UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Prunus avium vegetal parts as a potential source of health benefits

Versão Final Após Defesa

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Dissertação para obtenção do Grau de Mestre em

Ciências Biomédicas

(2º ciclo de estudos)

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Covilhã, outubro de 2017

Dedication

To my grandfather (in memoriam)!

Acknowledgments

Firstly, my grateful thanks to my supervisor Luís Rodrigues da Silva, for his dedicated guidance, availability and understanding, wise corrections, all scientific knowledge transmitted and constant support, being a fundamental person in the realization of this dissertation.

I am also grateful to my co-supervisor Branca Monteiro da Silva for her advice and availability.

I also want to express my deeply thanks to Universidade da Beira Interior (Covilhã) for providing all the facilities and equipment necessary. To Centro Hospitalar of Cova da Beira for the human blood samples provided, but also for their availability in the development of the experimental work. To Cerfundão, for providing all the samples of leaves, flowers and stems used in this work, and a special thanks to Engineer Filipe.

I am also thankful to the Project "Cereja do Fundão confitada com mel e carqueja como promotora da saúde" (Project CENTRO-01-0247-FEDER-017547), co-financed by the European Regional Development Fund (ERDF) through the Regional Operational Program of the Center (Portugal 2020) for funding this dissertation.

To Catarina and Carolina for the friendship and help in the accomplishment of this work. Thanks also for the advice, by the strength they gave me in the most complicated situations and by the positive criticisms.

I thank my Portuguese and Swiss friends who have always supported me in all the steps of this journey, for the joys and sorrows exchanged, and especially for the great friendship they have always offered me.

Lastly, and most importantly, I'm also gratefully thankful to my parents, my sister and my grandmother. Thank you for the unconditional support, unconditional love, friendship and strength you always given me. Thank you for always believe in me. I even thank my parents for having encouraged me to write and read when I was still in my childhood (that was essential for this challenge).

Resumo

Os benefícios das plantas edíveis e medicinais são conhecidos desde a antiguidade, estando intimamente relacionados com a capacidades destas de sintetizar uma grande variedade de compostos bioativos, que podem ser usados nas suas funções biológicas, como por exemplo na defesa contra predadores, mas também como suplementos e/ou novos fármacos devido ao seu grande poder antioxidante e capacidade anti-inflamatória. Entende-se como planta medicinal a própria planta, ou parte desta, como por exemplo folhas, frutos, raízes, flores, entre outros. Na espécie *Prunus avium* Linnaeus, o fruto tem sido alvo de vários estudos e o seu consumo incentivado. Para além de ser muito apreciado devido à sua cor, sabor doce, alto teor em água e baixo teor em calorias, apresenta ainda uma capacidade antioxidante notória. Relativamente à folha, à flor e ao pedúnculo da cerejeira, todos eles são usados como fertilizantes. A folha e o pedúnculo são ainda usados na preparação de infusões, em especial o pedúnculo, cujas funções diuréticas e sedativas são amplamente conhecidas. Nesta ordem de contexto e com o objetivo de se alargar o conhecimento e a aplicabilidade das folhas, flores e pedúnculos da cerejeira, foram preparados dois extratos, um hidroetanólico (50:50 *v*/*v*) e uma infusão das 3 matrizes. Procedeu-se então à análise do perfil fenólico e da atividade biológica dos mesmos. A análise por LC-DAD permitiu a identificação de trinta e um compostos fenólicos: um ácido hidroxibenzóico, treze ácidos hidroxicinâmicos, três flavanóis, três isoflavonas, três flavanonas e oito flavan-3-óis, sendo um derivado da sakuranetina, o ácido 5-*O*-caffeolquínico e o derivado 1 do ácido hidroxicinâmico, os compostos maioritários no pedúnculo, folha e flor, respetivamente. Em relação à atividade antioxidante, esta foi avaliada através de ensaios *in vitro* contra os radicais DPPH, superóxido e óxido nítrico. Todos os extratos mostraram grande potencial, numa dose-dependente da concentração para todos os ensaios. Os extratos hidroetanólicos dos pedúnculos e das folhas demonstraram ser os mais ativos contra o radical DPPH (IC_{50} = 22.37 ± 0.29 µg/mL e IC_{50} = 27.29 ± 0.77 µg/mL, respetivamente). Os mesmos extratos obtiveram os melhores resultados na captura dos radicais superóxido com (IC_{50} = 9.11 ± 0.16 µg/mL e IC_{50} = 13.87 ± 0.41 µg/mL, para o extrato hidroetanólico das folhas e dos pedúnculos, respetivamente). Por outro lado, a infusão dos pedúnculos e o extrato hidroetanólico das flores demonstraram ter a maior capacidade antioxidante contra o radical óxido nítrico (IC_{50} = 99.99 ± 1.89 µg/mL e IC_{50} = 123.38 ± 1.57 µg/mL, respetivamente). A atividade antidiabética foi testada utilizando a enzima *a*-glucosidade, sendo os extratos dos pedúnculos os que obtiveram o melhor resultado com IC_{50} de 3.18 ± 0.23 µg/mL para a infusão e 7.67 ± 0.23 µg/mL para o extrato hidroetanólico. Por fim, foi avaliado a capacidade protetora destas mesmas matrizes na proteção dos eritrócitos humanos contra o dano oxidativo, através de ensaios contra a oxidação da hemoglobina, peroxidação lipídica e hemólise. Todos os extratos mostraram grande capacidade protetora. O extrato hidroetanólico do pedúnculo foi o mais ativo na prevenção da peroxidação lipídica e da hemólise com IC_{50} = 26.20 ± 0.38 µg/mL e IC_{50} = 1.58 ± 0.18 µg/mL, respetivamente. Por outro lado, o extrato hidroetanólico da flor

demonstrou maior capacidade protetora contra a oxidação da hemoglobina (IC_{50}=12.85 ± 0.61 µg/mL). Através dos resultados obtidos neste trabalho, podemos considerar que as folhas, flores e pedúnculos da cerejeira são uma fonte promissora de compostos bioativos, com propriedades antioxidantes, protegendo as células dos danos causados por radicais livres, potencial antidiabético e de proteção dos eritrócitos humanos. Sendo, no entanto, necessário a realização de novos estudos para desmistificar o uso destes produtos como possibilidade de formulações naturais usadas como suplementos alimentares, produtos nutracêuticos e farmacêuticos.

Palavras-chave

Prunus avium L., partes vegetais, propriedades antioxidantes, propriedades antidiabéticas, proteção de eritrócitos.

Resumo alargado

As alternativas a medicamentos ou substâncias químicas têm vindo a ganhar grande destaque no mercado relativamente à escolha da terapêutica para diversas patologias, principalmente as que são baseadas em compostos naturais, pelo seu baixo custo, fácil acesso e reduzidos efeitos secundários.

As recomendações da organização mundial de saúde (OMS) é para que o consumo de frutos e vegetais aumente, devido à composição destes em numerosos compostos bioativos capazes de desempenhar diversas funções quando ingeridos, nomeadamente funções antioxidantes, antimicrobianas e anti-inflamatórias.

As plantas, ou parte destas, têm sido usadas ao longo dos anos, na chamada medicina tradicional, sob a forma de infusões ou decocções. As partes de plantas utilizadas para estes fins, podem ser as raízes, caules, folhas, flores, caroços dos frutos, entre outros.

Na espécie *Prunus avium* L., o fruto tem sido alvo de vários estudos e o seu consumo incentivado. Para além de ser muito apreciado devido à sua cor, sabor doce, alto teor em água e baixo teor em calorias, apresenta ainda uma capacidade antioxidante notória. Relativamente à folha, à flor e ao pedúnculo da cerejeira, todos eles são usados como fertilizantes. A folha e o pedúnculo são ainda usados na preparação de infusões, em especial o pedúnculo, cujas funções diuréticas e sedativas são amplamente conhecidas. Assim sendo, e com o objetivo de se alargar o conhecimento e a aplicabilidade das folhas, flores e pedúnculo da cerejeira, foram preparados dois extratos, um hidroetanólico (50:50 *v*/*v*) e uma infusão de cada uma das 3 matrizes. Seguidamente procedeu-se à análise do seu perfil fenólico e potencial biológico relativamente à atividade antioxidante, potencial antidiabético e proteção dos eritrócitos contra danos oxidativos. A análise por LC-DAD permitiu a identificação de trinta e um compostos fenólicos: um ácido hidroxibenzóico, treze ácidos hidroxicinâmicos, três flavanóis, três isoflavonas, três flavanonas e oito flavan-3-óis.

O conteúdo total de fenóis variou entre 20485.9 e 60916.6 μg/g. Os extratos da folha demonstraram ser os mais ricos em compostos fenólicos, em especial o extrato hidroetanólico. Por outro lado, os extratos da flor foram aqueles em que o conteúdo fenólico se apresentou mais baixo.

Quanto aos compostos apresentados por cada uma das matrizes, a classe de compostos fenólicos predominante nos pedúnculos foram os flavonóides (82.0-86.6%), enquanto que na flor e na folha foram os ácidos fenólicos a classe predominante (72.0 - 83.8%). O composto maioritário do pedúnculo foi um derivado da sakuranetina (representando em ambos os extratos 46.6% do teor total dos compostos fenólicos no extrato hidroetanólico e na infusão, respetivamente).

Enquanto que na folha, o composto predominante foi o ácido 5-*O*-cafeolquínico (representando 29.5% e 36.0% do teor total dos compostos fenólicos no extrato hidroetanólico e na infusão, respetivamente). Finalmente, o derivado do ácido hidroxicinâmico 1 foi o composto maioritário detetado na flor, representando 60.2% e 66.4% do teor total dos compostos fenólicos no extrato hidroetanólico e na infusão, respetivamente.

Os radicais livres são constantemente produzidos durante o metabolismo normal da célula, na maior parte sob a forma de espécies reativas de azoto (RNS) e de oxigénio (ROS), sendo que o organismo, perante a exposição a estes radicais, desenvolveu mecanismos de defesa endógenos, a fim de minimizar a concentrações destes, uma vez que quando em excesso causam danos nas células e contribuem para o desenvolvimento de doenças associadas ao stress oxidativo. Quando estes mecanismos falham ou são insuficientes, os antioxidantes presentes na dieta assumem grande importância como possíveis agentes protetores, reduzindo os danos oxidativos. Neste contexto a atividade antioxidante dos extratos das folhas, flores e pedúnculos da cerejeira foi avaliada, através de ensaios *in vitro* contra os radicais DPPH, superóxido e óxido nítrico. O extrato hidroetanólico dos pedúnculos demonstrou ser o mais ativo contra o DPPH• (IC_{50} = 22.37 ± 0.29 µg/mL), enquanto que o extrato hidroetanólico das flores apresentou o melhor resultado contra o radical superóxido (IC_{50} = 9.11 ± 0.16 µg/mL). Por outro lado, a infusão dos pedúnculos demonstrou maior capacidade na captura do radical do óxido nítrico (IC_{50} = 99.99 ± 1.89 µg/mL). A atividade dos extratos está fortemente relacionada com a sua composição fenólica, pois a presença de grupos hidroxilo e o grupo catecol na sua estrutura, aumentam a capacidade antioxidante, uma vez que eles têm facilidade em ceder átomos de hidrogénio.

A *diabetes mellitus* é uma doença metabólica, caracterizada por hiperglicemia e distúrbios no metabolismo dos hidratos de carbono, proteínas e gorduras. Uma das formas de tratamento está relacionada com a inibição da enzima *a*-glucosidase, sendo esta responsável pela conversão dos hidratos de carbono em monossacarídeos absorvíveis, obtendo-se assim uma redução do índice glicémico. A capacidade dos extratos de inibir esta enzima foi testada para todos os extratos, e praticamente todos eles mostram ser mais ativos que o controlo positivo, a acarbose, exceto a infusão das flores. A infusão dos pedúnculos com um IC_{50} = 3.18 ± 0.23 µg/mL revelou ser a mais ativa. A excelente atividade apresentada, deve-se à composição fenólica dos extratos, e em particular à estrutura dos compostos fenólicos, que lhe permitem captar radicais, que normalmente se encontram aumentados em doenças inflamatórias, como é o caso da diabetes *mellitus*.

A membrana dos eritrócitos é suscetível a danos causados pelas espécies reativas, devido ao seu envolvimento no transporte de oxigénio, mas também devido ao seu alto teor em ácidos gordos. Através da criação de um ambiente com radicais peroxilo, conseguiu-se avaliar a atividade dos extratos das folhas, flores e pedúnculos. Todos os extratos revelaram uma boa

atividade protetora dos eritrócitos humanos, sendo o extrato hidroetanólico dos pedúnculos o que demonstrou ser mais ativo, nos ensaios da peroxidação lipídica e hemólise com IC_{50} = 26.20 ± 0.38 µg/mL e 1.58 ± 0.18 µg/mL, respetivamente. No entanto, a maior atividade contra o ensaio da oxidação da hemoglobina foi obtida com o extrato hidroetanólico das flores (IC_{50}=12.85 ± 0.61 µg/mL). O efeito protetor associado aos compostos fenólicos deve-se à sua estrutura, que permite a ligação destes à membrana aumentando a sua estabilidade e capturando estes radicais antes que eles sejam capazes de a danificar.

Os resultados obtidos neste trabalho, revelam que as folhas, flores e pedúnculos de *P. avium* são uma fonte promissora de compostos bioativos, apresentando também uma excelente atividade antioxidante, potencial antidiabético e de proteção de eritrócitos, podendo ser valorizados no futuro através da possibilidade de preparação de formulações naturais usadas como suplementos alimentares, produtos nutracêuticos e farmacêuticos.

Abstract

The benefit effects of edible and medicinal plants are recognized since ancient times, being intimately linked with the capacity of them to synthesize a great variety of bioactive compounds, that can be used in their biological functions, for example in their defense against predators, but also as a supplement and/or medicinal drug due to their higher antioxidant power and anti-inflammatory capacity. Medicinal plant is defined as the entire plant, or parts, as for example leaves, fruits, roots, flowers, among others. In *P. avium* L. specie, the fruit, has been the target of several studies and its consumption has been encouraged. In addition to be largely appreciated due to its color, sweet taste, high contents of water and low-calorie value, sweet cherries also present a notorious antioxidant capacity. Relatively to leaves, flowers and stems of cherry tree, all of them are used as fertilizers. The flowers and stems are used as infusions, especially stems whose diuretic and sedative functions are well-known. Then and with the objective to extending the knowledge and applicability of stems, there were prepared two extracts, one hydroethanolic (50:50 *v*/*v*) and an infusion of the three matrices, and the phenolic profile and biological activity of the extracts were analyzed. LC-DAD analysis allowed the identification of thirty-one phenolic compounds: one hydroxybenzoic acid, thirteen hydroxycinnamic acids, three flavanols, three isoflavones, three flavanones and eight flavan-3-ols. Being sakuratenin derivative, 5-O-caffeolquinic acid and hydroxycinnamic derivative 1, the major compound in stems, leaves and flowers extracts, respectively. The antioxidant potential was evaluated by *in vitro* assays against DPPH, superoxide and nitric oxide radicals. All extracts revealed great potential in a dose-dependent manner for all assays. The hydroethanolic extracts of stems and leaves showed to be to the most active against DPPH radical (IC_{50} = 22.37 ± 0.29 µg/mL and IC_{50} = 27.29 ± 0.77 µg/mL, respectively). The same extracts obtained the best results to scavenging superoxide radicals IC_{50} = 9.11 ± 0.16 µg/mL and IC_{50} = 13.87 ± 0.41 µg/mL, for the hydroethanolic leaves and stems extracts, respectively. On the other hand, the infusion stems and hydroethanolic flowers extracts showed the highest antioxidant activity against nitric oxide radical (IC_{50} = 99.99 ± 1.89 µg/mL and IC_{50} = 123.38 ± 1.57 µg/mL), respectively. The antidiabetic activity was tested using the *a*-glucosidase enzyme, being the extracts of stems the most active, with an IC_{50} = 3.18 ± 0.23 µg/mL for infusion, and IC_{50} = 7.67 ± 0.23 µg/mL for hydroethanolic extract. Finally, the protective capacity of the human erythrocytes against oxidative damage of leaves, stems and flowers extracts was evaluated through the assays concerning the hemoglobin oxidation, lipid peroxidation and hemolysis. The hydroethanolic extract of stems was the most active against lipid peroxidation and hemolysis with IC_{50} = 26.20 ± 0.38 µg/mL and IC_{50}=1.58 ± 0.18 µg/mL, respectively. On the other hand, the hydroethanolic extract of flowers showed the greater activity against the hemoglobin oxidation assay (IC_{50}=12.85 ± 0.61 µg/mL). Considering the results obtained in this work, we can consider that leaves, stems and flowers of sweet cherry are a promising source of bioactive compounds, with antioxidant properties, protecting cells from the oxidative damage caused by free radicals,

possess antidiabetic potential and protect the human erythrocytes. However, it is necessary to carry out new studies to demystify the use of these products as a possibility of natural formulations used as food supplements, nutraceuticals and pharmaceutical applications.

Keywords

Prunus avium L., vegetal parts, antioxidant properties, antidiabetic potential, erythrocytes protection.

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List of Abbreviations

AAPH	2,2′- azobis (2-ethylpropionamidine) and dihydrochloride
AChE	Acetylcholinesterase
ASE	Accelerate solvent extraction
CE	Capillary electrophoresis
COMT	Catechol-*O*-methyltransferases
DAD	Diode array
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DPPH$^{\bullet}$	1,1-Diphenyl-2-picrylhydrazyl radical
DW	Dry weight
ESI-MS	Electrospray ionization mass spectrometry
FAB-MS	Fast atom bombardment mass spectrometry
FW	Fresh weight
GC-MS	Gas-chromatography with a mass detector
HHPE	High hydrostatic pressure extraction
HPLC	High performance liquid chromatography
IFN-*γ*	Interferon gamma
IL	Interleukin
iNOS	Nitric oxide synthase
LC-DAD	Liquid chromatography with diode-array detection
LLE	Liquid-liquid extraction
MAE	Microwave-assisted extraction

NADH	ß-nicotinamide adenine dinucleotide
NAD(P)H	Nicotinamide adenine dinucleotide phosphatase
NBT	Nitrotetrazolium blue chloride
NO	Nitric oxide
$^{\bullet}$NO	Nitric oxide radical
OH	Hydroxyl
$^{\bullet}$OH	Hydroxyl radical
PBS	Phosphate-buffered saline
PC	Paper chromatography
PEF	Pulsed electric field
PMS	Phenazine methosulfate
PPP	Penthose phosphate pathway
RNS	Reactive nitrogenous species
ROO$^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
SCF	Supercritical fluids
SCWE	Subcritical water extraction
SFE	Super fluid extraction
SLE	Solid-liquid extraction
(SGLT)-1	Sodium-dependent glucose transporter
$O_2^{\bullet -}$	Superoxide radical
SPE	Solid-phase extraction
SULTs	Sulfotransferases

TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-α
UAE	Ultrasound-assisted extraction
UGTs	UDP-glucuronosyltransferases
UHPLC	Ultra-high pressure chromatography
UMAE	Ultrasound/ microwave assisted extraction
USDA	United States Department of Agriculture
UV/VIS	Ultra-violet/Visible
WHO	World Health Organization

I. Theoretical Introduction

1. Introduction

Nowadays, fruits and vegetables are important foods in a healthy diet, helping to prevent chronic diseases, such as cardiovascular diseases and certain types of cancer [1]. Considering the nutritional facts, health promotion and disease prevention agents are looking at these food products from a different angle, in order to develop new types of food like "superfoods" and functional foods [2].

However, their byproducts (leaves, flowers, stems and pips) goes to the "trash" during preparation and processing. The land application is a frequently used option as organic and nutrient amendment, once the byproducts can provide an important number of nutrients for crops and improve soil properties. Currently, this situation is changing since researchers are discovering new applications to valorize this "waste", creating new potential value added ingredients [3].

The valorization of food industry byproducts and agricultural waste has been the focus of worldwide investigation. There has increased the interest about the therapeutic use of natural products, especially those derived from plants [4,5]. The focus is mainly on scientific evaluation of traditional drugs with plant origin, due to their effectiveness, presumably minimum side effects, and relatively low costs and toxicity [6,7].

Sweet cherry (P. *avium* L.) is one of the most popular temperate red fruits, being Portugal is a traditional producer of sweet cherries, particularly in the regions of Beira Interior (Fundão, Belmonte and Covilhã) [8,9].

Cherries are a red fruit characterized by a reduced level of calories, high levels of water and absence of sodium. They also present a considerable amount of organic acids, fibers, vitamins, potassium, fatty acids and phytochemicals, such as volatile compounds, carotenoids, flavonoids (flavonols, flavan-3-ols, flavanones, flavones, and anthocyanins), hydroxycinnamic and hydroxybenzoic acids [10-12].This fruit is known for their innumerous benefits on human health when ingested, mainly due to its antioxidant, anticancer, antidiabetic and anti-inflammatory properties [9-11,13,14].

The production and processing of sweet cherry produced large amounts of byproducts (leaves, flowers, stems and pits), several tones are produced, they have numerous technological applications, however, currently they are used manly as fertilizers and infusions [10]. Moreover, stems of *P. avium* were reported for their use in traditional medicine as diuretics, sedatives, draining, may reduce inflammation, affect the cardiovascular system and smooth muscle [15-

[19]. In relation to leaves, some sources indicate that they are useful to make infusions [20]. Additionally, as far as we know no reports exist about the possible exploitation of *P. avium* flowers. This way, cherry byproducts represent a relatively cheap raw material that could be used for recovery of valuable health benefit compounds.

Particularly, phenolic compounds contribute towards to the color and sensory characteristics of edible plants [21], and also play an important role in reproduction and growth, providing protection against pathogens and predators [22]. Obtaining these compounds from secondary metabolism involves several important and determinant processes, including plant selection, preparation of the extracts, separation, purification and finally identification of the chemical constituents [23-25]. The biological analysis can be performed with pure extracts, but also with obtained pure compounds [26]. After this, it is necessary to identify exactly which compounds contribute to the plant's biological activities, as well as determining their mode of action [26,27].

2. Methods for extraction, separation and determination of phenolics

Phenolics are widely distributed in plant foods (including fruits, cereal grains, vegetables and legumes), beverages (tea, fruit juices, cocoa and coffee), tobacco and others [28-30]. Their preparation and extraction depends mostly on the nature of the sample matrix and the chemical properties of the phenolics, including their polarity, concentration, molecular structure, hydroxyl groups and number of aromatic rings [31,32].

Firstly, solid samples may suffer freezing with liquid nitrogen or drying, lyophilization and are homogenized [33].

Relatively to identification, separation and quantification of phenolic compounds, there is no standardized methodology, since there is no approach that can determine simultaneously all phenolics in a sample with good recovery. Indeed, there are many extraction methods for phenolic compounds. The most common technique to extract phenolics implies either organic or inorganic solvents called solid-liquid extraction (SLE). This technique can be defined as a mass transport phenomenon, in which compounds contained in a solid matrix migrate into a solvent carried into contact with it [34]. The most common extraction solvents used in the preparation of the extracts from vegetal matrices are methanol, ethanol, ethyl acetate, acetone, diethyl ether and ethyl acetate. However, as hydro-alcoholic solvents, like methanol, are toxic in processed foods [35], ethanol and propanol, have been preferred. Besides these last ones allow to extract both hydrophilic and lipophilic compounds. Water, ethyl acetate, acetone and their mixtures also can be used to extract of phenolic compounds [36].

Solid-phase extraction (SPE) is used for purification and partial concentration of matrices. It is a simple preparation process, where compounds that are suspended or dissolved in a liquid mixture are separated from other compounds also present, according to their chemical and physical properties [37]. Several columns for analyte extraction and for enrichment from sample extracts and aqueous samples are used, such as alkyl-bonded silica or copolymer sorbents [38,39]. In the majority of cases, the preferred sorbent is the C18-bonded silica [33].

Nevertheless, there are other extraction methods like liquid-liquid extraction (LLE) and super fluid extraction (SFE) [40]. LLE is based on a mass transfer operation in which an immiscible or closely immiscible liquid (solvent) is thoroughly mixed with a liquid solution (the feed), that usually comprise one or more solutes [41]. On the other hand, SFE can be a good environmentally friendly alternative to conventional organic solvent extraction methods [42]. It is a rapid method, selective, automatable and avoid the use of large amounts of toxic solvents [43], where the usual supercritical fluids (SCF) applied are ethanol, ethane, methane, carbon dioxide, propane, ammonia, benzene and water [24].

More recently, more modern extraction techniques for phenolics have emerged, such as ultrasound-assisted extraction (UAE) [44,45], microwave-assisted extraction (MAE) [46], ultrasound/ microwave assisted extraction (UMAE) [47], subcritical water extraction (SCWE) [48], pulsed electric field (PEF) [49], accelerate solvent extraction (ASE) [50] and high hydrostatic pressure extraction (HHPE) [51]. Furthermore, there are other methods that allow the identification of phenolics, such as colorimetric arrays [52], electrospray ionization mass spectrometry (ESI-MS) [53], fast atom bombardment mass spectrometry (FAB-MS) [54], thin layer chromatography (TLC) [55], paper chromatography (PC) [55] and capillary electrophoresis (CE) [56].

As regards to gas-chromatography coupled with a mass detector (GC-MS), this is essentially used to separate components of a mixture of volatile compounds [57] and to identify qualitatively and quantitatively them, allowing the acquisition of molecular mass data and structural information together with the identification of compounds [28].

The extraction of phenolics depends of several conditions, like interfering compounds (e.g. fats, sugars and organic acids) that can be extracted at the same time of the interest compounds. In this situation, an additional step is normally required, which is the hydrolysis [58]. This one is applied to convert flavonoid glycosides into aglycones. Additionally, the use of acidified organic solvents before the high performance liquid chromatography (HPLC) analysis serves to break down complexes of anthocyanins with co-pigments and metals [59].

As regards to the extraction of the phenolic compounds from *P. avium* stems, this one is currently performed using solvents such as ethanol/water or methanol/water, using SPE [60,61] (Table 1). For the identification and quantification of their phenolics, chromatographic techniques joint with different detectors are preferred to identify and quantify phenolic

compounds, especially the high performance liquid chromatography (HPLC) [62,63] combined with a mass detector (LC-MS, that is nowadays the best analytical approach to study phenolic compounds from different biological matrix) or coupled with a diode array (DAD), ultraviolet/visible (UV/VIS) or with UV-fluorescence [23] [64-67]. Both HPLC-DAD [61] and liquid chromatography (LC)/LC-MS (Table 1) were already applied to characterize the phenolic content of *P. avium* leaves [68,69]. Nevertheless, the other techniques applied were GC, HPLC and thin layer chromatography (TLC) [70] (Table 1).

Indeed, HPLC techniques offer a unique opportunity to separate, at the same time, all analyzed components together with their derivatives. In HPLC mobile phase both isocratic and eluent elution are used for analyzing phenolic compounds. Organic modifiers are applied, mainly methanol and acetonitrile. Nevertheless, methanol is often preferable because its use on higher percentages could protect the HPLC column, however its toxic for the biological systems [23]. Appropriate column selection is another critical factor in phenolic compound identification. The choice is normally based on the polarity. Usually reverse phase (RP-C18) or normal phase C18 columns are the most used [11,71]. However, new types of columns (superficial porous particles columns and monolithic) are also currently used in phenolic detection, especially when using the latest HPLC techniques, as well as ultra-high pressure chromatography (UHPLC) and LC×LC two-dimensional separation systems [72]. Other relevant techniques for detected total phenolic content include spectrophotometric assays [73].

Concerning the determination of total phenolics, numerous methods have been applied to plant samples, like as colorimetry with iron samples, permanganate titration and Folin-Ciocalteu method [42]. The last one is described in several pharmacopeias, and is the most commonly used due to its reproducibility and simplicity. The reaction forms a blue chromophore based on the transfer of electrons from phenolic compounds to phosphotungstic/phosphomolybdic complex [74]. The maximum absorption of the chromophores depends on the concentration of the pH of the solution and the concentration of the phenolic compounds. The absorption is determined by spectrophotometry at 760 nm [25].

Table 1. Identification and quantification methods of phenolic compounds of *P. avium* vegetal parts.

Plant Part	Phenolic Compounds	Technique	Chromatographic conditions	Total phenolic compounds (TPC)	Reference
Stems	3-*O*-Caffeolyquinic acid Cis caffeic acid hexoside Taxifolin-7-*O*-hexoside *p*-Coumaroylquinic acid Trans caffeic acid hexoside Catechin Sinapic acid Aromadendrin-7-*O*-hexoside Coumaric acid hexoside Ferulic acid hexoside Quercetin-*O*-rutinoside-*O*-hexoside Kaempferol-*O*-rutinoside-*O*-hexoside Aromadendrin-*O*-hexoside Methyl-aromadendrin-*O*-hexoside Quercetin-3-*O*-rutinoside Quercetin-3-*O*-glucoside Genistein-7-*O*-glucoside Naringenin-7-*O*-glucoside Kaempferol-3-*O*-rutinoside Methyl quercetin-*O*-rutinoside Kaempferol-3-*O*-glucoside Pinocembrin-*O*-pentosylhexoside Dihydrowogonin/sakuranetin-*O*-pentosylhexoside Chrysin-7-*O*-glucoside Dihydrowogonin 7-*O*-glucoside/sakuranetin 5-*O*-glucoside Methyl genistein	HPLC-DAD (λ=280 and 370 nm)	Column: Waters Sperisorb S3 ODS-2 C18 (150 mm x 4.6 mm, 3 µm particle size) Flow rate: 0.5 mL/min Mobile phase: (A) Water with 0.1 % formic acid (B) acetonitrile		[61]
Stems	Caffeic acid Ferulic acid Ellagic acid Quercetin Pyrogallol *p*-Hydroxybenzoic acid Vanillin *p*-Coumaric acid Gallic acid Ascorbic acid	LC-MS	Column: Macherey-Nagel Nucleoder C18 (125 mm x 2 mm, 5 µm particle size) Flow rate: 0.3 mL/min Injection volume: 10 µL Vaporizer temperature :300 ºC Capillary voltage: 5000 V Mobile phase: (A) Methanol with 0.5 % formic acid in water (B) methanol with 0.5 % formic acid	TPC - Folin-Ciocalteu	[60]
Stems	3-*O*-Caffeolyquinic acid Catechin Cholorogenic acid *p*-Coumaric acid Epicatechin Quercetin-3-*O*-rutinoside Kaempferol-3-*O*-rutinoside Ferulic acid Hydroxycynnamic acid derivative *p*-Coumaroylquinic acid Apigenin Sakuranetin	HPLC-DAD-UV/VIS (λ=280, 320, 350 and 520 nm)	Column: C18 (250 mm x 46 mm, 5 µm particle size) Flow rate: 1 mL/min Mobile phase: (A) Water with 1 % trifluoracetic acid (TFA) (B) Acetonitrile with 1 % trifluoracetic acid (TFA)		[75]
Leaves	Naringenin Kaempferol 3-*O*-rutinoside Kaempferol 3-*O*-glucoside Quercetin 3-*O*-rutinoside Quercetin 3-*O*-glucoside Catechin 5-*O*-Caffeolyquinic acid	HPLC-UV	Column: LiChrosorb RP18 (250 mm x 20.5 mm, 5 µm particle size)		[70]
Leaves	Quercetin-3-rutinosyl-7,3'-bisglucoside Quercetin-3-rutinosyl-4'-diglucoside Quercetin-3-rutinosyl-4'-diglucoside Kaempferol-3-rutinosyl-4'-diglucoside	HPLC-UV, (λ=300 nm)	Column: Sperisorb LiChrosorb Si60 (600 mm x 3 mm, 5 µm particle size) Flow rate: 0.9 mL/min		[68]
Leaves	Quercetin 3-rutinoside-4'-glucoside Kaempferol 3-rutinoside-4'-glucoside Quercetin-3-rutinosyl-4'-glucoside Kaempferol-3-rutinosyl-4'-glucoside	HPLC-UV LC H-NMR (400 MHz)	Column: C-18 Polygosil (250 mm x 20 mm, 5 µm particle size) Flow rate: 10 mL/min Run time: 100 min		[76]
Leaves	Quercetin-3-rutinosyl-4'-glucoside Kaempferol-3-rutinosyl-4'-glucoside Dihydrowogonin- 7-glucoside Arbutin	HPLC-UV	Column: C-18 Polygosil (250 mm x 20 mm, 5 µm particle size) Injection volume: 10 µL Flow rate: 2 mL/min		[69]

2. *Prunus avium* L.

The cherry tree is characterized as a deciduous tree, which can reach 15-25 meters in height. This plant is originally from Asia, belongs to Rosaceae family, subfamily Prunoideae, genus *Prunus* and subspecies *avium* [77], in opposition of sour cherry, whose classification is *Prunus cerasus* L. [78].

P. avium (sweet cherry fruit) has a diploid number (2n=16), while *Prunus cerasus* L. (sour cherry) is tetraploid [79]. Furthermore, sweet cherry fruit is a small rounded fruit with a red color, being rich in anthocyanins [80] and possesses low caloric value [81,82]. It is very appreciated and known for its attractive chromatic characteristics and aromatic attributes, as well as for their richness in nutrients, namely in phenolic compounds [77].

In respect to leaves, these ones are alternate, simple ovoid-acute, glabrous matt or sub-shiny, variably finely downy beneath, with a serrated margin and an acuminate tip (Figure 1A). During the autumn, they change from green to orange color. The flowers are allogamous, actinomorphic, about 2-2.5 cm in diameter, produced in early spring at the same time as the new leaves, with five pure white petals, yellowish stamens and a superior ovary. Moreover, they are hermaphroditic and pollinated by bees [78,83] (Figure 1B). As for peduncles (stems), their green color is due to their chlorophyll content (Figure 1C). Water is lost along them with other temperature-dependent metabolic processes, inducing visible symptoms of desiccation. Furthermore, color change in peduncles may be used to evaluated the degree of fruit freshness [84].

All the plant parts of *P. avium* (Figure 1) possess several compounds like micronutrients (vitamins and minerals), macronutrients (organic acids, proteins, fibers, carbohydrates and fats) and phenolic compounds [61,70,80,85,86].

Figure 1. *Prunus avium* L. (A) leaves, (B) flowers and (C) fruit and stems.

2.1. Phenolic composition of cherry vegetal parts

Phenolic compounds are secondary natural bioactive compounds found in plants and in their parts, that act as a defense mechanism against diseases [87,88]. They are ubiquitously distributed in plants and in plant-derived foods and beverages [89].

In plants, they are mostly synthesized through the pentose phosphate pathway (PPP), and shikimate and phenylpropanoid pathways [90]. The oxidative PPP provides the erythrose-4-phosphate precursor for the shikimate pathway. Consequently, the shikimate pathway converts this sugar phosphate to aromatic amino acids like phenylalanine, which becomes the precursor for the phenylpropanoid pathway [89]. Continuing, the presence of phenolic compounds in plants protect them against poor soil-fertility, pathogens, ultraviolet radiation and climate variations [91]. They have also been reported to exhibit pharmacological properties such as antioxidant, antiviral, antitumor, anti-inflammatory, hypotensive and antimicrobial activities [92,93]. They are important factors in plant food quality because of their relevant role in taste, flavor, appearance and health-promoting properties of sweet cherries and byproducts [94]. The phenolic compounds found in *P. avium* byproducts (leaves, flowers and stems) are flavonoids (flavonols, flavanols, flavones, isoflavones and flavanones) and non-flavonoids (phenolic acids).

2.1.1. Non-flavonoids

2.1.1.1. Phenolic acids

Phenolic acids can be divided in two major substituted acid derivatives: hydroxybenzoic and hydroxycinnamic acids. The differences between them are in the methoxylation and hydroxylation pattern of their aromatic rings, in the number of carbons, and also in a higher antioxidant capacity exhibited by hydroxycinnamic acids when compared to hydroxybenzoic acids [73,95,96].

2.1.1.1.1. Hydroxybenzoic acids

Hydroxybenzoic acids is a class of phenolic acids, they have 7 carbons, a C_6.C_1 carbon skeleton, and their derivatives are e.g. protocatechuic (3,4-dihydroxy), 3,4-dihydroxybenzoic, syringic

(3,5-dimethoxy-4-hydroxy), vanillic (3-methoxy-4-hydroxy), *ρ*-hydroxybenzoic, gallic (conjugated as its dimer, trimer and tetramer: ellagic acid, tergallic acid and gallic acid, respectively) acids. They are found in minor contents in *P. avium* leaf and stem than hydroxycinnamic acids [75]. Pyrogallol (0.262 mg/g dw) is the major hydroxybenzoic acid described in stems. Vanillin (0.079 mg/g dw), gallic acid (0.041 to 0.050 mg/g dw) and *ρ*-hydroxybenzoic (0.011 to 0.020 mg/g dw) were also described in these plant constituents [60] (Figure 2). Nevertheless, there are no reports about the presence of hydroxybenzoic acids in leaves and flowers. In sweet cherry fruits, the *ρ*-hydroxybenzoic and gallic acids were reported as the majority ones [97-99].

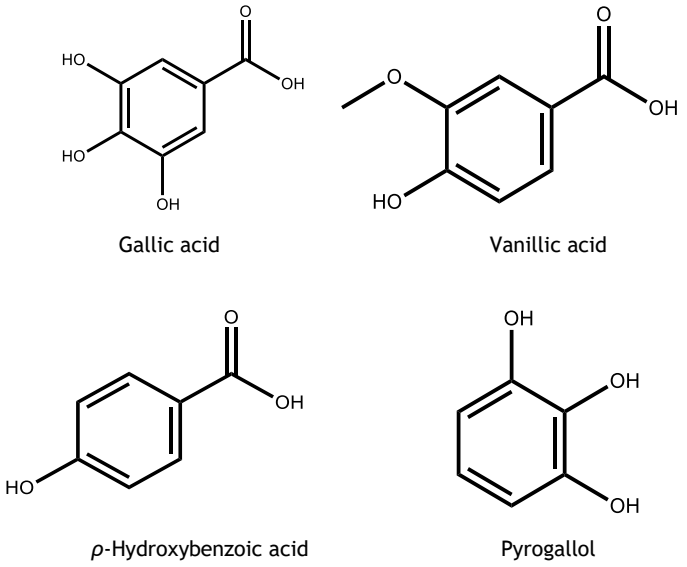


Figure 2. Hydroxybenzoic acids present in stems of *P. avium*.

2.1.1.1.2. Hydroxycinnamic acids

Hydroxycinnamic acids are phenolic acids derived from tyrosine and phenylalanine and have 9 carbons in their constitutions, a $C_6.C_3$ carbon skeleton with a characteristic double bound in the side chain, and may have a *trans* or *cis* configuration. They are one of the predominant classes of phenolic compounds found in nature [100-102]. The ones described in *P. avium* stems are *ρ*-coumaric acid (0.038-0.161 mg/g dw) which is the major one, or its derivative (*ρ*-coumaroylquinic acid) (0.53 mg/g dw), followed by 3-*O*-caffeoylquinic acid (0.43 mg/g dw), ferulic acids and derivatives (0.22-0.23 mg/g dw), caffeic acid and derivatives (0.011-0.091 mg/g dw) [60,61,75] (Figure 3). 5-*O*-caffeoylquinic acid was also described in *P. avium* leaves (17.06 mg/g dw) [70]. As far as we known, there are no hydroxycinnamic acids described in flowers of *P. avium*. In respect to sweet cherry fruits, the major acids reported are the 3-*O*-caffeoylquinic

acid, followed by *ρ*-coumaroylquinic and 5-*O*-caffeoylquinic acids. Also caffeic, synaptic, ferulic and *ρ*-coumaric acids have been referred [11,103,104].

Phenolic acids, like as cinnamic, caffeic, ferulic and 5-*O*-caffeoylquinic acids have been described in recent research as being able to prevent and treat diabetes, including insulin resistance, hyperlipidemia, hyperglycemia, obesity and inflammation [105]

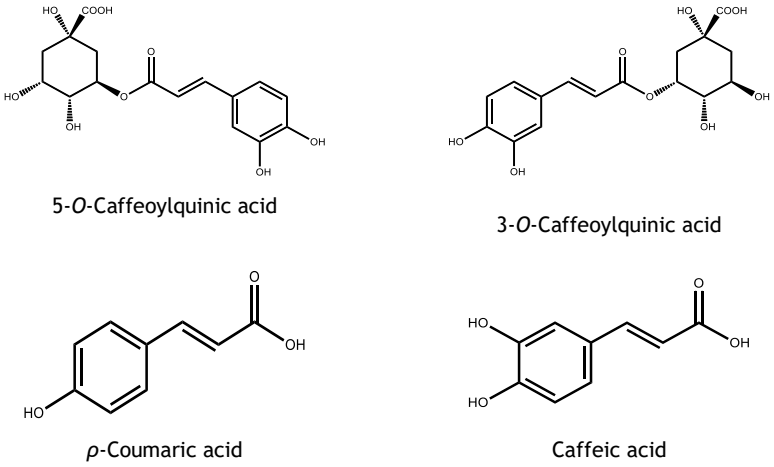


Figure 3. Main hydroxycinnamic acids present in leaves and stems of *P. avium*.

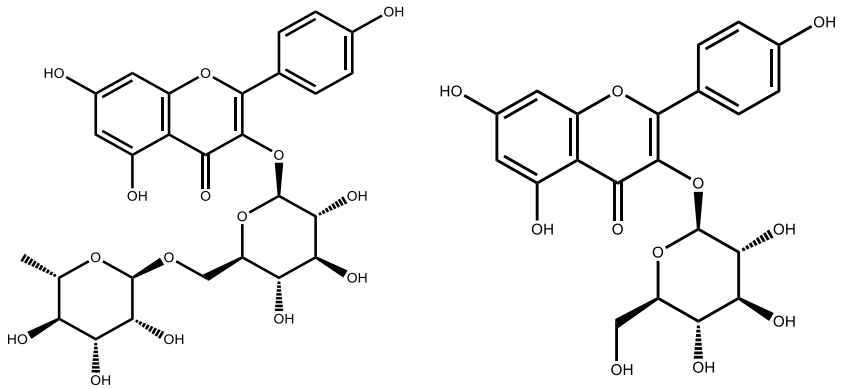
2.1.2. Flavonoids

The flavonoid class contains more than 10000 molecules based on a C_6-C_3-C_6 carbon skeleton [106]. These molecules are characterized by a phenylbenzopyrone structure and synthesized from phenylalanine [107], being derivatives of the 2-phenylchromone parent compound. They are composed by three phenolic rings (A, B and C). A and B rings derive from acetate/malonate and shikimate pathway, being linked by a 3-carbon chain that forms an oxygenated heterocyclic ring (C ring). Moreover, flavonoids are the most abundant polyphenols found in plants and edible fruits, including *P. avium* stems, leaves and sweet cherries [11,61,68,69,75]. They occur in diet primarily as aglycones or conjugated with sugar molecules (the most commons are L-ramnose, D-galactose, D-glucose and L-arabinose), thus ensuring the diversity of these compounds [108,109]. Their structural variability also insured by the degree of methoxylation, hydroxylation and glycosylation, dividing flavonoids into six subgroups: flavonols, flavanols, flavones, flavanones, isoflavones and anthocyanins [42].

The reported daily intake of flavonoids is approximately 100 mg [110]. Some of them possess antiplatelet [111,112], antitumoral [113-115], anti-ischaemic and anti-inflammatory [116-118] activities.

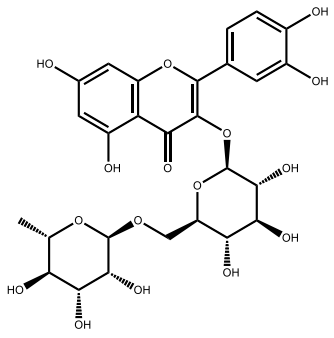
2.1.2.1. Flavonols

Flavonols are compounds with a chemical structure very identical to that of flavones, the only difference is the hydroxyl group at the 3-position of the C ring. These compounds are very common in higher plants, occurring usually in their outer parts [119]. The most frequent in *P. avium* stems, leaves and fruit are kaempferol and quercetin, being this last one the major flavonol found in sweet cherries in the form of quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside (rutin), being the last one the major representative [77,119], followed by kaempferol-3-*O*-rutinoside, myricetin-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside [98,120,121]. On the other hand, the reported compounds in *P. avium* leaves and stems are quercetin-3-*O*-glucoside (7.38 and 0.27 mg/g dw), quercetin-3-*O*-rutinoside (9.28 and 0.87 mg/g dw), kaempferol-3-*O*-rutinoside (6.60 and 0.88 mg/g dw) and kaempferol-3-*O*-glucoside (6.86 and 0.30 mg/g dw) [61,68-70,75,76], respectively (Figure 4). In stems were also described quercetin-*O*-rutinoside-*O*-hexoside (0.02-0.44 mg/g dw), methyl quercetin-*O*-rutinoside (0.15 mg/g dw) and quercetin (0.12 mg/g dw) [60,61].

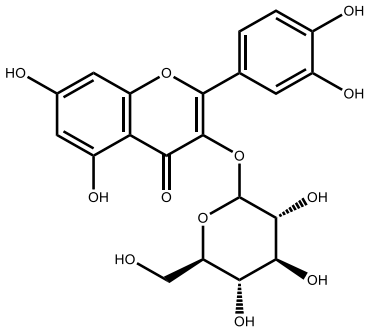


Kaempferol-3-*O*-rutinoside

Kaempferol-3-*O*-glucoside



Quercetin-3-*O*-rutinoside

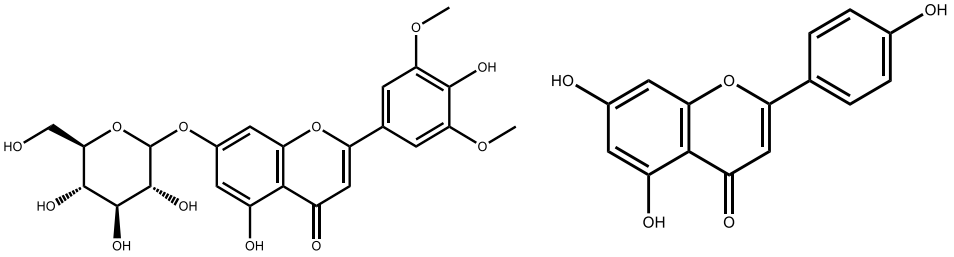


Quercetin-3-*O*-glucoside

Figure 4. Main flavonols present in leaves and stems of *P. avium*.

2.1.2.2. Flavones

Flavones are characterized by unsaturation at the C2-C3 position of the heterocyclic ring C, and given the absence of a hydroxyl group at the position 3 of the same ring, several substitutions are allowed [119,122]. The most common found in nature are apigenin, luteolin, diosmetin and they occur more frequently as 3-glycosides forms [123]. The major food sources of flavones are thyme, parsley and celery [124,125]. Chrysin-7-*O*-glucoside (0.50 mg/g dw) and apigenin (0.033 mg/g dw) are the only flavone described in stems [61,75] (Figure 5). In respect to flower and leaf, as far as we know, nothing has been reported so far. On the other hand, apigenin and luteolin were reported in sweet cherry [126].



Chrysin-7-*O*-glucoside

Apigenin

Figure 5. Main flavones present in stems of *P. avium*.

2.1.2.3. Flavanones

Flavanones are characterised primarily as flavones by the absence of hydroxyl group at the position 3 of C ring. In addition, they also present a double bond between the positions 2 and 3 and a chiral centre at position 2 [127].

The number of identified flavanones and their 3-hydroxy derivatives (flavanonols, which are also named as dihydroflavonols) has increased significantly since 2002 [128]. The most common found in nature are naringenin, hesperidin, naringenin-7-*O*-rutinoside and hesperitin [108]. On the other hand, a well-known flavanonol is taxifolin [129]. The higher sources of flavanones are citrus fruits [130,131]. Pinocembrin (0.03-0.23mg/g dw) and sakuranetin-5-*O*-glucoside (0.36-13.63 mg/g dw) are reported in stems, being the last one the major compound, while naringenin (0.74 mg/g dw) is found in leaves. Additionally, flavanonols such as taxifolin (0.19-0.79 mg/g dw), aromadendrin-*O*-hexoside (0.31 mg/g dw), methyl-aromadendrin-*O*-hexoside (0.06 mg/g dw) and aromadendrin-7-*O*-hexoside (0.86-2.66 mg/g dw) are reported in stems [61,69,70,75] (Figure 6). However, in flower the reports of these flavonoids as far as we known, remains unknow. Otherwise, naringenin and hesperitin are the only flavanones described in sweet cherry [126,132].

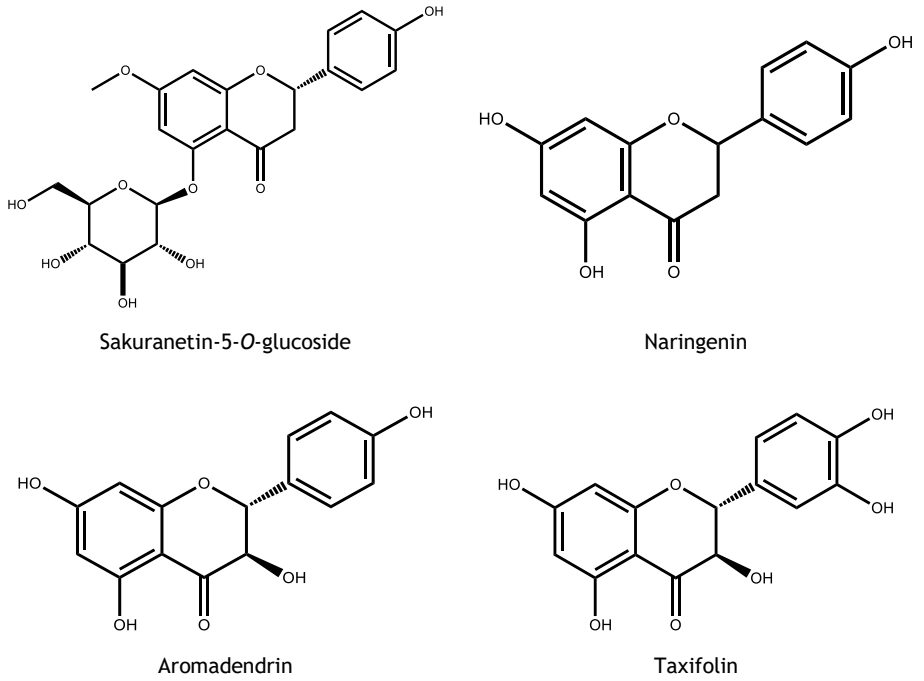
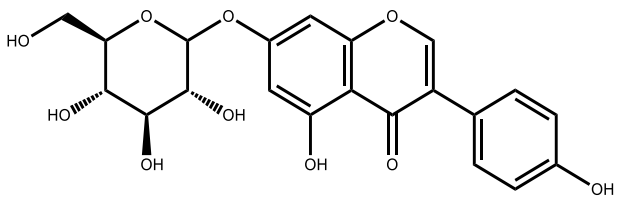


Figure 6. Main flavanones present in leaves and stems of *P. avium*.

2.1.2.4. Isoflavones

The isoflavonoids possess the phenylchroman B ring linked at position 3 instead of position 2 as presented in other flavonoids. This migration reaction is catalyzed by 2-hydroxyisoflavanone synthase and polyketide synthase chalcone synthase: both enzymes exist naturally in isoflavones [127,133]. The main food source of isoflavones is soy (*Glycine max L.*) [134]. To our knowledge, the only isoflavones described in *P. avium* components is genistin, also known as genistein-7-*O*-glucoside (0.42-3.74 mg/g dw) and methyl genistein (0.03-0.31 mg/g dw) in stems (Figure 7) [61].



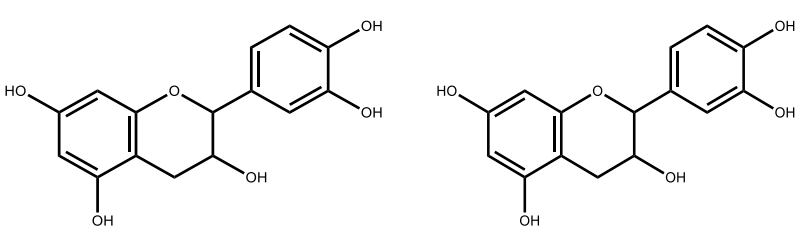
Genistein-7-*O*-glucoside

Figure 7. Genistein-7-*O*-glucoside present in stems of *P. avium*.

2.1.2.5. Flavanols

Flavanols are one of the major classes of flavonoids, including catechins, flavan-3-ols and pro-anthocyanidin dimers and trimers. Their daily intake has been estimated to be 18-50 mg, being the main sources of flavonols tea, grapes, pears and apples [135]. The two chiral centres at C2 and C3 of the monomeric flavanol produce four isomers, two of them, for each level of B-ring hydroxylation: (+)-catechin and (-)-catechin, (+)- epicatechin, (-)-epicatechin.(-)-epiafzelechin is another flavanol reported, however, it has a more limited distribution in nature [136]. They are biologically active molecules known as very strong antioxidants that can scavenge various forms of free radicals [137]. In addition, they also present ability to prevent platelet aggregation, to inhibit oxidation of low density lipoprotein (LDL), and lower plasma cholesterol levels [138].

Concerning the presence of these phenolic compounds in *P. avium* vegetal parts, catechin and epicatechin are the most common flavanol found in stems (0.42-3.74 mg/g and 0.087 mg/g dw, respectively) and leaves (0.740 mg/g dw)[61,70,75] (Figure 8). Concerning flower, as far as we known, there is no available information. Regarding sweet cherries, some reports described the presence of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin-gallate, (-)-epigallocatechin-3-gallate and (+)-gallocatechin in their constitution [98,103,132,139].



Catechin Epicatechin

Figure 8. Flavanols present in leaves and stems of *P. avium*.

2.1.2.6. Anthocyanins

Anthocyanins (of the Greek *anthos* and *Kianos* means flower and blue, respectively) are the most important pigments of vascular plants [140]. These pigments are responsible for the colours displayed by many fruits, flowers and leaves in the plant kingdom, but also for easily capturing free radical species and chelate metals [38]. Concerning sweet cherry vegetal parts, anthocyanins are only identified in fruits [141]. Cyanidin-3-*O*-rutinoside ranges from 98.996 mg/100 g to 197.889 mg/100 g of dw, being the main one, followed by cyanidin-3-*O*-glucoside, delphinidin, petunidin, peonidin-3-*O*-rutinoside, peonidin-3-*O*-glucoside, pelargonidin-3-*O*-rutinoside, peonidin, malvidin and small traces of pelargonidin [9,11,103,132,142-144]

3. Bioavailability

Polyphenols are abundant in our diet, however bioavailability differs greatly among them, and consequently, the most abundant polyphenols in our diet are not necessarily those leading to the highest concentrations of the active metabolites in target tissues [145]. After ingestion, these compounds can be modified by the hydrolyzing activity of saliva, during passage through the stomach or small intestine, before reaching the colon [146]. Their absorption is associated with the activity of a cascade of enzymes [146]. In the small intestine, located in the brush border of epithelial cells, the cleavage of sugar units is mediated by lactase phloridzin hydrolase (LPH), with a broad specificity for flavonoid-*O*-*β*-D-glucosides releasing the aglycones, which can enter by passive diffusion into epithelial cells [147]. Additionally, another alternative is the transport of flavonoids glycosides into epithelial cells by the active sodium-dependent glucose transporter (SGLT)-1 [148].

After absorption by epithelial cells, polyphenols are conjugated by sulfation, methylation and glucoronidation reaction catalyzed by sulfotransferases (SULTs), catechol-*O*-methyltransferases (COMT) and UDP-glucuronosyltransferases (UGTs), respectively [149]. SULTs are an important group of enzymes in the chemical metabolism of drugs, peptides and phenols, which catalyze the transfer of sulfo group (generally 3'-phosphoadenosine 5´-phosphosulphate) to an acceptor (for example phenol). This reaction occurs in the liver [150,151]. On the other hand, catechol-*O*-methyltransferases (COMT) catalyzes the transfer of a methyl group from S-adenosylmethionine to polyphenols [152]. The reaction of methylation occurs preferentially in position 3', although in a small number of situations it can occur in position 4'. This reaction occurs in liver and kidneys [151]. Finally, UDP-glucuronosyltransferase (UGT) family, located in the endoplasmic reticulum in several tissues, catalyzes the transfer of a glucuronic acid from UDP-glucuronic acid to polyphenols [125].

Before the passage to the systemic circulation, in the wall of the small intestine, the phase II of the metabolism takes place, where metabolites undergo further conversions before entering

to portal vein and going to the liver [146]. To be eliminated, these metabolites can follow two main pathways, being that the size of the metabolites is important to define the pathway in question. On one hand, the larger and widely conjugated metabolites are more likely eliminated by bile; on the other hand, small conjugates are preferably eliminated in the urine [153].

Summarizing, after ingestion dietary polyphenols reach the small intestine where they suffer glucoronidation and methylation processes. Then, they can go to the liver where they undergo methylation and sulfation processes, or they can go directly to the colon, suffering their hydrolysis by the action of esterases. In the colon, they can either return to the liver or be excreted. When they return to the liver, they can go back to the small intestine by bile action, or pass to the kidney, passing onto the target tissues or be excreted [154].

The bioavailability of flavonoids ranges between 1-26%. Only flavonoid aglycones are capable to pass the gut wall, and no enzymes can stop them [155]. *B*-Glycosidic bonds are secreted into the gut or are present in intestinal wall. Hydrolysis occurs in the colon by microorganisms, that, at the same time, degrade dietary flavonoids intake [124]. After absorption, the flavonoids are conjugated, in the liver, as glucuronide, methyl and sulfate conjugates, or combined as a result of phases I and II enzyme action in the small intestine, liver and plasma [156]. Inside the colon, enzymes (e.g.: *B*-glucosidase and *a*-rhamnosidase) produced by gastrointestinal bacteria, such as *Bacteroides* JY-6, *Streptococcus faecium* VGH-1, *Streptococcus* sp. strain FRP-17, *Escherichia coli* HGH21 and HGH6, *Eubacterium ramulus* and *Clostridium orbiscindens* also cleave the flavonoid ring, formatting products with low-molecular-weight as hydroxyphenylacetic acids and hydroxyphenylpropionic acids which are also absorbed, and have effects in systemically and in the gastrointestinal tract [108]. Particularly, flavanols have short life time in human body, appearing in the circulatory system 30 min after the consumption, being excreted 12h after ingestion [157].

Bioavailability of phenolic acids also has been widely study [158-161]. The major hydroxycinnamates found in the diet are ferulic, sinapic, *p*-coumaric and caffeic acids, normally as esters or glycosides of quinic acid [162]. Esterification of *trans*-cinnamic acids to quinic acids originates the famous family of chlorogenic acids [163].

Olthof and collaborators have demonstrated that the esterification of caffeic acid, as in chlorogenic acid, reduces its absorption [158]. The absorption of chlorogenic acid occurs principally in the colon, after their hydrolyzation by microbial esterase [164,165]. One-third of absorbed chlorogenic acids is absorbed in the upper gastrointestinal tract, and then subject to the action of esterase [166], releasing other compounds, such as free hydroxycinnamic acids. The remaining two-thirds of chlorogenic acids achieve the colon, where they are further metabolized [167]. Studies performed 4 and 5 hours after ingestion suggest a biphasic profile of absorption, with the primary absorption and metabolism taking place in the small intestine and

further metabolism occurring in the colon [168,169]. These hydroxycinnamic acids are highly bioavailable, with up to 30 % of intake excreted as metabolites within 24 hours of ingestion [167].

4. Health benefits of *P. avium* vegetal parts

Several studies have been showing the beneficial effects of fruits and vegetables daily intake against human several diseases [170-173]. In fact, fruits and vegetables are considered rich sources of numerous bioactive compounds, namely phenolic compounds, which are natural antioxidant substances, capable of reducing the oxidative stress, and consequently oxidative stress-related disorders [174,175]. Already many studies have reported that these secondary plant metabolites exhibit a wide range of powerful physiological properties, such as antimicrobial, anti-inflammatory, antioxidant, anti-allergenic, antithrombotic, vasodilatory and cardioprotective effects [176-180]. Some of their bioactive properties will be presented below.

Antibacterial properties

One of the health benefits of medicinal plants and specifically of cherry stems is the antibacterial activity. Considering that initially, antibiotics used to be called "phenomenon drugs", more than 60 years later, the efficacy of these drugs has reduced mainly due to the emerging drug resistant organisms [181,182]. Given this loss of antibiotics effectiveness, there is a growing interest in medicinal plants and their products' antimicrobial effects to control the growth of several microbial agents, and consequently its effects on human health [183,184]. Plants are rich in a wide variety of secondary metabolites which have antimicrobial properties, such as saponins, tannins, steroids, alkaloids, flavonoids, phenols, glycosides, alcohols and acids [185].

The antibacterial activity of extract from cherry stems was evaluated by minimum inhibitory concentration (MIC) bioassay against *Escherichia coli* isolates (*E. coli* MJS 260, *E. coli* MJS 294 and *E. coli* JH270). The research study was performed through a conventional (70 % methanol) and ultrasound assisted extraction (UAE) extracts. The results proved that the extracts obtained by UAE proved to be the most active, with the lowest MIC values (0.125-0.250 mg/mL) against all *E.coli* isolates [75].

Regarding the anti-microbial properties of *P. avium* flower and leaf, as far as we know, no reports exists, nevertheless there are few studies using the *P. avium* fruits [186] and others *Prunus* species leaves and fruits [186-189].

A study using stem and leaf extracts of Ginja, also known as tart cheery (*P. cerasus*), which its phenolic composition is similar to sweet cherry [132]. Four extracts were used ethyl acetate, water, ethanol and acetone. Their activity was evaluated using eight bacterial strains: *Bacillus subtilis*, methicillin sensitive *Staphylococcus aureus* (MSSA) and methicillin resistant *Staphylococcus aureus* (MRSA), *Pseudomona sp.*, *Pseudomonas aeruginosa*, *Flavobacterium sp.*,

E. coli and *Salmonella spp*. The results obtained revealed that the most active extracts were those from stems in ethanol, not in ethyl acetate like for the antibacterial behavior. Furthermore, the leave extracts in water revealed better activity than those prepared in acetone and ethanol [187].

Antimicrobial activity of sweet cherry extracts (methanol juice and pomace extracted, whole juiced and pomace) was tested in a recent study carried out by Hanbali and collaborators. They reported that the same extracts can inhibit *Gram-negative bacteria* (*Citrobacter koseri, Enterobacter cloacae, E. coli, E. coli* and extended-spectrum *β*-lactamase type*, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa*), as well as Gram-positive bacteria included: *Bacillus subtilis, Enterococcus* Group D*, Staphylococcus aureus, Streptococcus* Group A and *Streptococcus* Group B type. The results reported that these extracts are able to diminish the resistance and growth of the various microorganisms. Besides, the referred extracts of sweet cherries also exhibited antimicrobial efficacy against fungus *Candida albicans* [189].

Antioxidant properties

One of the most important recommendations to the general public is to increase the intake of foods, preferably the Mediterranean foodstuffs, which are rich in antioxidants due to their well-known healthy effects [190]. The overproduction of reactive species, such as hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), superoxide ion (O_2^-) and hydroxyl radical ($^{\bullet}OH$) species results in DNA mutation, cell membrane disintegration and membrane protein damage [191], increasing the risk of of development of several pathological situations, such as cancer, diabetes, neurological pathologies and cardiovascular problems [192]. Given the aforementioned, there is a growing demand for the use of natural antioxidant substances.

Plants with antioxidant properties have been gaining great interest considering their potential against oxidative radicals [193,194]. Currently, some studies concerning cherry leaves and stems are already found [75,195,196].

In a study led by Bastos and collaborators, the activity of hydromethanolic and infusion extracts of *P. avium* fruits and stems have been tested against 1,1-diphenyl-2-picryl-hydrazyl radical ($DPPH^{\bullet}$), to inhibit *β*-carotene bleaching, thiobarbituric acid reactive substances (TBARS), and their ability to convert ferric ions (Fe^{3+}) into ferrous ions (Fe^{2+}) (reducing power) [61]. In this study hydroethanolic stem extract demonstrated higher antioxidant potential in all the assays, followed by stem decoctions, infusions and sweet cherry hydromethanolic extract. For $DPPH^{\bullet}$ scavenging assay, the half maximal inhibitory concentrations (IC_{50}) obtained were: 0.36 ± 0.01, 0.54 ± 0.01, 0.63 ± 0.01 extract weight and 0.99 ± 0.01 mg/mL for stem hydromethanolic extract, decoction, infusion and for sweet cherry extract, respectively [61]. In another study, the antioxidant activity of acetone leaf extract was tested using the DPPH assay. The results showed the ability of the extract to reduce $DPPH^{\bullet}$ (IC_{50} = 461.11 ± 4.67 mg/mL), however in a less

effective away when compared to acetone stem extract (IC_{50} = 48.91 ± 0.44 mg/mL), possibly due to their lower phenolic content [196]. Concerning to the capacity to bleach *β*-carotene inhibition, the IC_{50} obtained were 0.30 ± 0.01, 1.80 ± 0.04, 0.35 ± 0.04 mg and 0.42 ± 0.06 mg/mL for stem and sweet cherry hydromethanolic extract, decoction and infusion, respectively. Additionally, the results for TBARS inhibition proved that the stem hydromethanolic extract was the most active (IC_{50} = 0.07 ± 0.00 mg/mL), followed by stem decoction (IC_{50} = 0.13 ± 0.01 mg/mL), infusion (IC_{50} = 0.24 ± 0.01mg/mL) and finally, sweet cherry hydromethanolic extract (IC_{50} = 1.46 ± 0.09 mg/mL)[61]. The reducing power was evaluated by the ability to convert ferric ions (Fe^{3+}) into ferrous ions (Fe^{2+}). Once again, stem hydromethanolic extract, decoction and infusion showed more effectiveness than fruit hydromethanolic extract (IC_{50}= 0.18 ± 0.02 mg/mL, 0.18 ± 0.02 mg/mL, 0.44 ± 0.03 mg/mL and 0.57 ± 0.01 mg/mL respectively). These results might be related with the higher non-colored phenolic compounds content found in stems, when compared to those found in fruits [61].

In another study using water and ethanol extracts of tart cherries' stem (*Cerasus avium* L.), the antiradical and antioxidant properties were examined, through a ferric thiocyanate method, Fe^{3+} and cupric ion (Cu^{2+}) reducing assays, and by Fe^{2+} chelating assay. Considering the results obtained in Fe^{3+} reducing assay and Fe^{2+} chelating assay, infusion extract exhibited great antioxidant activity (IC_{50} scores of 0.523 ± 0.049 mg/mL and 11.59 mg/mL, respectively). However ethanolic extract showed the best result in cupric ion (Cu^{2+}) (IC_{50} = 0.269 ± 0.041 mg/mL) [60].

The total antioxidant activity of sweet cherry stems was also tested using the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate ($ABTS^{\bullet+}$) radical cation assay. The study was performed through a conventional (70 % methanol) and ultrasound assisted extraction (UAE). UAE yielded the best activity (IC_{50} = 2.2 mg/mL) compared with the conventional extraction (IC_{50} = 4.2 mg/mL) [75].

Another study concerning methanol extracts of cherry leaves, resorting to deoxyribose assay, reported that they are extremely effective to scavenging $^{\bullet}OH$. The analyzed extract exhibited 97 % scavenging capacity at 0.02 mg/mL concentration. This study also proves that extracts from cherry plant parts are a great source of natural antioxidants [195].

One study was carried out to clarify the effects of the oral administration of cherry's stem on hormones of reproductive organs, using albino male mice. The obtained results demonstrated that the extract not only prevents oxidative stress thereby reducing cell damage, but also modulates hormone production, enhancing the levels of progesterone and testosterone, and declining the level of estradiol [197].

Anti-inflammatory properties

Inflammation is a normal biological process of the immune system against pathogens [198]. Numerous inflammatory mediators are synthetized and secret during the inflammatory process, these substances are often divided in two main categories: pro- and anti- inflammatory mediators [199]. Lipopolysaccharide (LPS), a bacterial endotoxin (*Escherichia Coli*) that stimulates the inflammation in macrophages, is responsible for the increase of the biosynthesis of inducible nitric oxide synthase (iNOS), and consequently for the production of nitric oxide (NO). This radical is essential for the living state of cells and organisms [200], however when over-produced, rises the risk of occurrence of several degenerative diseases such as rheumatoid arthritis, gout arthritis, shoulder tendinitis, heart disease, asthma, cancer and inflammatory bowel disease.

In the last decades, hundreds of researches and review papers were published concerning the effects of several plants on the anti-inflammatory activities. In respect to *P. avium* flower, stem and leaf, as far as we known, no reports exist about their anti-inflammatory properties, however there are few studies that used sweet cherry fruits and *Prunus persica* flower [198,201]

Sweet cherries are rich in polyphenols, they can inhibit inflammatory pathways. This fact was proved by Jacob and collaborators, where they evaluated inflammatory markers in ten healthy women volunteers. The volunteers consumed 280 g per day of *Bing* cherry cultivar during 28 days. The results obtained proved that they exhibited reduced levels of C-reactive protein and nitric oxide radicals, but also in plasma uric acid, LDL cholesterol (associated to heart attacks) and on tumor necrosis factor (TNF-α) [201].

In another study with *Prunus persica* var. *dadiviana* flower, the authors evaluated the anti-inflammatory effects. The flowers were extracted with methanol and fractionated into ethyl acetate and butanol fractions, then were tested by *in vitro* assays using RAW 264.7 cells. The results obtained revealed that all extracts suppressed the lipopolysaccharide-induced nitric oxide production. Furthermore, methanolic and butanol fractions showed to be able to significantly reduce the levels of interleukin (IL)-1β, IL-6 and TNF-α, proved their high anti-inflammatory effects [198]. These results are quite promising and alert for the possibility to use phytochemicals to be incorporate into new drugs to treat related inflammatory diseases and symptoms.

Neuroprotective properties

The term “neuroprotection” encloses homeostatic mechanisms that occur in the central nervous system, whose principal objective is to protect the neurons from apoptosis and degeneration, after an acute brain injury [202]. In fact, inflammation and oxidative stress are

major contributors of neurodegenerative conditions (as Alzheimer's disease, Down syndrome and multiple sclerosis) [203].

To our best knowledge, no reports exist about *P. avium* flower, leaf and stem concerning their neuroprotective activities, nevertheless there are few studies about sweet and sour cherries [120,204,205]. Kim and co-workers studied some cultivars of sweet and sour cherries, in PC-12 neuronal cell line, and they concluded that oxidative damage in cells was reduced after their exposure to cherry phenolic extracts. It was also verified a direct relationship between the dose of anthocyanins and the protective effects displayed, concluding that the higher concentration of anthocyanins raises the cell viability and neuroprotection [120].

However, *P. avium* plant parts can reduce oxidative stress levels in cells, protecting them against the occurrence of neurodegenerative pathologies [206]. This fact is intensively associated with the presence of quercetin or derivatives, which are a potent metal chelators and antioxidative radical scavengers that can effectively diminish free radical species involved in neuronal damage [205], inhibit acetylcholinesterase (AChE) activity [204] and also ameliorate hypoxia induced memory dysfunction in rats through its antioxidant and anti-apoptotic activity [207].

Cardiovascular protective properties

Cardiovascular protection is other area of research in the field of medicinal plants [208]. More than 17 million people die annually due to cardiovascular problems, including heart attack and stroke, being these ones the world´s leading cause of death. They are a consequence of unhealthy behaviors, such as smoking, adopting a sedentary lifestyle and eating foods with high salt content [209]. To attenuate this pandemic diseases, supplements of natural antioxidants such as ascorbic acid and vitamin E have been prescribed, given that they show to be capable to offer protection in patients with heart diseases [210]. Furthermore, other bioactive compounds, as carotenoids and phenolics have gained prominence, since they are associated to the prevention of LDL oxidation and are able to reduce oxidative stress levels involved in plaque formation [211].

Although there is a lack of specific studies concerning the role of vegetal parts of *P. avium* species in cardiovascular protection. A study evaluated the effect of *P. avium* stem extract on concentrations of triglyceride, cholesterol and total protein in serum of 15 white albino male mice aged between 2-3 months. The rats were divided into 3 groups (5 animals each): the first group was the control; the second group included animals treated with 1 mL of cherry stem extract at a concentration of 0.04 mg/kg/day, while the third group consisted of animals fed with 1 mL of cherry stem extract at a concentration of 0.08 mg/kg/day. After 2 weeks of treatment, it was verified that the extract can reduce the lipid levels by decreasing total cholesterol and triglyceride concentrations. Moreover, it was also observed that mice which

consumed the stem's extract had less fat storage in the liver and lower oxidative stress levels [212].

Antidiabetic properties

Diabetes *mellitus* is an emerging health problem in western societies, is a metabolic disorder responsible for abnormal glucose levels in the blood, being several times associated with obesity problems [213,214]. Another problem linked to diabetes is the oxidative stress which can damage lipids, nucleic acids and proteins [215]. The scientific community is searching for new natural compounds with antidiabetic properties to overcome any resistance developed by patients to the currently used drugs [216], as *a*-glucosidase inhibitors. They act by reversible inhibition of this enzyme. This inhibition reduces the levels of glucose, by preventing that the enzyme breaking down the α-glycosidic bonds in complex carbohydrates to release absorbable monosaccharides [217,218].

Several plants have been reported to contain antidiabetic properties [219,220]. Recently, it has been proven by our research team, the antidiabetic potential of sweet cherry [11], using hydroethanolic extracts of five sweet cherry cultivars (*Saco, Sweetheart, Satin, Maring* and *Hedelfinger*) from Fundão region (Portugal). These ones have been tested against *a*-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[11]. All the extracts were most active than acarbose control (IC_{50}= 306.66 ± 0.84 μg/mL), a drug commercialized as an enzyme inhibitor for type 2 diabetes, but whose use causes several undesirable effects like intestinal pain, flatulence and diarrhea [11].

In a study performed with 63 alloxan induced diabetic albino mice weighing 20-23 g showed that oral administration of stem's hydroethanolic extract at a concentration of 250 mg/kg body weight for 15 days exhibited beneficial effects on glycoproteins. In this work, 45 adult male albino mice were divided equally into 3 groups: group 1: control; group 2: diabetic mice; and group 3: diabetic mice treated with cherry stem hydroethanolic extract. Diabetes was induced in the mice by an intraperitoneal injection with 100 mg/kg body weight of alloxan, which is a drug that can produce a massive pancreatic insulin releases, thus leading to fatal hypoglycemia. The authors verified that the administration of stem hydroethanolic extract can reduce glucose, glycosylated hemoglobin, urea and creatinine levels in diabetic mice treated with the cherry stem as compared to untreated diabetic mice. The results proved that *P. avium* stems showed effectiveness in attenuating diabetes. Additionally, 18 mice were fed with cherry stem hydroethanolic extract only for toxicity test. There are no adverse effects reported [221].

The effect of ethanolic extracts of *P. avium* stems in serum of alloxan induced diabetic rats was also evaluated. A total of 25 diabetic rats were divided in 5 groups (5 rats per group). The first one was the control group. In the other four groups, mice ingested extract of stem via

oral, that were dissolved in water at doses of 25, 75, 125 and 200 mg/kg. The results obtained revealed that the stem extracts might stimulate a release of insulin, allow lipase activity, improve insulin sensitivity, normalize blood glucose levels and reduce glucose production [222].

Anticancer properties

Finally, *P. avium* vegetal parts have shown no activity results in the remission of cancer cells [61], however sweet cherry hydromethanolic extract showing activity and against colon carcinoma. Cancer is a disease or a group of diseases characterized by uncontrolled growth and proliferation of abnormal cells [223]. Cancer can be caused by external factors, like tobacco, unhealthy diet and infectious organisms, and by endogenous factors, such as inherited mutations, hormones and immune mutations [224].

There are many pathways involved in cancer diseases development, but phosphoinositide 3-kinase (PI3K) pathway is one of the most often activated signal transduction pathways in human cancer, and may be responsible for drug sensitivity or resistance to specific therapeutic agents [225]. Studies were established to understand the role of the PI3K pathway in various cellular processes, such as inflammation, cell survival, metabolism, motility and cancer risk [226].

In respect to *P. avium* stems and fruits hydromethanolic extracts anticancer properties, they have been tested against five human tumor cell lines: HCT-15 (colon carcinoma), MCF-7 (breast adenocarcinoma), HeLa (cervical carcinoma), NCI-H460 (non-small cell lung cancer) and HepG2 (hepatocellular carcinoma) cells. The fruit extract was the only one showing activity and revealed selectivity against HCT-15 cells, possibly due to the presence of anthocyanins, however no activity was observed above the concentration of 400 µg/mL for the remaining cell lines [61]. None of the tested preparations have shown hepatoxicity against primary cells.

Relatively to anticancer abilities of flower and leaf of *P. avium*, as far as we know, no studies were reported. However, it is expectable that *P. avium* leaves also exhibit properties against cancer, due to the presence of high amounts of 5-*O*-caffeoylquinic acid [70]. Indeed, this hydroxycinnamic acid already demonstrated to be one the chemical entities able to suppress the hepatoma invasion *in vitro*, thus being an important aid in tumor control [227].

II. Aims of the study

The objective of this study was to improve the knowledge about the phenolic profile of *P. avium* stem, leaf and flower from Saco cultivar collected from Fundão region (Portugal), and evaluated their bioactive properties, using infusions and hydroethanolic extracts (ethanol/water 50:50 *v*/*v*). To accomplish this aim, specific objectives were delineated:

- Analysis and characterization of the phenolic compounds of *P. avium* vegetal parts by liquid chromatography with diode-array detection (LC-DAD);
- Determination of the antioxidant capacity of *P. avium* vegetal parts extracts through free radical scavenging assays against DPPH•, $O_2^{\bullet-}$ and •NO radicals;
- Evaluation of the inhibitory effect of hydroethanolic and infusion extracts of *P. avium* vegetal parts against *a*-glucosidase enzyme; and
- Determination of the ability of *P. avium* vegetal parts infusion and hydroethanolic extracts to prevent $ROO^{\bullet}$-induced oxidative damage in human erythrocytes, concerning to inhibit hemoglobin oxidation, lipid peroxidation and hemolysis.

III. Materials and Methods

5. Reagents

All chemicals used were of analytical grade. Sakuranetin, kaempferol-3-*O*-rutinoside, genestein-7-O-glucoside and genestein were from Extrasynthese (Genay, France). 3-*O*-Caffeoylquinic acid, *p*-coumaric acid, kaempferol-3-*O*-glucoside, quercetin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, catechin and epigallocatechin were obtained from Sigma-Aldrich (St. Louis, MO, USA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH•), *β*-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), thiobarbituric acid (TBA), trichloroacetic acid (TCA) and *tert*-butyl hydroperoxide (*t*BHP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, 4-nitrophenyl-alpha-D-glucopyranoside (pNPG) and sodium nitroprusside dihydrate (SNP) were obtained from Alfa Aesar (Karlsruhe, Germany). Methanol and acetonitrile were from Fisher Chemical (Leicestershire, United Kingdom). Water was deionized using a Milli-Q water purification system (Millipore Ibérica, S.A.U., Madrid).

6. Plant samples

Fresh stems, leaves and flowers of *Saco* sweet cherry cultivar were collected in fresh during the year of 2016 from an orchard of an associated farmer of the Cerfundão company (Fundão, Portugal). They were immediately frozen with liquid nitrogen and maintained at -20 ºC. Then, *P. avium* vegetal parts were lyophilized and powdered in order to obtain a mean particle size lower than 910 μm and used for the preparation of the two different extracts, namely hydroethanolic extracts and infusions. Dried vegetal parts corresponding to a mixture of different individuals. Voucher specimen for leaves, flowers and stems were deposited at CICS Health Sciences Research Centre, Faculdade de Ciências da Saúde, Universidade da Beira Interior (LEA-CE-072016, ST-CE-072016, FLO-CE-042017).

7. Extracts Preparation

Infusion: 1 g of each dried material (leaves, flowers and stems) was boiled with 100 mL of water for 5 minutes. Thus, it mimics the way how it is regularly prepared for human consumption. The resulting extract was filtered through a membrane filter (0.45 μm), frozen and lyophilized and kept in a desiccator, in the dark, until analysis. The yields obtained from starting dry material were 28.14 ± 1.78 g, 26.00 ± 1.80 g and 40.93 ± 0.36 g for leaves, stems and flowers, respectively. The extractions were performed in triplicate.

Ethanolic/water: 1 g of each dried material (leaves, flowers and stems) was extracted with ethanol: water (1:1) mixture, as follows: 30 min of sonication, maceration during 1 h (stirring at 200 rpm) at room temperature, plus 30 min of sonication [228]. The obtained extracts were filtered through 0.45 mm polytetrafluoroethylene (PTFE) membrane (Millipore, Bedford, MA), evaporated under reduced pressure and kept at -20 ºC until further analysis. The yields of hydroethanolic extracts were 26.04 ± 1.64 g, 30.42 ± 1.96 g and 38.29 ± 1.38 g for leaves, stems and flowers, respectively, from starting dry material. Extractions were carried out in triplicate.

8. Phenolic compounds

Both lyophilized extracts (infusion and hydroethanolic) from leaves, flowers and stems (1 mg) were dissolved in 1 mL of methanol. The obtained extracts were filtered through a 0.45 mm polytetrafluoroethylene membrane. 20 µL of each sample were analyzed on a LC model Agilent 1260 system (Agilent, Santa Clara, California, USA) as previously described [11], using a Nucleosil® 100-5 C18 column (25.0 cm × 0.46 cm; 5 µm particle size waters; Macherey-Nagel, Düren, Germany). Detection was achieved with an Agilent 1260 Infinity Diode Array Detector (DAD) using the ChemStation software supplied by Agilent Technologies (Waldbronn, Germany). 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 fixing 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. The total run was 80 min. The established solvent flow rate was 1.0 mL/min. The injection volume was 20 µL. Spectral data from all peaks were accumulated in the range of 200–400 nm. Phenolic compound quantification was completed through the absorbance recorded in the chromatograms relative to external standards at 280 nm for flavan-3-ols and hydroxybenzoic acids, 320 nm for hydroxycinnamic acids and 350 nm for flavonols. The compounds found in each extract were identified by comparing their retention times and UV–VIS spectra with those of authentic standards. The compound hydroxybenzoic acid derivative was quantified as *p*-hydroxybenzoic acid. 3-*O*-Caffeoylquinic acid and hydroxycinnamic acid derivatives (1-8) were quantified as 5-*O*-caffeoylquinic acid. *p*-Coumaroylquinic acid and *p*-coumaric acid derivatives 1 and 2 were quantified as *p*-coumaric acid. Sakuranetin derivative was quantified as sakuranetin. Genistein derivative was quantified as genistein. Catechin derivative was quantified as catechin.

9. Biological activity

9.1. DPPH assay

The capacity of infusion and hydroethanolic extract from leaves, stems and flowers to scavenging the DPPH radical was prepared in a 96-well plate (seven different concentrations

were prepared, ranging between 6.94-111.11 µg/mL). All dilutions of the samples were previously redissolved in methanol (25 µL) and placed in the different wells of the microplate, followed by the addition of 200 µL of 150 mM methanolic DPPH• [229]. The mixture was placed in the dark during 30 min, and the absorbance was measured at 515 nm. Three experiments were performed in triplicate. Ascorbic acid was used as positive control.

9.2. Superoxide radical assay

The effect of the lyophilized extracts on superoxide radical-induced reduction was generated by the NADH/phenazine methosulfate system, as previously reported by Silva et al. (2014) [229]. Six different concentrations for each extract were tested, ranging between 0.49 - 125.0 µg/mL. The assay was performed using NBT and monitored at 562 nm. All components were dissolved in potassium phosphate buffer (KH_2PO_4, 19 mM, pH 7.4). Ascorbic acid was used as positive control. Three experiments were performed in triplicate.

9.3. 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 (15.63-500.00 µg/mL). The reaction mixture in each 96-well plate consisted on 100 µL of each extract dissolved in buffer (KH_2PO_4 100 mM, pH 7.4) and 100 µL of SNP (20 mM). Then, the mixture, was incubated during 60 min at room temperature and under light. After, 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 2% H3PO4) was added. 10 min latter and away from the light, the absorbance of the chromophore formed with Griess reagent was read at 562 nm. Three experiments were performed in triplicate. Ascorbic acid was used as positive control.

9.4. *a*-Glucosidase inhibitory activity

The inhibition of *a*-glucosidase activity of all extracts from cherry vegetal parts was determined based on Ellman's method [230]. Six different concentrations (0.92-468.75 µg/mL) were tested using a 96-well-plate. Each well contained 50 µL of the extract dissolved in potassium phosphate buffer, 150 µL of 100 mM potassium phosphate buffer and 100 µL of 2 mM 4-nitrophenyl-*a*-D-glucopyranoside (PNP-G). The reaction was initiated by the addition of 25 µL of the enzyme solution (0.44 U/ mL). The reaction mixture was incubated at 37ºC during 10 min. Then, the absorbance of 4-nitrophenol released from PNP-G was measured. The increase of absorbance was compared with that of the control (buffer instead of sample solution) to calculate the inhibitory activity. Acarbose was used to positive control. Three experiments were performed in triplicate.

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

Considering the evaluation of the *in vitro* ROO•-induced oxidative damage in human erythrocytes, extracts were dissolved in PBS and six different concentrations were prepared. Lyophilized extracts (1 mg) were dissolved in 1 mL of PBS. Four experiments were performed in duplicate in each microplate, and the results were expressed as IC_{50} values (µg/mL).

9.5.1. Isolation of human erythrocytes

Venous human blood was collected from randomized patients of Centro Hospitalar of Cova da Beira (Covilhã), by antecubital venipuncture into K_3EDTA vacuum tubes. Erythrocytes were isolated based on the procedure described by Gonçalves et al. (2017) [11,231] and after written informer consent. Approximately 4 mL of the blood samples were transferred to sterile 15 mL conical centrifuge tubes, previously containing phosphate buffer saline (PBS) at pH 7.4. The mixed mixture was centrifuged at 1500xg for 5 min at 4 °C. After centrifugation, erythrocytes (red portion) were separated from buffy coat and plasma (supernatant), followed by a washing step. This procedure was repeated twice, erythrocytes were re-suspended using the same buffer and the supernatant was discarded. The next step was re-suspended erythrocytes in 6 mL of the same buffer, and prepared a suspension of the mixture (diluted 200x) in trypan blue solution. 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 resulting suspension of isolated erythrocytes were kept on ice until use.

9.5.2. Inhibition of hemoglobin oxidation

The inhibition of hemoglobin (Hb) oxidation was determined by monitoring the ability of the lyophilized extracts on the formation of methemoglobin (metHb) [231]. The decomposition of AAPH (dissolved in PBS) generated by the water-bath temperature (37ºC) react with oxyhemoglobin forming methaemoglobin. Six dilutions (7.5–120 µg/mL) of each extract were prepared with PBS. 100 µL of the sample solution and 200 µL of erythrocytes solution formed the reaction mixture. In case of control or blank, solution sample is replaced by 100 µL of PBS. Next step is incubation in water-bath at 37ºC, under slow agitation (50 rpm). After incubation, 200 µL of AAPH (50 mM, final concentration) were added to the mixture (except in the blank), followed by incubation in the same conditions previous described, for 4 hours. The entire volume (500 µL) was transferred to 1.5 mL conic eppendorfs and centrifuged at 1500 g, during 5 min at 4 ºC. The supernatant (300 µL) was placed in a 96-well plate and the absorbance was read at 630 nm. Five experiments were performed in duplicate. Quercetin was used as positive control.

9.5.3. Inhibition of lipid peroxidation

Lipid peroxidation in erythrocytes was indirectly assessed by the formation of thiobarbituric acid-reactive substances (TBARS) [231]. Six different concentrations of infusion and hydroethanolic extracts of *P. avium* leaves, stems and flowers dissolved in PBS (15–480 µg/mL), were mixed with the suspension of human cells (500 × 10^6 cells/mL, final density) at 37 ºC during 30 min with slow agitation (≈ 50 rpm). After incubation, *tert*-butyl hydroperoxide (tBHP) (0.2 mM, final concentration) was added to the media, which was then further incubated at 37 °C under slow agitation for 30 min. After incubation, whole content was collected, and transfered to 1.5 mL-connic eppendorfs and trichloroacetic acid (TCA) 28 % (*w*/*v*) was added to precipitate the proteins before centrifugation at 16,000 × g for 10 min at 18 °C. For the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form TBARS, the supernatant was positioned in a 2 mL-conical test tube (with screw cap), succeeded by the addition of TBA 1% (w/v), and the mixture was heated for 15 min at 100 ºC in a water bath. Finally, the test tubes were cooled at room temperature and the absorbance was measured at 532 nm. Five experiments were performed in duplicate. Quercetin was used as positive control.

9.5.4. Inhibition of hemolysis

Once the thermal decomposition of AAPH is responsible by the ROO• generation and for lysis events, hemolysis of human erythrocytes was evaluated by monitoring the release of Hb after membrane disruption caused by the hemolytic process, according to the optimized procedure described by Chisté et al., (2014) [231]. Briefly, six different concentrations (0.94–30 µg/mL) of the lyophilized infusion and hydroethanolic extracts from vegetal parts were dissolved in PBS. 200 µL of suspension of human erythrocytes (1775 × 10^6 cells/mL) were incubated at 37 ºC, with 100 µL of the sample solution in water-bath during 30 min, under slow agitation (≈ 50 rpm). After incubation, 200 µL of AAPH (17 mM, final concentration) were added to the mixture (except in the blank), followed by incubation in the same conditions previous described, for 3 hours. The entire volume (500 µL), was transferred to 1.5 mL conic-eppendorf and centrifuged at 1500 g, during 5 min at 4ºC. The supernatant (300 µL) was placed in a 96-well plate and the absorbance was read at 540 nm. Five experiments were performed in duplicate. Quercetin was used as positive control.

10. Statistical analysis

Statistical comparison was determined using one-way ANOVA and the means were classified by Tukey's test at a 95% level of significance. Differences were considered significant for $P < 0.05$. To determine the contribution of the total phenols, on their antioxidant activity, Pearson's correlation coefficients were calculated. All analyses were performed using Graph Pad Prism Version 5.01 (GraphPad Software, Inc., San Diego, CA).

IV. Results and Discussion

11. Phenolic compounds analysis

Phenolic compounds are secondary metabolites widely found in fruits and plants, mostly represented by phenolic acids and flavonoids. They have been reported as having health benefits and a wide range of physiological properties, such as antioxidant, anti-inflammatory, cardioprotective and anti-microbial effects [178,180,232,233].

The LC-DAD analysis in leaves, stems and flowers allowed the determination of thirty-one compounds, which can be divided into six groups: one hydroxybenzoic acid (**2**), thirteen hydroxycinnamic acids (**1**, **3-5**, **8-10**, **12-15**, **24** and **25**), three flavan-3-ols (**6**, **7** and **11**), three isoflavones (**21**, **29** and **30**), three flavanones (**23**, **28** and **31**) and eight flavonols (**16-20**, **22**, **26** and **27**) (Table 2, Figure 9). The total amounts of the vegetal parts ranged between 20485.9 and 60916.6 μg/g of dried extract, being hydroethanolic extract of leaves the richest one, followed by leaves infusion, hydroethanolic and infusion of stems. Flowers extracts presented the lowest amounts in phenolic compounds (Table 2).

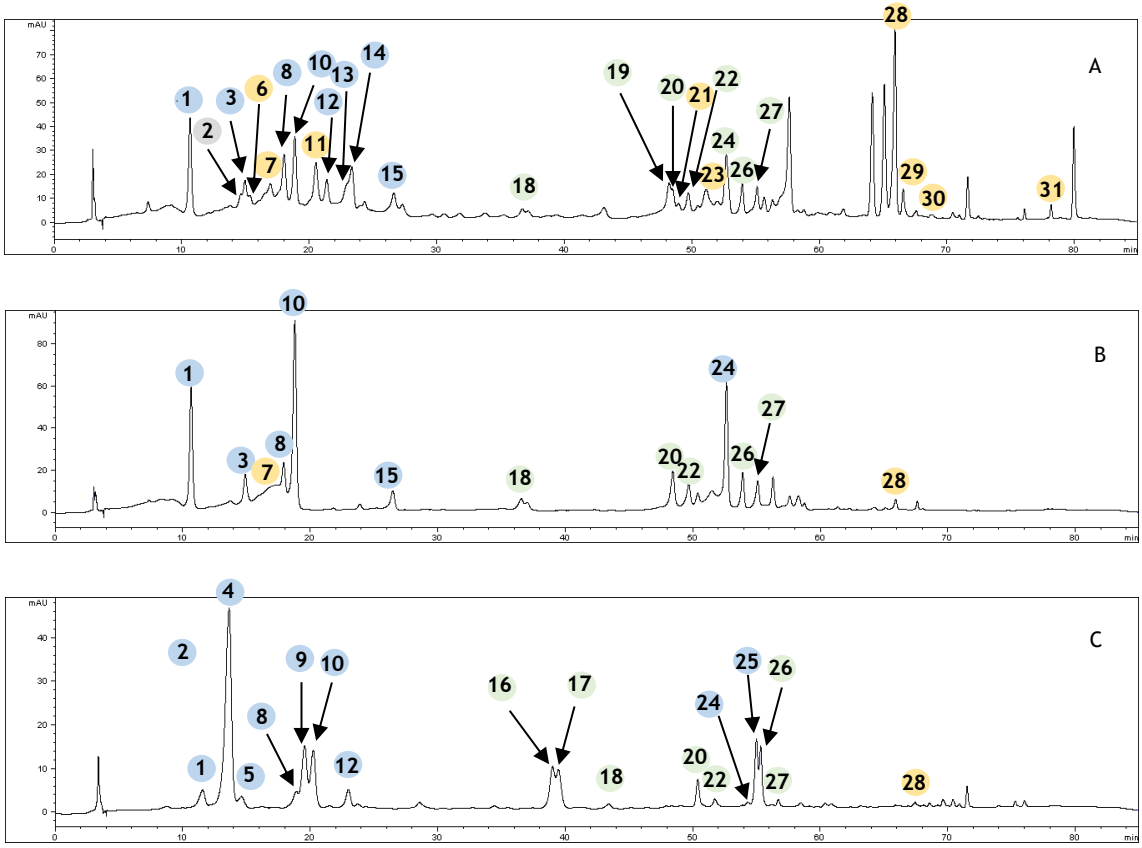


Figure 9. Phenolic profile of *P. avium* hydroethanolic vegetal parts extracts obtained by LC-DAD. Detection at 320 nm, stems (A), leaves (B) and flowers (C).

Table 2. Phenolic contents of leaves, flowers and stems of *P. avium* (µg/g of lyophilized sample).

	Non-coloured phenolic	Regression equations	R^2	Stems		Leaves		Flowers	
				Hydroethanolic	Infusion	Hydroethanolic	Infusion	Hydroethanolic	Infusion
1	**3-*O*-Caffeoylquinic acid**	Y =46.371X + 170.34	0.9955	1154.9 ± 8.5	1178.2 ± 67.6	11281.4 ± 334.4^{a,b}	8374.8 ± 274.6^{a,b,c}	nq	nq
2	**Hydroxybenzoic acid derivative**	Y = 36.182X + 121.9	0.9900	299.3 ± 6.4	918.4 ± 31.2	nd	nd	nd	nd
3	***p*-Coumaroylquinic acid**	Y = 260.73x – 137.54	0.9966	115.2 ± 1.8	103.0 ± 2.1	927.7 ± 17.1^{a,b}	759.7 ± 3.7^{a,b,c}	nd	nd
4	**Hydroxycinnamic acid derivative 1**	Y = 46.371x + 170.34	0.9955	nd	nd	nd	nd	16467.8 ± 460.5	13605.4 ± 419.9
5	**Hydroxycinnamic acid derivative 2**	Y = 46.371x + 170.34	0.9955	nd	nd	nd	nd	nq	nq
6	**Epigallocatechin**	Y = 46.72x + 89.687	0.9990	950.6 ± 59.8	951.2 ± 57.2	nd	nd	nd	nd
7	**Catechin**	Y = 34.519x + 134.38	0.9984	5259.5 ± 61.4	5014.0 ± 202.4	nd	nq	nd	nd
8	**Hydroxycinnamic acid derivative 3**	Y = 46.371x + 170.34	0.9955	361.8 ± 42.9	481.3 ± 48.4^{a}	nq	327.4 ± 32.0^{b}	nq	nq
9	**Hydroxycinnamic acid derivative 4**	Y = 46.371x + 170.34	0.9955	nd	nd	nd	nd	3015.8 ± 190.5	1167.7 ± 137.3
10	**5-*O*-Caffeoylquinic acid**	Y = 46.371x + 170.34	0.9955	708.9 ± 10.6	763.4 ± 58.2	17950.4 ± 570.8^{a,b}	15854.6 ± 655.7^{a,b,c}	2127.0 ± 51.1^{a,b,c,d}	1252.9 ± 25.2^{c}
11	**Catechin derivative**	Y = 34.519x + 134.38	0.9984	197.0 ± 4.0	179.3 ± 20.0	nd	nd	nd	nd
12	***p*-Coumaric derivative 1**	Y = 260.73x – 137.54	0.9966	135.1 ± 0.4	119.4 ± 2.2	nd	nd	nq	nq
13	***p*-Coumaric derivative 2**	Y = 260.73x – 137.54	0.9966	125.7 ± 5.6	151.9 ± 4.7	nd	nd	nd	nd
14	**Hydroxycinnamic acid derivative 5**	Y = 46.371x + 170.34	0.9955	356.6 ± 5.9	367.9 ± 25.4	nd	nd	nd	nd
15	***p*-Coumaric derivative 3**	Y = 260.73x – 137.54	0.9966	nq	nq	791.0 ± 21.2	689.0 ± 5.5	nd	nd
16	**Kaempferol derivative 1**	Y = 86.842x + 165.39	0.9995	nd	nd	nd	nd	1053.3 ± 98.9	744.4 ± 20.0
17	**Kaempferol derivative 2**	Y = 86.842x + 165.39	0.9997	nd	nd	nd	nd	396.9 ± 17.7	146.6 ± 4.9
18	**Quercetin derivative**	Y = 48.564x – 11.222	0.9950	313.2 ± 18.4	249.2 ± 16.0	2537.3 ± 165.6^{a,b}	1742.9 ± 82.2^{a,b,c}	462.8 ± 16.8^{b,c,d}	252.1 ± 14.2^{c,d,e}
19	**Quercetin-3-*O*-galactoside**	Y = 79.529x + 162.67	0.9914	294.0 ± 32.2	200.7 ± 7.7	nd	nd	nd	nd
20	**Quercetin-3-*O*-rutinoside**	Y = 71.047x – 168.96	0.9929	643.4 ± 9.1	559.1 ± 14.0	6728.0 ± 328.8^{a,b}	5154.1 ± 28.6^{a,b,c}	2547.3 ± 57.3^{a,b,c,d}	2318.8 ± 18.1 ^{a,b,c,d}
21	**Genistein-7-*O*-glucoside**	Y = 32.909x + 268.12	0.9995	nq	nq	nd	nd	nd	nd
22	**Quercetin-3-*O*-glucoside**	Y = 97.326x + 172.07	0.9931	140.0 ± 10.8	61.0 ± 9.7^{a}	1794.9 ± 28.5^{a,b}	900.4 ± 20.3^{a,b,c}	nq	nq
23	**Narigenin-7-*O*-glucoside**	Y = 39.27x + 299.97	0.9995	4036.2 ± 103.5	2836.4 ± 194.3	nd	nd	nd	nd
24	**Hydroxycinnamic acid derivative 6**	Y = 46.371x + 170.34	0.9955	905.3 ± 85.3	686.8 ± 24.7	12932.2 ± 651.0^{a,b}	8852.6 ± 69.4^{a,b,c}	nq	nq
25	**Hydroxycinnamic acid derivative 7**	Y = 46.371x + 170.34	0.9955	nd	nd	nd	nd	1308.2 ± 165.3	997.9 ± 82.4
26	**Kaempferol-3-*O*-rutinoside**	Y = 39.796x + 292.6	0.9996	155.1 ± 20.1	nq	2428.64 ± 410.9 ^{a}	579.6 ± 25.4 ^{b}	nq	nq
27	**Kaempferol-3-*O*-glucoside**	Y = 86.842x + 165.39	0.9959	243.6 ± 3.4	74.7 ± 10.8	1467.7 ± 175.9 ^{a,b}	594.2 ± 7.6 ^{a,b,c}	nq	nq
28	**Sakuranetin derivative**	Y = 67.02x + 469.06	0.9983	13500.3 ± 239.1	11555.9 ± 610.8^{a}	2077.3 ± 61.8^{a,b}	196.5 ± 5.6^{a,b,c}	nq	nq
29	**Genistein derivative**	Y = 48.087x + 172.74	0.9971	1044.8 ± 25.4	nd	nd	nd	nd	nd
30	**Genistein**	Y = 48.087x + 172.74	0.9971	nq	nq	nd	nd	nd	nd
31	**Sakuranetin**	Y = 67.02x + 469.06	0.9983	nq	50.66 ± 8.6	nq	nq	nd	nd
			Σ	30940.6	26502.3^{a}	60916.6^{a,b}	44025.7^{a,b,c}	27352.2^{a,c,d}	20485.9^{a,b,c,d,e}

Values are expressed as mean ± standard deviation of three assays. Σ, sum of the determined phenolic compounds; nq, not quantified; nd, not detected. Significant results (P<0.05) are indicated as: a – *vs* hydroethanolic stems; b – *vs* infusion stems; c – *vs* hydroethanolic leaves; d – *vs* infusion leaves; e – *vs* hydroethanolic flowers

A total of 13 compounds were identified in leaves by LC-DAD, seven of them were previously described, being 3-*O*-caffeolyquinic acid, *p*-coumaroylquinic acid, *p*-coumaric acid derivative 3 and hydroxycinnamic acid derivatives 1 and 6 described herein for the first time [69,70,76].

Regarding the hydroxycinnamic acids in leaves, a total of 34858.10 and 43882.80 μg/g were obtained for infusion and hydroethanolic extract, representing 79.2% and 72.0% of total phenolic compounds, respectively (Table 2). 5-*O*-caffeoylquinic acid was the major one found in both extracts, representing 29.5% and 36.0% of total phenolic compounds for hydroethanolic and infusion extract, respectively, followed by 3-*O*-caffeoylquinic acid proved to be the second main one (Table 2). Our results were similar to those obtained by Bauer et al. (1989), that reported the 5-*O*-caffeoylquinic acid as the major one with a content of 17060 μg/g [70,234]. The high content of phenolic acids in leaves are in accordance with previous works [234,235].

In respect to flavonoids, leaves revealed a total of 9167.62 and 17033.80 μg/g, which represents 20.8% and 28.0% of total phenolic compounds in infusion and hydroethanolic extracts, respectively (Table 2). Quercetin-3-*O*-rutinoside was the major flavonoid found in leaves extracts, representing 11.1% and 11.7% of total phenolic compounds content for infusion and hydroethanolic extract, respectively. Followed by quercetin derivative and kampferol-3-*O*-rutinoside (Table 2). The main class of flavonoids in leaves extracts was flavonols with 87.8% and 97.9% of total flavonoids in infusion and hydroethanolic extract, respectively (Table 2). Our results are in agreement with previous works, once the authors reported the flavonols as the major class of flavonoids found in leaves, highlighting the presence of quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside [70,76].

Comparing the obtained results with leaves from *Prunus cerasus*, we can see that the amounts of phenolic compounds observed in our leaves extracts were 5-8 times higher than leaves extracts of *P. cerasus* studied by Oszmiański et al. (2014) [234] (Table 2). When comparing with hydromethanolic extracts of raspberry and blackberry leaves, that presented a phenolic content lower than in our leaves extracts of *P. avium* (4191.94 – 5668.18 μg/g of dw, respectively) (Table 2). The major phenolic compound found in these extracts was ellagic acid followed by catechin, being that the first one was not identified in the extract of leaves of *P. avium* [235].

Relatively to flowers, a total of 17 compounds were identified for the first time in this work. Flowers proved to be the plant part richer in hydroxycinnamic acids with 17023.90 and 22918.90 μg/g, representing 83.8% and 83.1% of total phenolics quantified in infusion and hydroethanolic extract, respectively (Table 2). Hydroxycinnamic acid derivative 1 was the major compound identified, representing 66.4% and 60.2% of total phenolic compounds in infusion and hydroethanolic extract of flowers, respectively, followed by hydroxycinnamic derivative 4 and 5-*O*-caffeoylquinic acid (Table 2).

Furthermore, flowers showed the lowest content in flavonoids with 3461.97 and 4433.32 μg/g, corresponding to 16.9% and 16.2% of total phenolic content in infusion and hydroethanolic extracts, respectively (Table 2). Quercetin-3-*O*-rutinoside was the major flavonoid identified in flowers, representing 9.3% and 11.3% of total phenolic compounds in infusion and hydroethanolic extracts, respectively, followed by kaempferol derivative 1 and quercetin derivative (Table 2).

Comparing *P. avium* flowers with *Prunus persica* flowers, *P. avium* flowers showed higher amounts of phenolics (11228 μg/g expressed of dw) (Table 2).The major compound identified in flowers of *P. persica* was 5-*O*-caffeoylquinic acid, being 3-6 times higher than obtained for hydroethanolic extract and infusion of *P. avium* flowers, respectively [236].

Finally, a total of 25 compounds were identified in stems. All compounds were previously described, except the quercetin-3-*O*-galactoside and epigallocatechin, herein reported for the first time [60,61,75].

In respect to hydroxybenzoic acids, one compound was identified as hydroxybenzoic acid derivative with a total amount of 299.29 and 918.41 μg/g, representing 0.97-3.47% of total phenolic content. Additionally, the total content of hydroxycinnamic acids in stems was 3851.78 and 3863.49 μg/g, they corresponded to ca. 12.5%-14.5% of total phenolic compounds, being 3-*O*-caffeoylquinic acid the major one in both extracts (3.7%-4.4%), followed by 5-*O*-caffeoylquinic acid (Table 2). The content of hydroxycinnamic acids in stems is in accordance with a previous works, that reported 3-*O*-caffeoylquinic acid as the major one and hydroxycinnamic acids representing 0.91% of total phenolic contents [61].

The total amounts of flavonoids (flavonols, flavanols, flavanones and isoflavones) in stems extracts ranged from 21732.10 to 26777.90 μg/g de , representing 82.0%-86.6% of total phenolic compounds, in infusion and hydroethanolic extract, respectively (Table 2), being similar than those previously reported [61]. Sakuranetin derivative was the major flavonoid identified, representing 43.6% and 43.6% of total phenolic compounds in infusion and hydroethanolic extract of stems, respectively. Followed by catechin and narigenin-7-*O*-glucoside (Table 2). Previous works reported sakuranetin-5-*O*-glucoside was the major compound found in stems of *P. avium* [61,75].

Comparing our stems extract with *P. avium* heartwood, we can see that, heartwood possess catechin as the major compound, followed by *p*-coumaric acid and *p*-hydroxybenzoic acid being the content of catechin six times higher than stems extracts [237]. On the other hand, the plant parts proved to be richer in non-coloured phenolics than sweet cherry fruits, whose total non-coloured phenolic content range between 389.10 and 2024.44 μg/ g of dw). 3-*O*-Caffeoylquinic acid was reported as the main hydroxycinnamic acid found in fruits, being also the major one in stems extracts [11]. In addition, quercetin-3-*O*-rutinoside, which was one of the most

representative flavonol in leaves and flowers, was also the most predominant flavonol in fruits [9,11]. Relatively to other phenolic classes, sakuratenin and derivatives that belongs to flavanones and were largely identified in stems and leaves extracts, were also reported in fruits, but they only represent 1% of total phenolic compounds [12,238]. Anthocyanins are only present in fruits, corresponding from 51.9% to 73.5% of total phenolics [11].

Phenolic acids are aromatic secondary metabolites with considerable antioxidant effect against several pathologies, like cancer, neurological and cardiovascular diseases [124,239]. Among these acids, 5-*O*-caffeoylquinic and 3-*O*-caffeoylquinic present more antioxidant capacity than *ρ*-coumarolyquinic acid, due to the number of OH group. In fact, while *ρ*-coumarolyquinic acid present only one OH group, 5-*O*-caffeoylquinic and 3-*O*-caffeoylquinic present a diOH group [142]. In respect to flavonoids , they can act as hepatoprotective, anti-inflammatory, antibacterial, antiviral and anticancer agents [240]. Quercetin and derivatives have been showed to inhibit 15-lipoxygenase, an enzyme important in the oxidative modification of low density lipoprotein (LDL), leading to the development of atherosclerosis [241,242], but also preventing neurodegenerative diseases due to their free radical scavenging and antioxidant properties, which protect neuronal cell membrane against hydrogen peroxide (H_2O_2) neurotoxicity [242,243].

12. Biologic potential of leaves, flowers and stems of *P. avium*

Few studies reported the traditional use of infusion extracts of *P. avium* stems, namely as sedatives, draining and diuretics [15,16], but also leaves have been used as infusions [20]. The antioxidant and antimicrobial potential of stems was previously studied, being reported in these studies the high concentration of phenolic compounds [60,61,75].

12.1. Antioxidant activity

The increase of interest in phytochemicals as bioactive components is due to their characteristics, such as metal-chelating activities, reduction potential as electron donors, being hence powerful natural antioxidants [244]. By literature, it is possible to verify that the dietary intake of them contributes to improve the quality of life, by increasing the defence levels in the organism against reactive species and free radicals, therefore preventing or postponing the onset of several diseases.

The antioxidant activity of *P. avium* vegetal parts was assessed against $DPPH^{\bullet}$, $^{\bullet}NO$ and $O_2^{\bullet-}$ species. As far as we know, the scavenging activity against $\bullet NO$ and $O_2^{\bullet-}$ in *P. avium* stems and leaves was herein reported for the first time, as well as the antioxidant activity of *P. avium* flowers.

12.1.1. DPPH• assay

The antioxidant capacity of phenolic compounds can be evaluated by many trials, like 1.1-diphenyl-2-picrlhydrazine (DPPH), first described by Blois in 1958 and later modified by many researchers. This is a stable and synthetic free radical that reacts with compounds that easily donate a hydrogen atoms. Basically this method is based on the scavenging of DPPH through the addition of an antioxidant or radical species that decolorizes the solution (violet color), and the results takes into account the decrease in absorption at 515 nm [245-248]. All extracts showed satisfactory antioxidant activity in a concentration dependent-manner (Figure 10). The hydroethanolic extract of stems was the most active with an IC_{50} = 22.37 ± 0.29 µg/mL, followed by hydroethanolic extract of leaves (IC_{50} = 27.29 ± 0.77 µg/mL) and infusion of stems (IC_{50} = 38.29 ± 0.84 µg/mL) (Figure 10, Table 3). Additionally, all extracts exhibited lower activity than ascorbic acid positive control (IC_{50} = 11.83 ± 0.31 µg/mL) (Table 3). The results obtained revealing that vegetal parts of *P. avium* are very effective against DPPH•. (Table 3).

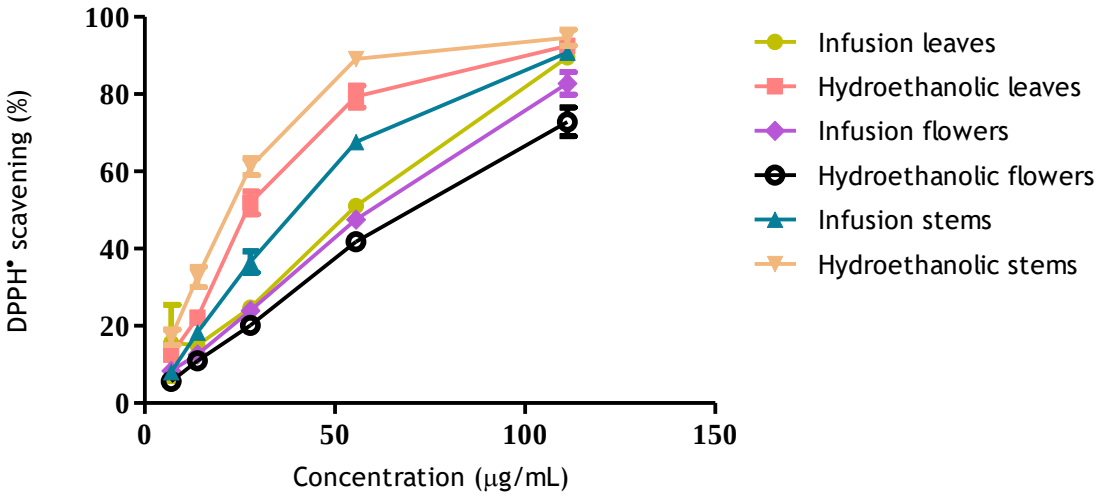


Figure 10. Antioxidant potential of leaves, flowers and stems extracts against DPPH•.

Our results are in accordance with Bursal et al. (2013), that reported a IC_{50} = 23.38 µg/mL (expressed as dried extract) of stems infusion, proved their great capacity to scavenger DPPH• [60].

Table 3. IC_{50} (µg/mL) values obtained in antioxidant activity and α-glucosidase assays for leaves, flowers and stems of *P. avium* extracts.

	Stems		Leaves		Flowers	
Assay	**Hydroethanolic**	**Infusion**	**Hydroethanolic**	**Infusion**	**Hydroethanolic**	**Infusion**
DPPH•	22.37 ± 0.29	38.29 ± 0.84^{a}	27.29 ± 0.77^{b}	54.37 ± 0.34 ^{a,b,c}	61.59 ± 0.71^{a,b,c,d}	54.70 ± 0.72^{a,b,c,e}
SO•	13.87 ± 0.41	23.28 ± 0.30^{a}	9.11 ± 0.16^{b}	27.11 ± 0.63 ^{a,b,c}	27.18 ± 0.46^{a,b,c}	30.48 ± 0.41^{a,b,c,d,e}
•NO	224.18 ± 2.16	99.99 ± 1.89^{a}	335.94 ± 3.63^{a,b}	381.63 ± 2.33 ^{a,b}	123.38 ± 1.57^{a,c,d}	180.77 ± 1.37^{b,c,d}
α-glucosidase	7.67 ± 0.23	3.18 ± 0.23	15.61 ± 0.48	106.92 ± 0.87 ^{a,b,c}	59.83 ± 0.68^{a,b,c,d}	535.52 ± 2.16^{a,b,c,d,e}

Values are expressed as mean ± standard derivation of three assays; difference between each activity for the tested extracts were tested for significance using the one-away analysis of variance (ANOVA). Significant results (P<0.05) are indicated as: a – *vs* hydroethanolic stems; b – *vs* infusion stems; c – *vs* hydroethanolic leaves; d – *vs* infusion leaves; e – *vs* hydroethanolic flowers.

Comparatively with other works, our extracts from leaves revealed more activity than crude methanol extracts of sweet cherry leaves (87.80 ± 1.73% of inhibition at 450 µg/mL) [195]. Additionally, our values obtained for stems extracts are less effectiveness than ethanolic extracts of stems (IC_{50} = 17.36 µg/mL, expressed as dried extract) [60]. Additionally, our extracts showed better activity than hydromethanolic extract, infusions and decoctions from stems (IC_{50} = 360 ± 10 µg/mL, IC_{50} = 630 ± 10 µg/mL and IC_{50} = 540 ± 10 µg/mL, respectively, expressed as dried extract) [61].

Furthermore, the activity revealed by the both extracts from leaves, stems and flowers proved to be less active than that revealed by hydroethanolic extracts of sweet cherry cultivars, namely *Saco*, *Satin*, *Maring* and *Hedelfinger* (IC_{50} scores of 16.24 ± 0.46 µg/mL,10 ± 0.43 µg/mL, 20.66 ± 0.52 µg/mL and 12.12 ± 0.37 µg/mL dw, respectively) [11].

Concerning some medicinal plants, the activity demonstrated by hydroethanolic extract of flowers was similar than the exhibited by leaves from *Ficus carica* cultivar *Branca Traditional* (IC_{50} = 59.60 µg/mL of dried aqueous extract). On the other hand, all the extracts tested in this study showed better results than leaves from *Ficus carica* cultivar *Pingo de Mel* (IC_{50} = 75.20 µg/mL of dried aqueous extract) [249].

Indeed, the obtained results in this research indicated that the activity of extracts from stems against DPPH• was positively correlated with the total phenolic acids (r=0.9767), but negatively correlated with the total phenolics amounts (r=-0.9615) and total flavonoids contents (r=-0.9743), (p<0.05). Furthermore, a negative correlation was also obtained for extracts from leaves and total phenolics, total phenolic acids and flavonoids content (r=-0.9709, r=-0.9591 and r=-0.9802, respectively) (p<0.05). Nevertheless, both flowers extracts proved to have excellent antioxidant capacity against DPPH•, and this one can be supported by the positive

correlation obtained between its extracts and total phenolic content (r=0.9230), total phenolic acids (r=0.9200) and total flavonoids (r=0.9358) (p<0.05).

Other studies already established a positive correlation between the concentration of phenolics and the antioxidant potential of plant extracts [250-252].

12.1.2. Superoxide radical

Superoxide radical ($O_2^{\bullet-}$) is recognized because it plays important roles in many pathophysiologic states including inflammation, oxygen toxicity and phagocyte-mediated activity [253]. This one results of several physiological processes, as purine metabolism and electron leakage from the respiratory chain that will reduce oxygen [251]. There are many reactive oxygen species (ROS), such as hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2) and superoxide ion (O_2^-). The interaction of superoxide with certain transition metal ions, yield a highly-reactive oxidizing species, the hydroxyl radical (OH) [192]. The oxidation produced by these reactive species can result in DNA mutation, cell membrane disintegration and membrane protein damage, leading to the development of many diseases [191].

In the present study, the analyzed extracts were particularly active against $O_2^{\bullet-}$ (Figure 11), being leaves hydroethanolic extract the most active (IC_{50} = 9.11 ± 0.16 µg/mL), followed by hydroethanolic extract and infusion from stems (IC_{50} = 13.87 ± 0.41 µg/mL and 23.28 ± 0.30 µg/mL, respectively). Comparatively to the positive control ascorbic acid, all tested extracts showed better or similar results (IC_{50} = 30.04 ± 0.34 µg/mL) (Table 3).

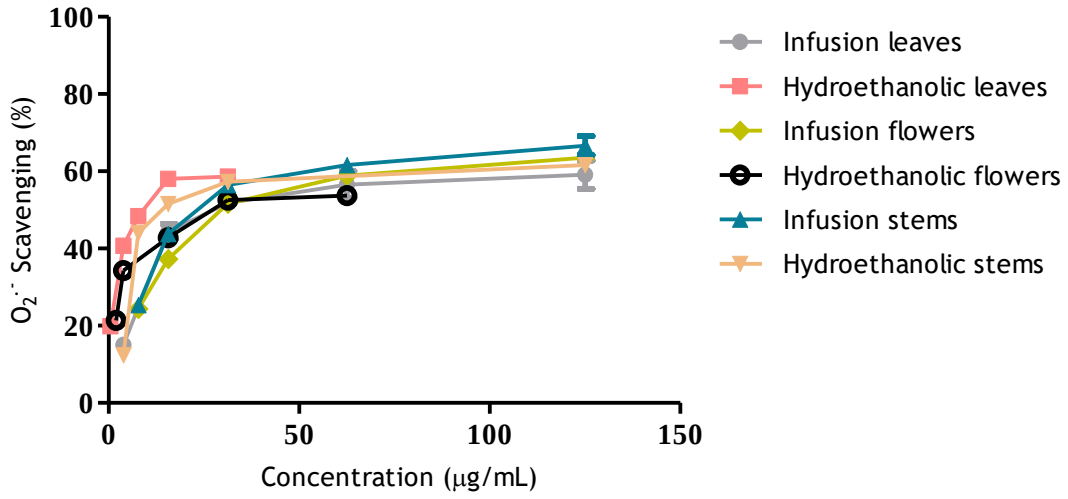


Figure 11.Scavenging activity of leaves, flowers and stems extracts against $O_2^{\bullet-}$ radical.

Comparatively with other plant parts, infusion from leaves, flowers hydroethanolic extract and infusion from flowers demonstrated similar activity to the infusion of *Endopleura uchi* (*E. uchi*)

bark extract (IC_{50} = 27 µg/mL, expressed as dry weight), and *E. uchi* hydroethanolic extract (IC_{50} = 33 µg/mL) [254]. Furthermore, infusion and hydroethanolic flowers extracts and infusion from leaves were obtained better results than acetone extracts of leaves and stems from *Cassia auriculata* (*C. auriculata*) (IC_{50} = 167 µg/mL and IC_{50} = 115 µg/mL, expressed as dried extract), respectively, as well as, *Cassia fistula* acetone extract of stems (IC_{50} = 145 µg/mL, expressed as dried extract) [255].

The correlation between antioxidant activity and the total phenolic content, total phenolic acids and total flavonoids were evaluated by calculating Pearson's correlation coefficients. Negative correlation was found between flower extracts and the total phenolic amounts (r=-0.8788), the total flavonoid content (r=-0.8610) and the total phenolic acids (r=-0.8889) (p<0.05). Similar results were presented by leaves extracts: r=-0.9784 for the total phenolic, r=-0.9711 for the total phenolic acids and r=-0.9825 for the total flavonoids content, respectively (p<0.05). On the other hand, the total phenolic acids content of stems extracts has demonstrated a positive correlation with the antioxidant activity against O_2^- (r=0.9380). Nevertheless, negative correlations were found with the total phenols amount (r=-0.9799), and the total flavonoids (r=-0.9858) (p<0.05).

Therefore, the observed data are coherent with other reports, that reported the influence of phenolic amounts in antioxidant activity [256-258]. In fact, this beneficial property is mainly associated with their redox potential, which plays an important role in neutralizing free radical [259]. Phenolics hydroxyl groups are good hydrogen donors: antioxidants with these antioxidant properties can react with RO and RN species [260]. Hydroxycinnamic acids activity is related with the number of hydroxyl groups (OH), and their position [261]. Free radical scavenging by flavonoids is increased by the presence of conjugation between the B and A rings allows a resonance effect of the aromatic nucleus that provides stability to the radical and the unsaturated 2,3-bond in conjugation with a 4-oxo function [262,263].

12.1.3. Nitric oxide

The nitric oxide (**NO**) is an important chemical mediator widely used by cells in the human body, like endothelial, nerve, epithelial and immune cells like macrophages, which release this compound in order to combat organisms "foreign" to the body [264]. It also behaves as a neurotransmitter between nerve cells, acting in the presynaptic to the postsynaptic sense, and it is thought that it may be involved in memory formation too. The thinner, more internal layer of our blood vessels (endothelium) also uses NO as to control the smooth muscle relocation of the blood vessel causing it to dilate and thus lowering blood pressure [264]. The synthesis of NO occurs from the arginine that through the enzyme nitric oxide synthase (iNOS) that produces nitric oxide and citrulline. This reaction is carried out in the presence of oxygen (O_2) and the phosphate nucleotide adenine nicotinamide (NADPH) [265]. The affected nitric oxide synthesis,

means negative feedback in the synthesis of NOS, being this one associated with the increase of the oxidative stress of the cells, as well as the increase of the reactive oxygen species (ROS) [266]. Insufficient synthesis of NO, or its reactions with oxygen and superoxide species, originates an overproduction of more toxic radicals, and consequently to a lack of nutrients such as amino acids, leading to a decrease the synthesis of proteins in cells, and thus way contributing to the development of neurodegenerative and chronic diseases such as diabetes, cancer, rheumatoid arthritis and atherosclerosis [267].

Concerning the scavenging potential of nitric oxide radical (•NO), the extracts from vegetal parts showed activity in a concentration dependent-manner (Figure 12), being stems infusion, hydroethanolic and infusion from flowers the most active (IC_{50} = 99.99 ± 1.89 , IC_{50} =123.38 ± 1.57 , IC_{50} = 180.77 ± 1.37 µg/mL, expressed as dried extract, respectively), followed by stems hydroethanolic extracts (IC_{50} = 224.18 ± 2.16 µg/mL), leaves hydroethanolic extract (IC_{50} = 335.94 ± 3.63 µg/mL) and infusion from leaves (IC_{50} = 381.63 ± 2.33 µg/mL). In addition, infusion of stems and hydroethanolic extracts of flowers showed better ability to capture •NO than ascorbic acid positive control (IC_{50} = 158.18 ± 0.79 µg/mL), while all other extracts revealed less activity comparatively to control (Table 3).

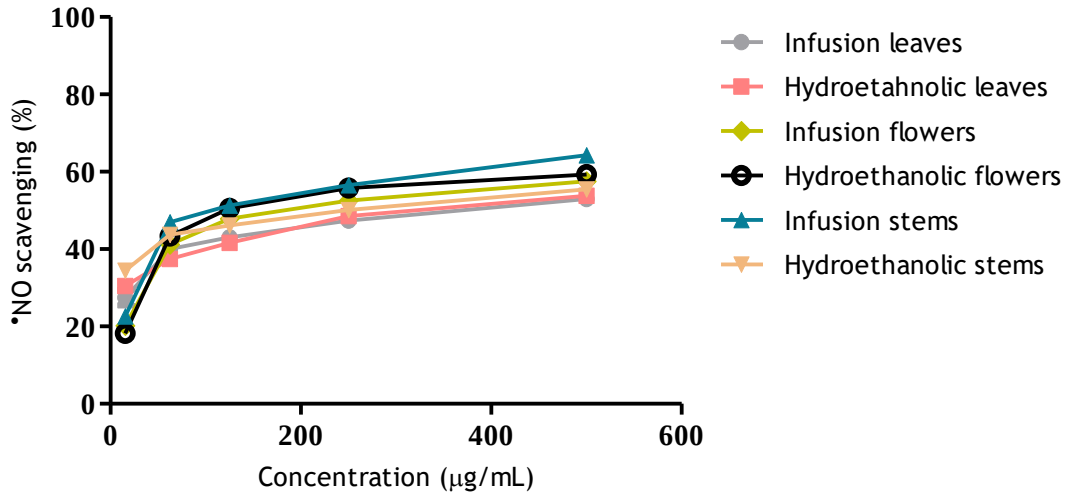


Figure 12. Antioxidant potential of leaves, flowers and stems extracts against •NO radical.

All studied extracts showed a promising activity when compared with sweet cherry cultivar *Satin* (IC_{50} = 439.40 ± 2.44 µg/mL) [11]. Nevertheless, comparatively with some medicinal plants, *Peltophorum pterocarpum* extract shown better scavenging activity than all extracts presented in this study (IC_{50} = 84.25 ± 1.18 µg/mL, expressed as dried methanolic extract) [268]. Furthermore, the activity of hydroethanolic flower and infusion stems was similar to strawberry fruits (IC_{50} = 118.00 ± 45.20 µg/mL, express as ethanolic dried extract), but less effective than blueberry and blackberry fruits (IC_{50} = 2.20 ± 0.01 µg/mL and IC_{50} = 2.60 ± 0.02 µg/mL express as ethanolic dried extract, respectively) [269].

The results obtained indicated the excellent activity of all extracts from *P. avium* vegetal parts against •NO. In respect to *P. avium* stems, there was found a positive correlation between both stem extracts and the total phenolic amounts (r=0.9443), and the total flavonoid content (r=0.9496). However, a negative correlation was found considering the total phenolic acids (r=-0.9002) (p<0.05). In case of flower extracts, a negative correlation was obtained for all parameters: r=-0.9345 for total phenolics, r=-0.9319 for total phenolic acids and r=-0.9453 for total flavonoid content, respectively (p<0.05). These data are another evidence about the behavior of phenolic compounds as free radical terminators and metal chelators [270], mainly attributed to their chemical structure, for example the antioxidant activity of hydroxycinnamic acids seems to be influenced by the number of hydroxy groups (OH) presented on the aromatic rings. So, high number of hydroxy groups usually results in a higher antioxidant capacity [271-273]. On the other hand, flavonoids have showed best antioxidant activity than phenolic acids, due to their chemical structure with the missing 2,3-double bond and conjugated 4-*oxo* group on C ring, consider important for their free radical scavenging activity, preventing the electron transfer from B ring to A ring [274,275]

12.2. *a*-Glucosidase inhibitory activity

Diabetes mellitus is a very prevalent disease. In 2000 the estimated number of people affected was 171 million, and is expected to increase to 366 million by the year 2030 [276]. This mainly affects people by diabetes type 2 around the age of 30, being type 2 the most common type of diabetes, also called adult-onset diabetes [277]. This pathology is caused by abnormalities in carbohydrate metabolism related with low blood insulin level or insensitivity to target organs to insulin [216], originating hyperglycemia effects which is the main responsible for the development of diabetic complications [214]. An important factor in carbohydrate digestion is *a*-glucosidase enzyme, located in the brush border of the small intestine and responsible for the breakdown of carbohydrates to absorbable molecules, namely monosaccharides [278]. Another problem linked with diabetes is oxidative stress, given that the difference between the generation of oxygen-derived radicals and an organism´s antioxidant potential can main to oxidative damage to cell components, such as lipids, nucleic acids and proteins [215].

According to our knowledge and as far as we known, this is the first report concerning the inhibitory effect of *P. avium* extracts from stems, leaves and flowers against α-glucosidase enzyme. All analyzed extracts could inhibit this enzyme in a dose dependent-manner (Figure 13). The *P. avium* vegetal parts with most inhibitory capacity was infusion (IC_{50} = 3.18 ± 0.23 µg/mL) and hydroethanolic (IC_{50} = 7.67 ± 0.23 µg/mL) extracts of stems, followed by leaves and flowers hydroethanolic extracts (IC_{50} = 15.61 ± 0.48 µg/mL and IC_{50} = 59.83 ± 0.68 µg/mL, respectively). Infusion of flowers was the less active (IC_{50} = 535.52 ± 2.16 µg/mL). Except infusion flowers, all other analyzed extracts, revealed better activity when compared to the therapeutic drug acarbose (IC_{50} = 296.83 ± 1.63 µg/mL) (Table 3).

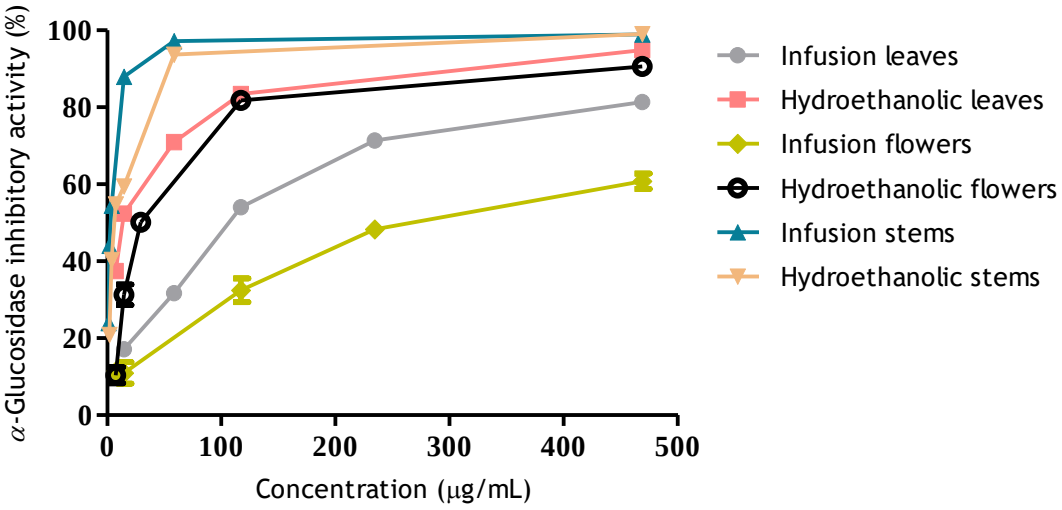


Figure 13. Inhibitory activity of leaves, flowers and stems against *a*-glucosidase.

The stems extracts showed higher activity than those obtained with *Saco* and *Hedelfinger* sweet cherries (IC_{50} = 10.79 ± 0.40 μg/mL and IC_{50} = 10.25 ± 0.49 μg/mL expressed as dried hydroethanolic extracts, respectively) [11], as well to that achieved relatively to *Lepisanthes alata* (Malay cherry) leaves aqueous extract (IC_{50} = 10.83 ± 0.67 μg/mL expressed as dried extract) [279]. In regards to leaves, hydroethanolic extract presented better results than leaves and stems from *Neptunia oleracea* (IC_{50} = 19.09 μg/mL and IC_{50} = 19.74 μg/mL expressed as crude extract, respectively) [280]. On the other hand, ethanolic extracts of seven pomegranate flowers cultivars *Chetoui* (IC_{50} = 32.66 ± 0.90 μg/mL dw), *Espagnoule* (IC_{50} = 31.54 ± 0.50 μg/mL dw), *Gabsi* (IC_{50} = 35.56 ± 0.67 μg/mL dw), *Garsi* (IC_{50} = 42.62 ± 2.05 μg/mL dw), *Rafrari* (IC_{50} = 29.77 ± 1.50 μg/mL dw), *Zaghwani* (IC_{50} = 42.59 ± 0.65 μg/mL dw) and *Zehri* (IC_{50} = 48.02 ± 1.30 μg/mL dw), showed better results in respect to inhibition of *a*-glucosidase enzyme than our flowers extracts, but lower activity than stems and hydroethanolic extracts of leaves [281].

Differences obtained in vegetal parts results may be due to the solvent used [281], as well as the total phenolic amounts [282]. To reinforce this latter fact, the Pearson's test was performed take into account the IC_{50} values found in the assay. Consequently, a positive correlation was found in stems extracts for the total phenolic amounts (r=0.9741), and the total flavonoid content (r=0.9822), however a negative correlation was found with the total phenolic acids (r=-0.9500) (p<0.05). In case of flowers extracts, a negative correlation was obtained for all parameters (r=-0.9759, r=-0.9744 and r=-0.9798, for the total phenolics, total phenolic acids and flavonoid content, respectively) (p<0.05). Similar results were presented by leaves extracts (r=-0.9880, -0.9834 and - 0.9889, for the total phenolics, total phenolic acids and flavonoid content, respectively) (p<0.05). These correlations between the phenolic composition and the inhibitory activity of *a*-glucosidase enzyme were previously described [283-285].

The antidiabetic activity may be due to the phenolic acids content, especially hydroxycinnamic acids, but also due the flavonoids content, like quercetin in which there potential to inhibit *a*-glucosidase enzyme was previously reported [286]. Considering that, the inhibitory activity of flavonoids, increase considerably with an increase in the number of the hydroxyl on the B ring. Then evaluation the activity of different group of flavonoids, the reported data were: in 4' –hydroxylated flavonoids, the decreasing order the inhibitory activity was genistein>kaempferol>naringenin, while in 3',4'–dehydroxylated flavonoids, the order was quercetin>catechin>epicatechin. So, the decreasing order of the α-glucosidase inhibitory activity of flavonoids class was concluded to be isoflavone>flavonol>flavone>flavanone>flavan-3-ol. Concluding that, the structures of the A, B and C rings were related to the inhibitory activity. As well to the A and C rings, hydroxylation at the 3 and 5 positions enhanced the activity of the flavonoid [287]. Another study proved that the flavonoids action depends of the number, type and position of hydroxyl groups. But also, that the flavonoids regulate glucose homeostasis through a multitude of actions and by a complex intracellular signaling related by a particular structure of O- or C-glycosides flavonoids [288-290].

12.3. Protective effects of flowers, leaves and stems from *P. avium* against oxidative damage in human blood samples

Erythrocytes are highly abundant circulating cells in the vertebrates, and their major function is the respiratory gas exchange [291]. In addition, these cells also participate in the immune complex reactions (antibody, complement and bacteria) [292]. However, its composition rich in polyunsaturated fatty acid in the membrane, coupled with their major function associated with oxygen transportation, make these blood cells are specific targets for free radical species, being potential promoters of reactive oxygen species (ROS) [293,294]. Despite erythrocytes membrane has an efficient antioxidant mechanism that maintain their integrity, this not is not sufficient, and normally detoxifying antioxidants such as, catalases, glutathione and glutathione-related enzymes also act to prevent oxidative damage [295].

Considering all the benefits of the vegetal parts of *P. avium*, t, and as far as we known, we evaluated for the first time the effects of leaves, flowers and stems against ROO•-induced damage. The aqueous solution containing AAPH at 37ºC generates oxidative stress, causing quickly damage on the erythrocytes membrane due to the chain reaction [296]. Then occurs hemoglobin oxidation, lipid peroxidation and later hemolysis, by the generation of alkyl radicals in the presence of oxygen [231].

12.3.1. Hemoglobin oxidation inhibition

One of the most fascinating hemoprotein is hemoglobin (Hb). This one is responsible for transporting oxygen from the lungs to the tissues. In addition, extracellular Hb is a nitric oxide

scavenger [297]. However, by their tendency to undergo oxidation, it is recognized by its interference in cellular damage [298]. The result of hemoglobin oxidation is methemoglobin (MHb), that occurs when the iron in the heme group is in the form Fe^{3+} and not as normally Fe^{2+} state [299]. It is known that the altered state is related to oxidative stress, however this phenomenon is not fully understood.

With the aim of studying the inhibitory capacity of hemoglobin oxidation by flowers, stems and leaf extracts, peroxyl radicals ($ROO^{\bullet}$) were generated by AAPH thermal decomposition to induce hemoglobin oxidation, forming methemoglobin. As far as we know, the hemoglobin oxidation inhibitory assay using flowers, stems and leaves extracts was determined for the first time. All extracts could inhibit hemoglobin oxidation in a dose-dependent manner (Figure 14), being hydroethanolic extracts from flowers and stems the most active with an IC_{50} values of 12.85 ± 0.61 and 13.73 ± 0.68 µg/mL, respectively, followed by infusion (IC_{50} = 14.27 ± 0.73 µg/mL) and hydroethanolic (IC_{50} = 23.88 ± 0.78 µg/mL) extracts of leaves. Infusion of stems and flowers showed the lower activity with an IC_{50} = 25.77 ± 0.74 µg/mL and 30.56 ± 0.33 µg/mL, respectively (Table 4).

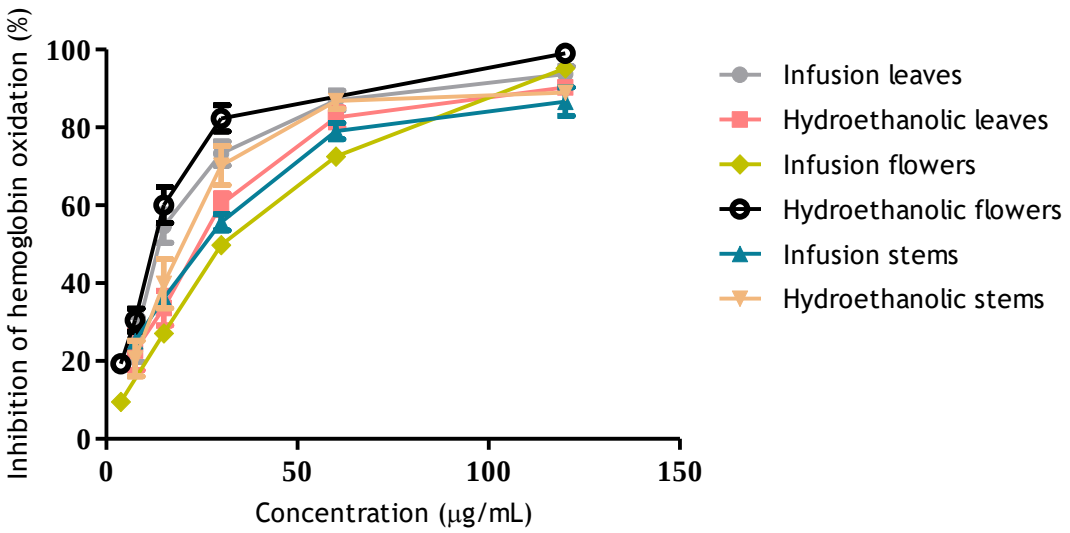


Figure 14. Inhibitory of hemoglobin oxidation (%) by leaves, flowers and stems of *P. avium* extracts.

All extracts were less active, than positive control quercetin (IC_{50} = 2.16± 0.12 µg/mL) (Table 4).

Table 4. IC_{50} **(µg/mL) values obtained for protection of the human erythrocytes against ROO• assays for leaves, flowers and stems of *P. avium* extracts.**

	Stems		Leaves		Flowers	
Assay	**Hydroethanolic**	**Infusion**	**Hydroethanolic**	**Infusion**	**Hydroethanolic**	**Infusion**
Hemoglobin Oxidation	13.73 ± 0.68	25.77 ± 0.74[a]	23.88 ± 0.78[a]	14.27 ± 0.73[b,c]	12.85 ± 0.61[b,c]	30.56 ± 0.33[a,c,d,e]
Lipid Peroxidation	26.20 ± 0.38	47.29 ± 0.55	70.91 ± 1.44	429.80 ± 2.39 [a,b,c]	292.40 ± 2.68[a,b,c,d]	363.74 ± 3.05[a,b,c,d,e]
Hemolysis	1.58 ± 0.18	3.36 ± 0.32[a]	2.79 ± 0.39	3.21 ± 0.34[a]	5.76 ± 0.42[a,b,c,d]	6.51 ± 0.33[a,b,c,d]

Values are expressed as mean ± standard derivation of three assays; difference between each activity for the tested extracts were tested for significance using the one-away analysis of variance (ANOVA). Significant results (P<0.05) are indicated as: a – *vs* hydroethanolic stems; b – *vs* infusion stems; c – *vs* hydroethanolic leaves; d – *vs* infusion leaves; e - *vs* hydroethanolic flowers.

Furthermore, all extracts showed a more considerable activity than those obtained with *Saco* sweet cherry cultivar (IC_{50} = 38.57 ± 0.96 µg/mL expressed as dried hydroethanolic extract) [11].

Relatively to other vegetal parts activity from other plants, the leaves, flowers and stems extracts from *P.* avium showed to be more active than stems and leaves of *Barringtonia racemose* (IC_{50} = 100 and 500 µg/mL expressed as dried aqueous extract, respectively) [300].

A few studies showed the ability to flavonoids to prevent the oxidative damage induced in the erythrocyte membrane by radical species [301,302]. The OH substitutions presented in flavonoid derivatives are related to their protective ability for erythrocytes, noting that the higher number of OH groups, the higher the antioxidant capacity [301,303]. Considering that, flowers extracts showed a negative correlation between the total flavonoids amount and the hemoglobin oxidation inhibition (r=- 0.9646), however in stems and leaves extracts a positive correlation was found (r=0.9518 and r=0.8121, respectively (p<0.05)).

In particular, it has been reported that quercetin and derivatives may inhibit the oxidation of hemoglobin, inhibiting potential enzymatic reactions [304,305]. However, for the compounds quercetin derivative and quercetin-3-*O*-rutinoside, a negative correlation was found between stems (r=- 0.8249 and r=-0.9821), flowers (r=-0.9935) and no correlation was found for extracts of the leaves (p<0.05). As regards to quercetin-3-*O*-rutinoside, this one demonstrated similar results, a negative correlation for stems (r=-0.9821), flowers (r=-0.9342), and no correlation was obtained for leaf extract (p<0.05). A positive correlation was found between leaves and quercetin-3-*O*-galactoside (r=0.8540) for stems and leaves, nevertheless a negative correlation was presented by stems (r=-0.8527) (p<0.05). Quercetin-3-*O*-galactoside, only detected in stems showed a negative correlation (r=-0.9575) (p<0.05). Our results are according to those reported in the literature [304,305].

12.3.2. Lipid peroxidation inhibition

The hemoglobin oxidation leads to the formation of methemoglobin (MHb), that occurs when the iron in the heme group is not in its normal state [299], causing oxidative stress events, damaging lipids and modifying proteins interactions, and consequently affecting the balance and resistance of the erythrocytes membrane [306]. The first dangerous step is when ROS remove a hydrogen atom from fatty acid side chains [307]. As it contains a large number of double bounds in their side chains, the polyunsaturated fatty acids (PUFA) become more susceptible to peroxidation [308], and the reaction between the resulting lipid radicals and molecular oxygen yields $ROO^{\bullet}$, which are strong pro-oxidant radicals capable to remove hydrogen atoms from adjacent fatty acids, forming lipid hydroperoxides. These events trig the decomposition of lipid hydroperoxides in secondary lipid peroxidation products like malondialdehydes (MDAs), which are markers of oxidative stress. Additionally, the process of lipid peroxidation products is catalyzed by the presence of transition metal, like iron [307-309].

The most commonly method used for the measurement of lipid peroxidation in biomaterials is thiobarbituric acid reactive substances (TBARS) [309]. Therefore, the capacity of flowers, leaves and stems extracts to inhibit the TBARS formation was determined when *tert*-butyl hydroperoxide (*t*BHP) was added. This one induces lipid peroxidation in erythrocytes, being the reaction with thiobarbituric acid (TBA) and malondialdehyde (MDA) read at 532 nm [231]. As far as we know, the lipid peroxidation inhibitory assay using leaves, flowers and stems extracts was determined for the first time in this work on human erythrocytes.

All extracts showed activity against lipid peroxidation in a dose-dependent manner (Figure 15), being stems extracts the most active with an IC_{50} = 26.20 ± 0.38 and IC_{50} = 47.29 ± 0.55 µg/mL for hydroethanolic extract and infusion, respectively. Followed by leaves hydroethanolic extract (IC_{50} = 70.91 ± 1.44 µg/mL) and flowers hydroethanolic extract (IC_{50} = 292.40 ± 2.68 µg/mL), respectively. Leaves and flowers Infusion showed the lower activity with IC_{50} values of 429.80 ± 2.39 µg/mL and 363.74 ± 3.05 µg/mL, respectively. Comparing the obtained results with the positive control quercetin (22.79 ± 0.70 µg/mL), we can see that the stems hydroethanolic extract possess activity close to quercetin, and all other extracts were less active than positive control (Table 4).

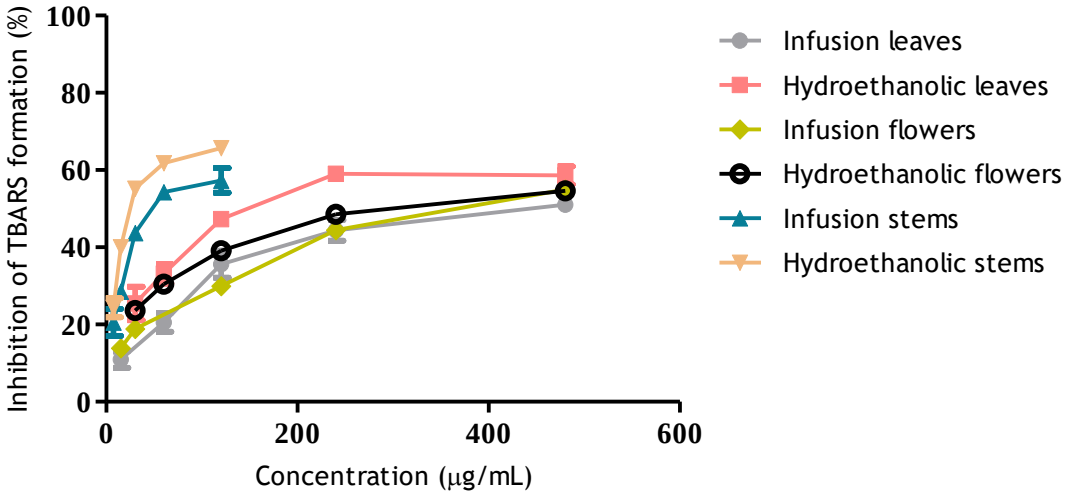


Figure 15. Inhibitory of lipid peroxidation (%) by leaves, flowers and stems of *P. avium* extracts.

Comparatively this activity with another study using *P. avium* stems extracts, namely hydromethanolic, infusion and decoction in porcine (Sus scrofa) brain homogenates, these ones showed lower activity than our stems extracts (IC_{50} = 70 µg/mL, IC_{50} = 240 ± 0.10 µg/mL, IC_{50} = 130 ± 0.10 µg/mL, respectively, expressed in dried extract) [61].

Relatively to hydrophilic extracts of murici (*Byrsonima crassifolia*), a fruit native to the North and Northeast regions of Brazil, *P.* avium stems, flowers and leaves extracts showed to be more active than these fruits (IC_{50} = 1000 µg/mL expressed as dried hydromethanolic extract) [310]. A few studies showed a correlation between the total phenolics of the extracts and their activity against lipid peroxidation [311,312]. In fact, phenolic compounds are powerful chain-breaking antioxidants, scavenging ROO• by donating a phenolic hydrogen atom to them, forming a resonance-stabilized antioxidant radical and a lipid hydroperoxide [313,314]. In especially flavonoids exhibit metal chelation properties, capturing these ones, which were formed in the aqueous phase before they can attack the membrane of erythrocytes, protecting it from these radicals [294,315].

Once more, the correlation between the total amount of phenolics and the activity of the extract was tested. Stems extracts showed a negative correlation for the total flavonoid amount and the total phenolic content (r=-0.9708 and r=-0.9675, respectively) (p<0.05). Similar results were presented by leaves extracts with a negative correlation for the total phenolic amount and total flavonoid content of r=-0.9805 and r=-0.9826, respectively) (p<0.05). Additionally, flowers extracts showed no correlation between the total phenolics and flavonoid amount and the lipid peroxidation inhibitory activity.

The correlation between the quercetin and their derivatives with the antioxidant activity against lipid peroxidation inhibition was also tested. For the compound quercetin derivative, a

negative correlation was found in stems (r=-0.9820) and leaves (r=-0.9693) extracts ($p<0.05$). Quercetin-3-*O*-rutinoside amounts demonstrated similar results, a negative correlation with stems (r=-0.9636) and leaves (r=-0.9601) extracts ($p<0.05$). Also, a negative correlation was obtained between quercetin-3-*O*-glucoside of stems and leaves (r=-0.9827 and r=-0.9959, respectively) and the antioxidant activity ($p<0.05$). Quercetin-3-*O*-galactoside, only detected in stems showed a negative correlation (r=-0.9059) ($p<0.05$). Flower extracts do not show any correlation with their antioxidant activity. Even so, the relation between quercetin amounts and the extract activity against lipid peroxidation assay was reported in the literature [315].

12.3.3. Hemolysis of human erythrocytes inhibition

Free radical species formed, attack the membrane of erythrocytes, and in the presence of transition metals, lipid peroxidation is induced, leading to hemolysis, and eventually to cell death [311,316].

The extracts of *P. avium* vegetal parts inhibited hemolysis in a concentration-dependent manner (Figure 16), being hydroethanolic stems and leaves extracts the most effective, with IC_{50} values of 1.58 ± 0.18 µg/mL and 2.79 ± 0.39 µg/mL, respectively. Flowers extracts showed the lower activity with an IC_{50} = 5.76 ± 0.42 µg/mL for hydroethanolic extract, and IC_{50} = 6.51 ± 0.33 µg/mL for infusion. All extracts were less active than positive control quercetin (IC_{50} = 0.67 ± 0.13 µg/mL) (Table 4).

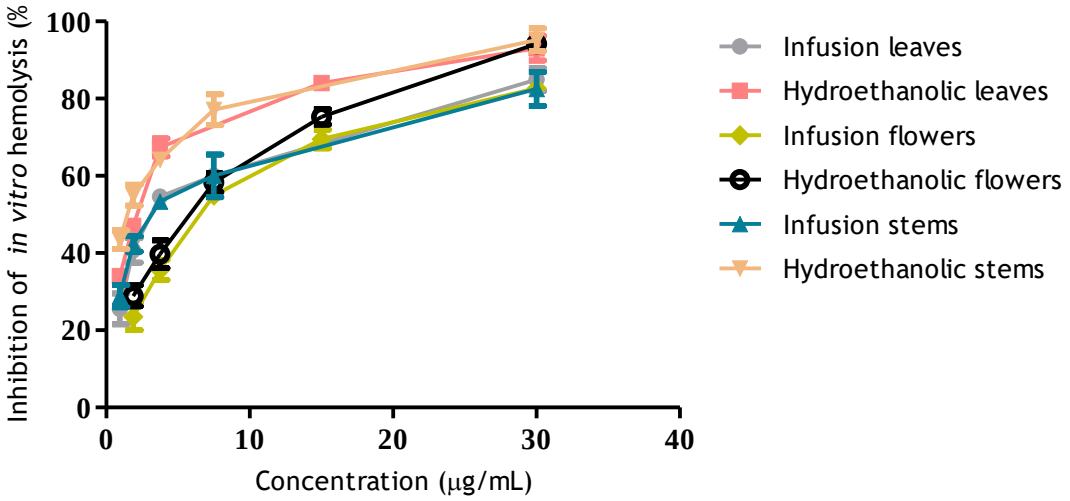


Figure 16. Inhibitory of erythrocytes hemolysis (%) by leaves, flowers and stems of *P. avium* extracts.

Comparatively to *Saco* sweet cherry cultivar (IC_{50} = 73.03 ± 1.48 µg/mL expressed as dried hydroethanolic extract), all extracts of leaves, flowers and stems revealed more activity [11]. Additionally, comparing with pulp of *Cydonia oblonga* Miller (quince) extract, this one had a activity 100 times lower than the infusion flowers extract of *P. avium* (IC_{50} = 652 µg/mL,

expressed as dried methanolic extract), while peel of *Cydonia oblonga* Miller (quince) methanolic extract presented an activity 120 times lower than hydroethanolic flowers extract of *P. avium* (IC_{50} = 695 µg/mL, expressed as dried extract) [317].

Parallel with these, the antihemolytic activity measured for the *Cydonia oblonga* Miller (quince) leaves extract was 7 times lower (IC_{50} = 24.3 ± 9.6 µg/mL, expressed as dried extract) than that of infusion from stems [318].

According to a previously reported by Magalhães et al. (2009) an extract that demonstrated higher activity against the DPPH assay, also showed higher inhibitory activity against hemolysis. To reinforce this fact, correlation tests were done [317]. Within this context, correlation was sought between total phenols and flavonoids and their anti-hemolytic activity. A negative correlation was found for the total phenolics amount in leaf extracts (r=-0.8659), however no correlation was found in flowers and stems extracts (p<0.05). While for the total flavonoid content, a negative correlation was demonstrated in leaves and stems extracts with (r=-0.8323 and r=-0.8208, respectively). Once again, no correlation was presented by flowers extracts (p<0.05).

Besides the compounds reported in *P. avium* vegetal parts, it is important to consider their antioxidant activity as the result of interaction between them [251]. As for, flavonoids they had high activity owing to their chemical structure (C2=C3 bond in C3 ring increases antioxidant capacity) [262]. This feature together with the recognize liposolubility of phenolic compounds rises anti-hemolytic protections [319]. This allows them to acting as antioxidant agents, being strongly incorporate into the membrane, capturing harmful species them before they can attack the membrane, leading to minimization of the concentration of reactive species and consequently preventing lysis events [320].

V. Conclusions

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

- The interest in therapeutic use of natural products and alternative therapies, particularly those derived from plants, containing antioxidants and health-promoting phenolic compounds have been growing, due their minimum side effects, lower costs and toxicity.

- The phenolic composition by LC-DAD analysis, of *P. avium* vegetal parts (leaves, flowers and stems), allowed the identification of 31 phenolic compounds. Leaves showed the highest amounts. Stems was the richest in flavonoids, while leaves and flowers revealed higher contents in phenolic acids, more specifically hydroxycinnamic acids.

- Sakuranetin derivative was the main phenolic compound present in stems. 5-*O*-caffeoylquinic acid was the major one in leaves, while hydroxycinnamic acid derivative 1 was the main phenolic in leaves.

- All the analyzed extracts have demonstrated a satisfactory antioxidant activity. The hydroethanolic extracts of stems and leaves showed the highest antioxidant activity against DPPH$^{\bullet}$ and $O_2^{\bullet-}$ radicals. On the other hand, infusion of stems and hydroethanolic of flowers have demonstrated the best activity to scavenging $^{\bullet}NO$. The activity demonstrated by all extracts in this work may be due to their richness in phenolic compounds.

- The activity of leaves, flowers and stems against α-glucosidase enzyme revealed that all the extracts used in this work showed best inhibitory activity than the positive control acarbose, one drug commercialized for the treatment of diabetes type 2, except the infusion of flowers.

- Hydroethanolic stems extract showed the highest activity in the hemolysis and lipid peroxidation assays in human erythrocytes, however, hydroethanolic flower was the most active in hemoglobin oxidation. The high activity observed for the analyzed extracts may be due to their phenolic composition, especially flavonoids, which were reported by their ability to capture free radicals and preventing the membrane's damage.

- The extracts analyzed in the present work demonstrated to be composed by great number of phenolic compounds, high antioxidant capacity to capture free radical

species, and consequently to protect membrane of human erythrocytes against cells damage induced by ROO•. The high biological potential demonstrated allows the extrapolation of possible health benefits in several diseases, like cardiovascular, diabetes, inflammatory and hemolytic pathologies. However, more studies are needed to explore the phenolic composition and biological potential, as well to confirm the real benefits to human health, and to improve their application in pharmaceutical and nutraceutical formulations.

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