Saco* hydroethanolic phenolic rich extract to prevent $ROO^{\bullet}$ induced oxidative damage in human erythrocytes, concerning to inhibit hemoglobin oxidation and hemolysis.

III. Materials and Methods

1. Standards and reagents

All chemicals used were of analytical grade. The standard compounds were purchased from various suppliers. Cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, pelargonidin-3-*O*-rutinoside and peonidin-3-*O*-rutinoside were from Extrasynthese (Genay, France). 3-*O*-caffeoylquinic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, kaempferol-3-*O*-glucoside, quercetin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, catechin, epicatechin and caffeic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH•), *B*-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), *a*-glucosidase from *Saccharomyces cerevisiae* (type I, lyophilized powder), phosphate-buffered saline (PBS), trypan blue and 2,2′- azobis (2-ethylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma- Aldrich (St. Louis, MO, USA). N-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, 4-nitrophenyl-alpha-D-glucopyranoside and sodium nitroprusside dihydrate (SNP) were obtained from Alfa Aesar (Karlsruhe, Germany). Methanol and acetonitrile were from Fisher Chemical. Water was deionized using a Milli-Q water purification system (Millipore Ibérica, S.A.U., Madrid).

2. Cherry samples

Five sweet cherries (*Prunus avium* L.) cultivars, namely *Saco, Sweetheart, Satin, Maring* and *Hedelfinger* (Figure 5) were collected from Fundão region at the same stage of ripness. Approximately, 1 kg of fruits were collected by hand between may and june 2015. Cherries were immediately transported to the laboratory of Faculty of Health Sciences. Pits were removed and separated from pulp. Cherries pulp were immediately frozen with liquid nitrogen and maintained at -20ºC, lyophilized (SCANVAC CoolSafe^{tm}, Frilabo, Portugal) and powdered (mean particle size lower than 910 µm), being divided into three aliquots, extracted, and analysed separately for chemical composition and biological activity.

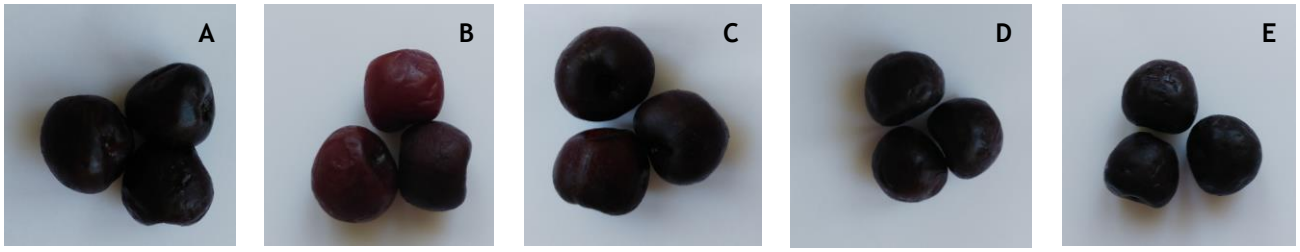


Figure 5. Sweet cherries samples analysed in this study: *Saco* (A), *Sweetheart* (B), *Satin* (C), *Maring* (D) and *Hedelfinger* (E).

3. Determination of phenolic compounds

3.1. Extraction

The non-coloured and coloured phenolic compounds were extracted according to the procedure described by Silva and Queiroz (2016). Aliquots of 1 g of powder sample were weighed and extracted with 100 mL of EtOH (80%) along 2 h under stirring after flushing with nitrogen to avoid oxidations. Then, the extract was centrifuged for 10 min at 4000 rpm/ min. Then, the material was again extracted during 15 min with 100 mL of EtOH (80%). The both supernatants were evaporated to dryness under reduced pressure at 30ºC. The resultant extract was dissolved with 50 mL of deionised water and placed into the column. The solid-phase extraction cartridge was preconditioned with 20 mL of ethyl acetate, 20 mL of ethanol and 20 mL of 0.01 mol/L HCl. After passage of the sample, the column was washed with 3 mL of 0.01 mol/L HCl. Then, the fraction I, designed by non-coloured phenolics was eluted with 20 mL of ethyl acetate. The fraction II, designed by anthocyanins was eluted with 40 mL of ethanol containing 0.1% HCl. The fractions I and II were evaporated under reduced pressure, and the dried extracts obtained were redissolved with 4 mL of methanol (non-coloured phenolics) and in 20 mL of acidified water, pH 3.0 (anthocyanins), and filtered using a PTFE membrane (0.45 µm). The average yield of extractions was 0.79 ± 0.03% and 0.39 ± 0.03% for fraction I and II, respectively. Phenolic compounds quantification was achieved by measuring the absorbance recorded in the chromatograms relative to external standards. This procedure was performed in triplicate.

3.2. LC-DAD analysis

The extracts were analysed using a HPLC model Agilent 1260 system (Agilent, Santa Clara, California, USA). Chromatographic separation was achieved on a Nucleosil® 100-5 C18 column (25.0 cm ×0.46 cm; 5 µm particle size waters; Macherey-Nagel, Düren, Germany).

3.2.1. Determination of anthocyanins

The method used for anthocyanins extraction was adopted from Silva and Queiroz (2016). The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v/v; eluent A) and water/formic acid/acetonitrile (40:10:50, v/v/v; eluent B) using a gradient program as follows: from 10% to 25% B (10 min), from 25% to 31% B (5 min), from 31% to 40% (5 min), from 40% to 50% B (10 min), from 50% to 100% B (10 min), from 100% to 10% B (5 min). Total run time was 50 min. Flow rate was 0.8 mL/min. The injection volume was 20 µL. Detection was achieved with an Agilent 1260 Infinity Diode Array Detector. The compounds in each sample were identified by comparing their retention times and UV–Vis spectra in the 200–600 nm range with the library of spectra previously compiled by the authors. Peak purity was checked by means of the ChemStation software supplied by Agilent Technologies (Waldbronn,

Germany). Anthocyanin quantification was achieved by the absorbance recorded in the chromatograms relative to external standards at 500 nm. Compounds Unknown 1 and Unknown 2 were quantified as cyanidin-3-*O*-rutinoside.

3.2.2. Determination of non-coloured phenolic compounds

The method for quantification of the non-coloured phenolic was previously described by Silva and Queiroz (2016). The mobile phase used is composed by 2% (v/v) acetic acid in water (eluent A) and 0.5% (v/v) acetic acid in water and acetonitrile (50:50, v/v, eluent B). The solvent system starting with 10% of B, and installing a gradient to obtain (24% B at 20 min, 30% B at 40 min, 55% B at 60 min, 70% B 65 min, 80% B at 70 min), 100% B at 75 min, and maintain 100% B isocratic during 5 min (80 min). A solvent flow rate was 1,0 mL/min. The injection volume was 20 µL. Detection was achieved with an Agilent 1260 Infinity Diode Array Detector. Spectral data from all peaks were accumulated in the range of 200-400 nm and chromatograms were recorded at 280, 320 and 350 nm. The data were processed on ChemStation software supplied by Agilent Technologies (Waldbronn, Germany). Peak purity was checked by the software contrast facilities. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The quantification of phenolic compounds was achieved by the absorbance recorded in the chromatograms relative to external standard at 350 nm for flavonols, at 320 nm for hydroxycinnamic acids. At 280 nm was used for flavan-3-ols and hydroxybenzoic acids. The compound hydroxybenzoic acid derivative was quantified as *p*-hydroxybenzoic acid. 3-*O*-caffeoylquinic acid and hydroxycinnamic acid derivative were quantified as 5-*O*-caffeoylquinic acid. *p*-Coumaric acid derivative 1, *p*-coumaroylquinic acid and *p*-coumaric acid derivative 2 were quantified as *p*-coumaric acid. Catechin derivative was quantified as catechin.

4. Biological activity

The extract used for the biological assays were the mixture of both fractions I and II used for the evaluation of the phenolic compounds described above. The fractions were dried, being observed an average yield of 3.1% ± 0.006%. The biological assays were determined in a Microplate Spectrophotometer Reader (Bio-Rad Laboratories).

4.1. Antioxidant activity

4.1.1. FRAP assay

Ferric Reducing Antioxidant Power (FRAP) assay was performed according to the colorimetric method described by Benzie and Strain (1996), by monitoring the changes in absorbance at 593 nm at T0' and T40' due to the reduction of the Fe^{3+}-2,4,6-tripyridyl-s-triazine (TPTZ) complex to a blue coloured Fe^{2+}-TPTZ complex, induced by the extracts. This antioxidant

potential was determined against the standards of L-ascorbic acid, and the absorbance was corrected using a blank of H_2O instead of the sample. The changes in absorbance values of the tested reaction mixtures were used to calculate the ferric reducing-antioxidant power value of the samples (FRAP value in μM). For each cherry extract was used the final concentration of 1.5 mg/mL. It was tested and the experiments were performed in duplicate. The stock solution contains 300 nM acetate de buffer, pH 3.6; 10 mM 2, 4, 6-TPTZ solution in 40 mM HCL and 20 mM $FeCl_3 \bullet H_2O$. The frap solution is prepared, in a 96 well microplate, by mixing 6 μL of sample with 180 μL of working frap reagent. The working frap reagent is prepared by mixing 2.5 mL acetate buffer, 0.25 mL TPTZ and 0.25 ml $FeCl_3 \bullet H_2O$ (Thaipong et al. 2006). Ascorbic acid was used as control.

4.1.2. DPPH• assay

The ability of sweet cherries extracts to act as free radical scavenger against DPPH• radical was prepared in a 96-well plate (seven different concentrations, ranging between 5.39 μg/mL and 86.12 μg/mL). The reaction mixtures in the sample wells consisted of 25 μL of extract (redissolved in methanol) and 200 μL of 150 mM methanolic DPPH• (Silva et al. 2014) After addition of DPPH•, the plate was incubated during 30 min at room temperature, and the absorvance was determined at 515 nm. Ascorbic acid was used as control. Three experiments were performed in triplicate.

4.1.3. Nitric oxide assay

Antiradical activity was determined following a previously described method Silva et al. (2013) (Silva et al. 2013). This activity was measured spectrophotometrically in a 96-wells plate reader at 560 nm, using different sample extracts concentrations (8.060-1023 μg/mL).

The reaction mixture in each well consisted on 100 μL of extract dissolved in buffer (KH_2PO_4 100 mM, pH 7.4) and 100 μL of SNP (20 mM). The plates were incubated at room temperature for 60 min, under light. 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 2% H_3PO_4) was then added, and 10 min latter the observance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was determined at 562 nm. Ascorbic acid was used as control. Three experiments were performed in triplicate.

4.2. *a*-Glucosidase inhibitory activity

The activity of cherry extracts against *a*-glucosidase activity was evaluated based on Ellman's method previously described by Silva and Teixeira (2015). Six different extract concentrations (1.82-29.06 μg/mL) were tested. Each well contained 100 μL of 2 mM 4-nitrophenyl-*a*-D-glucopyranoside (PNP-G), in 150 μL of 100 mM potassium phosphate buffer (pH 7.0) and 50 μL of the extract dissolved in buffer. The reaction was initiated by the addition of 25 μL of the

enzyme solution (0.44 U/ mL). The plates were incubated at 37°◦C for 10 min. After this time, the absorbance of 4-nitrophenol released from PNP-G at 405 nm was measured. The increase in absorbance was compared with that of the control (buffer instead of sample solution) to calculate the inhibitory activity. Acarbose was used as positive control. Three experiments were performed in triplicate.

4.3. *In vitro* ROO•-induced oxidative damage in human erythrocytes

For the evaluation of the *in vitro* ROO•-induced oxidative damage in human erythrocytes was used only one cherry cultivar extract. *Saco* was selected, taking into consideration that is the cultivar most important in Fundão region (Portugal). The lyophilized extract from *Saco* (1 mg) was dissolved in 1 mL of PBS and six different concentrations were tested. Each result corresponds to four experiments (n=4), at six concentrations, performed in duplicate in each microplate. The IC_{50} values were calculated from the curves of percentage of hemolysis inhibition versus extract concentration, using the GraphPad Prism software.

4.3.1. Isolation of human erythrocytes

After written informed consent, venous human blood was collected from randomized patients of Centro Hospitalar Cova da Beira (Covilhã), by antecubital venipuncture into K_3EDTA vacuum tubes. Erythrocytes were isolated based on a procedure described in detail by Chisté et al. (2014a). Briefly, the collected blood (approximately 4 mL) was transferred to sterile conic tubes (15 mL) and they were mixed with 6 mL of PBS (pH 7.4) and centrifuged at 1500 ×*g* for 5 min at 4 °C. After centrifugation, the supernatant (containing plasma and buffy coats) was discarded; the erythrocytes (red portion in the bottom of the conic tube) were washed with 6 mL of PBS and centrifuged again. This procedure was repeated twice and the supernatant was discarded. The erythrocytes were then resuspended in 6 mL using the same buffer, and an aliquot of this suspension was diluted (200x) in 0.4% trypan blue solution, gently mixed. The number of cells (cells/ mL) and viability (always above 98%) were obtained by the Trypan blue exclusion method, using a Neubauer chamber and an optic microscope (40x). The suspension with the isolated erythrocytes was kept on ice until use.

4.3.2.1. Inhibition of hemoglobin oxidation

The inhibition of hemoglobin (Hb) oxidation was assessed by monitoring the effects of the lyophilized *Saco* extract on the formation of methemoglobin (metHb) after the reaction of oxyhemoglobin (HbO_2, a complex formed by the interaction of hemoglobin with oxygen in the air) with ROO• generated by AAPH (Chisté et al. 2014b). The lyophilized *Saco* extract at 6 different concentrations (6.3–100 µg/ mL) (100 µL) dissolved in PBS, was mixed with 200 µL of the suspension of human erythrocytes (1250×10^6 cells/mL, final density) in a 48-well plate and incubated in a water-bath at 37 °C, for 30 min, under slow agitation (≈50 rpm). After

incubation, 200 µL of AAPH (50 mM, final concentration) was added to the media and then incubated again at 37 °C, under slow agitation, for 4 h. The entire volume of the reaction mixture was transferred to 1.5 mL-conic eppendorfs and centrifuged at 1500 ×*g* for 5 min at 4 °C. The supernatant (300 µL) was placed in a 96-well plate and the absorbance was read at 630 nm (Mariutti et al., 2014). The results were expressed as IC_{50} (µg/mL) from the plots as the antioxidant concentration required for inhibiting 50% of hemoglobin oxidation.

4.3.2.2. Inhibition of hemolysis

ROO• were generated by AAPH and the prevention of ROO•-induced hemolysis of human erythrocytes was evaluated by monitoring the release of hemoglobin after membrane disruption caused by the hemolytic process, according to the optimized procedure previously reported Chisté et al. (2004a). Briefly, six different concentrations of the lyophilized *Saco* extract, dissolved in PBS (31-500 µg/ mL) and the suspension of human erythrocytes (1775 × 10^6 cells/ mL), in a 48 well-plate, were incubated in a water-bath at 37 °C, for 30 min, under slow agitation (≈50 rpm), followed by the addition of AAPH solution (17 mM) and once more incubated for 3h at 37 °C under slow agitation. After incubation in the presence of AAPH, the entire volume of the reaction mixture was transferred to 1,5 mL-connic microtubes and centrifuged at 1500 ×*g* for 5 min at 4°C. The supernatant (300 µL) was placed in a 96-well plate and the absorbance was obtained at 540 nm. The results were expressed as IC_{50} values (µg/mL).

4.4. Antibacterial activity

Minimum inhibitory concentration (MIC) was determined by employing broth microdilution methods based on the Clinical and Laboratory Standards Institute (CLSI) guidelines, reference documents M07-A8 and M100-S19, with minor modifications (CLSI, 2009; Silva & Teixeira, 2015). Two bacterial species were used for the experiment: *Staphylococcus aureus* (ATCC 20231) and *Escherichia coli* (ATCC 30083). Cherry extracts were dissolved in sterile water with 5% of DMSO (v/v), and after were filtered with a sterile membrane for incorporation in a 96 wells plate reader. The MIC of extracts was determined by two-fold serial dilution method, in 96-well plates according to (CLSI, 2009; Teixeira & Silva, 2013). Well-plates were incubated for 24h at 37ºC. The MIC was determined as the lowest concentration of dried extracts inhibiting the visual growth of the test culture on the microplate. Gentamicin MIC for *S. aureus* (ATCC 20231) was determined as quality control, and the result was within the recommended limits (CLSI, 2009). Sterility and positive controls in MHB medium alone and with 5% of DMSO (v/v) were included. Positive control wells contained microorganisms without antibiotics. The experiments were performed in duplicate and repeated independently three times, yielding essentially the same results. The higher concentration of cherry extracts tested was 200 µg/mL of dried extract for the tested concentrations no antibacterial activity was founded.

5. Statistical analysis

All data were recorded as mean ± standard deviation of triplicate determinations. Mean values were compared using one-way analysis of variance (one-way ANOVA) (Graph Pad Prism Version 6.01, GraphPad Software, Inc., San Diego, CA) and the means were classified by Tukey's test at a 95% level of significance. Differences were considered significant for $P<0,05$.

IV. Results and Discussion

In this work, it was studied 5 samples of sweet cherries from Fundão region (*Saco*, *Sweetheart*, *Satin*, *Maring* and *Hedelfinger*). The cherries were analysed relative to coloured and non-coloured phenolic compounds by LC-DAD analysis. We also evaluated antioxidant capacity against FRAP, DPPH$^{\bullet}$ and $^{\bullet}$NO radicals, antidiabetic capacity based on *a*-glucosidase inhibition activity. Additionally, the *Saco* hydroethanolic extract was used to evaluate the protection capacity against ROO$^{\bullet}$ in human erythrocytes, by preventing hemoglobin oxidation and hemolysis.

1. Phenolic characterization

1.1. Anthocyanins

The analysis obtained by LC-DAD allowed the identification of six anthocyanins: unknown 1 **(1)**, cyanidin-3-*O*-glucoside **(2)**, cyanidin-3-*O*-rutinoside **(3)**, unknown 2 **(4)**, pelargonidin-3-*O*-rutinoside **(5)** and peonidin-3-*O*-rutinoside **(6)** (Figure 6 and Table 4).

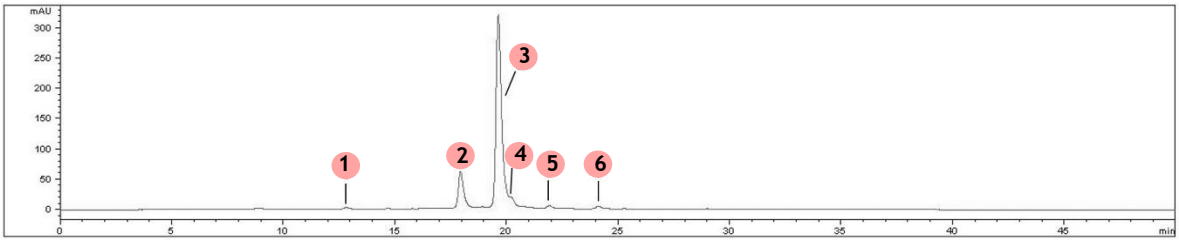


Figure 6. Coloured phenolic profile of *Saco* sweet cherry obtained by LC-DAD. Detection at 500 nm. (1) unknown 1, (2) cyanidin-3-*O*-glucoside, (3) cyanidin-3-*O*-rutinoside, (4) unknown 2, (5) pelargonidin-3-*O*-rutinoside and (6) peonidin-3-O-rutinoside.

All of these compounds were previously described in sweet cherries cultivars (Chaovanalikit & Wrolstad 2004; Gonçalves et al. 2004; Kim et al. 2005; Usenik et al. 2008; Jakobek et al. 2009; Kelebek & Selli 2011; Serra et al. 2011; Ballistreri et al. 2013; Bastos et al. 2015), except the unknown 1 and 2, despite the fact that we cannot identify the both compounds, they are reported herein for the first time (Table 4). Although the differences observed in the amounts of each anthocyanin, the analysed samples exhibited similar profile. The anthocyanin identified as unknown 1 was only identified in *Saco* and *Hedelfinger* (Table 4). On the other hand, pelargodinin-3-*O*-rutinoside was identified in all samples, except in *Satin* (Table 4).

The linearity was obtained with standard solutions at seven different concentrations, selected as representative of the range of concentrations of the compounds in sweet cherries. The calibration plots showed a good correlation, as indicated by correlation coefficient (R^2) values

higher than 0.99 for all anthocyanins (Table 4). The limit of detection (LOD=$3S_0/b$), and limit of quantification (LOQ=$10S_0/b$) (S_0 is the standard deviation of the signal-to-noise ratio of a low concentration standard and b is the slope of the calibration curve) (Oliveira et al. 2010) are also shown in Table 4. The LOD and LOQ measured in sweet cherries cultivars ranged between 0.21–0.63 ng/mL and 0.63–1.91 ng/mL, respectively. These results suggested that the proposed method is appropriated for the detection and quantification of the six anthocyanins and our results are in agreement with a previous work that reported LOD and LOQ for anthocyanins around 0.30 and 0.50 ng/mL and 0.10 and 1.25 ng/mL, respectively (Sandhu et al. 2016).

To evaluate the anthocyanins recovery, aliquots of cyanidin-3-*O*-glucoside standard solution were treated as the same method and quantified by LC-DAD, obtaining a recovery of 101.93 ± 0.27%. Repeatability was performed by analysing the same samples five times in the same day by the same analyst. The coefficients of variation obtained were lower than 5% (Table 4), proving that the repeatability of the procedure was good (Bayram et al. 2013).

Furthermore, the interday precision was determined by analysing the samples on five different days (one injection per day), and coefficients of variation found were lower than 14% (Table 4), indicating that interday precision of the procedure was satisfactory (Almeida et al. 2012).

By quantitative analysis, it was observed that the total amounts of anthocyanins ranging between 1076.97 and 2183.55 μg/g of lyophilized sweet cherries. *Maring* was the richest cultivar followed by *Hedelfinger* and *Saco*, being *Satin* the poorest one (Table 4). In a general way, our results are in accordance with Serra et al. (2011), where anthocyanins content varied between 560-3720 of μg/g of dw of fruit.

Cyanindin-3-*O*-rutinoside was the major compound in all sweet cherries cultivars, representing 87.74%-91.92% of total contents of anthocyanins, and 42.48%-68.55% of total phenolic compounds (Table 4 and Table 5), being the second phenolic more common in sweet cherries, after phenolic acids. Our data are in accordance with other previous works that reported the cyanindin-3-*O*-rutinoside as the main anthocyanin present in sweet cherries (Gonçalves, Landbo, Knudsen, et al. 2004; Usenik et al. 2008; Kelebek & Selli 2011; Serra et al. 2011; Grigoras et al. 2012). As far as we know, this is the first report about the anthocyanins profile of *Satin*.

Several other works also proved that cyanindin-3-*O*-rutinoside and cyanidin-3-*O*-glucosidase were found in major amounts, while peonidin-3-*O*-rutinoside and pelargonidin-3-*O*-rutinoside were found in lower concentrations in sweet cherries (Diaz-Mula et al. 2009; Gonçalves et al. 2004; Chaovanalikit & Wrolstad 2004; Usenik et al. 2008).

Table 4. Anthocyanins of five sweet cherries from Fundão (µg/g of lyophilized sample).

	Anthocyanins	Regression equations	R^2	LOD (ng/mL)	LOQ (ng/mL)	Repeatability (CV%)	Inter day precision (CV%)	*Saco* (µg/g)	*Sweetheart* (µg/g)	*Satin* (µg/g)	*Maring* (µg/g)	*Hedelfinger* (µg/g)
1	Unknown 1	Y=37.77X+23.53	0.9991	0.21	0.63	4.385	13.96	7.72 ± 0.19	nq	nq	nq	3.85 ± 0.14[a]
2	Cyanidin-3-*O*-glucoside	Y=113.64X+35.70	0.9992	0.21	0.63	0.523	0.81	83.76 ± 0.06	10.41 ± 0.06[a]	24.12 ± 0.02[a,b]	76.12 ± 0.65[a,b,c]	164.60 ± 1.90[a,b,c,d]
3	Cyanidin-3-*O*-rutinoside	Y=37.77X+23.53	0.9991	0.63	1.91	0.720	0.82	1462.91 ± 11.79	1050.78 ± 2.91[a]	989.96 ± 2.99[a,b]	1978.89 ± 15.21[a,b,c]	1667.23 ± 4.46[a,b,c,d]
4	Unknown 2	Y=37.77X+23.53	0.9991	0.60	1.84	2.780	9.62	67.52 ± 1.88	49.61 ± 0.67[a]	54.23 ± 0.14[a,b]	69.91 ± 3.85[b,c]	45.52 ± 2.81[a,c,d]
5	Pelargonidin-3-*O*-rutinoside	Y=39.22X+20.56	0.9990	0.60	1.84	0.834	1.03	13.48 ± 0.15	9.47 ± 1.31[a]	nq	16.02 ± 1.73[b]	15.71 ± 4.09[b]
6	Peonidin-3-*O*-rutinoside	Y=58.90X+32.24	0.9991	0.40	1.22	1.281	1.35	9.38 ± 0.07	23.40 ± 0.13[a]	8.66 ± 0.27[b]	42.62 ± 0.59[a,b,c]	3.21 ± 0.29[a,b,c,d]
							Σ	**1644.77**	**1143.66**[a]	**1076.97**[a,b]	**2183.55**[a,b,c]	**1900.12**[a,b,c,d]

Values are expressed as mean ± standard deviation of three assays. Σ, sum of the determined anthocyanins; nq, not quantified. Significant results ($P<0.05$) are indicated as: a – *vs Saco*; b – *vs Sweetheart*; c – *vs Satin*; d – *vs Maring*.

Comparing the sweet cherries with other red fruits, we can see that they were richer in anthocyanins than tart cherries (*Prunus cerasus* Linnaeus) (618.1 μg/g expressed as dw), and also presented higher contents in cyanidin-3-*O*-rutinoside, however tart cherries presented other anthocyanins not reported in sweet cherries, like as cyanidin-3-glucosylrutinoside and cyanidin-3-sophoroside (Seymour et al. 2008).

The Portuguese red grapes, *Jaen*, *Alfrochadeiro* and *Syrah* showed similar anthocyanins contents to sweet cherries, except *Touriga* that showed two times more anthocyanins than *Maring* which was the sweet cherry analysed with major contents in anthocyanins (Table 4). The man anthocyanin found in grapes was the malvidin-3-*O*-glucoside (Silva & Queiroz 2016).

Sweet cherries also showed a similar content to blackberry fruits (*Rubus Fruticosus* Linnaeus) (1760 μg/g expressed as dw) and cranberries fruits (1214 μg/g expressed as dw) (White et al. 2010; Zia-Ul-Haq et al. 2014). As observed for sweet cherries, cyanidin-3-*O*-glucoside is the major anthocyanin found in blackberries, followed by cyanidin-3-xyloside, cyanidin-3-malonylglucoside, cyanidin-3-dioxalylglucoside and cyanidin-3-sambubioside (Zia-Ul-Haq et al. 2014), while cranberries are richer in cyanidin-3-*O*-arabinoside and peonidin-3-O-arabinoside (White et al. 2010).

On the other hand, blackcurrant extracts (*Ribes nigrum* Linnaeus) presented more anthocyanins than sweet cherries (ranging between 7560-10640 μg/g expressed as dw) (Kähkönen et al. 2001), being delphinidin-3-*O*-rutinoside the major one, followed by cyanidin-3-*O*-rutinoside (Bonarska-Kujawa et al. 2014).

Furthermore, sweet cherries showed more anthocyanins than gooseberries (*Ribes grossularia* Lepaan punainen) (830 μg/g expressed as dw) (Kähkönen et al. 2001). Cyanidin-3-*O*-rutinoside was found in higher amounts (Jordheim et al. 2007).

In human nutrition, anthocyanins are known as the major antioxidant molecules, due to the number of free OH around the ring B (Miguel 2011), being responsible for 90% of antioxidant capacity revealed by sweet cherries (Jakobek et al. 2009). They are mostly found in red fruits like grapes, strawberries, blueberries, red vegetables and others, increasing them free-radical scavenger properties (Patras et al. 2010). In addition, they also show other beneficial effects, as antimicrobial (Hanbali et al. 2012), anti-inflammatory (Seymour et al. 2014), neuroprotective effects (Youdim et al., 2000), cellular signalling activity (Bell, Gaze, et al. 2014), cardiovascular and against cancer preventions and, anti-diabetic and obesity control (He & Giusti 2010). These effects are due to the ability of anthocyanins to scavenge radical species, chelate metals, establish direct binding with proteins and active receptors on peroxisome proliferator, altering its activity and affecting the energy of the substrate metabolism and inflammation (Wang & Stoner 2008). All of these capacities improve pathologic threats, as cancer, diabetes and cardiovascular pathologies.

1.2. Non-coloured phenolic compounds

In respect to non-coloured phenolic compounds, the analysis of sweet cherries by LC-DAD allowed the identification and quantification of seventeen non-coloured different phenolics, comprising one hydroxybenzoic acid **(1)**, eight hydroxycinnamic acids **(**peaks **2-4, 6-9** and **11)**, three flavan-3-ols (peaks **5**, **10** and **12**) and five flavonols (peaks **13-17**) (Figure 7 and Table 5).

All compounds were previously reported in sweet cherries (Chaovanalikit & Wrolstad, 2004; Gonçalves et al. 2004, Taylor et al. 2009, Kelebek & Selli, 2011; Serra et al. 2011; Bastos et al. 2015; Cao et al. 2015), except quercetin-3-*O*-galactoside herein reported for the first time. Nevertheless, the five cultivars showed qualitative and quantitative differences (Table 5). Quercetin-3-*O*-galactoside was only detected in *Maring* and *Hedelfinger*. Additionally, *p*-coumaric acid and quercetin were only identified in *Saco* and *Hedelfinger*. Catechin was not detected in *Satin* and *Hedelfinger*.

Calibration curves were obtained with standard solutions at seven different concentrations, selected as representative of the range of concentrations of the compounds in sweet cherries. The calibration plots showed a good correlation, as indicated by R^2 values higher than 0.99 for all non-coloured phenolics (Table 1). The LOD and LOQ determined ranged between 0.16–1.18 ng/mL and 0.50–3.59 ng/mL, respectively (Table 3). Both values of LOD and LOQ showed the lowest value obtained for *ρ*-coumaric acid derivatives 1 and 2, *ρ*-coumaroylquinic and *ρ*-coumaric acids, and the highest value for hydroxybenzoic acid derivative. These results suggested that the proposed method is appropriated for the detection and quantification of the seventeen phenolics identified, even in low concentration levels, being in agreement with Bayram et al. (2013) that reported values between 0.03 and 1.70 ng/mL for LOD and 0.30-5.40 ng/mL for LOQ (Bayram et al. 2013).

To evaluate the non-coloured phenolics recovery, aliquots of chlorogenic acid and quercetin-3-*O*-rutinoside were treated and quantified by the same method, obtaining a recovery of 86.10 ± 0.045% and 100.39 ± 1.81%, respectively.

Repeatability and interday precision were determined, and the coefficients of variation obtained were lower than 10% and 15%, respectively (Table 5), indicating that the repeatability and interday precision of the procedure were good (Bayram et al. 2013).

By quantitative analysis, it was observed that the non-coloured phenolic contents of sweet cherries cultivars ranged between 389.10 and 2024.44 μg/g of dried fruit, being *Hedelfinger* the richest one, followed by *Saco* and *Maring*. *Sweetheart* presented the lowest amounts in non-coloured phenolic compounds, with contents five times less than *Hedelfinger* and four

times less than *Saco* (Table 5). Our results were similar to those obtained by Serra et al. (2011), where non-coloured content varied between 542-1957 of µg/g of dw. Curiously, the same study, also reported that the total flavonoids in *Saco* was 1957 µg/g, alike to those obtained in this work (Table 3).

Phenolic acids corresponded to 95.17%, 93.60%, 93.55%, 90.69%, 86.32% of total non-coloured phenolic compounds for *Hedelfinger, Saco, Satin, Maring* and *Sweetheart*, respectively (Table 3). The hydroxybenzoic acid derivative was the only hydroxybenzoic acid identified and quantified in all cultivars (Table 5). Relatively to hydroxycinnamic acids, they correspond to 61.85%-91.47% of the total of non-coloured phenolic compounds, being 3-*O*-caffeoylquinic acid the major one found in all cherry samples studied, except in *Satin*, where *p*-coumaroylquinic acid was the biggest one.

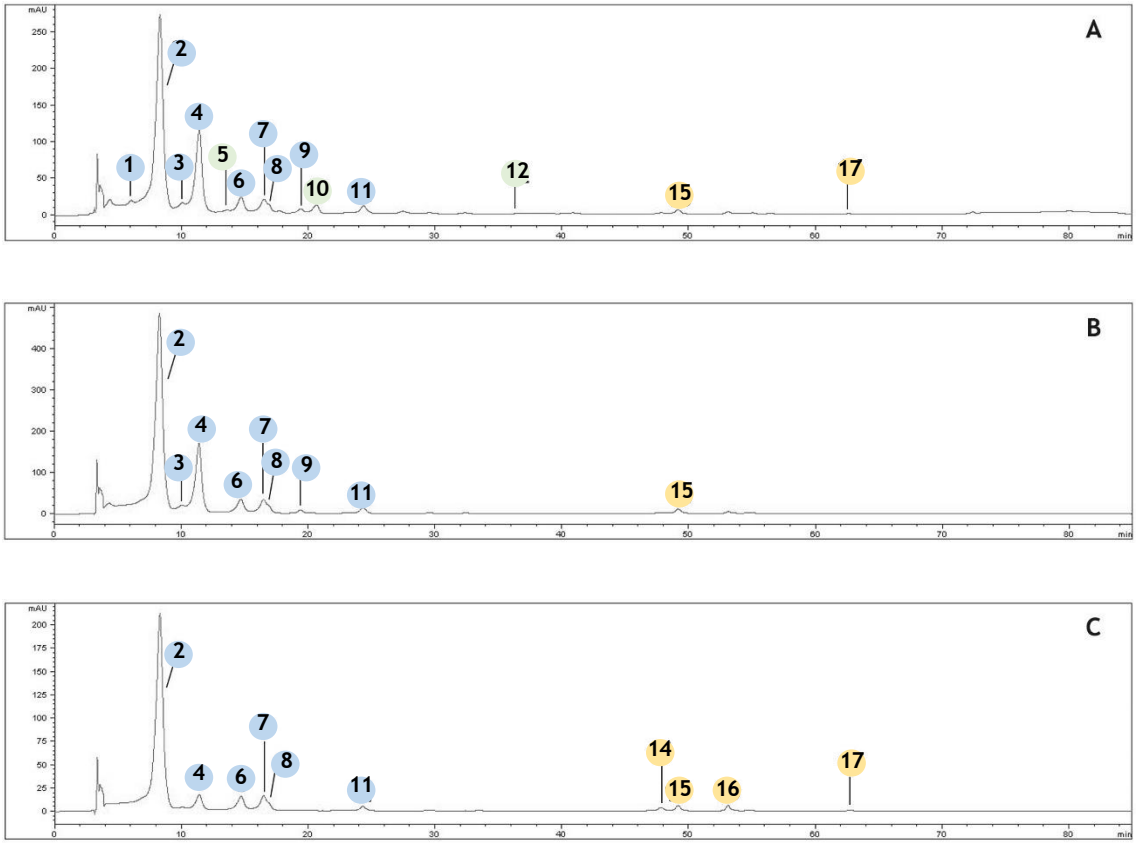


Figure 7. Non-coloured phenolic profile of Saco sweet cherry obtained by LC-DAD. Detection at 280 nm (A), 320 nm (B) and 350 nm (C).

Table 5. Non-coloured phenolic contents of five sweet cherries from Fundão (µg/g of lyophilized sample).

	Non-coloured phenolic	Regression equations	R^2	LOD (ng/mL)	LOQ (ng/mL)	Repeteability (CV%)	Inter day precision (CV%)	*Saco* (µg/g)	*Sweetheart* (µg/g)	*Satin* (µg/g)	*Maring* (µg/g)	*Hedelfinger* (µg/g)
1	Hydroxybenzoic acid derivative	Y = 20.16X + 47.964	0.9953	1.18	3.59	2.41	12.04	36.02 ± 1.08	33.26 ± 0.05	40.71 ± 0.47^{b}	49.45 ± 5.10^{a,b,c}	674.66 ± 27.69 ^{a,b,c,d}
2	3-*O*-Caffeoylquinic acid	Y = 57.75X + 74.07	0.9997	0.41	1.25	1.17	8.84	1170.74 ± 19.78	145.81 ± 3.06^{a}	255.08 ± 3.25^{a,b}	540.68 ± 11.88 $^{a, b, c}$	940.17 ± 11.79$^{a,b,c, d}$
3	*p*-Coumaric acid derivative 1	Y = 142.47X + 153.30	0.9997	0.16	0.50	1.55	14.90	18.19 ± 0.36	11.58 ± 1.04^{a}	28.49 ± 0.18^{a,b}	4.06 ± 0.05^{a,b,c}	16.47 ± 3.46^{b,c,d}
4	*p*-Coumaroylquinic acid	Y = 142.47X + 153.30	0.9997	0.16	0.50	1.26	10.36	175.03 ± 2.69	81.95 ± 0.74^{a}	349.12 ± 1.06^{a,b}	60.56 ± 0.26^{a,b,c}	161.14 ± 1.88^{a,b,c,d}
5	Catechin	Y = 21.72X + 16.74	0.9983	1.09	3.33	1.70	3.45	14.70 ± 0.32	2.67 ± 0.21^{a}	nq	4.97 ± 0.22^{a}	nq
6	Hydroxycinnamic acid derivative	Y = 57.75X + 74.07	0.9997	0.41	1.25	1.41	5.75	86.56 ± 1.53	33.95 ± 0.07^{a}	71.22 ± 0.41^{a,b}	57.25 ± 0.29^{a,b,c}	63.59 ± 0.28^{a,b,c,d}
7	5-*O*-Caffeoylquinic acid	Y = 57.75X + 74.07	0.9997	0.41	1.25	2.07	7.60	76.41 ± 0.92	26.98 ± 0.10^{a}	100.99 ± 0.40^{a,b}	53.35 ± 0.24^{a,b,c}	48.76 ± 0.26^{a,b,c,d}
8	Caffeic acid	Y = 136.66X + 18.19	0.9999	0.17	0.52	5.40	9.90	11.19 ± 0.52	nq	nq	nq	nq
9	*p*-Coumaric acid derivative 2	Y = 142.47X + 153.30	0.9997	0.16	0.50	2.53	12.69	3.45 ± 0.11	2.35 ± 0.03^{a}	17.73 ± 0.12^{a,b}	nq	5.06 ± 0.04^{a,b,c}
10	Epicatechin	Y = 27.10X + 34.96	0.9994	0.88	2.67	1.44	7.56	54.51 ± 0.95	2.81 ± 0.09 ^{a}	12.98 ± 0.06^{a,b}	7.94 ± 0.25^{a,b,c}	7.39 ± 0.20 $^{a, b, c}$
11	*p*-coumaric	Y = 142.47X + 153.30	0.9997	0.16	0.50	2.02	7.09	11.32 ± 0.27	nq	nq	nq	16.91 ± 0.08^{a}
12	Catechin derivative	Y = 21.72X + 16.74	0.9983	1.09	3.33	9.86	8.97	7.80 ± 0.99	4.07 ± 0.31^{a}	13.86 ± 0.15^{a,b}	12.50 ± 1.15^{a,b}	8.79 ± 0.30^{b,c,d}
13	Quercetin-3-*O*-galactoside	Y = 40.21X + 89.12	0.9951	0.59	1.80	--	--	nq	nq	nq	10.24 ± 0.82	8.32 ± 0.86^{d}
14	Quercetin-3-*O*-rutinoside	Y = 38.29X - 20.98	0.9999	0.62	1.89	1.35	4.33	13.09 ± 0.16	24.43 ± 0.06^{a}	12.66 ± 0.96^{b}	24.04 ± 0.96^{a,c}	44.10 ± 2.37^{a,b,c,d}
15	Quercetin-3-*O*-glucoside	Y = 59.44X + 8.18	0.9998	0.40	1.21	1.15	2.77	9.22 ± 0.13	4.99 ± 0.05 ^{a}	14.72 ± 0.11^{a,b}	4.96 ± 0.06$^{a, c}$	6.33 ± 0.79^{a,b,c,d}
16	Kaempferol-3-*O*-rutinoside	Y = 53.35X + 65.41	0.9995	0.44	1.35	2.67	6.76	6.98 ± 0.22	14.27 ± 0.05^{a}	5.29 ± 0.18^{b}	14.45 ± 0.05^{a,c}	13.53 ± 1.39^{a,c}
17	Quercetin	Y = 29.01X + 26.77	0.9997	0.82	2.49	3.82	7.72	2.32 ± 0.09	nq	nq	nq	9.22 ± 1.09^{a}
							Σ	**1697.53**	**389.10**^{a}	**922.86**^{a,b}	**844.44**^{a,b,c}	**2024.44**^{a,b,c,d}

Values are expressed as mean ± standard deviation of three assays. Σ, sum of the determined phenolic compounds; nq, not quantified. Significant results ($P<0.05$) are indicated as: a – *vs Saco*; b – *vs Sweetheart*; c – *vs Satin*; d – *vs Maring*.

These results are in accordance with previous works which reported the 3-*O*-caffeoylquinic acid as the majority non-coloured phenolic in *Saco, Maring* and *Sweetheart* cultivars (Serra et al. 2011; Hayaloglu & Demir 2016).

Phenolic acids are aromatic secondary metabolites widely spread in the plant kingdom, with considerable antioxidant effect, preventing cancer, cardiovascular and neurological pathologies (Laranjinha et al. 1994). 5-*O*-caffeoylquinic and 3-*O*-caffeoylquinic, ferulic and caffeic acids present more antioxidant capacity than *ρ*-coumarolyquinic acid because they have a diOH group while *ρ*-coumarolyquinic acid only has one (Gonçalves et al. 2004; Tokusoglu 2011). Hydroxybenzoic acids show less antioxidant capacity than hydroxycinnamic acids due to the steric hindrance of the carboxylate group near to the OH groups on phenolic ring. This fact affects their capacity to donate hydrogens (Denardin et al. 2015).

In respect to flavonoids (flavan-3-ols and flavonols), their percentages in sweet cherries ranged from 4.46% to 13.00% of the total contents of non-coloured phenolics. Epicatechin was the flavonoid found in higher amounts in *Saco*, corresponding to 3.21% of total amounts of non-coloured phenolics. On the other hand, quercetin-3-*O*-rutinoside was the most abundant in *Sweetheart, Maring* and *Hedelfinger*, ranging between 6.28%, 2.85% and 2.18% of total non-coloured phenolics, respectively; while quercetin-3-*O*-glucoside was the main one in *Satin,* representing 1.60% of total non-coloured phenolics. Flavonoids like epicatechin, catechin, quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside were previously reported in sweet cherries *Maring, Saco* and *Sweetheart* (Serra et al. 2011; Hayaloglu & Demir 2016). As far as we know, this is the first study that reported the non-coloured phenolic profile of *Satin* cultivar.

Previous works also proved that phenolic acids were the major non-coloured phenolic compounds found in sweet cherries (mainly 3-*O*-caffeoylquinic and *ρ*-coumaroylquinic acids), followed by flavan-3-ols and flavonols (Chaovanalikit & Wrolstad 2004; Kim et al. 2005; Usenik et al. 2008; Kelebek & Selli 2011; Serradilla et al. 2011; Bastos et al. 2015).

Comparatively with other red fruits, tart cherries and lingonberries showed larger amounts of non-coloured phenolics than sweet cherries (5103-7813 μg/g and 461 000 μg/ expressed as dw, respectively) (Olsson et al. 2004; Kirakosyan et al. 2009). Tart cherries presented isorharmnetin-3-*O*-rutinoside (not identified in sweet cherries), quercetin and kaempferol as the main ones (Seymour et al. 2008). Lingonberries are richer in benzoic acids, quercetin and quercetin glycosides (Olsson et al. 2004). Rowanberries (20 900 μg/g of total phenolics expressed as dw) (Kähkönen et al. 2001) also showed higher contents of 3-*O*-caffeoylquinic 5-*O*-caffeoylquinic as sweet cherries (Kylli et al. 2010).

Red grapes proved to be poorer than sweet cherries (ranged between 343.80 and 1328.30 μg/g expressed as dw), being rich in epigallocatechin, catechin and quercetin-3-*O*-glucoside (Silva & Queiroz 2016).

As sweet cherries, blueberries and black chokeberries (total amount of non-coloured phenolics = 26 700 μg/g and 68 200 μg/g, respectively) are very rich in hydroxycinnamic acids, particularly caffeic acid, presenting lower amounts in quercetin than sweet cherries (Olsson et al. 2004, Jakobek et al. 2007).

Flavonoids are the largely responsible for inducing of systemic acquired resistance against a wide range of microbial pathogens and herbivores and for provide protection against ultraviolet radiation (Cushnie & Lamb 2005). They also demonstrate antiallergic, anticancer, anti-inflammatory and anti-hemorrhagic properties due to be chemically one-electron donors (Wang et al. 1996) Particularly, flavan-3-ols are known due to their antimicrobial power (Daglia 2012) and antioxidant capacity (Scalbert et al. 2005) and flavonols for the neuroprotection offered (Andres-Lacueva et al. 2005) and anti-inflammatory activity (Dajas et al. 2013). Particularly, picatechin and quercetin are reported to be the best antioxidants flavonoids due to the presence of a catechol group (Hatia et al. 2014), highlight quercetin (and its derivatives) that are able to inhibit tyrosine kinases involved in cells growth and immune and inflammatory responses (Ren et al. 2003), showing to be 4 times more efficient to scavenge free radicals than ascorbic acid (Szajdek & Borowska 2008), showing also anti-mutagenic and carcinogenic capacities by the Ames test (Erlund 2004).

2. Biologic potential of *Prunus avium* L.

Several studies reported the health benefits of these summer fruits, being closely linked to their phenolic constitution. Anthocyanins and non-coloured phenolic compounds interact among them, increasing the colours' diversity and altering stability, flavour, nutritional values and other food qualities (Dixon & Steele 1999) and also work collaboratively, increasing health benefits, as antioxidant effects, reducing the oxidative stress which is involved in some pathologies, such as Alzheimer's disease and diabetes mellitus (He & Giusti 2010; Duarte & Silva 2014).

2.1. Antioxidant activity

The antioxidants properties of the hydroethanolic sweet cherries extracts were evaluated against FRAP, DPPH$^{\bullet}$ and $^{\bullet}$NO. To our knowledge, the antioxidant activity against $^{\bullet}$NO was herein reported for the first time in Portuguese cherries.

2.1.1. FRAP

The FRAP assay offers a total antioxidant power of food extracts and plants, estimating the ability of the extract or compound to reduce the colourless ferric ion complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ), with a yellow colour (Blasa et al. 2007). This assay gives quickly and reproducible results, and it is simple to perform (Benzie & Strain 1996). Ferric species can transfer an electron to H_2O_2, forming $^{\bullet}OH$ (Fenton reaction), oxidizing most organic molecules and proteins (Imlay 2003). An overproduction of ferric species is related to be involved in Alzheimer's disease (Andjelkovic et al. 2006).

All tested extracts exhibited antioxidant capacity in a dose-dependent effect. There were no statistically significant ($P<0.05$) differences, but we observed that *Satin* and *Saco* were the most active, with values at 26.74 µM Fe^{2+} and 25.84 µM Fe^{2+} of antioxidant power at a concentration of 1.5 mg/mL, respectively, followed by *Hedelfinger* (24.92 µM Fe^{2+}), *Maring* (20.98 µM Fe^{2+}) and *Sweetheart* (15.84 µM Fe^{2+}) (Figure 8).

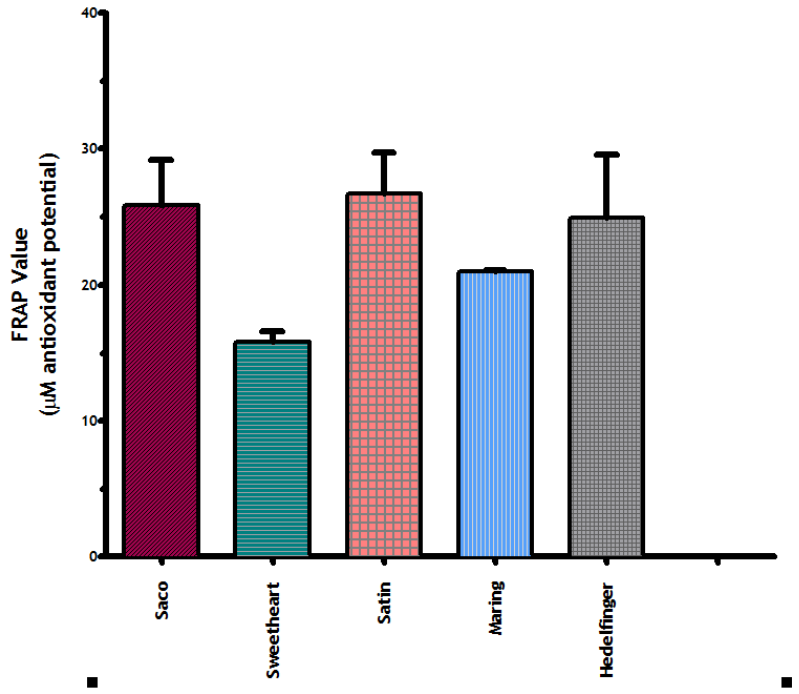


Figure 8. Antioxidant potential of sweet cherries dried extracts against ferric species.

Our values were similar to the FRAP value obtained by Cornelian cherry juice (23.50 µM) (Gastol et al. 2013). However, sweet cherries showed less capacity to reduce ferric species than blackberries (190.78 ± 0.78 µM expressed as dried ethanolic extracts) (Ivanovic et al. 2014) and harworths (79.20 µM expressed as dried methanolic extracts) (Barzegar et al. 2015). Relatively to fruit juices, our results were also lower than apple, plum and pear juices (1.97 µM, 2.37 µM and 3.92 µM, respectively) (Gastol et al. 2013).

The different values of sweet cherry cultivars obtained for FRAP assay may reflect a relative difference in the ability of antioxidant compounds in the extracts to quench ferric iron in *in vitro* systems. The antioxidant capacity of the extracts is enhanced by OH groups; 3',4'-orthodihydroxy configuration in B ring and 4-carbonyl group in C ring. In addition, the catechol group present in C ring due to the presence of 3-OH group or 3- and 5-OH groups also increase this biologic potential, the presence of the C2–C3 double bond configured with a 4-keto arrangement (Wojdyło et al. 2007). Anthocyanins are the large responsible for the capture of ferric ions due to the catechol group that enhances not only the capture of free radicals, but also the electron donating (Ali et al. 2016), converting them into stable products and terminate the free radical chain reaction (Mendes et al. 2011). The enhance of OH groups (principally in A and C rings) in other phenolics (that work in a synergestic way with anthocyanins), increasing the ferric reducing power (Benzie & Szeto 1999). Hydroxycinnamic acids, mainly caffeic and 5-*O*-chlorogenic acids, also have capacity to chelate metals. Additionally, chlorogenic acid revealed to be the best hydroxycinnamate to reduce ferric species, due to its structure, a caffeic acid ester linked to a quinic acid. Hydroxybenzoic acids also show capacity due to its carboxylic group (Andjelkovic et al. 2006).

So, it was expected that *Sweetheart* had the weakest antioxidant activity because it was the poorest cultivar in total phenolics. On the other hand, *Satin* showed the highest reducing power despite not being one of the rich cultivars in phenolic compounds. This may be explained by the presence of synergistic and antagonist effects between the compounds and due to the presence of other phytochemicals not identified in this work, their presence cannot be ignored, that might also have influence in the antioxidant activity, particularly organic acids (mainly quinic and malic acids) which possess the ability to chelate metals (Mendes et al. 2011). The other cultivars with more redactor power were *Saco* and *Hedelfinger*, the cultivars richer in total phenolics.

2.1.2. DPPH

DPPH$^{\bullet}$ is a stable radical, which allowed us to make the general screening of antioxidant capacity of hydroethanolic sweet cherries extracts, due to its stability and simplicity, involving only the reaction between the radical and an antioxidant based on hydrogen donation to scavenge DPPH radicals, changing from violet to yellow (Ebrahimzadeh et al. 2010; Teixeira & Silva 2013).

All tested extracts exhibited a dose-dependent effect against DPPH$^{\bullet}$. *Hedelfinger, Satin* and *Saco* were the most active (IC_{50} = 12.1 ± 0.37; 14.1 ± 0.43 and 16.2 ± 0.46 µg/mL of dried extract, respectively) (Table 6 and Figure 9), displaying similar activity to the positive control ascorbic acid (IC_{50}= 16.92 ± 0.69 µg/mL), revealing that hydroethanolic extracts of sweet cherries are very effective against DPPH$^{\bullet}$.

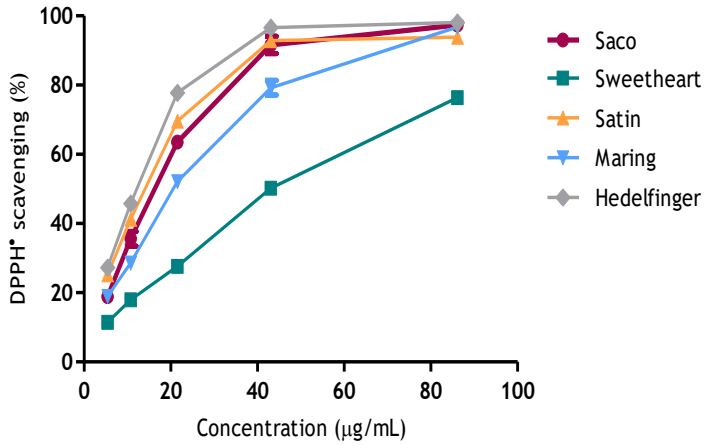


Figure 9. Antioxidant potential of sweet cherries extracts against DPPH•.

Our values are in accordance with Prvulović et al. (2011) that reported the aqueous:acetone (70:30) extract of *Hedelfinger* showed great capacity to scavenger DPPH• (showing an activity of 86.94% at 0.02 g/mL) (Prvulović et al. 2011).

Table 6. IC_{50} (µg/mL) values found in the antioxidant activity and α-glucosidase assays for sweet cherries dried extracts.

Assay	*Saco*	*Sweetheart*	*Satin*	*Maring*	*Hedelfinger*
DPPH•	16.24 ± 0.46	43.03 ± 0.53^{a}	14.10 ± 0.43^{b}	20.66 ± 0.52^{a,b,c}	12.12 ± 0.37$^{a,b\,d}$
•NO	176.69 ± 3.35	227.90 ± 1.55^{a}	439.40 ± 2.44^{a,b}	140.91 ± 1.85^{a,b,c}	185.11 ± 1.52^{b,c,d}
***α*-Glucosidase**	10.79 ± 0.40	14.34 ± 0.56^{a}	16.31 ± 0.71^{a,b}	11.38 ± 0.48^{b,c}	10.25 ± 0.49^{b,c}

Values are expressed as mean ± standard deviation of three assays; Significant results (P<0,05) are indicated as: a – *vs Saco*; b – *vs Sweetheart*; c – *vs Satin*; d – *vs Maring*.

Comparatively with other red fruits, sweet cherries revealed more activity than blackberry *Čačanska Bestrna* culivars (IC_{50} ranged between 96.0 and 118.1 µg/mL expressed as dried aqueous extracts) (Ivanovic et al. 2014) and less activity than calafate berries, blueberries (IC_{50} = 2.33 ± 0.21 µg/mL and 3.32 ± 0.18 µg/mL expressed as dried aqueous extract, respectively) and strawberries (*Fragaria* × *ananassa* Duch.) (IC_{50} = 7.6 ± 2.1 µg/ml expressed as dried aqueous extract) (Brito et al. 2014; Mandave et al. 2013). Furthermore, the activity showed by *Hedelfinger*, *Satin* and *Saco* were similar to bilberries (*Vaccinium myrtillus* Linnaeus) and pomegranates (*Punica granatum*) (IC_{50} = 14.87 ±0.52 µg/mL and 14.53 µg/ml expressed as dried methanolic and aqueous extract, respectively) (Moraes et al. 2013; Güder et al. 2015).

In respect to medicinal plants, the activity obtained for *Maring* was similar to quince (*Cydonia oblonga*) leaves (IC_{50} = 21.6 ± 3.5 μg/ml of dried methanolic extract), while *Hedelfinger* showed a close value to green tea extracts (IC_{50} = 12.7 ± 0.1 μg/ml of dried methanolic extract) (Costa et al. 2009).

The antioxidant capacity observed may be in large part due to phenolic content, whose anti-radical properties are known (Jakobek et al. 2007). Furthermore, additive or synergistic interactions occur between phenolic compounds and other non-determined active compounds. In a general way, the antioxidant capacity of fruits is proportional to their phenolic content: richer phenolic content, bigger antioxidant activity (Kelebek & Selli 2011). Anthocyanins, flavonoids and phenolic acids are the major responsible for anti-radical activity demonstrated by sweet cherries. These phenolic compounds can act as reducing agents, and also donate hydrogens, scavenge free radical species and quench singlet oxygen (Costa et al. 2009), due to their three structural groups: the *orto*-diOH structure or the catechol group in the B ring; the B ring conjugation with the 4-oxo group via the 2,3-double bond, and finally due the additional presence of both 3- and 5- OH groups in the 4-oxo-group (Hirsch et al. 2013). This is the reason why quercetin is the flavonol that presents the best antioxidant capacity (Majewska et al. 2011). Furthermore, also phenolic acids showed antioxidant activity. Particularly, 5-*O*-caffeoylquinic and 3-*O*-caffeoylquinic, ferulic and caffeic acids present more antioxidant capacity than *ρ*-coumarolyquinic acid because they have a diOH group, while *ρ*-coumarolyquinic acid is the weakest antioxidant because only has one OH group (Gonçalves et al. 2004; Tokusoglu 2011). Hydroxybenzoic acids show less antioxidant capacity than hydroxycinnamic acids due to the steric hindrance of the carboxylate group near to the OH groups on phenolic ring that affects their capacity to donate hydrogens (Denardin et al. 2015).

In addition, anthocyanins also possess great phenolic compounds that are responsible for the antioxidant activity showed by fruits and vegetables, mainly due to the OH group in position 3 of ring C and also in the positions 3', 4' and 5' in ring B, that increases not only hydrogen donation, particularly cyanidin (and its glycosides) that shows more OH groups than the others (Wang & Stoner 2008; Ali et al. 2016). This fact may explain why *Saco* and *Hedelfinger* were the most efficient cultivars against this radical.

2.1.3. Nitric oxide

The nitric oxide is an important chemical mediator generated by endothelial cells, involved in several physiological effects to defend the organism, as vascular, gastrointestinal and nervous systems, lung vasodilation, and in tumoral, microbial and inflammatory processes. They have a negative impact in mitochondria and proteins when overproduced, activating pro-inflammatory transcription factors (Sathya & Siddhuraju 2012; Bernardes et al. 2014; Silva &

Teixeira 2015), causing neurodegenerative and chronic diseases, as rheumatoid arthritis, inflammatory bowel disease, diabetes, atherosclerosis and cancer (Bor et al. 2006). During inflammatory processes, the •NO released can cause hemoglobin oxidation (conversion of hemoglobin to methemoglobin), and consequently increases the rigidity of the erythrocytes, leading to an increase of their hemolysis (Umbreit 2007). Furthermore, nitric oxide itself is not very reactive, however this free radical is able to react with oxygen and superoxide species, producing more toxic radicals, increasing cells damage (Silva & Teixeira 2015).

The Figure 10 revealed the capacity of hydroethanolic extracts from sweet cherries against •NO. All tested extracts exhibited a dose-dependent effect, being *Maring*, *Saco* and *Hedelfinger* (IC_{50} =140.91 ± 1.85; 176.68 ± 3.35 and 185.11 ± 1.52 µg/mL of dried extract, respectively) the most actives, followed by *Sweetheart* (IC_{50} = 227.89 ± 1.55 µg/mL) and *Satin* (IC_{50} = 439.39 ± 2.44 µg/mL) (Table 6 and Figure 10). Our results revealed good capacity to scavenge NO radical. *Maring*, *Saco* and *Hedelfinger* showed similar effect when compared to positive control ascorbic acid (IC_{50}= 162.66 ± 1.31 µg/mL). The positive effect of sweet cherry against •NO was proved in a study performed with eighteen healthy men and women. They consumed 280 g/day of *Bing* sweet cherries during 28 days, producing a significant reduction (about 18%) in the NO levels, after analysed blood samples (Kelley et al. 2006).

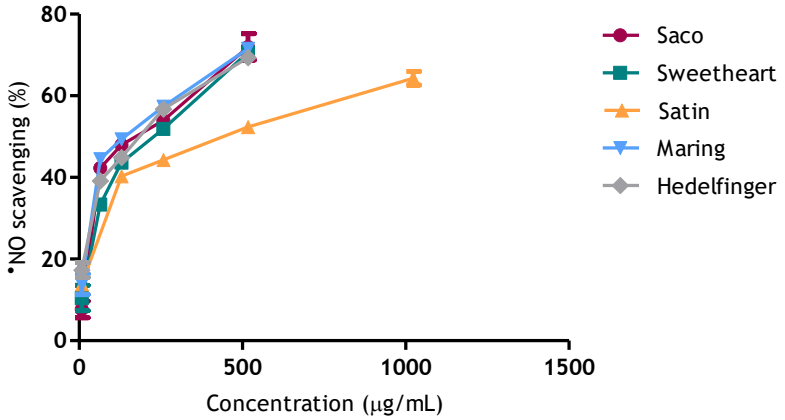


Figure 10. Antioxidant potential of sweet cherries dried extracts against •NO.

Our results are much higher than the reported by Bhattacharjee et al. (2016) (IC_{50} = 21.1 ± 2.31 µg/ml expressed as dried ethanolic extract) for Indian sweet cherries (Bhattacharjee et al. 2016). This difference of activity between sweet cherries could be due to the genotype of sweet cherries, geographical region, time of ripeness and storage conditions.

In addition, sweet cherries showed less efficiency than blackberries (IC_{50} = 2.6 ± 0.2 µg/ml expressed as dried ethanolic extract) (Basu & Maier 2016), cranberries (IC_{50} = 4.4 ± 0.4 µg/ml expressed as dried ethyl acetate extract) (Lapshina et al. 2015), strawberries guava *(Psidium*

cattleianum) peel and pulp (6.8 ± 0.2 μg/mL and 2.2 ± 0.1 μg/mL expressed as ethanolic dried extracts, respectively) (Ribeiro et al. 2014).

On the other hand, cherries were more actives comparatively with blueberries (IC_{50} = 1500 μg/mL expressed as dried ethanolic extract) (Samad et al. 2014), peel and pulp of Pingo de Mel fig (IC_{25} = 2425.5 and 3154.8 μg/mL expressed as dried aqueous extract, respectively) (Oliveira et al. 2009).

Comparatively to other fruits and plants with anti-inflammatory properties, sweet cherries proved not to be so active as bael fruits (*Aegle marmelos)* (IC_{50} = 66.9 μg/ml expresses as dried methanolic extract) (Hamid et al. 2012), *Dolichos biflorus* seeds (IC_{50} = 74.77 ± 1.73 expressed as dried 70:30 methanolic aqueous extract) (Hazra et al. 2009) and *Schinus terebinthifolius* fruits (IC_{50} = 19.23 ± 1.64 μg/ml expressed as dried methanolic extracts) (Bernardes et al. 2014). However, sweet cherries showed to be more active than leaves of *Laser trilobum* Linnaeus (IC_{50} = 517.7 ± 23.1 μg/mL expressed as dried hydroethanolic extracts) (Ebrahimzadeh et al. 2010).

The potential of plants and fruits to scavenge •NO are mainly due to flavonoids and have great impact in cells protection from damages, preventing inflammatory processes, diabetes, cancer, hemoglobin oxidation and hemolysis (Ebrahimzadeh et al. 2010; Parul et al. 2012).

This ability to scavenge •NO is better in hydroxylated flavonoids (especially 3-OH, 5-OH, 7-OH, 4'-OH and 3'-OH) and in flavonoids with double bonds between C2 and C3 (Bernardes et al. 2014), because they can easily arrest the chain of reactions initiated by •NO overproduction that causes damages (Ebrahimzadeh et al. 2010). Particularly quercetin plays an important role supressing NO• accumulation by reducing the expression of inducible nitric oxide synthase (iNOS) in a competitive manner with the substrate (Maldonado-Rojas & Olivero-Verbel 2012; Babu et al. 2013). Anthocyanins also show great ability to scavenge these radicals, inhibiting iNOS enzymatic activity and COX II (Miguel 2011). By docking, Maldonado-Rojas and Olivero-Verbel (2012) discovered that cyanidin-3-*O*-rutinoside is the anthocyanin that easily interact with iNOS, by the substrate-inhibitor binding site, regulating iNOS expression (Maldonado-Rojas & Olivero-Verbel 2012). On the other hand, Hu et al. (2003) also reported that diOH groups in the A-ring are strongly active in the •NO capture, while the 4' substitition, namely by a methyl group, affects negatively this activity. So, cyanidin-3-*O*-glucoside presents more scavenger capacity than peonidin-3-*O*-glucoside (Hu et al. 2003). Additionally, anthocyanins and flavonols also inhibit the NO production in macrophages (Kelley et al. 2006). All of these evidences explain why *Maring* and *Hedelfinger* are the richest sweet cherries in cyanidin-3-*O*-rutinoside and in cyanidin-3-*O*-glucoside, that are the best to scavenge •NO. The presence of quercetin in *Saco* and *Hedelfinger* also enhanced their efficiency against these radicals.

It is also important to emphasize the contribute of other non-identified compounds to the observed antioxidant capacity, for example, melatonin was described in sweet cherries and was also reported to be a potent scavenger of •NO (González-Gómez et al. 2009).

2.2. *α*-Glucosidase inhibitory activity

Diabetes *mellitus* type 2 is one of the most prevalent inflammatory diseases without cure, affecting more than 382 million people worldwide (Langenberg et al. 2014). This disease is characterized by hyperglycaemia resulting from defects in insulin secretion with or without insulin resistance, or from predominant insulin resistance with a relative insulin deficiency (Silva & Teixeira 2015). One of the approaches to treat this pandemic disease is to retard the absorption of glucose, by inhibiting carbohydrate-hydrolyzing enzymes, such as *α*-glucosidase, a membrane-bound enzyme located in the brush border of the small intestine required for the breakdown of carbohydrates (disaccharides and oligosaccharides) to facilitate the absorption of monosaccharides (glucose) (You et al. 2012). Inhibitors of these enzymes can delay absorption of ingested carbohydrates, reducing postprandial glucose and insulin peaks, adjusting blood sugar levels and improving insulin sensitivity (Yin et al. 2012; Silva & Costa 2014).

As far as we know, this is the first report concerning the capacity of sweet cherries extracts to inhibit *α*-glucosidase activity. All tested extracts were able to inhibit this enzyme in a dose-dependent manner. *Hedelfinger* (IC_{50}= 10.25 ± 0.49 μg/mL) was the most active, followed by *Saco* (IC_{50}= 10.79 ± 0.40 μg/mL) and *Maring* (IC_{50}= 11.38 ± 0.48 μg/mL) (Table 6 and Figure 11).

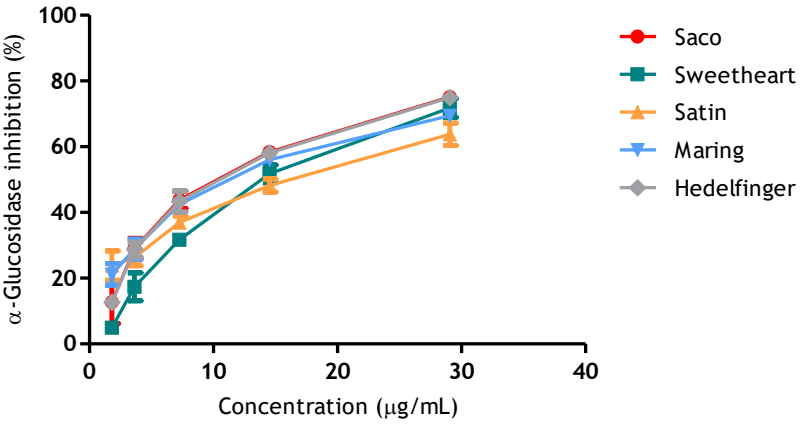


Figure 11. Inhibition capacity of sweet cherries extracts against α-glucosidase enzyme.

The obtained results are much lower compared to positive control acarbose (IC_{50}= 306.66 ± 0.84 μg/mL), a drug commercialized as enzyme inhibitor for type 2 diabetes, however its use can cause various unwanted side effects, such as intestinal pain, flatulence and diarrhea (Sathya & Siddhuraju 2012). Given that, there is an increasingly demand for inhibitors from

natural sources. Our results revealed that hydroethanolic extracts of sweet cherries high in phenolic compounds have a great potential to inhibit this enzyme, and may be used as functional food or incorporated in antidiabetic drugs. In respect to antidiabetical potential of sweet cherries, a previous study already reported that diabetic rats fed with 200 mg/kg body weight of sweet cherries extracts, during 30 days, showed blood glucose and urinary microalbumin levels reduced, and also an increase of creatinine secretion, proving that sweet cherries consumption could protect *β*-cells of pancreas from glucose-induced oxidative stress (Lachin 2014).

Comparatively with other red fruits, the inhibition of *a*-glucosidase by sweet cherries proved to be more active than the reported for raspberries (*Rubus* spp) fruits (IC_{50} = 67.7 μg/mL expressed as dried ethyl acetate extract) (Yin et al. 2012) and strawberries (*Fragaria* × *ananassa* Duch.) (IC_{50} = 76.83 ± 0.93 μg/ml expressed as dried aqueous extract) (Mandave et al. 2013). Relatively to uchuva *Physalis peruviana* fruits, very used since ancient times to threat diabetes, our extracts proved to be much more efficient than this last one (IC_{50} = 4114.7 μg/ mL expressed as dried ethanolic extract) (Rey et al. 2015). On the other hand, the average results obtained for cherries extracts revealed to be ten times better than bilberries (*Vaccinium myrtillus* Linnaeus) (IC_{50}= 138.41 ± 1.05 μg/mL expressed as dried methanolic extract) (Güder et al. 2015) and fifty-six times more than lovi-lovi (*Flacourtia inermis*) fruits (IC_{50} = 710.69 μg/mL expressed as methanolic extract) (Alakolanga et al. 2015).

Other reports exist with medicinal plants used in folk medicine for the treament of diabetes, only *polygonum senegalensis* leafs (IC_{50} = 1.5 μg/ml expressed as dried hydroalcoholic extract) (Bothon et al. 2013), *Rhus chinensis (*IC_{50} = 0.9 μg/ml expressed as dried aqueous extract) (Benalla et al. 2010) and *Endopleura uchi* leaves (IC_{50} = 2.4 μg/mL of dried infusion and IC_{50} = 2.2 μg/mL of dried hydroethanolic extract) (Silva & Teixeira 2015) proved to be more active than sweet cherries extracts. On the other hand, our extracts showed more efficiency than *Acosmium panamense* leaves (IC_{50} = 109 μg/ml expressed as butanolic dried extract**)** (Benalla et al. 2010), *Artocarpus altilis, Cinnamomum zeylanicum, Piper betel* and *Artocarpus heterophyllus* leaves (IC_{50} = 129.85 ± 10.29; 140.01 ± 10.08; 96.56 ± 12.93 and 76.90 ± 9.55 μg/mL, expressed as dried methanolic extracts, respectively). This activity was related to flavonols and phenolic acids contents found in their composition (Nair et al. 2013).

The positive results obtained for sweet cherries against *a*-glucosidase may be attributed, at least partially, to their phenolic composition, since several studies reported the antidiabetic potential of phenolic compounds (Wang et al. 2010; Kumar et al. 2011; You et al. 2012; Silva & Teixeira 2015). Both anthocyanins and non-coloured phenolics can inhibit *a*-glucosidase activity in a mixed competitive and non-competitive type inhibition, working together to enhancing antidiabetic properties (Tadera et al. 2006; Benalla et al. 2010; Adisakwattana et al. 2011; Rey et al. 2015; Kazeem et al. 2013;). A study proved that exist a direct relationship

between anthocyanins content and *α*-glucosidase activity, after analysing the content of coloured phenolics in extracts of strawberries, raspberries, blueberries, blackcurrants and red cabbages, they concluded that blueberries and blackcurrants extracts, which had the highest anthocyanin content, also were the most effective inhibitors of *α*-glucoside enzyme (McDougall et al. 2005). Tadera et al. (2006) revealed that the unsaturated C ring, 3-OH, 4-CO, 5-OH , the linkage of the B ring at the position 3, the OH substitution on the B ring, the double 2,3-double bond while 3-OH reduced it, besides increasing antioxidant capacity, also enhances inhibitory capacities, thus justifying the reason of anthocyanins being more potent to inhibit *α*-glucosidase than flavonols, flavanones and flavan-3-ols (Tadera et al. 2006), and may be the most responsible for andiabetic properties of fruits.

In this activity was also verified that the phenolic content increases the antidiabetic capacity, so *Hedelfinger,* which was the richest in phenolic content, also showed the highest activity against *α*-glucoside enzyme, followed by *Saco*.

Nevertheless, two specific studies revealed that cyanidin-3-*O*-rutinoside, catechins and other flavonoids, besides protecting cells from oxidative damage, slowing the development of diabetic complications, also competes with glucose for binding sites in sodium-dependent transporter 1, delaying glucose absorption (Iwal et al. 2006; Adisakwattana et al. 2011).

More evidences suggest that flavonoids act as ligands of peroxisome proliferator activating receptor subtype *γ,* involved in genes expression of many biological processes, particularly insulin and glucose metabolism and glucose transporter (GLUT) 4 expression, activating its expression in adipose tissue, inhibiting the expression of several anti-inflammatory enzymes, as TNF-*α*, IL-6. They also act as ligand of insulin receptor substrate-1 (this receptor that plays an important role in the insulin-stimulated signal transduction) in skeletal muscle and hepatic tissue, increasing insulin sensitivity (Zheng et al. 2011; Babu et al. 2013). Flavonoids also reduce apoptosis and promote pancreatic *β*-cells proliferation and translocation of GLUT4 via Phosphatidylinositol 3-kinase/ Protein kinase B and AMP-activated protein kinase pathways (Vinayagam & Xu 2015). Moreover, flavonoids also interact with GLUT2 and GLUT4, inhibiting intestinal glucose uptake by enhancing hepatic glucose uptake via GLUT2, or increasing the glucose uptake in adipocytes and muscles via GLUT4 (Iwal et al. 2006). Consequently, flavonoids protect pancreas function and prevent the development of diabetic complications, inhibiting the process of proteins and lipids glycosylation (Yin et al. 2012).

In another study, it was observed that catechin (IC_{50} = 3.1 μM for *α*-glucosidase inhibition) (De Souza Schmidt Gonçalves et al. 2010) improves the tolerance to glucose by starch or sucrose ingestion in rats (Scalbert et al. 2005) and also increases insulin secretion (Coman et al. 2012). Accordingly, quercetin displayed better *α*-glucosidase inhibition results (IC_{50} = 0.1 μM) than its conjugated form with rutinose (IC_{50} = 1.3 μM) (De Souza Schmidt Gonçalves et al.

2010). Diabetic rats orally administered with quercetin-3-*O*-rutinoside during 24h presented blood glucose levels reduced in 50.19% (Verma et al. 2013). This flavonol also stimulates glucose uptake via the phosphoinositide 3-kinase, a typical protein kinase C, and MAPK pathways, causing decreased glycated hemoglobin (a form of haemoglobin associated to blood glucose stability) IL-6, TNF-*a* and caspase-3, accompanied by an increase of glutathione peroxidase and B-cell lymphoma 2 (Vinayagam & Xu 2015). In respect to hidroxycinnamic acids, it was also demonstrated that 5-*O*-caffeoylquinic acid (IC_{50} = 7.6 μM) was less active than quercetin concerning *a*-glucosidase inhibition (De Souza Schmidt Gonçalves et al. 2010), causing the inhibition of glucose-6-phosphate translocase in hepatic glucose-6-phosphatase systems and contributing to reduce glucose absorption (Bräunlich et al. 2013).

Furthermore, kaempferol-3-*O*-rutinoside, *ρ*-coumaric and caffeic acids have also been gaining interest concerning diabetes, improving insulin sensitivity (Coman et al. 2012).

2.3. Protective effects of *Saco* extracts against ROO• in human blood samples

Erythrocytes contribute to immune responses, as inducing vasodilatory nitric oxide by promoting endothelial nitric oxide synthase and preventing neutrophils apoptosis (Iyer et al. 2013), but they are also considered as major targets for free radicals species for being constituted by a high concentration of polyunsaturated fatty acids and also for their specific role in oxygen transportation, being closely linked with redox active hemoglobin molecules, which are promotors of ROS (Carvalho et al. 2010). Due to their high concentrations of oxygen and ferrous ions, they generated easily ROS, such as ROO•, which are involved in membrane damages and hemolysis promotion, hindering the transport of oxygen (Chisté et al. 2014a). Although, erythrocytes contain antioxidants, as catalase, glutathione and glutathione-related enzymes, the excessive production of ROS results in oxidative damages (Mendes et al. 2011), which can be prevented by natural antioxidants from the diet.

Then, in this experimental work, and knowing the numerous benefits of noticed cherry bioactive compounds, we evaluated for the first time the preventive effect of dried hydroethanolic extracts of *Saco* sweet cherries against ROO•-mediated toxicity, using an *in vitro* model to evaluate the free radical-induced damage of biological biomarkers. APPH at 37ºC in aqueous solutions promotes oxidative stress, causing degradation of erythrocytes membrane proteins, followed by hemoglobin oxidation and last hemolysis, by the generation of alkyl radicals, that in the presence of oxygen forms ROO• (Chisté et al. 2014b).

2.3.1. Inhibition of hemoglobin oxidation

The oxidation of hemoglobin (resulting in methemoglobin (MHb), where the iron in the heme group is in the Fe^{3+} state and not as in normal state (Fe^{2+})) is not yet completely understood, but it is related to oxidative stress, in perturbations of protein interactions and damages in

lipids, that makes the membrane of erythrocytes more susceptible to be degraded (Jarolim et al. 1990; Umbreit 2007). MHb causes hipoxia events due to the inability of hemoglobin to bind or carry the oxygen and an increase of ROS and RNS. MHb is also related to erythrocytes lysis and inflammatory processes, because enhance IL-6 and IL-8 cytokines, and E-selectin release (adhesion molecule), by activating endothelial cells (Umbreit 2007; Iyer et al. 2013).

Saco dried extracts proved to have the capacity to protect this protein, supported by the results obtained for inhibition of hemoglobin oxidation in a concentration dependent manner (IC_{50} = 38.57 ± 0.96 µg/mL) (Figure 12), being twelve times less effective than quercetin (IC_{50} = 3.10 µg/mL) analysed in the same conditions, this flavonol backs to take a leading role, being reported as the most efficient phenolic against hemoglobin oxidation, preventing methemoglobinemia (Chisté et al. 2014b; Samad et al. 2014).

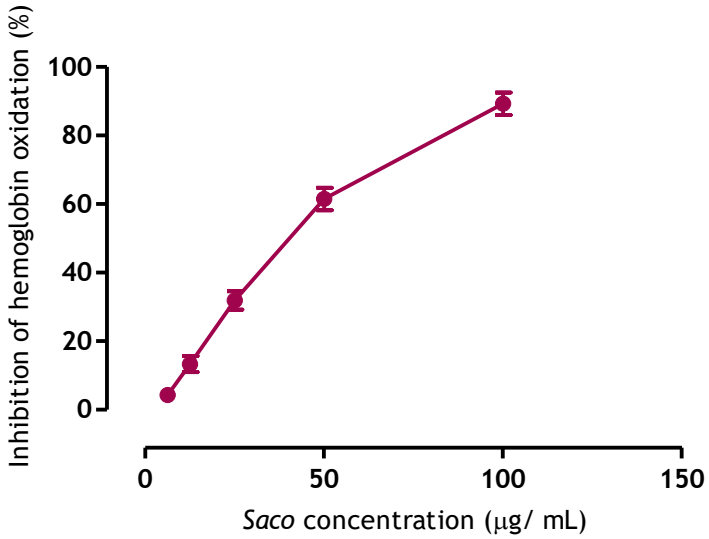


Figure 12. Protective effects of Saco against hemoglobin oxidation.

Few studies were found about the capacity of fruit extracts to protect hemoglobin against hemolysis. As far as we know, this is the first work that reported the effect of sweet cherries extracts against hemoglobin oxidation.

Relatively to other fruits and plants, *Saco* extracts showed to be seven times more efficient to prevent hemolysis than hydrophilic extracts of murici fruits (*Byrsonima crassifolia*), a fruit native from the North and Northeast regions of Brazil very rich in carotenoids (lutein and zeaxanthin), quercetin and gallic acid (IC_{50} = 271 ± 44 µg/ml expressed as dried extract) (Mariutti et al. 2014), and also more active than *Barringtonia racemose* stems and leaves (IC_{50} = 100 µg/mL and 500 µg/mL expressed as dried aqueous extract, respectively), a gallic acid rich plant very used to reduce blood pressure (Kong et al. 2014).

The flavonoids ability to prevent erythrocytes from damages is due to OH substitutions. The ones containing multiple OH substitutions, show more powerful capacity to scavenge $ROO^{\bullet}$,

protecting erythrocytes. One single OH substitution at position 5 does not provides activity, but di-OH substitutions at 3' and 4' increase the $ROO^{\bullet}$ absorbing activity of flavonoids (Cao et al. 1997). Recently Han et al. (2007), reported that phenolics with catechol rings protect better hemoglobin against oxidation than phenolics with only a phenol ring (Han et al. 2007).

Cyanidins, besides being great compounds to scavenge radicals, can easily bind to erythrocytes membrane, improving it strength and enhancing its protection against oxidation (Bonarska-Kujawa et al. 2014). Relatively to non-coloured phenolics, Kitagawa *et al.* (2004) reported that quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside and (-)epicatechin present in *Saco* cherry can inhibit hemoglobin oxidation, oxidizing the heme iron of erythrocytes, inhibiting their enzymatic reactions, as cyclooxygenase and cytochrome P450, preventing the oxidation of hemoglobin (Kitagawa et al. 2004).

However, it is necessary to perform further studies about the contribution of sweet cherries for erythrocytes protection.

Our investigation provided informations that supports the potential of sweet cherries as preventing agents against $ROO^{\bullet}$ toxicity.

2.3.2. Inhibition of hemolysis by $ROO^{\bullet}$

Free radical species attack erythrocytes, inducing the lipid peroxidation, which alters fluidity and the links established between enzymes and membranes receptors (Cheung et al. 2003), leading eventually to hemolysis, involved in cell injury and death (Alvarez-Suarez et al. 2012), associated with some hemoglobinopathies, oxidative drugs, transition metal excess, radiation and deficiencies in some erythrocyte antioxidant systems (Yang et al. 2006).

Saco extracts were able to inhibit hemolysis in a concentration dependent manner (IC_{50} = 73.03 ± 1.48 µg/mL) (Figure 13), despite their protective potential, *Saco* revealed a value 104 times less effective than quercetin control (the most efficient phenolic for erythrocytes protection reported until now) analyzed in the same conditions (IC_{50} = 0.7 µg/mL) (Chisté et al. 2014b) and three times better than vitamin C (IC_{50} = 235 ± 9 µg/ml) (Ebrahimzadeh et al. 2010). Our results proved that sweet cherries extracts could be used as therapeutic effects, namely in the treatment of hemolytic anemia.

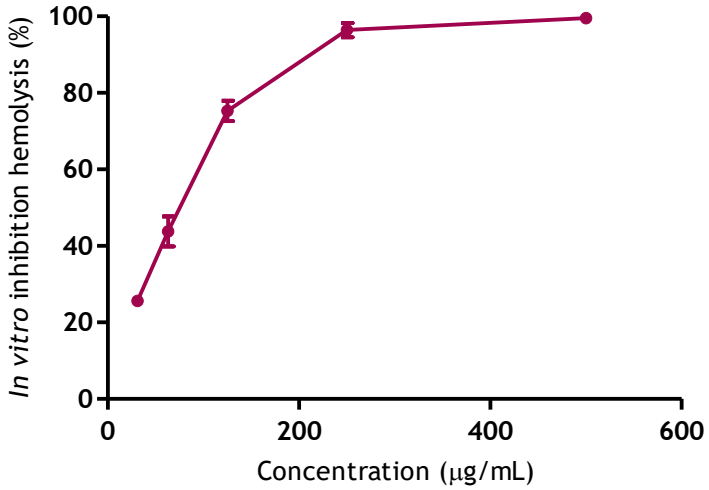


Figure 13. Protective effects of *Saco* against hemolysis.

Despite of the few studies concerning the capacity of fruits to inhibit hemolysis, our knowledge allowed us to conclude that the obtained values for hemolysis inhibition of sweet cherries were less efficient than Mexican grape (*Ruby Cabernet*) pomaces (IC_{50} = 11.62 μg/mL, expressed as dried methanolic extracts) (García-Becerra et al. 2016). Our extracts were five times more efficient than strawberries fruits (*Arbutus unedo* Linnaeus) (IC_{50} = 430.00 μg/mL expressed as dried aqueous extract) (Mendes et al. 2011) and four times less effective than hawthorn fruits (*Grataegus sinaica*) (IC_{50} = 18.00 μg/mL expressed as dried ethyl acetate extract) (Shahat et al. 1995).

Regarding to other plants, our values were similar to strawberries (*Arbutus unedo* Linnaeus) leaves (IC_{50} = 62.00 μg/mL expressed as dried aqueous extract) (Mendes et al. 2011). Nevertheless, sweet cherry extracts were less effective than green tea (*Camellia sinensis*, a plant very rich in 5-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid and flavan-3-ols) (IC_{50} = 24.30 ± 9.6 μg/ml expressed as dried methanolic infusion) (Costa et al. 2009).

It is well-known that anthocyanins and principally flavonoids enhance erythrocytes resistance against free radical species, mainly due to their capacity to capture free radicals, by donating electrons to H_2O_2 (neutralizing them into water) and also due to their metal-chelation properties, quenching the radicals formed in the aqueous phase before them can damage the erythrocytes membrane, preventing hemolysis event (Carvalho et al. 2010; Ebrahimzadeh et al. 2010), so it was expected that *Saco* was effective in erythrocytes protection against $ROO^{\bullet}$. These results are supported by other experimental work performed by Blasa et al. (2007) which proved that phenolics protect erythrocytes membrane due to its liposolubility and can donate electrons to H_2O_2, leading to its decrease (Blasa et al. 2007).

Anthocyanins increase integrity and function of erythrocytes, due to their double bond and to their structure of an *o*-diphenol in B ring, that allows them to scavenge $ROO^{\bullet}$ before they can damage the membrane of erythrocytes (Mohamed et al. 2013), while quercetin showed anti-

hemolytic properties, due to their hydrophobic nature (which facilitates their incorporation into erythrocytes), which protects lipids from the erythrocytes membrane against oxidation (Alvarez-Suarez et al. 2012). Catechin (the most flavan-3-ol present in *Saco*) can block the radical chain reaction, preventing the peroxidation process (Geetha et al. 2004). Another study, conducted by Edenharder & Grünhage (2003), demonstrated that epicatechin has the capacity to inhibit erythrocytes lysis in 86% (Edenharder & Grünhage 2003).

Chaudhuri et al. (2007) reported by *in vitro* studies via absorption and fluoresce spectroscopy, the effect of five flavonoids (namely fisetin, quercetin, chrysin, morin, and 3-hydroxyflavone) in ghost membranes of erythrocytes, concluding that these compounds can be incorporated in blood red cells, increasing their membrane integrity, decreasing its fluidity and also the diffusion of free radicals into them, and preventing hemolysis. Quercetin showed to be the most active (Chaudhuri et al. 2007). The same study also reported that quercetin was the compost that presented the best antioxidant capacity by thiobarbituric acid reactive substances assay. This compound also showed high capacity to penetrate erythrocytes and to chelate iron, protecting erythrocytes against hemolysis (Ferrali et al. 1997).

Kitagawa *et al.* (2004) reported the effect of quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, (-)epicatechin and morin against hemolysis in bovine erythrocytes induced by AAPH, and they observed that all compounds, mainly quercetin and its glycosides, can retard hemolysis, by a dose-dependent manner, obtaining values of IC_{50} = 31 ± 9 μM for quercetin, IC_{50} = 37 ± 5 μM for quercetin-3-*O*-rutinoside and IC_{50} = 47 ± 4 μM for quercetin-3-*O*-glucoside (Kitagawa et al. 2004).

V. Conclusions

The data obtained in this dissertation enabled to retire some conclusions:

- By LC-DAD, it was identified a total of six anthocyanins and seventeen non-coloured phenolic compounds in sweet cherries. Cyanidin-3-*O*-rutinoside was the anthocyanin present in larger amounts, while in relation to non-coloured phenolics, phenolic acids were the main constituents, particularly 3- and 5-*O*-caffeoylquinic acids. *Hedelfinger* has shown the highest total amounts of phenolic compounds, being also the richest sweet cherry cultivar concerning to non-coloured phenolics content. *Maring,* another sweet cherry cultivar, revealed the higher levels in anthocyanins.

- *Satin* and *Saco* exhibited the best antioxidant activity against FRAP, *Hedelfinger* against DPPH$^{\bullet}$ and *Maring* against $^{\bullet}$NO. This antioxidant potential is due to phenolic composition, highlighting cyanidin-3-*O*-rutinoside, quercetin, kaempferol and hydroxycinnamic acids properties. Their chemical structure makes them natural electron donors so they will possess the capacity to easily capture free radical species and chelate metals. Additionally, none extract revealed antibacterial activity for the tested concentrations.

- Our data revealed that sweet cherries extracts, namely *Hedelfinger*, *Saco* and *Maring*, were more effective in the inhibition of *a*-glucosidase activity than acarbose, one of the most well-known drugs commercialized as enzyme inhibitors for type 2 diabetes.

- *Saco* proved to be able to inhibit hemoglobin oxidation and hemolysis in a concentration dependent manner, being obtained promising values. Anthocyanins and non-coloured phenolics are able to incorporate at erythrocytes membrane due to their liposolubility. This process will enhance erythrocytes resistance to oxidation by capturing ROO$^{\bullet}$ before they can attack this membrane.

- The obtained results led us to conclude that sweet cherries extracts have a great biological potential, mainly due to their antioxidant activity against free radical species, protecting cells against oxidative damages and may even be used as a therapeutic in the treatment of inflammatory diseases (as diabetes, gout and arthritis), hemolytic anemia, cancer, neurological and cardiovascular pathologies. Nevertheless, more studies are needed to unravel other positive benefits of sweet cherries in our health.

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