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Assessment of the biological propertiesof *Eugenia uniflora* related with prevention of cardiovascular diseases

MASTER DISSERTATION

Jéssica Mariana Abreu Gonçalves
MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA
A Nossa Universidade
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SUPERVISOR
José de Sousa Câmara

Originality Statement

"Plagiarism consists of presenting the ideas, opinions, sentences/texts, results or conclusions of others as your own, even if they have been translated. The practice of plagiarism is a serious breach of academic ethics and can lead to failure or withdrawal of the degree, as well as civil, criminal, and disciplinary liability."

I declare on my honour that this dissertation is my exclusive authorship, it is original, and I have referenced and cited all the sources used.

September 2024

Jéssica Mariana Abreu Gonçalves

Acknowledgments

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Abstract

Plant-based foods constitute a significant source of phytochemical compounds that can help to prevent the occurrence of several diseases, including cardiovascular diseases (CVDs), which currently constitute the leading cause of death worldwide. This work aimed the analysis of two different varieties (orange and purple) of *Eugenia uniflora* L. (*Myrtaceae*) fruits (pitanga) and *Eugenia uniflora* L. leaves, to evaluate their biological properties namely those related with the prevention of CVDs, including the antioxidant, anti-inflammatory, and antihypertensive activities. The antioxidant activity was assessed using spectrophotometric techniques applied to two different *in vitro* assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging activity. The antioxidant activity was correlated with total polyphenol content (TPC), total flavonoid content (TFC), and total anthocyanin content (TAC). The antihypertensive activity was carried out based on the angiotensin-converting enzyme (ACE) assay while the anti-inflammatory capacity was evaluated using the protein denaturation inhibition assay. The study revealed that both varieties of *Eugenia uniflora* L., demonstrated comparable values across the measured parameters, with the purple variety generally displaying slightly higher values, except for DPPH and ABTS assays. However, these differences were not statistically significant. Among the tested samples, *Eugenia uniflora* L. leaves consistently showed the lowest values in all assays.

Free polyphenols were identified using the μ-QuEChERS extraction technique combined with ultrahigh-performance liquid chromatography equipped with a photodiode array detection system (UHPLC-PDA). Notably, UHPLC-PDA analysis allowed the identification of gallic acid, syringaldehyde, resveratrol, quercetin, cinnamic acid, and kaempferol, in the target samples. Gallic acid was found as the most dominant polyphenol across all samples, with the orange pitanga variety presenting the highest concentration (13.1 ± 0.5 mg/100 g DW). This exploratory study showed that *Eugenia uniflora* L. fruits and leaves extracts hold potential to be used namely as natural antioxidant, anti-inflammatory and antihypertensive agents, with different applications in food, pharmaceutical and cosmetic industries, for instance. These findings underscore the value of *Eugenia uniflora* L. extracts as a robust source of bioactive compounds highlighting their potential role in promoting human health, particularly in the prevention of cardiovascular diseases (CVDs).

Keywords: *Eugenia uniflora* L.; biological activities; Cardiovascular diseases; Polyphenols.

Resumo

Os alimentos de origem constituem uma fonte significativa de compostos fitoquímicos muitos dos quais podem contribuir para prevenir a ocorrência de diversas doenças, incluindo as doenças cardiovasculares (DCV), que constituem atualmente a principal causa de morte em todo o mundo. Este trabalho teve como objetivo avaliar as propriedades biológicas, nomeadamente as relacionadas com a prevenção de CVDs, incluindo as propriedades antioxidantes, anti-inflamatórias e anti-hipertensivas, de frutos (pitangas) das variedades laranja e roxa de *Eugenia uniflora* L. (*Myrtaceae*) (pitangueira) e das folhas. A atividade antioxidante foi avaliada utilizando técnicas espectrofotométricas aplicadas a dois ensaios *in vitro*, método de eliminação de radicais livres 2,2-difenil-1-picril-hidrazil (DPPH), e atividade de eliminação de catiões radicalares 2,2'-azino-bis(3-etilbenzotiazolina-6-ácido sulfônico) (ABTS), correlacionado-se a sua atividade antioxidante com o conteúdo total de polifenóis (TPC), conteúdo total de flavonoides (TFC) e conteúdo total de antocianinas (TAC). A atividade anti-hipertensiva foi realizada com base no ensaio da enzima conversora de angiotensina (ECA), enquanto a capacidade anti-inflamatória foi avaliada pelo ensaio de inibição da desnaturação de proteínas. O estudo revelou que as variedades de *Eugenia uniflora* L. demonstraram valores comparáveis em todos os ensaios realizados, com a variedade roxa geralmente exibindo valores ligeiramente superiores, exceto para os ensaios DPPH e ABTS. No entanto, essas diferenças não foram estatisticamente significativas. Entre as amostras testadas, as folhas de *Eugenia uniflora* L. apresentaram consistentemente os valores mais baixos em todos os ensaios.

Os polifenóis livres das amostras em estudo foram extraídos, identificados e quantificados utilizando a técnica de extração µ-QuEChERS combinada com cromatografia líquida de ultra eficiência equipada com sistema de detecção de fotodiodos (UHPLC-PDA). Este procedimento analitico permitiu a identificação de alguns polifenóis tais como ácido gálico, siringaldeído, resveratrol, quercetina, ácido cinâmico e kaempferol. O ácido gálico foi o polifenol maioritário em todas as amostras em estudo, com a variedade da pitanga laranja a apresentar a maior concentração (13,1 ± 0,5 mg/100 g DW) deste composto. Este estudo exploratório mostrou que os extratos dos frutos e folhas da *Eugenia uniflora* L. têm potencial para serem utilizados nomeadamente como agentes antioxidantes, anti-inflamatórios e anti-hipertensores, em diferentes aplicações nas indústrias alimentar, farmacêutica e cosmética, por exemplo. Estas descobertas sublinham o valor dos extratos de *Eugenia uniflora* L. como uma fonte robusta de compostos bioativos, destacando o seu papel potencial na promoção da saúde humana, particularmente na prevenção de doenças cardiovasculares (CVDs).

Palavras-chave: *Eugenia uniflora* L.; biological activities; Cardiovascular diseases; Polyphenols.

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Abbreviatures

%Rec.	Percentage of recovery
%RSD	Percentage of relative standard deviation
µ-QuEChERS	Miniaturized quick, easy, cheap, effective, rugged, and safe
ABTS	2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid
ACE	Angiotensin-converting enzyme
ACN	Acetonitrile
BACs	Bioactive compounds
CNS	Central nervous system
CVDs	Cardiovascular diseases
dSPE	Dispersive solid-phase extraction
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
EtAc	Ethyl acetate
FA	Formic acid
GAE	Gallic acid equivalents
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
HTLC	High-resolution thin-layer chromatography
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
NMR	Nuclear magnetic resonance
NO	Nitric oxide
PLE	Pressurized liquid extraction
QE	Quercetin equivalents
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
RT	Retention time
SD	Standard deviation
TA	Total anthocyanins
TAC	Total anthocyanins content

TFC	Total flavonoid content
TE	Trolox equivalents
TPC	Total phenolic content
UHPLC	Ultra-high performance liquid chromatography
UV-Vis	Ultraviolet-visible

Chapter I

INTRODUCTION

1. Introduction

CVDs are a group of multifactorial disorders of the heart and blood vessels and include coronary heart disease, cerebrovascular disease, among other diseases (Figure 1) [1]. CVDs constitute a major public health issue worldwide, claiming 17.1 million lives a year and being the leading cause of mortality [2]. The major risk factors contributing to CVDs include unhealthy lifestyles such as poor diet, smoking, physical inactivity, excessive alcohol intake, polluted environments, among others. These risk factors are associated with changes in biochemical parameters leading to increased blood pressure, high cholesterol, and hypertension. The incidence of CVDs has been increasing continuously, and this upward trend is projected to continue in the next decades [3], especially in developed countries, where the fast socioeconomic progress has greatly affected lifestyle, urbanization, and accelerated population aging [4,5].

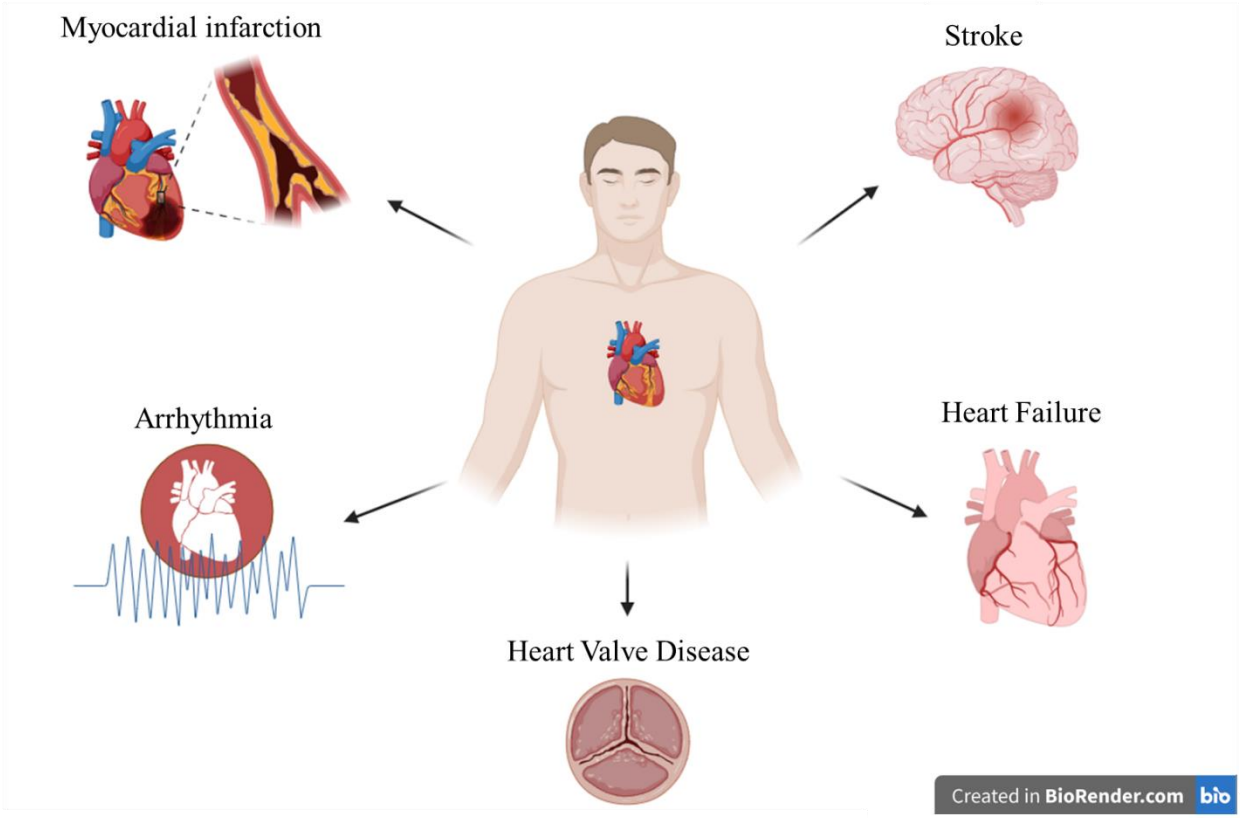


Figure 1. Different types of CVDs.

All CVDs are caused by atherosclerosis, a complex, chronic, and progressive condition that affects large and medium-sized arteries. It is characterized by the accumulation of cholesterol, fatty substances, cellular waste products, calcium, and fibrin, which accumulate inside the artery lining, leading to thickening of the arterial walls and reduced blood flow to organs and tissues (Figure 2) [6].

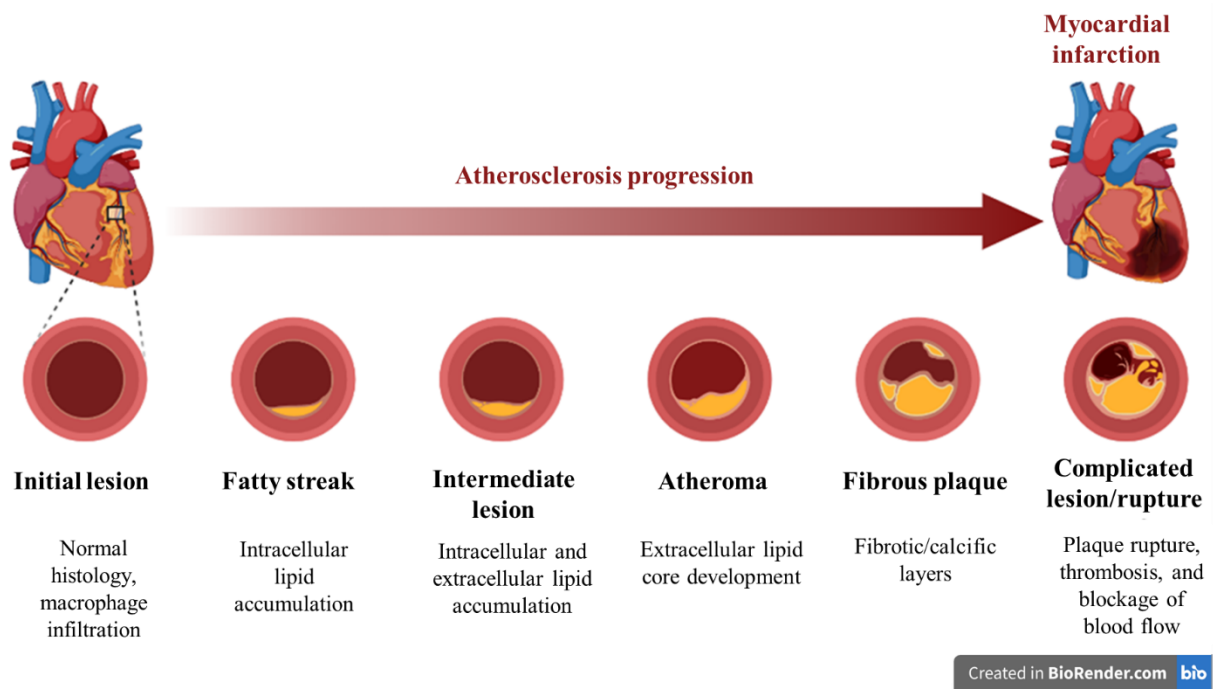


Figure 2. Progression of atherosclerosis.

The lesions of the disease (atheroma or atherosclerotic plaques) have three distinct components: the atheroma, the underlying accumulation of cholesterol crystals, and the calcification of older or more advanced lesions. Atherosclerosis is an inflammatory condition and high plasma concentrations of cholesterol [7], in particular low-density lipoprotein (LDL), are one of the main risk factors for atherosclerosis [8]. The pathogenesis of atherosclerosis remains incompletely understood, but emerging evidence suggests that it may involve multiple cellular events, including endothelial cell dysfunction, inflammation, proliferation of vascular smooth muscle cells (SMCs), matrix alteration, and neovascularization [9]. Autophagy, along with chronic and acute overproduction of reactive oxygen species (ROS), is an integral part of disease development and progression and may represent fruitful avenues for biological investigation and the identification of new therapeutic targets [10].

Oxidative stress induced by free radicals is directly related to the development of various diseases and their associated complications. Increased oxidative stress has been identified as one of the probable common causes of CVD. The normal operation of the cell depends on a delicate balance between the presence of ROS and antioxidants. Increasing ROS causes vasoconstriction and reduced nitric oxide (NO) availability, which lead to arterial hypertension. Additionally, myocardial calcium handling is negatively impacted by ROS, leading to arrhythmia, and cardiac remodeling is accelerated by hypertrophic signaling and apoptosis [11]. Potential nutraceuticals and diets that might be beneficial in decreasing the burden of oxidative stress in CVD include omega-3 fatty acids and polyphenols, among others [11]. Recent research has focused on the long-term cardiovascular outcomes of COVID-19, which has been shown to increase the risk of several CVDs, including heart failure, ischemic heart disease, and thromboembolic disease [12].

1.1. Risk factors

Several risk factors are associated with the development of CVDs. A risk factor is any characteristic or behavior that increases the likelihood of developing a particular disease or condition. In the context of CVD, some common risk factors include physical inactivity, poor diet, smoking, and alcohol intake (Figure 3).

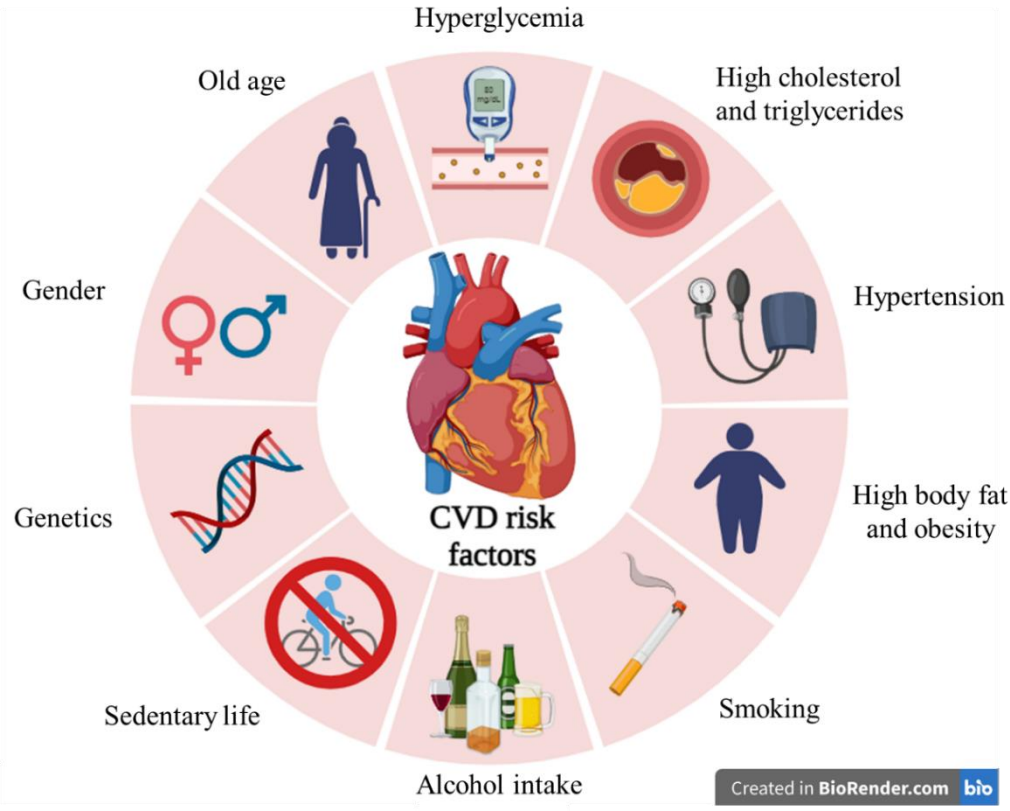


Figure 3. Important risk factors for CVDs.

These risk factors are associated with changes in biochemical parameters leading to increased blood pressure, high cholesterol, and hypertension. Identifying and managing risk factors is an important way to prevent and reduce CVDs [13,14]. Risk factors are classified into two major categories: modifiable and non-modifiable risk factors.

1.1.1. Modifiable risk factors

Modifiable risk factors for CVD are those that can be changed or controlled through lifestyle modifications or medical interventions. These include poor diet, physical inactivity, smoking, alcohol intake, and stress [13–16].

Hypertension, is a common condition in which the force of blood against the walls of arteries is consistently too high [17]. This can lead to damage to the artery walls. Our body tries to repair these injuries, but the repaired tissue ends up attracting white blood cells, cholesterol, and other substances, which lead to thickening and loss of elasticity in the artery walls, sometimes forming clots that lead to their occlusion and compromised blood circulation [18]. These situations can lead to the onset of atherosclerosis and other CVDs, such as stroke. Increased blood pressure also means that the heart must work harder to pump blood, causing the heart walls to thicken (hypertrophy). It is a major risk factor for CVD [19]. When lifestyle modifications alone are not enough to control blood pressure, the use of medication is necessary [20].

Another important risk factor for CVD is high blood cholesterol levels. Cholesterol, a fat-like substance carried in the blood, is found in every cell of the body. The liver is the primary site of cholesterol synthesis, producing the necessary amounts for various physiological functions, including the formation of cell membranes and the biosynthesis of steroid hormones such as estrogen, testosterone, progesterone, aldosterone, and cortisol [21]. The increase in cholesterol levels can be related with the consumption of foods of animals origin, which are rich in saturated fats [22]. There are two main types of cholesterol: the "bad cholesterol " – LDL, which builds up inside the arteries and causes atherosclerosis, leading to serious CVDs; and the "good” cholesterol – high-density lipoprotein (HDL), which helps remove the excess cholesterol from the bloodstream and transporting it back to the liver for excretion [23,24]. Epidemiological studies have established the association between serum cholesterol, specifically serum LDL, and coronary heart disease [25]. Low HDL levels are also a potent risk factor for atherosclerosis [26]. In addition to cholesterol, high levels of serum triglycerides have been recognized as risk factors for heart disease [27]. Triglyceride levels are very diet-dependent, and high values usually indicate a high-fat diet [28]. High blood sugar

levels are also a risk factor to be considered. Heart problems are the leading cause of death among people with diabetes, especially in type 2 diabetes [29].

Sedentary lifestyle is also an important modifiable risk factor. Sedentary behaviour is associated with a greater predisposition to the development of CVDs [30]. Exercise not only burns calories, which helps maintain a healthy weight, but also helps control cholesterol levels and diabetes, and can lower blood pressure. In addition, exercise also strengthens the heart muscle and makes the arteries more flexible, improving cardiovascular health [31]. Sedentarism in addition to an unhealthy diet leads to overweight and obesity, which are already considered the epidemic of this century [32]. These conditions can lead to an increase in high cholesterol levels, high blood pressure, and diabetes, all of which are risk factors for heart disease [33,34]. Stress can also be considered a risk factor for CVDs and this connection can occur through direct mechanisms, such as the body's physiological response to stress, but also indirectly, through behaviours associated with stress [35].

Another modified important factor risk for CVDs is smoking which is associated with vascular damage resulting in endothelial dysfunction, increased oxidative stress, and inflammation, which can increase arterial stiffness. Smoking, in both its active and passive exposure, is a toxicosis and therefore should be considered a systemic disease with marked cardiovascular impairment [36,37].

1.1.2. Non-modifiable risk factors

Non-modifiable risk factors for CVDs are those that cannot be altered or modified, such as age, gender, and family history [15]. Older age is a risk factor for CVDs. As a person ages, the heart's function tends to decline. The heart walls may thicken, and the arteries can stiffen, reducing the heart's efficiency in pumping blood to the body's muscles [38]. These changes increase the risk of developing CVDs with age [39]. Because of sex hormones, women are generally protected from heart disease until menopause, when the risk increases [40]. Several genetic risk factors predispose to CVDs. Besides familial hypercholesterolemia and diabetes, there are also changes in genes involved in coagulation, which are essential for the proper functioning of the heart and blood vessels [41].

The overall cardiovascular risk is particularly important and means that attention must be paid to all risk factors at the same time, and not just to each one in isolation. The fight against risk factors must be done together, to try to control them all at the same time [42]. It is important to note that while non-modifiable risk factors cannot be changed, lifestyle modifications and

medical interventions can effectively manage and reduce the impact of modifiable risk factors on CVDs risk. Making positive lifestyle changes, such as adopting a healthy diet, engaging in regular physical activity, quitting smoking, controlling weight, and controlling blood pressure and cholesterol levels, can significantly reduce the risk of developing CVDs [43].

1.2. Angiotensin-converting enzyme

Angiotensin-converting enzyme (ACE) is an enzyme that is found in the lungs but is also present in other tissues, such as the blood vessels and kidneys. It plays a crucial role in regulating blood pressure and fluid balance in the body. The main function of ACE is to convert angiotensin I, a hormone produced in the liver, into angiotensin II. Angiotensin II is a potent vasoconstrictor, which means that it narrows blood vessels leading to an increase of blood pressure. In addition, angiotensin II is a major component of the renin-angiotensin-aldosterone system (RAAS), so it stimulates the release of aldosterone, a hormone that promotes sodium and water retention, leading to increased blood volume (Figure 4).

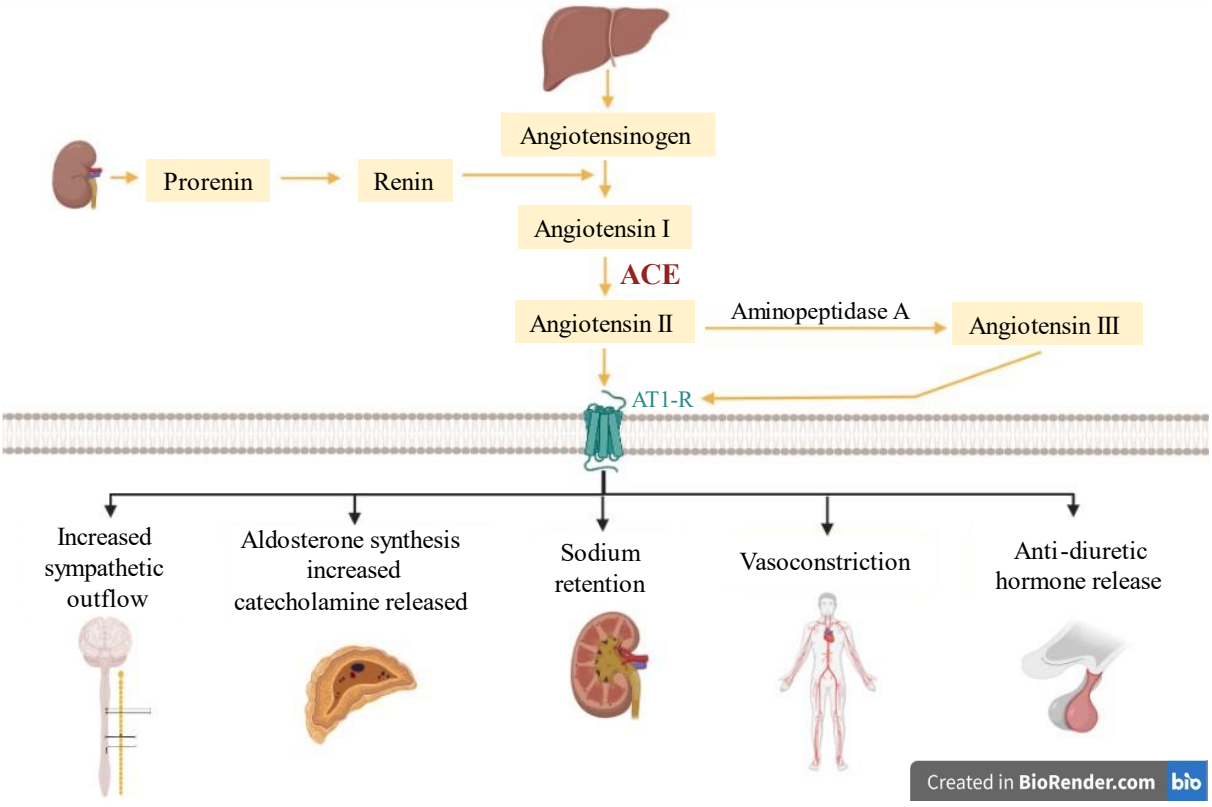


Figure 4. Renin-angiotensin system. ACE: Angiotensin-converting enzyme. AT1-R: Angiotensin II type 1 receptor.

The RAAS is a neurohormonal system that is activated in response to decreased blood pressure or decreased renal blood flow [44]. Combined, these actions contribute to the regulation of blood pressure and the maintenance of homeostasis [45].

In the treatment of CVDs, ACE inhibitors are commonly prescribed. These drugs act by inhibiting the action of ACE, thus reducing the conversion of angiotensin I to angiotensin II. By blocking this pathway, ACE inhibitors cause vasodilation, which leads to a decrease in blood pressure. In addition, ACE inhibitors decrease aldosterone secretion, leading to reduced fluid retention [46]. Angiotensin-converting enzyme 2 (ACE2) is an ACE homologous enzyme that converts angiotensin II to angiotensin 1-7, a vasodilator peptide that has opposite effects to angiotensin II. ACE2 plays a critical role in controlling cardiac physiology and altered ACE2 expression is linked to the progression of heart failure [47]. By lowering blood pressure and reducing pressure on the heart, ACE inhibitors are effective in treating conditions such as hypertension, heart failure, and even certain types of kidney disease [48]. Studies have been conducted on alternative drug therapies based on natural products to inhibit the action of ACE and thus prevent CVDs [49,50].

1.3. The influence of diet on CVDs

Diet is a fundamental factor in disease prevention and overall health [51]. The intricate relationship between nutrition and health means that a well-balanced diet can be instrumental in warding off illness, while dietary habits profoundly impact the risk factors associated with various diseases [52]. Recent research refers to the Mediterranean dietary pattern, rich in fruits and vegetables, as the most cardioprotective, due to its high concentration of bioactive compounds such as polyunsaturated fatty acids, polyphenols, fibers, phytosterols, and vitamins, which exert antioxidant, anti-inflammatory, and antithrombotic effects contributing to delay the onset and progression of CVDs [53]. In this way, it is possible to identify potential targets (dietary patterns, single foods, or individual nutrients) to prevent CVDs and quantify the magnitude of these beneficial effects [54].

1.3.1. Bioactive compounds in foods

Bioactive compounds (BACs) are low molecular weight compounds (< 500 g/mol) with biological activities that occurs in foods, herbs, and spices. These compounds include polyphenols, vitamins, minerals, and polyunsaturated fatty acids, among others (Figure 5) [55]. BACs from food play an important role in the prevention of illnesses, such as CVDs, cancer, diabetes, and metabolic disorders [56].

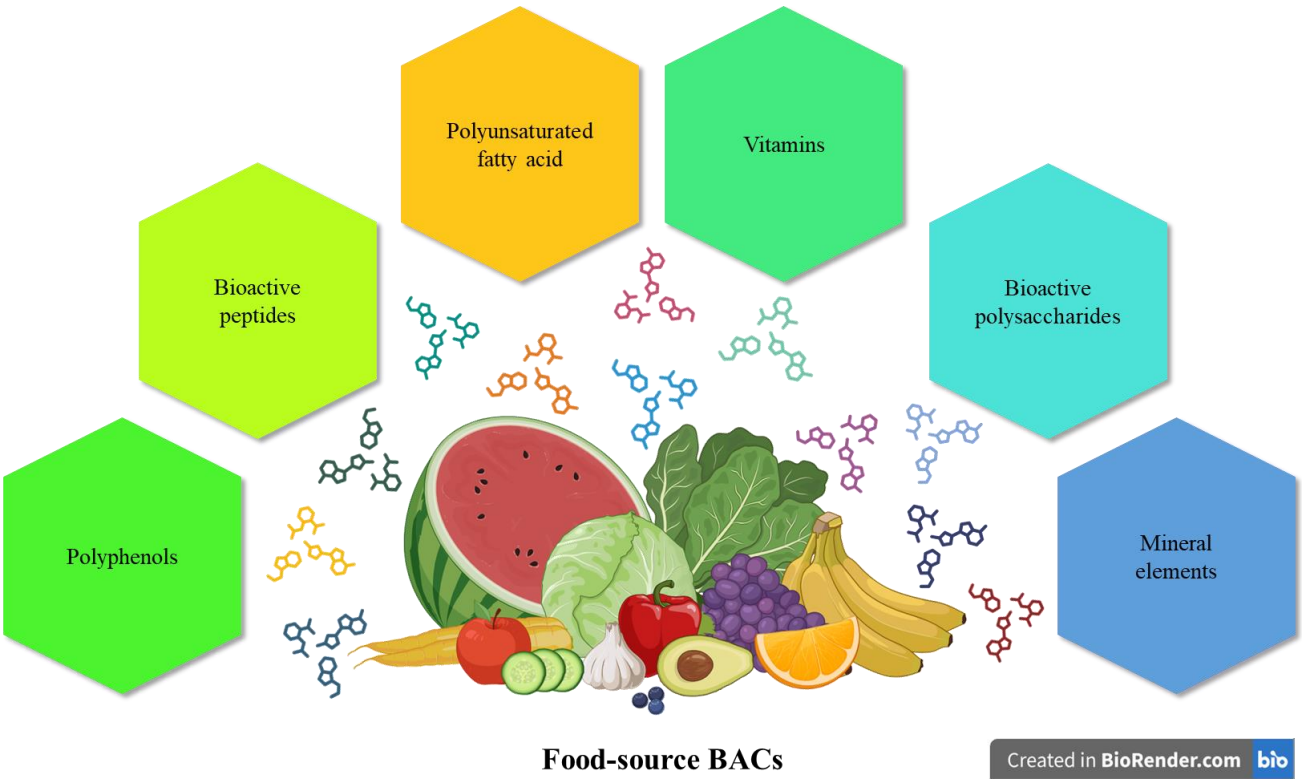


Figure 5. Most common BACs found in foods.

1.3.1.1. Polyphenols

Some studies have demonstrated the preventive benefits of a polyphenol-rich diet against most chronic illnesses [57]. Have been associated with a reduced incidence of CVD [58,59], by improving the function of the inner lining of the heart and blood vessels, increasing HDL-cholesterol, and decreasing LDL-cholesterol, thus promoting antiplatelet and anti-inflammatory effects [60]. However, the extent of health benefits of polyphenols are strongly related to their nature, levels, and bioavailability, which depend on their origin, food matrix, processing, digestion, and cellular metabolism [61]. Plant polyphenols (Figure 6) are a suitable alternative to synthetic preservative agents, with antioxidant and antimicrobial properties [62], but their uses in the food industry are undermined by a series of limitations such as low solubility and stability during food processing and storage, lack of standardization, and undesirable organoleptic properties [63].

From a chemical point of view, polyphenols can be classified into flavonoids (flavones, flavanols, flavanones, isoflavones, and anthocyanins) and non-flavonoids, namely phenolic acids, stilbenes, coumarins, and lignans. Dietary polyphenols also aid in reducing systemic inflammation, insulin resistance, blood pressure, and lipid profiles. Resveratrol, a stilbene, and the flavonoid quercetin have both been related to better cardiovascular health [64].

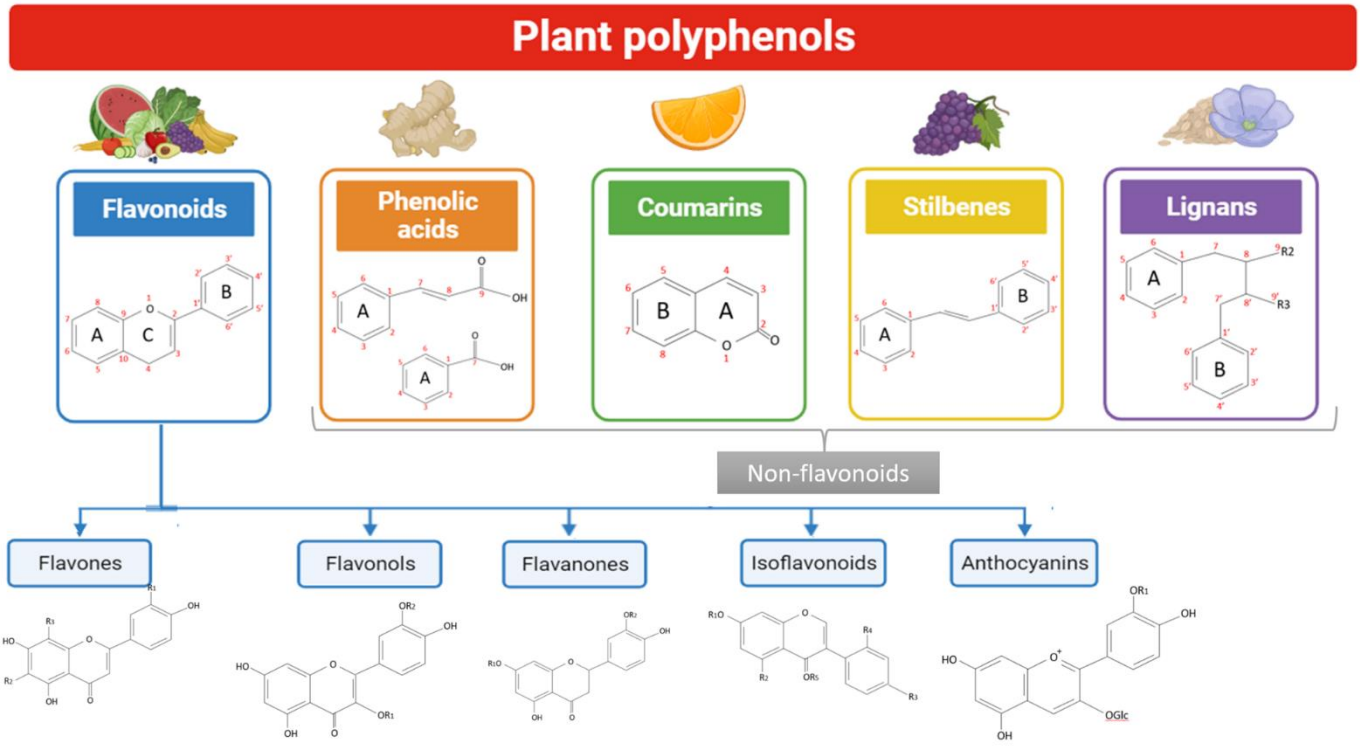


Figure 6. Polyphenol classification into flavonoids (flavones, flavanols, flavanones, isoflavones, and anthocyanins) and non-flavonoids (phenolic acids, coumarins, stilbenes, and lignans).

i) Flavonoids and non-flavonoids

Flavonoids are a group of natural substances with variable phenolic structures that are found in fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine (Figure 7) [65]. These natural products are well known for their beneficial effects on health, including antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic properties, coupled with their capacity to modulate key cellular enzyme function [66]. The effect of food processing on flavonoid content and bioavailability in fruits and vegetables has also been studied by Ruiz-Cruz et al. [67]. Several techniques for extraction and analysis of flavonoids in foodstuffs and biological fluids have been reported, as well as their occurrence in foods and beverages [68]. Although most studies about the effects of polyphenols on CVDs are focused on flavonoids,

non-flavonoid polyphenolic compounds have also been shown to possess potential preventive effect [69].

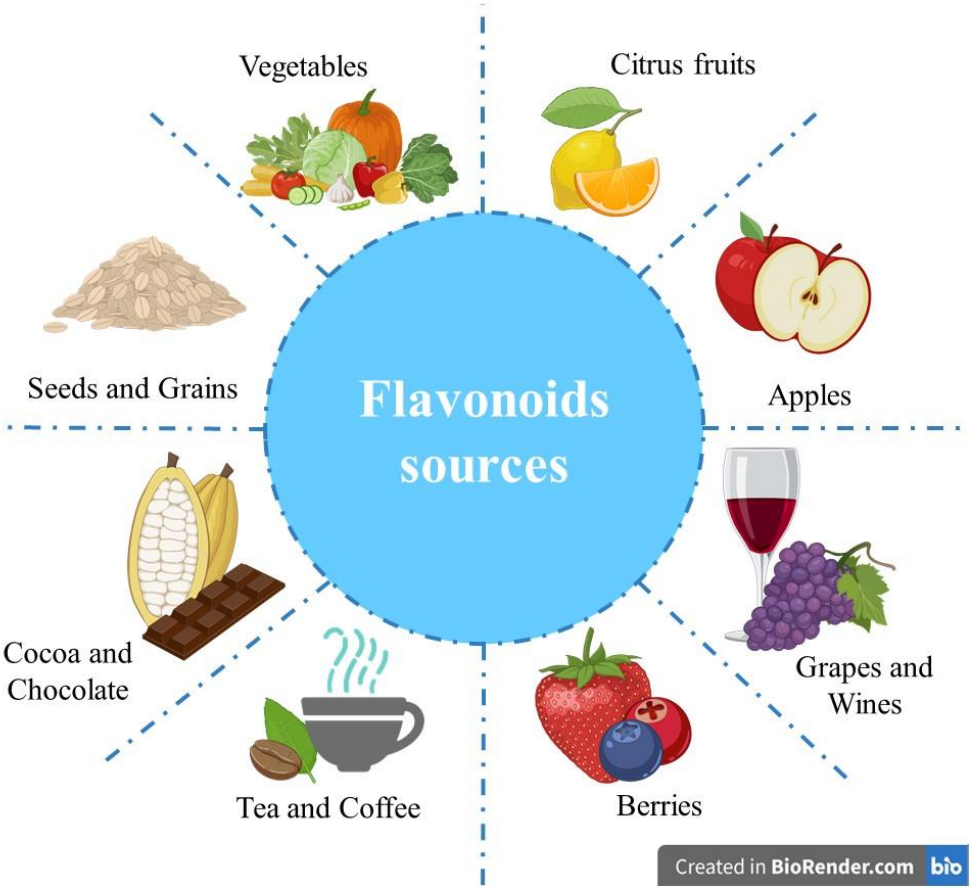


Figure 7. Important souces of flavonoids.

ii) Bioaccessibility and bioavailability of polyphenols

Polyphenol intake is important to maintain good health. However, the metabolism, transportation, and distribution to the target organs are a complex process not yet completely understood. The bioaccessibility and bioavailability of polyphenols in the gastrointestinal system are the key determinants of their absorption. In contrast to bioavailability, which refers to a substance's capacity to be digested and dispersed by the body, bioaccessibility refers to the amount of these compounds that are accessible for metabolic processes and can be modified by the interaction of polyphenols with dietary components [55]. Polyphenols often have poor bioavailability since their metabolism is influenced by a variety of parameters, including their solubility, chemical structure, degree of polymerization, and interactions with other molecules, among others. Phases I and II of the polyphenols metabolization, which occur in the cells of the liver and gut, respectively, may be split into two categories. Phase I of polyphenol

oxidation, reduction, and hydrolysis results in modifications to the amino, carboxyl, and hydroxyl groups that make up their structure; On the other hand, in phase II, the chemicals' toxicity is decreased, and they are eliminated by enzymatic processes (Figure 8) [55].

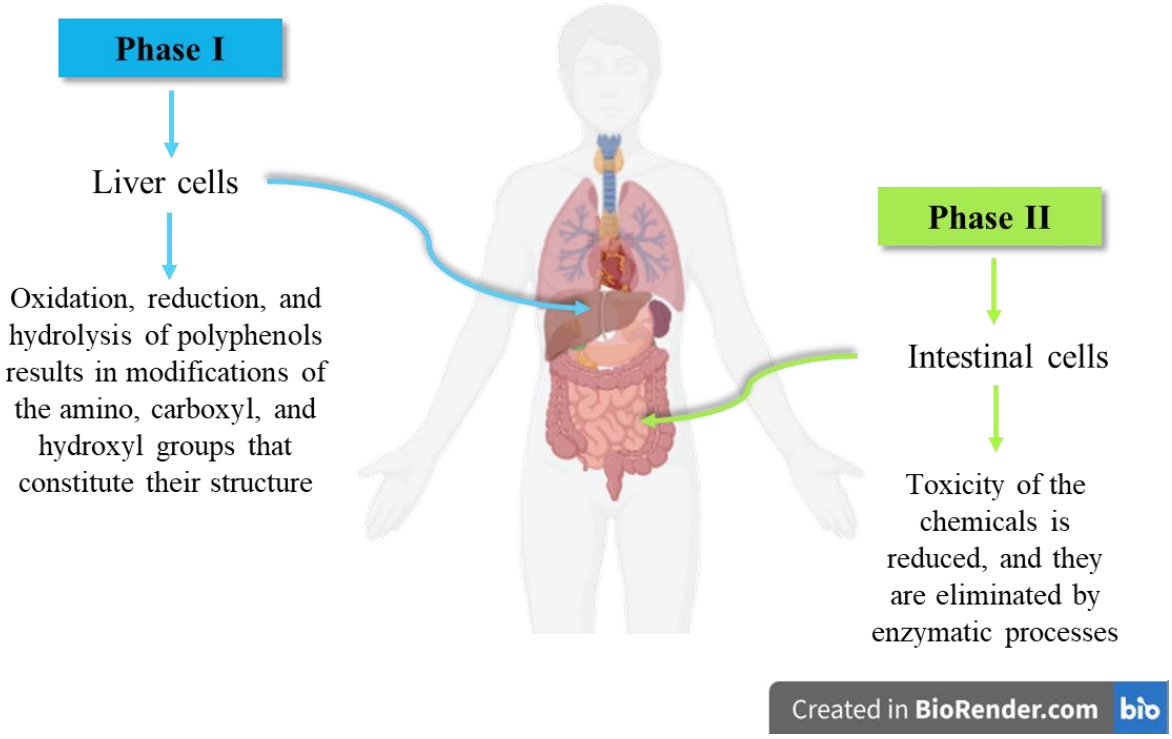


Figure 8. Bioaccessibility and bioavailability of polyphenols.

1.4. *Eugenia uniflora* L.

1.4.1. Origin, botany, morphology, and applications

Eugenia uniflora L. is an indigenous Brazilian plant of the *Myrtaceae* family. The family *Myrtaceae* is one of the major commercial fruit families in the world [70]. In addition to their ecological importance, representative species of the *Myrtaceae* family present great agro-industrial potential [71]. It is used in folk medicine to prevent and treat some symptoms related to hypertension, flu, fever, cough, and diarrhea [72]. The most studied species of *Eugenia* is *Eugenia uniflora* L. This name was given to the fruit by the Tupi Indians who inhabited the Brazilian region before the arrival of the Europeans [73].

The *Eugenia uniflora* L. (pitangueira) (Figure 9) is a tree with a dense crown that ranges in height from 2 to 9 m, has a rounded form, and a deep root system. It is predominantly found in South America [74]. Mature leaves of *Eugenia uniflora* L. are dark green, as opposed to the young leaves, which are brownish green in color and membranous in texture. After about a

year, blooms start to appear at the base of the branches. They consist of 4 to 8 hermaphrodite blooms with little smell and little to no nectar production. The flower has four free petals that have a creamy white tint [74]. It is a plant with good resistance to wind and cold, with moderate tolerance to waterlogging and drought, and poor tolerance to saline soils. The fruit from *Eugenia uniflora* L. is commonly referred to as the pitanga. Pitanga often feature berry-like shapes and 8–10 longitudinal furrows in their epidermis. Depending on the degree of ripeness the fruit can be green, yellow, orange, or purple. They have a distinctively sweet, acidic flavor with a strong scent. Pitanga can be eaten fresh or added to beverages, desserts, ice cream, juices, and jellies [74]. Along with syrups and wines with therapeutic properties, liqueurs are also made from fruit. Pitanga has furthermore been utilized by the cosmetic industry as a phytocosmetic to create shampoos, hair conditioners, soaps, and even fragrances [73].

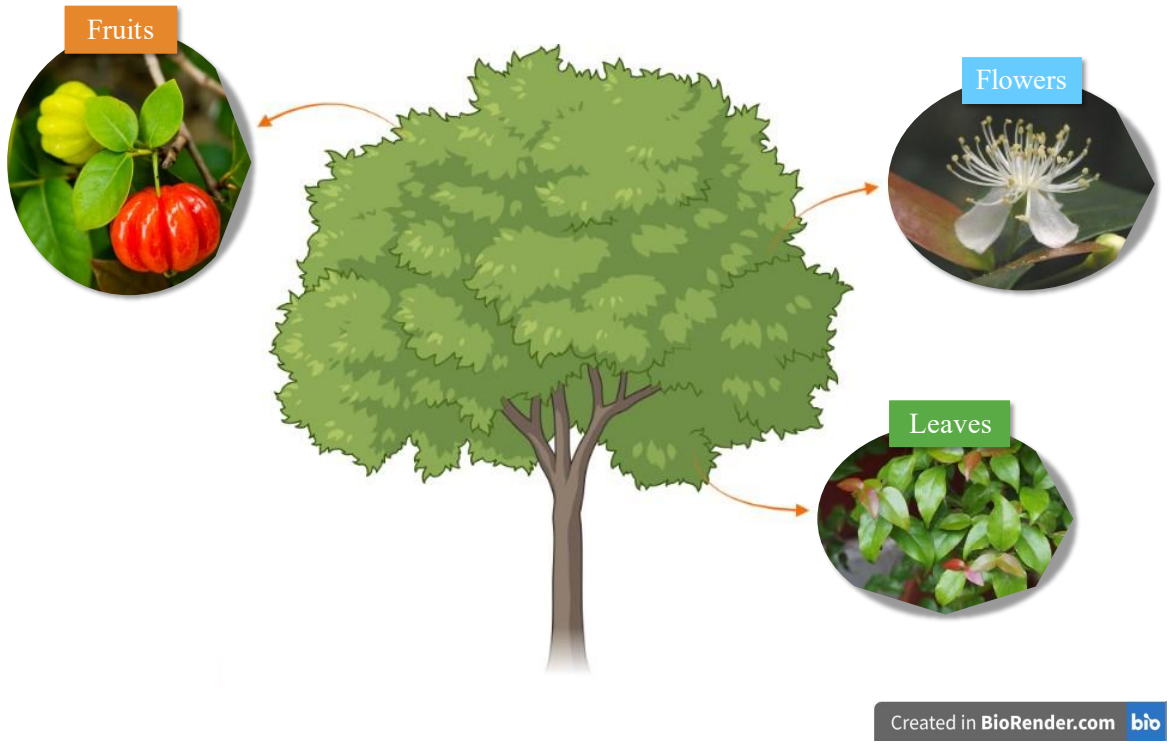


Figure 9. *Eugenia uniflora* L. fruits, flowers, and leaves.

1.4.2. Physical-chemical characterization of *Eugenia uniflora* L.

Anthraquinones, steroids, triterpenes, flavonoids, saponin heterosides, and tannins have all been shown to be present in *Eugenia uniflora* L. leaves [74]. According to Helt et al. [75], the pulp has few calories and is rich in calcium, phosphorus, iron, and vitamins B1, B2, and C. The sugar content ranges from 8.3 to 11.6 º Brix, with an average acidity of 1.8%, and a moisture content of approximately 80 %.

The purple pitanga is much less common than the orange variety, however present higher antioxidant levels [76]. Tambara et al. [76] reported the presence of five anthocyanins: delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, pelargonidin 3-O-glucoside, cyanindin 3-O-pentoside, and cyanidin derivative, in purple pitanga. In addition, its rind contains more anthocyanin flavonoids and carotenoids compared to the pulp [74]. Comparing the orange and purple varieties, the orange variety presents lower pH, acidity, carbohydrate levels, and phenolic content than the purple variety. The orange variety contains carotenoids, natural pigments synthesized during the photosynthetic process that give to fruit its typical color. Among carotenoids, some of them present an important activity on cancer and CVDs prevention. Gallic acid and derivatives, quercetin and derivatives, quercetin-3-β-D-glucoside, quercetin-3-rhammoside, kaempferol derivative, cyanidin-3-glucoside, cyanidin derivative, and malvidin derivative, were found in the ethanolic extract of the orange variety of pitanga [77]. In the yellow variety, the flavonoid profile is characterized by the presence of quercetin, myricetin, and their derivatives, with these compounds, particularly myricetin and quercetin, maintaining relatively consistent levels across maturation stages. However, the overall phenolic content undergoes significant changes as the plants mature [78]. Orange and purple pitanga contain higher levels of phenolic compounds, anthocyanins, and carotenoids than yellow pitanga, providing photoprotective properties that may help prevent diseases related to oxidative damage. Research on this type of pitanga, however, is limited due to its low carotenoid content [74,79].

In general, the orange variety of pitanga has the highest concentrations of carotenoids like β-carotene and lycopene as well as flavonols like myricetin, kaempferol, and quercetin. These BACs are also present in trace amounts in yellow pitanga. Due to the high levels of carotenoids and phenolic compounds, the purple variety frequently presents the highest antioxidant potential [75]. Pre-extraction steps (drying, grinding, pressing, etc.), the extraction method, and factors like the storage time that can affect pulp characteristics and result in a reduction in the amount of carotenoids need to be carefully considered as they affect the quantity and stability of the phytochemicals [80, 81].

1.4.3. Biological effects of *Eugenia uniflora* L. fruits and leaves

Eugenia uniflora L. medicinal properties are widely established. It has anti-inflammatory [82], antihypertensive, and antioxidant properties [74], which prevents lipid peroxidation and the formation of free radicals. Additionally, it possesses antiproliferative, antiviral, antifungal,

and antibacterial properties [74]. These properties have a great impact on the prevention of CVDs, cancer, and neurodegeneration diseases [74]. Recent research suggests that *Eugenia uniflora* L. is a viable natural source of phytochemicals used in the formulation of novel drugs that target the central nervous system (CNS) [83–85]. Some studies report the antihypertensive capacity of *Eugenia uniflora* L. [86,87], resulting in the reduction of ROS overproduction, normalization serum lipids (cholesterol, LDL, and HDL), regulation anti-inflammatory and anti-apoptosis processes that protect against myocardial injuries, inhibition ACE activity (overactivation of the RAAS causes cardiovascular dysfunction), and consequently lowers blood pressure [88–90]. The pharmacological basis of this practical usage has been investigated in several studies. Anconatani et al. [91] work reported that *Eugenia uniflora* L. has a hypotensive action that is mediated by a direct vasodilatory activity. A diuretic effect was also reported by the same authors, which may be brought on by an increase in renal blood flow [91].

1.5. Analytical techniques for the extraction and quantification of polyphenols

As previously mentioned, polyphenols play a significant role in the prevention of some diseases. It is therefore important to determine their nature and levels that occur in different samples. To accomplish this, several extraction and analytical techniques have been investigated [92–97].

Extraction techniques such as conventional solid-liquid extraction [92], pressurized liquid extraction (PLE) [93], and ultrasound-assisted extraction (UAE) coupled with the HPLC-UV method [94] have been reported for the extraction of polyphenols in different samples. In the investigation carried out by Lazzarotto-Figueiró et al. [95], UAE was used to extract polyphenols from plants of the *Myrtaceae* family, including *Eugenia* species. Another study by Bagatini et al. [93] investigated the extraction of polyphenols from *Eugenia uniflora* L. leaves using aqueous infusion and PLE [93]. An emerging technique for extracting polyphenols from *Eugenia uniflora* L. leaves is energised dispersive guided extraction (EDGE®), which was developed utilizing response surface methodology [96]. Some of these studies also used spectrophotometric tests to determine polyphenolic content and antioxidant potential, such as the Folin-Ciocalteau, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [92,93,95,96,98]. Silva et al. [97] employed the QuEChERS extraction technique, combined with dispersive solid-phase extraction (dSPE), to extract polyphenols from tropical fruits, including orange pitanga. The HPLC-DAD-ESI-MS^n

technique allowed them to identify myricetin arabinopyranoside and quercetin rhamnose, as most dominant compounds.

1.5.1. Extraction technique - µ-QuEChERS

QuEChERS is a high-throughput extraction method developed by Anastassiades et al. [99] in 2003 for the measurement of multi-residue pesticides in fruits and vegetables. Since then, it has been employed to extract different chemical compounds from a wide range of matrices. This method has recently been used to extract polyphenols and other BACs from foods. Furthermore, miniaturized quick, easy, cheap, effective, rugged, and safe (µ-QuEChERS), an updated version of the original technique, reduces the quantity of sample and organic solvents, making it more environmentally friendly. It is a two-phase technique.The first step involves the extraction of the target analytes using an organic solvent, with the addition of partitioning salts (e.g., anhydrous magnesium sulfate ($MgSO_4$), sodium chloride (NaCl)). Several organic solvents can be employed for extraction depending on the chemical nature of the target compounds. Acetonitrile (ACN) has been shown to successfully extract nonpolar and moderately polar analytes while also reducing the extraction of lipophilic material. However, as the organic phase may contain water-soluble residues, which can be eliminated by adding $MgSO_4$ (a drying agent) (Figure 10).

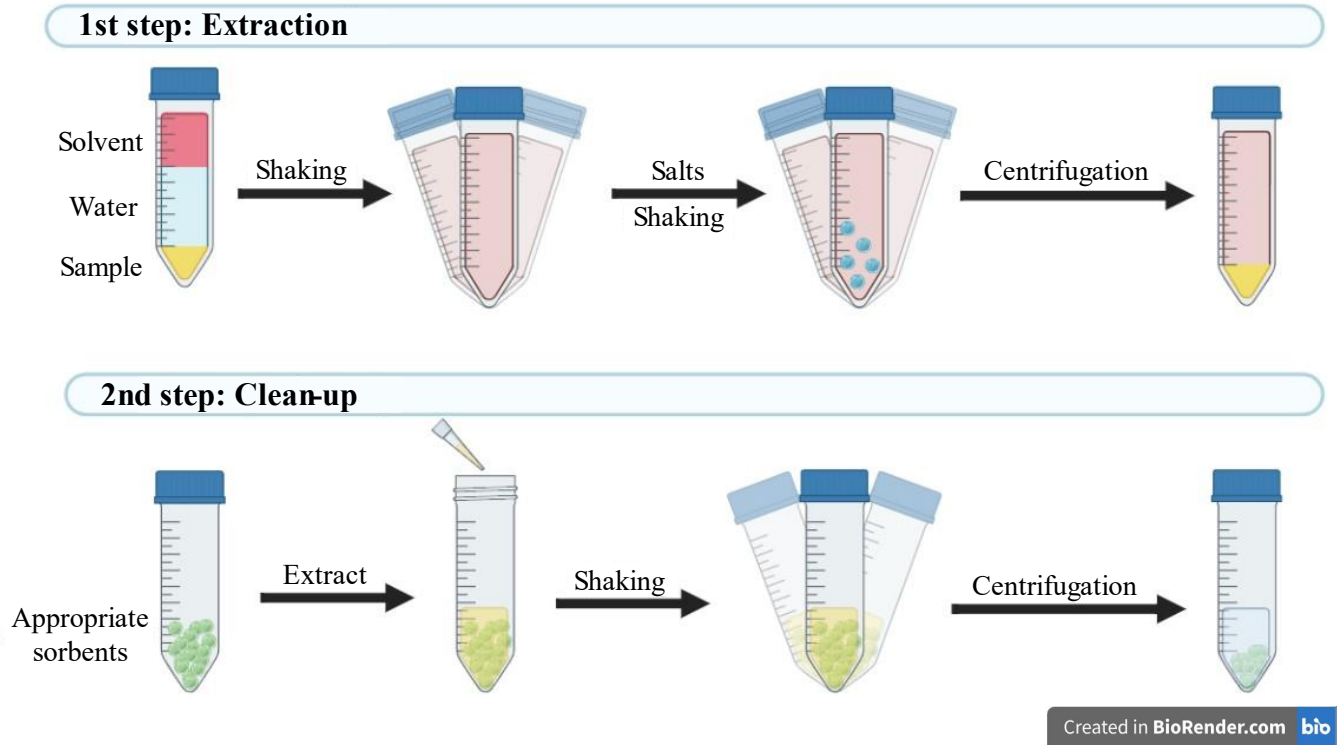


Figure 10. Schematic diagram of µ-QuEChERS extraction process.

Extraction and partitioning are typically facilitated by vortex homogenization and ultrasonication [100]. The second stage includes the clean-up stage, through a dSPE. The use of SPE in this stage seeks to remove unwanted matrix-coextracted components (e.g., organic acids, fatty acids, pigments, and sugars) as well as traces of water. The advantage of employing dSPE instead of traditional SPE is that it may be done in mass without needing arduous and time-consuming methods. The sorbents used in the clean-up stage are generally primary secondary amine (PSA), octadecylsilane (C18), graphitized carbon black (GCB), and $MgSO_4$. The sorbents employed in this step can be selected according to the complexity of the sample matrix and the nature of interferences to be removed [100,101]. The μ-QuEChERS extracts can be analysed by different analytical high-performance techniques such as high-performance liquid chromatography (HPLC), gas chromatography-mass spectroscopy (GC-MS), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR) [102].

1.5.2. Ultra high-performance liquid chromatography

Chromatography is a process for separating, identifying, and quantifying the constituents of a mixture. Chromatographic separation is based on the distribution of components between two phases: a porous bed, bulk liquid, or film phase that is generally fixed, known as the stationary phase, and a fluid or gaseous phase, also known as the mobile phase, which passes through the stationary phase and over all the system. The separation is determined by component properties such as adsorption, partition, affinity, and molecular weight differences. Certain components are interact more strongly in the stationary phase and take longer to be eluted, whilst others move quickly through the mobile phase [103].

Oliveira et al. [104] conducted research using ultra-high resolution mass spectrometry combined with ionization methods to analyse the chemical profile of plant extracts. In a study carried out by Assunção et al [105], HPLC equipped with an ultraviolet detection system was used to quantify specific compounds, such as gallic acid, ellagic acid, and catechin in ethanolic extracts of *Eugenia uniflora* L. fruits. In another investigation, *Eugenia uniflora* L. leaves were used to evaluate the performance of polyphenolic extraction by using solvent systems, different extractive conditions, and two distinct chromatographic techniques: HPLC and high-resolution thin-layer chromatography (HTLC) [106]. Souza et al. [107] also used an efficient HPLC approach on *Eugenia uniflora* L. leaves, utilizing bioethanol as a mobile phase as a sustainable and non-toxic solvent.

Regarding ultra-high-performance liquid chromatography (UHPLC) (Figure 11), it offers greater efficiency than HPLC. It employs columns with particle sizes smaller than 1.8 µm, providing a higher separation and resolution power than HPLC. In general, UHPLC is frequently used with mass spectrometry to analyze BACs [108]. This technique is known for its flexibility, excellent chemical durability, and wide range of application fields, which makes it a valuable tool for analyzing drugs, pharmaceutical formulations, natural products, among others [109].

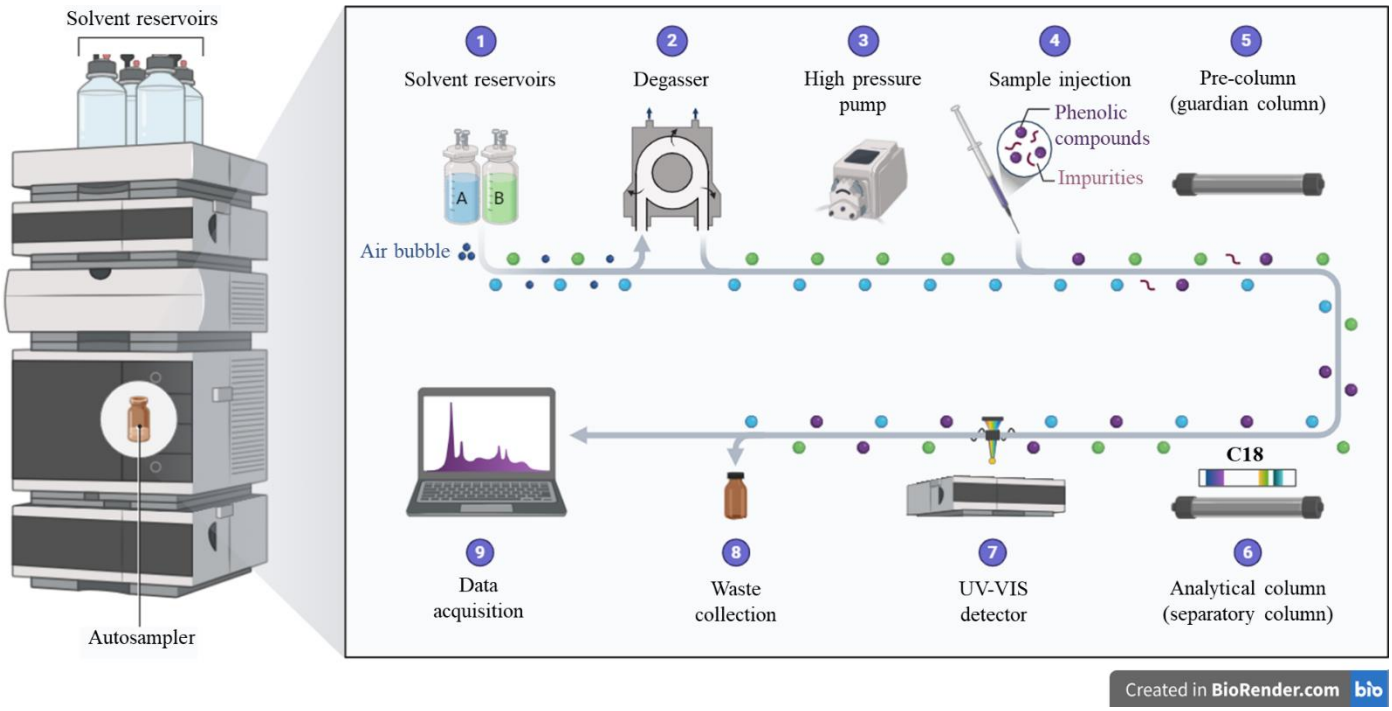


Figure 11. Different components of a UHPLC system.

1.6. Aims and scope

The main objective of this work is to deepen knowledge about the contribution of food on CVDs prevention, with a particular focus on *Eugenia uniflora* L. fruits, often referred to as "pitanga". The study will particularly compare the biological properties, namely the antioxidant, anti-inflammatory, and antihypertensive capacity of different varieties of pitanga (orange and purple) and leaves of *Eugenia uniflora* L. to evaluate their potential CVDs prevention. In addition, we aim to identify and quantify the polyphenols from the investigated matrices using the µ-QuEChERS/UHPLC-PDA methodology.

Chapter II

MATERIALS AND METHODS

2. Materials and methods

2.1. Reagents and standards

All used reagents and standards were of analytical grade. Potassium persulfate ($K_2S_2O_8$, 99.0%) and potassium phosphate dibasic trihydrate (K_2HPO_4•$3H_2O$) were acquired from Merck® (Buchs, Switzerland). Sodium chloride (NaCl, 99.5%), trisodium citrate dihydrate ($C_6H_9Na_3O_9$, 99.0%), potassium dihydrogen phosphate (KH_2PO_4, 99.5%), N-[3-(2-furyl)acryloyl]-Phe-Gly-Gly (FAPGG) and formic acid (FA, CH_2O_2, 98.0%) were acquired from Panreac Applichem (Barcelona, Spain). Aluminum chloride ($AlCl_3$), potassium chloride (KCl, 99.5%), ethyl acetate (EtAc, $C_4H_8O_2$, 99.7%), and quercetin ($C_{15}H_{10}O_7$•$2H_2O$, 99.1%) were supplied by Riedel-de Haën® (Seelze, Germany). 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, $C_{18}H_{24}N_6O_6S_4$, 98.0%), resveratrol ($C_{14}H_{12}O_3$, 99.0%), and kaempferol ($C_{15}H_{10}O_6$, 97.0%) were acquired from Sigma-Aldrich (Buchs, Switzerland). Sodium carbonate (Na_2CO_3, 99.7%) was supplied by Labsolve® (Lisbon, Portugal). Syringaldehyde was acquired from Acros Organics (Geel, Belgium). HPLC grade ACN (CH_3CN) and methanol (MeOH, CH_3OH) were acquired from Fisher Scientific (Loughborough, United Kingdom). Angiotensin-converting enzyme (ACE, from human, 95.0%), hydrochloric acid (HCl, 37.0%) and trisodium citrate dihydrate ($C_6H_5Na_3O_7$•$2H_2O$, 99%). The Folin-Ciocalteu solution, 2,2-diphenyl-1-picrylhydrazyl (DPPH, $C_{18}H_{12}N_5O_6$), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, $C_{14}H_{18}O_4$, 98.0%), gallic acid ($C_7H_6O_5$, 98.0%), and cinnamic acid ($C_9H_8O_2$, 99%) were supplied by Fluka (Munich, Germany). The 2 mL DisQuE™ dSPE tubes containing the sorbents (150 mg of $MgSO_4$ and 25 mg PSA) used in the μ-QuEChERS clean-up setup were obtained from Waters (Milford, MA, USA). Ultrapure water (H_2O) (18 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Burlington, MA, USA).

2.2. Samples and sample treatment

The *Eugenia uniflora* L. fruits, from orange and purple varieties, used in this study were purchased in Mercado dos Lavradores (Madeira Island, Portugal) at mature stage, as used for consumption. First, and after washing, the pitanga seeds were removed from the fruit. Then the fruits from orange and purple varieties of *Eugenia uniflora* L. as well as the leaves (Figure 12) were lyophilized, powdered, and stored in amber glass vials at room temperature until the extraction.


Figure 12. *Eugenia uniflora* L. fruits and leaves.

2.3. Extraction by µ-QuEChERS

The µ-QuEChERS extraction technique used for isolation of polyphenols from, *Eugenia uniflora* L. fruits and leaves, was based on the described by Casado et al. [110]. Briefly, 0.5 g of lyophilized sample was added to 0.4 g of the µ-QuEChERS buffer salts mixture in a ratio of 4:1:1:0.5 ($MgSO_4$, NaCl, $C_6H_9Na_3O_9$, $C_6H_9NaO_8$, respectively). Then 2 mL of an ACN:EtAc solution (1:1, v/v) containing 0.1% FA was added and the flask was vortexed. The mixture was then subjected to ultrasonic agitation for 5 min, in an ultrasonic bath, and centrifuged for 5 min at 5000 rpm. The supernatant (~1000 µL) was then transferred to a 2 mL DisQueTM dSPE clean-up tube with 150 mg of $MgSO_4$ and 25 mg of PSA. Everything was vortex before being centrifuged for 5 min at 4000 rpm. The extract was filtered using 0.22 µm PTFE syringe filters (BGB Analytik, VA, USA) into a vial and kept at -20 °C for analysis.

2.4. Total phenolic content

The total phenolic content (TPC) of *Eugenia uniflora* L. fruits and leaves extracts was determined using the Folin-Ciocalteu procedure described by Figueira et al. [111] with some modifications. Briefly, 3 mL of Folin-Ciocalteu solution (1:10 (v/v)) and 2.4 mL of 7.5% (w/v) Na_2CO_3 solution in ultrapure water were added to 0.6 mL of extract. The mixture was homogenized and incubated for 30 min in the dark and at room temperature (25 ± 1ºC). After the incubation, the absorbance was measured using a UV-Vis spectrophotometer (Lambda 25, Perkin Elmer, Waltham, MA, USA) at a wavelength of 765 nm. The results were expressed in mg of gallic acid equivalents (GAE)/100 g of dried weight (DW) after the recorded absorbance was interpolated into the calibration curve ($y = 0.0123x - 0.0298$, where y is the absorbance and x the concentration, $R^2 = 0.9918$) prepared with standard solutions with different

concentrations of gallic acid (15 to 76 mg/L). For each sample, the TPC was evaluated in triplicate.

2.5. Total flavonoid content

The total flavonoid content (TFC) was measured using the aluminum chloride ($AlCl_3$) colorimetric method described by Figueira et al. [111] with a few adjustments. 3 mL of 2% (w/v) of $AlCl_3$ solution in methanol was added to 3 mL of sample extract. This solution was homogenized in a vortex and left to react for 10 min in a dark environment at room temperature (25 ± 1ºC). After the incubation, the absorbance was measured using a UV-Vis spectrophotometer at 300 nm. The results were expressed in mg of quercetin equivalents (QE)/100 g DW after the recorded absorbance was interpolated into the calibration curve ($y = 0.0218x - 0.008$, where y is the absorbance and x the concentration, $R^2 = 0.9981$) prepared with standard solutions with different concentrations of quercetin (5 to 40 mg/L). The TFC was evaluated in triplicate for each sample.

2.6. Total anthocyanin content

The total anthocyanin content (TAC) was determined by the pH-differential method described by Sudarat et al. [112] with some modifications. Two dilutions of the extracts were prepared: ii) potassium chloride buffer, pH 1.0; ii) sodium acetate buffer, pH 4.5. In the case of the orange pitanga and the *Eugenia uniflora* L. leaves, 500 µL of extract was diluted in 2 mL of each of the buffers. For purple pitanga, 25 µL of extract in 2.5 mL of buffer. The solutions were left to equilibrate for 15 min. The absorbance of each dilution was measured at 510 and 700 nm against a blank (distilled water). These should be clean and free of sediment. However, some colloidal materials may be suspended in the sample which will cause light scattering which must be corrected by reading the absorbance at a wavelength where no absorption of the sample occurs (700 nm). The absorbance (A) of the diluted sample was calculated as follows: $A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$, where A510 is the absorbance used to quantify the concentration of anthocyanins in the sample and the absorbance at 700 nm to correct for possible interferences.

The concentration of monomeric anthocyanin pigments in the original sample is calculated using the following formula:

$$\text{Monomeric anthocyanin pigment (mg/L)} = \frac{(A \times MW \times DF \times 1000)}{(\varepsilon \times 1)},$$

and it was converted to mg of total anthocyanin content (TAC)/100 g DW. Where MW is the molecular weight, DF is the dilution factor, and ε is the molar absorptivity, calculate pigment content as cyanidin-3-glucoside, where MW = 449.2 g/mol and ε = 26900.

2.7. Determination of biological properties from *Eugenia uniflora* L. fruits and leaves

2.7.1. Antioxidant capacity

i) 2,2-Diphenyl-1-picrylhydrazyl scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) test was carried out following Figueira et al. [135], with slight changes, to determine the free radical scavenging capacity of the *Eugenia uniflora* L. fruits and leaves extracts under study. A stock solution of DPPH was prepared in methanol at a concentration of 0.015 g/L. Before the reaction, the stock solution was diluted to obtain a working solution with an absorbance of ~0.9. Then 0.1 mL of the extract was added to a reaction tube containing 3.9 mL of the DPPH working solution. The solution was homogenized and incubated for 45 min in the dark at room temperature (25 ± 1ºC). The absorbance was measured at a wavelength of 515 nm using a UV-Vis spectrophotometer. The free radical scavenging capacity to DPPH (AAR(DPPH)) was calculated using the following formula (calibration curve): $\ln(\%\Delta A515) = 1.3134 \times \ln(\mathrm{AAR(DPPH)}) - 4.287$ ($R^2 = 0.9881$), where $\%\Delta A515 = [(A515(0) - A515(45))/A515(0)] \times 100$, where A515(0) is the absorbance value measured at the start of the reaction and A515(45) is the absorbance value measured after 45 minutes of reaction. The calibration curve was obtained by carrying out the reaction procedure using standard solutions with different concentrations of Trolox (from 25 to 600 μg/mL), and the results were expressed as μM of Trolox equivalents (TE)/100 g DW. The antioxidant capacity against DPPH was assessed in triplicate for each sample.

ii) 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) was modified from the method published by Paixão et al. [113] to determine the antioxidant capacity *Eugenia uniflora* L. fruits and leaves extracts against the stable $ABTS^{\cdot+}$ radical cation. Briefly, a stock solution of ABTS (20 mM) was prepared in 50 mL of phosphate buffer saline (PBS, pH 7.4), and 200 μL of 70 mM potassium persulfate solution was added. The solution was stored in the dark at room temperature (25 ± 1°C) for 16 h. The ABTS solution was diluted with PBS until an absorbance value of ~0.9 was obtained. Then, 12 μL of the extracts were added to 3 mL of the diluted ABTS solution. The mixture was then homogenized and incubated for 20 min in the dark at room temperature. After this period, absorbance was measured at 734 nm using a UV-

Vis spectrophotometer. The free radical scavenging capacity against ABTS (AAR(ABTS)) was calculated using the following formula (calibration curve): $I = 0.0974 \times (AAR(ABTS) - 0.8523$ $(R^2 = 0.9992)$, where $I = [(AB - AA)/AB] \times 100$, with I being the percentage of inhibition of ABTS* and AB is the absorbance of a blank sample (t = 0 min) and AA being the absorbance after 20 min of adding the extracts. The calibration curve was obtained by performing the reaction procedure using standard solutions with different concentrations of Trolox (from 100 to 1500 µg/mL), and the results were expressed in µM TE/100 g DW. The antioxidant capacity against ABTS was evaluated in triplicate for each sample.

2.7.2. Antihypertensive capacity

The antihypertensive capacity was evaluated using the ACE- inhibition activity assay reported by Figueira et al. [111] with some modifications. Briefly, 50 µL of FAPGG (2 mM) was diluted in 450 µL of Tris-HCl buffer (50 mM, with 300 mM NaCl and 0.1 M HCl at pH 8.3). After vortex homogenization (1 min), 400 µL of water was added, then 50 µL of extract, followed by homogenization before adding 50 µL of ACE (0.5 U diluted from a 5 U stock solution in a potassium phosphate buffer – KH_2PO_4 9.3 mM and $K_2HPO_4 \cdot 3\ H_2O$ 0.7 M; with 300 mM NaCl at pH 8.3) and incubating for 3 min at 37 °C. Finally, the absorbance was measured every 2 min for 20 min at a wavelength of 328 nm. The inhibition rate was calculated using the following formula:

$$\%\ \text{Inhibition ACE} = \left(1 - \left(\frac{\text{Activity with inhibition}}{\text{Activity without inhibition}}\right)\right) \times 100,$$

where "Activity with inhibition" means the measurement of enzymatic activity in the presence of the inhibitor, and "Activity without inhibition" is the measurement of biological or enzymatic activity without the presence of the inhibitor.

2.7.3. Anti-inflamatory capacity

The anti-inflamatory capacity was evaluated by the the inhibitory capacity of the extracts relative to protein denaturation (inhibition of protein denaturation assay), according to Gunathilake et al. [114], with some modifications. Briefly, 100 µL of each extract was previously diluted in 4 mL of PBS (pH 6.4). Then 2 mL of 2% albumin was added to 2 mL of the diluted sample. The mixture was vortexed for a few seconds and incubated at 37 °C for 30 min. They were then incubated at 70 °C for 20 min and cooled. The absorbance was measured

at 660 nm using a UV-Vis spectrometer. The results are expressed as a percentage of inhibition of protein denaturation using the following formula:

$$\%\ \text{Inhibition of protein denaturation} = ((A_1 - A_2)/A_1) \times 100,$$

where A_1 represents the absorbance of the control (PBS, pH 6.4) and A_2 the absorbance of the extracts.

2.8. Conditions for the UHPLC-PDA analysis

A UHPLC system (Waters Ultra-High Performance Liquid Chromatography Acquity H-Class system) (Milford, MA, USA) equipped with a quaternary solvent manager (QSM), an Acquity sample manager (SM), a column heater, a degassing system, and a photodiode array detector (2996 PDA) was used for the chromatographic analysis of pitanga and *Eugenia uniflora* L. leaves. The column used to separate the analytes is an Acquity UPLC® CSH™ C18 analytical column (2.1 mm × 150 mm, 1.7 μm particle size) (Waters, Milford, MA, USA). The analytes of interest were separated chromatographically at a column temperature of 40 °C, using a mobile phase of acidified water (0.1% FA) (solvent A) and ACN (solvent B). The gradient conditions applied are described in Table 1. Each chromatographic run was followed by a 2 minutes re-equilibration time before the next injection. The injection volume was 5 μl and the sample manager compartment was kept at 20°C. The PDA data was registered at 280, 320, and 360 nm, according to the maximum wavelength of the analysed compounds. The Empower software 2.0 (Waters, Milford, MA, USA) was utilized to control the whole UHPLC system and collect data. The target analytes were identified by comparing the retention times and UV spectrum to those obtained for pure standards under identical experimental conditions. Each extract was analyzed in triplicate.

Table 1. Gradient conditions applied for the UHPLC-PDA analysis of polyphenols in *Eugenia uniflora* L. leaves and orange and purple pitanga.

Time (min)	Flow (mL/min)	%A	%B
0.00	0.250	80.0	20.0
3.00	0.250	60.0	40.0
6.00	0.250	55.0	45.0
7.00	0.250	30.0	70.0
7.50	0.250	20.0	80.0
8.00	0.250	80.0	20.0

2.9. Validation of the μ-QuEChERS/UHPLC-PDA methodology

To verify the method suitability for the determination of the target analytes in the investigated samples, the μ-QuEChERS/UHPLC-PDA methodology was validated in terms of selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision (intra-day and inter-day, expressed as percentage of relative standard deviation, %RSD), and accuracy (expressed as percentage of recovery, %Rec.).

2.9.1. Selectivity

The selectivity of an analytical technique refers to its ability to distinguish analytes in a complex mixture without interference from other components in the mixture [115]. The selectivity was determined by comparing the sample extracts to the standard solutions using the μ-QuEChERS/UHPLC-PDA technique. The absence of interferences in the retention time and wavelength of the target analytes demonstrates the selectivity of the proposed method.

2.9.2. Linearity

The linearity of a method is the ability of an analytical method to deliver findings that are linearly proportional to the concentration of an analyte within a certain range of concentrations. It is measured by how closely a calibration plot of response (typically chromatographic area of the peak) *versus* concentration approximates a straight line [116]. The linearity of the μ-QuEChERS/UHPLC-PDA technique was established by creating a calibration curve with seven points within the concentration range described in Table 3.

2.9.3. Limits of detection (LOD) and quantification (LOQ)

The limit of detection (LOD) is the lowest concentration of an analyte from which its identification in a sample may be determined. Similarly, the limit of quantification (LOQ) is the minimum concentration of an analyte that may be measured in a sample within a limit of confidence. The calculations for LOD and LOQ are similar, and one can be deduced from the other [117]. The LOD and LOQ of the μ-QuEChERS/UHPLC-PDA technique used in this study were obtained using the formulas: (3.3*Sb)/a and (10*Sb)/a, respectively, where *Sb* refers to the standard deviation of the ordinate at the origin, and *a* is the slope of the calibration curve.

2.9.4. Precision

Precision is a measure of the method's ability to provide repeatable results. Precision can be categorized as repeatability, intermediate precision, and reproducibility (interlaboratory precision), and it should be evaluated on homogeneous samples [118]. In this study, precision

was measured in terms of repeatability, which was achieved by completing a series of repeated analyses in a short period (intra-day), and intermediate precision, which was achieved by repeating the analyses on various, non-consecutive days (inter-days). Intra-day precision was evaluated through the analysis of two extractions in duplicate (n=6) for each spiking level, whereas inter-day precision was determined by analyzing three different extractions in triplicate (n=9) for each spiking level. The precision was given as the percentage of the relative standard deviation (%RSD).

2.9.5. Accuracy

The degree of agreement between the recognized real value or a reference value and the actual result achieved is defined as accuracy. Typically, accuracy is measured by evaluating the analyte recovery in the spiked sample [118]. In this study, the accuracy was assayed by spiking *Eugenia uniflora* L. orange fruits at three different concentration levels: low, medium, and high (Table 4). The following equation was used to compute the percentage of recovery (% Rec):

$$\%\text{Rec} = \left(\frac{[\text{Spiked sample}]-[\text{Sample}]}{[\text{Standard added}]}\right) \times 100,$$

where [Spiked sample] is the analyte concentration in the fortified sample, [Sample] is the analyte concentration in the non-fortified sample, and [Standard added] is the concentration of the analyte added to the sample.

2.10. Statistical analysis

MetaboAnalyst 6.0 was used for statistical analysis, which includes data pre-processing to normalization (data transformation utilizing data scaling by mean-center and cubic root). To identify significant differences between samples, the normalized data was analyzed using one-way ANOVA followed by Turkey's test for post hoc multiple comparisons of means.

Chapter III

RESULTS AND DISCUSSION

3. Results and discussion

This section presents the key results of our study on assessment of the biological properties of *Eugenia uniflora* L. fruits and leaves related with the prevention of CVDs. The findings are organized according to the main research questions outlined in Chapter II, and their implications are discussed in relation to existing literature.

3.1. Evaluation of total polyphenols, flavonoids, and anthocyanins from extracts of fruits and leaves of *Eugenia uniflora* L.

TPC was determined using the Folin-Ciocalteu method. The Folin-Ciocalteu reagent (phosphomolybdenum/phosphotungsten complex), which exhibits a yellow coloration, interacts with phenolic compounds for the determination of total polyphenol content by transferring electrons to the complex, resulting in a blue coloration [119]. The TPC results for the *Eugenia uniflora* L. leaves and orange and purple pitanga extracts are presented in Figure 13. The TPC values are 13.2 ± 0.01 mg (GAE)/100 g DW for the *Eugenia uniflora* L. leaves, 61.3 ± 0.03 mg (GAE)/100 g DW for the orange pitanga and 113.9 ± 0.01 mg (GAE)/100 g DW for the purple pitanga. These values may be explained by the presence of anthocyanins in the purple pitanga.

The TFC assay was carried out using the aluminum chloride colorimetric method. This method evaluates the approximate number of flavonoids in the sample. In this reaction, a complex is formed between the carbonyl and hydroxyl groups of the flavonoid and the aluminum ion (Al^{3+}), giving rise to a yellowish color; the more flavonoids in the matrix, the darker the solution [120]. As can be seen in Figure 13, the TFC values for the *Eugenia uniflora* L. leaves are 4.1 ± 0.01 mg (QE)/100 g DW, for the orange pitanga 20.9 ± 0.10 mg (QE)/100 g DW, and for the purple pitanga 30.0 ± 0.03 mg (QE)/100 g DW. Similar to the TPC, all of the samples showed statistically significant differences ($p < 0.05$) among them. In brief, the purple pitanga had higher values in both the TPC and TFC assays than the orange variety. The values obtained are slightly lower than those reported in the literature, which may be explained by the solvent used in the extraction procedure, and by the composition of pitangas which is influenced by geographic region of production, climatic conditions, maturation stage, and variety. In a study carried out by Rodrigues et al. [121], orange pitanga showed TPC values of 231 mg GAE/100 g for the fruit pulp. Other comparable results of 179.0 and 201.8 mg GAE/100 g of orange pitanga pulp were obtained by Jacques et al. [122] and Bagetti et al. [123], respectively. Orange pitanga was found to have a value of 107 mg GAE/100 g in another

investigation [124]. Chaves et al. [125] found highest levels of TPC (11 400 mg kg^{-1}) for purple pitanga.

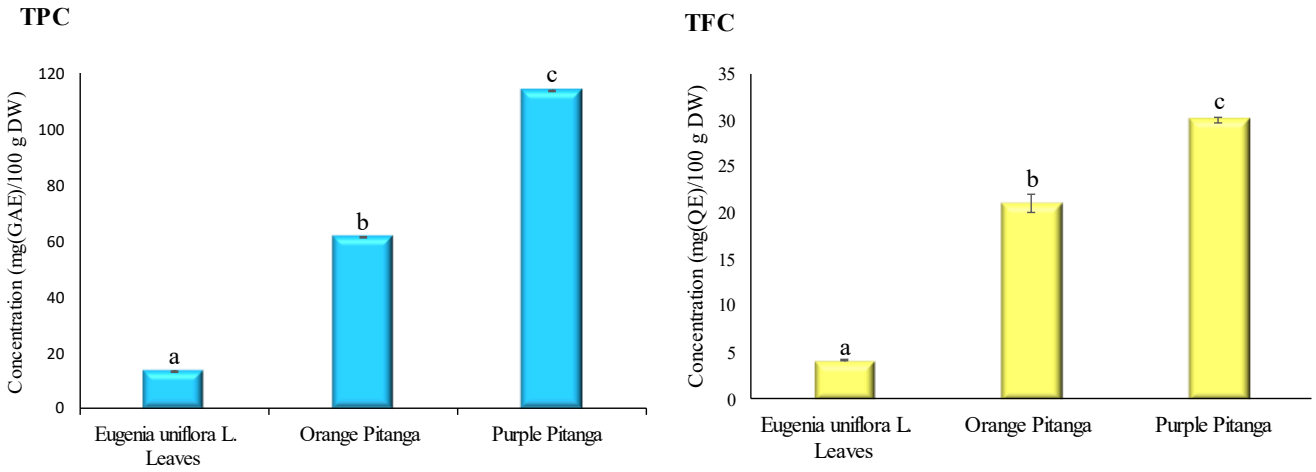


Figure 13. TPC and TFC values determined in orange and purple pitanga and *Eugenia uniflora* L. leaves extracts. Different superscript letters indicate statistically significant differences ($p < 0.05$) between the *Eugenia uniflora* L. leaves and pitanga (orange and purple varieties).

The TAC was determined by the pH-differential method described by Sudarat [112]. Anthocyanin's pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra. The colored oxonium form predominates at pH 1.0 and the colorless hemiketal form at pH 4.5. The differential pH method is based on this reaction and allow accurate and rapid measurement of the total anthocyanins, even in the presence of polymerized degraded pigments and other interfering compounds. Table 2 shows that the purple pitanga has an anthocyanin content eight times higher than that found in orange pitanga. In *Eugenia uniflora* L. leaves do not contain anthocyanins (n.d.). Also in the TAC test, the values are below those reported. Oliveira et al. [126], also determined the TAC in red pitanga having obtained a value significantly lower (1.72 ± 0.05 mg/g) than that obtained in this study (Table 2). Differences in variety and *terroir* may explain these differences.

Table 2. TAC of *Eugenia uniflora* L. leaves and orange and purple fruits.

***Eugenia uniflora* L.**	**Anthocyanins concentration (mg TA/100 g DW)**
Leaves	n.d.
Orange fruit	18.6 ± 0.2
Purple fruit	154.7 ± 0.3

3.2. Evaluation of the biological activities from extracts of fruits and leaves of *Eugenia uniflora* L.

3.2.1. Antioxidant Capacity

The antioxidant capacity of the investigated *Eugenia uniflora* L. samples (leaves, orange, and purple fruits) was determined using the DPPH and ABTS assays. These two assays use synthetic radicals that are not directly associated with food and biological systems and for this reason, objections are often raised to their use. However, they are commonly applied due to their simplicity, acceptable repeatability, and low cost. In addition, the literature shows that DPPH and ABTS are the synthetic radical tests most commonly used. The DPPH test is an overly sensitive technique for determining how well sample antioxidant defenses can scavenge the DPPH free radical. This approach is based on the donation of hydrogen atoms from the antioxidant to the radical and the transfer of electrons from the radical to the antioxidant. As it is decreased, its distinctive hue vanishes, making the solution lighter [127]. The obtained values for the DPPH assay, shown in Figure 14, are 3.4 ± 0.01 μM (TE)/g DW for the *Eugenia uniflora* L. leaves, 21.9 ± 0.01 μM (TE)/g DW for the orange pitanga, and 21.1 ± 0.01 μM (TE)/g DW for the purple pitanga. The statistical analysis showed that the DPPH scavenging capacity of the two varieties of fruits did not present statistically significant differences.

ABTS assay was also carried out to measure the antioxidant capacity of *Eugenia uniflora* L. leaves and fruits. The ABTS solution has a green coloration. Phenolic compounds reduce their free radicals, the greater the reduction of these radicals, the clearer the solution becomes, thus classifying the antioxidant capacity of the samples under analysis [128]. Figure 14 shows the obtained results for the *Eugenia uniflora* L. leaves (25.5 ± 1.4 μM (TE)/g DW), orange pitanga (278.5 ± 3.4 μM (TE)/g DW), and purple pitanga (257.4 ± 3.2 μM (TE)/g DW). The two varieties of fruits showed no statistically significant differences, in terms of the antioxidant capacity evaluated by the ABTS assay, however, the ABTS values obtained for the leaves are statistically different that those obtained for the orange and purple pitanga. All analysed samples showed values for the DPPH assay lower than those obtained from the ABTS assay. According to research conducted by Ferreira et al. [129], *Eugenia uniflora* L. leaves extracts showed values of 78.5 μM TE/g extract for the DPPH assay and 76.7 μM TE/g extract for the ABTS assay. Moreover, the study carried out by Figueira et al. [130] reported that pitanga seeds present a higher antioxidant capacity, based on DPPH and ABTS assays, than lemon

(*Citrus limon* var. *eureka*), tangerine (*Citrus reticulata* var. *setubalense*), tomato (*Solanum lycopersicum* var. *gordal*) and uva-da-serra (*Vaccinium padifolium* Sm.).

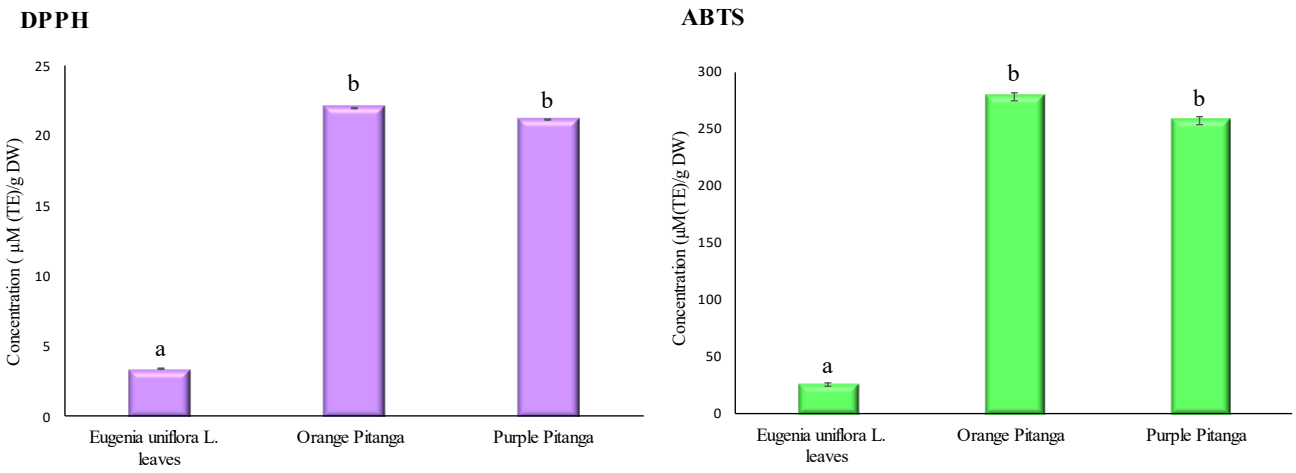


Figure 14. Antioxidant activity of *Eugenia uniflora* L. leaves and orange and purple pitanga extracts determined by DPPH and ABTS assays. Different superscript letters indicate statistically significant differences ($p < 0.05$) between the *Eugenia uniflora* L. leaves and pitanga (orange and purple varieties).

3.2.2. Antihypertensive capacity

The antihypertensive capacity was evaluated using the ACE activity inhibition assay (Figure 15). ACE is an enzyme that converts angiotensin I to angiotensin II which presents as a potent vasoconstrictor meaning that it narrows blood vessels and increases blood pressure. Purple and orange pitanga have an acceptable inhibition rate. The purple variety had a slightly higher inhibition rate than the orange variety, with 46.9 ± 0.1% and 42.6 ± 0.2%, respectively. In the case of *Eugenia uniflora* L. leaves, the inhibition rate lowers by half to around 25.2 ± 0.5%, indicating a decreased ability to block the ACE enzyme. In a study performed by Figueira et al.[130] the orange pitanga showed around 90% enzyme inhibition, a value sightly higher than that obtained in this study. The study carried out by Sensu et al. [131] on red barberries showed an inhibition of 73.8%. Das et al. [132] conducted a study evaluating ACE inhibition activity in various fruits. More than 75% ACE inhibition was shown by the aqueous fruit extracts of the red form of *Trapa bispinosa, Phoenix sylvestris, Cicca acida, Achras sapota,* and *Averrhoa carambola*. Conversely, nearly 50% inhibition was shown by *Ziziphus mauritiana*, *Spondias pinnata*, *Trapa bispinosa* (green), and *Punica granatum*. Low activity (< 50% inhibition) was demonstrated by *Aegle marmelos, Annona squamosa, Annona reticulata, Feronia elephantum, Physalis peruviana,* and *Syzygium jambos* [132].

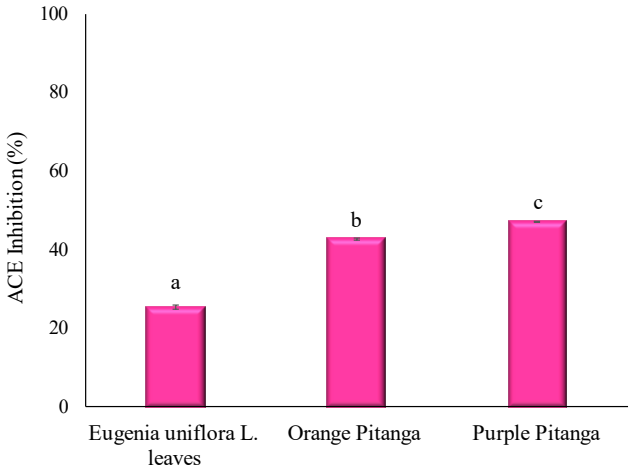


Figure 15. Antihypertensive capacity of *Eugenia uniflora* L. leaves and orange and purple pitanga extracts. Different superscript letters indicate statistically significant differences ($p < 0.05$) between the *Eugenia uniflora* L. leaves and pitanga (orange and purple varieties).

3.2.3. Anti-inflammatory capacity

The protein denaturation inhibition assay is used to measure the anti-inflammatory capacity (Figure 16) from leaves and purple and orange fruits of *Eugenia uniflora* L. Protein denaturation occurs when a protein loses its native structure and becomes unfolded, which can lead to loss of function. This assay is useful for identifying molecules that can stabilize proteins and prevent denaturation, which is important in various biological processes. This would suggest that the higher the percentage of inhibition of protein denaturation, the greater the anti-inflammatory capacity of the extracts. *Eugenia uniflora* L. leaves (25.7 ± 1.3%) had a lower inhibition percentage than the orange pitanga, which had the highest protein denaturation inhibition percentage (82.5 ± 1.5%), similar to the purple pitanga (81.7 ± 1.2%). In this way, both varieties of pitanga have a good ability to inhibit protein denaturation, which leads to a greater anti-inflammatory power having the capacity to prevent diseases. Gomathi et al. [133] carried out an anti-inflammatory assay to evaluate the raspberry extracts on prevention of diseases driven by inflammatory processes. The obtained results showed that raspberry that raspberry extracts have a protein denaturation inhibition of 77%. The study of Lehfa et al. [134] evaluated the anti-inflammatory capacity of *Arbutus unedo* L., an evergreen plant belonging to the *Ericaceae* family, having observed a percentage of protein denaturation inhibition of 70.1 ± 0.7%.

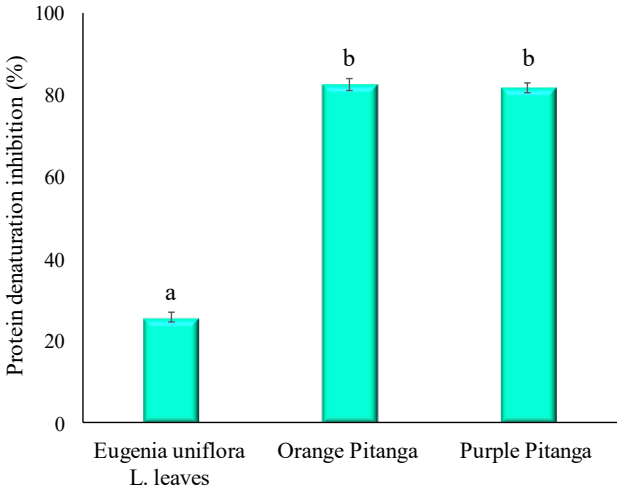
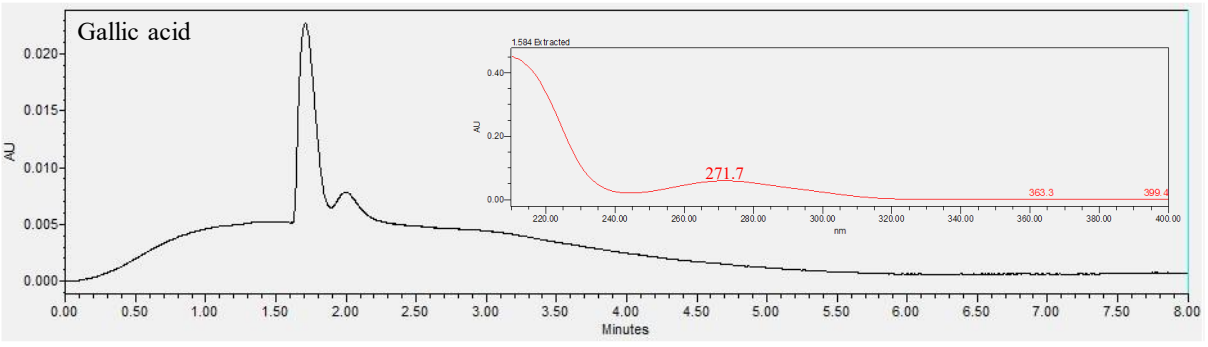


Figure 16. Anti-inflammatory capacity of *Eugenia uniflora* L. leaves and orange and purple pitanga extracts. Different superscript letters indicate significant differences ($p < 0.05$) between the *Eugenia uniflora* L. leaves and pitanga (orange and purple varieties).

3.3. Validation of the µ-QuEChERS/UHPLC-PDA methodology for polyphenols analysis

To determine the suitability of the µ-QuEChERS/UHPLC-PDA methodology for measuring polyphenols in pitanga and *Eugenia uniflora* L. leaves. The figures of merit of the method: selectivity, linearity, LOD, LOQ, precision, and accuracy, were determined.

The selectivity was evaluated by comparing the chromatograms and PDA spectra obtained for the target *Eugenia uniflora* L. extracts to those of analytical standards. The used standards, previously selected based on literature, were gallic acid, syringaldehyde, resveratrol, quercetin, cinnamic acid, and kaempferol. The chromatograms obtained from the individual standards used to identify polyphenols are shown in Figure 17. There were interferences in the retention times at which the analytes of interest are eluted, demonstrating that the approach is selective and allows for the identification of these analytes in the target *Eugenia uniflora* L. samples.



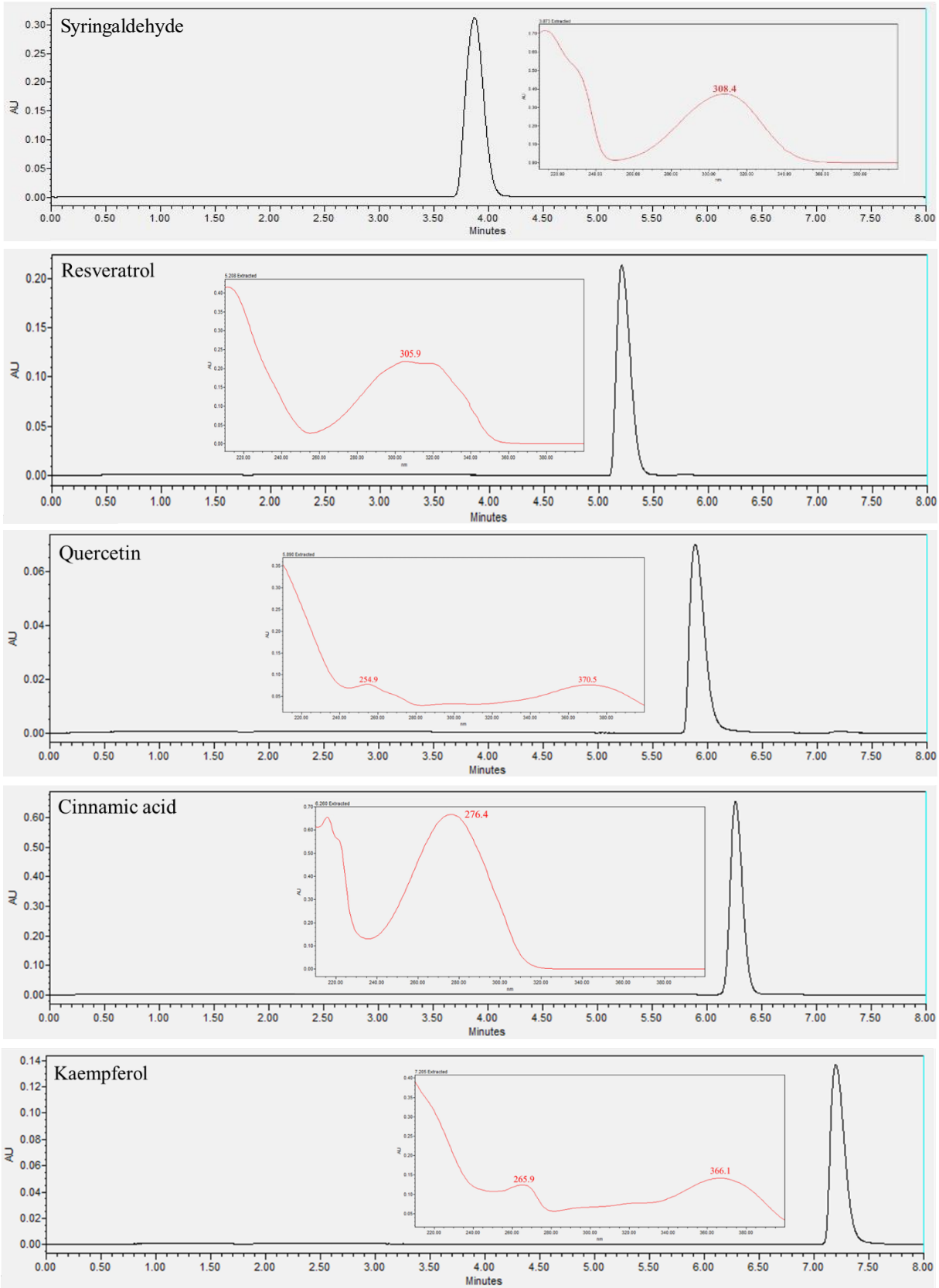


Figure 17. Chromatograms obtained for the individual standard solutions of polyphenols and PDA spectra acquired for each compound used for the identification of the compounds in *Eugenia uniflora* L. leaves and orange e purple pitanga (acquired at 280 nm for gallic acid and cinnamic acid, 320 nm for syringaldehyde and resveratrol, and 360 nm for quercetin and kaempferol).

Furthermore, the performance of the method to provide results directly proportional to the concentration of the analytes was assessed. The calibration curves were generated by using a standard solution with different concentrations of gallic acid (5 to 400 mg/L), syringaldehyde (1 to 40 mg/L), resveratrol (3 to 50 mg/L), quercetin (2 to 20 mg/L), cinnamic acid (6 to 400 mg/L), and kaempferol (4 to 50 mg/L). Table 3 shows the obtained results. Linearity was observed for all the compounds, within the concentration range studied. The coefficients of determination were greater than 0.994, indicating an acceptable fit of the obtained value to the calibration curve.

Table 3. Figures of merit of the analytical methodology µ-QuEChERS/UHPLC-PDA linearity, and limits of detection and quantification.

RT (min)	Analyte	λ_{max} (nm)	studied range (mg/L)	Calibration curve		LOD (mg/L)	LOQ (mg/L)
				Equation	R^2		
1.60	Gallic acid	280	5 - 400	y = 2014× + 5948.2	0.995	0.139	0.421
3.86	Syringaldehyde	320	1 - 40	y = 11098× – 8122.6	0.994	0.022	0.067
5.21	Resveratrol	320	3 - 50	y = 25342× – 9861.2	0.997	0.011	0.033
5.92	Quercetin	360	2 - 20	y = 4333.3× + 3181.8	0.994	0.068	0.206
6.23	Cinnamic acid	280	6 - 400	y = 36651× + 2090.3	0.997	0.012	0.037
7.21	Kaempferol	360	4 - 50	y = 13711× + 12969	0.995	0.022	0.066

RT – Retention time; LOD – Limit of detection; LOQ – Limit of quantification.

The LOD and LOQ values represent the lowest concentrations at which analytes can be identified and quantified in the sample, respectively. The LOD and LOQ values calculated for each compound are shown in Table 3. The obtained LODs ranging from 0.011 mg/L (resveratrol) to 0.139 mg/L (gallic acid), while the LOQ values ranged between 0.033 mg/L (resveratrol) and 0.421 mg/L (gallic acid). Similar results were observed in previous works [135], suggesting the proposed methodology is acceptable for the quantification of small amounts of these polyphenols in *Eugenia uniflora* L. samples.

The precision and accuracy of the µ-QuEChERS/UHPLC-PDA method were also verified by spiking orange pitanga at three different concentration levels (Table 4). Precision was employed to verify the method's capacity to generate reproducible results. It was evaluated in terms of repeatability (intra-day), in which analyses were done on the same day again, and intermediate precision (inter-day), in which analyses were undertaken on several days. The obtained data were expressed as a percentage of relative standard deviation (RSD%). The

results obtained for repeatability and intermediate precision were less than 20%, showing that the methodological approach performed well on these parameters. The accuracy results were represented as a percentage of recovery (Rec%). The procedure is increasingly accurate as the value approaches 100%. Analytical methods typically have an acceptable limit of ± 25% accuracy [136]. The accuracy values for the analytes employed in this study (Table 4) varied from 75% (syringaldehyde) to 117% (quercetin). The validation results indicate that the µ-QuEChERS/UHPLC-PDA methodology is a useful analytical approach to extract and analyse the target polyphenols from *Eugenia uniflora* L. fruit varieties and leaves.

Table 4. Results obtained for the precision and accuracy of the µ-QuEChERS/UHPLC-PDA method.

Analyte	Spiking level (mg/L)	Precision (RSD%)		Accuracy (Rec%)
		Intra-day	Inter-day	
Gallic acid	5	4.2	19.4	81.3 ± 3.8
	100	0.9	4.1	92.9 ± 5.6
	400	1.7	3.3	91.3 ± 0.6
Syringaldehyde	5	0.7	16.6	75.0 ± 2.4
	25	2.5	6.9	99.9 ± 3.9
	40	3.2	5.6	99.1 ± 5.6
Resveratrol	3	0.3	14.4	110.6 ± 4.7
	20	0.4	1.2	93.8 ± 2.4
	50	2.3	2.4	94.6 ± 0.7
Quercetin	4	2.1	8.2	117.0 ± 7.7
	10	1.3	3.8	101.1 ± 8.5
	20	3.7	3.7	111.0 ± 1.9
Cinnamic acid	6	0.4	9.1	92.5 ± 0.9
	100	0.3	0.3	90.5 ± 2.9
	400	0.3	1.7	96.0 ± 0.3
Kaempferol	4	0.7	9.6	107.0 ± 3.9
	25	0.9	1.9	98.3 ± 1.4
	50	3.0	3.4	98.8 ± 5.2

3.4. Analysis of polyphenols from *Eugenia uniflora* L. fruits and leaves by µ-QuEChERS/UHPLC-PDA method

The *Eugenia uniflora* L. samples (orange and purple fruits, and leaves) were analyzed (extracted, identified, and quantified) using the µ-QuEChERS/UHPLC-PDA methodology, which has been demonstrated to be effective for this purpose. The UHPLC-PDA chromatograms obtained for each sample are represented in Figure 18.

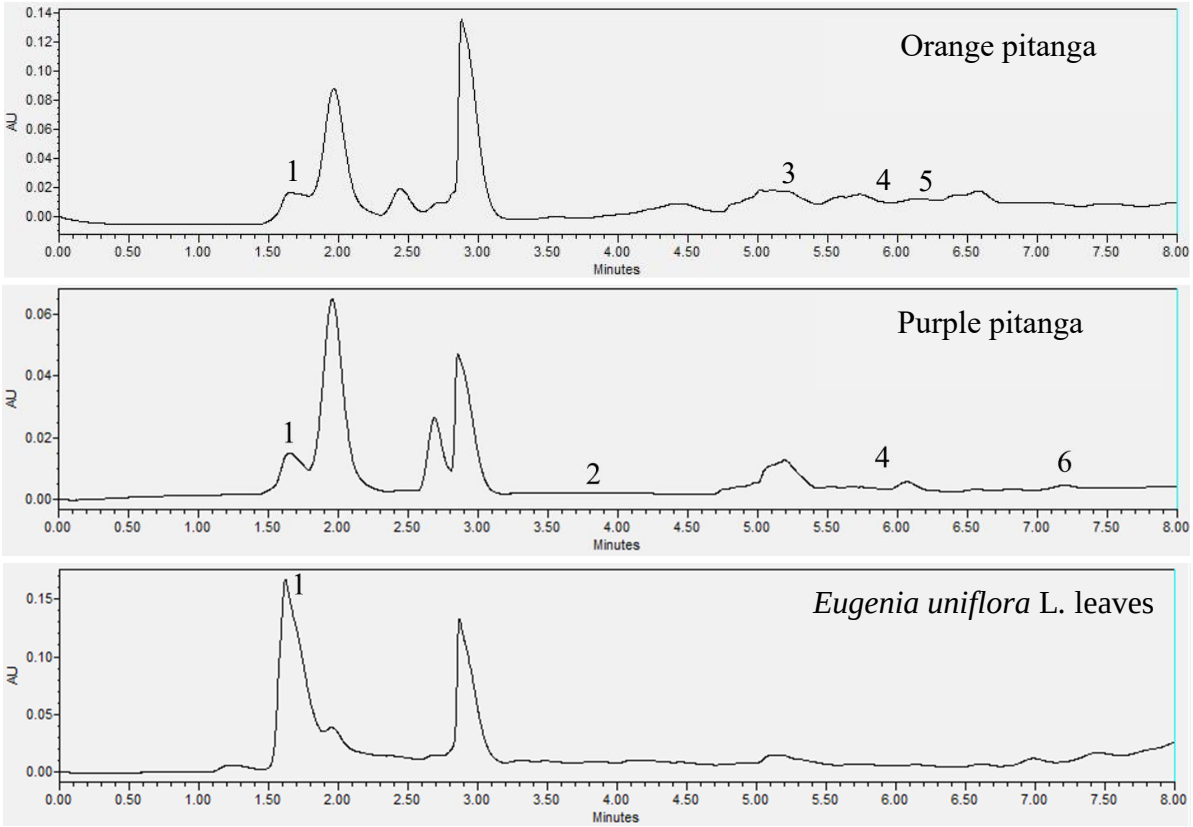


Figure 18. Chromatograms obtained for the *Eugenia uniflora* L. leaves and orange and purple pitanga extracts, acquired at 280 nm. Peak number 1: Gallic acid; 2: Syringaldehyde; 3: Resveratrol; 4: Quercetin; 5: Cinnamic acid; 6: Kaempferol.

Table 5 shows the concentrations of each polyphenol identified in the samples. The chromatographic areas for the polyphenols were obtained using the maximum wavelength of each compound: 280 nm for gallic acid and cinnamic acid, 320 nm for syringaldehyde and resveratrol, and 360 nm for quercetin and kaempferol.

Table 5. Results obtained for the identification and quantification of polyphenols in *Eugenia uniflora* L. leaves and orange and purple fruits through µ-QuEChERS/UHPLC-PDA.

RT (min)	Analyte	λmax (nm)	Concentration (mg/100 g DW) ± SD		
			Orange fruit	Purple fruit	*Leaves*
1.70	Gallic acid	280	13.13 ± 0.51	7.19 ± 0.51	2.78 ± 0.04
3.86	Syringaldehyde	320	-	0.09 ± 0.002	-
5.21	Resveratrol	320	1.72 ± 0.003	-	-
5.90	Quercetin	360	2.22 ± 0.03	0.27 ± 0.02	-
6.23	Cinnamic acid	280	0.26 ± 0.19	-	-
7.20	Kaempferol	360	-	0.63 ± 0.41	-

RT – Retention time; SD - Standard deviation.

As can be seen in Figure 18 and Table 5, the polyphenols profiles change depending on the variety of pitanga. In orange pitanga, it was possible to identify gallic acid (13.1 ± 0.5 mg/100 g DW), resveratrol (1.7 ± 0.003 mg/100 g DW), quercetin (2.2 ± 0.03 mg/100 g DW), and cinnamic acid (0.3 ± 0.2 mg/100 g DW).

Gallic acid (7.2 ± 0.5 mg/100 g DW), and quercetin (0.3 ± 0.02 mg/100 g DW) were also identified in the purple pitanga, but two other polyphenols, syringaldehyde (0.09 ± 0.002 mg/100 g DW), and kaempferol (0.6 ± 0.4 mg/100 g DW) were identified in this variety. In the case of the *Eugenia uniflora* L. leaves, only gallic acid (2.8 ± 0.04 mg/100 g DW) was identified. In a study carried out by Schumacher et al. [92], gallic acid was quantified in *Eugenia uniflora* L. by HPLC at 210 nm having obtained different concentrations according to the used solvent as follows: 6402 ± 222.7 mg/L for aqueous extract, 7361 ± 115.7 mg/L for ethanol extract; and 2677 ± 32.2 mg/L for methanol/acetone extract. Migueis et al. [98], identified 12 compounds such as cyanidin-3-hexoside, myricetin-hexoside 1, and quercetin rhammoside through HPLC-DAD-ESI/MS. The different results found can be explained by the fact that different solvents and different analysis methods were applied.

3.4.1. Properties of polyphenols identified in the *Eugenia uniflora* L. fuits and leaves

The results show that orange and purple pitanga and *Eugenia uniflora* L. leaves contain gallic acid. This is a compound present in several natural sources. It is considered a valuable compound for human health and also industrially due to its antioxidant potential. It is widely used as a UV radiation protector, an astringent in cosmetics, and a food preservative [137].

Concerning the influence of this compound on the cardiovascular system, some works have shown that gallic acid can have protective effects against cardiotoxicity and arrhythmias [138,139].

In orange pitanga it was possible to identify resveratrol, quercetin and cinnamic acid. Resveratrol has a wide range of health benefits, which explains the extensive *in vivo* and *in vitro* studies. It presents antioxidant and anti-inflammatory properties, helping in protection against oxidative stress and inflammation [140,141], antiglycation properties, by inhibiting the formation of advanced glycation end products, which are associated with aging and diabetic complications [142], antimicrobial properties against a diverse range of bacteria, viruses, and fungi [143,144], in addition, anticancer properties [141]. Moreover, resveratrol has been studied for its potential in the prevention of various CVDs, including atherosclerosis, hypertension, stroke, myocardial infarction, and heart failure [145–147].

Quercetin is a flavonoid that occurs naturally in fruits, vegetables, and juices [148,149]. It has several remarkable properties such as antioxidant and anti-inflammatory properties, acting as a free radical scavenger and inhibiting lipid peroxidation [149–151], in addition to anticancer and neuroprotective properties [149,152]. Quercitin has also been associated with cardiovascular health through the decrease of blood pressure, and cholesterol levels, and improving endothelial function [153,154].

Another polyphenol identified in orange pitanga was cinnamic acid, which also has some biological properties, such as anti-inflammatory, antimicrobial, anticancer, and antidiabetic properties [155,156]. Cinnamic acid also has hepatoprotective and neuroprotective properties [155]. Cinnamic acid has been shown to have cardioprotective effects, particularly in diabetic cardiomyopathy, and can help improve heart function, reduce inflammation, and alleviate dyslipidemia [156].

Syringaldehyde and kaempferol were identified in purple pitanga. Syringaldehyde is a natural aromatic aldehyde that has antifungal, antibacterial, and anticancer properties, making it a promising compound for several therapeutic applications [157,158]. It has also been shown to exert neuroprotective effects, reducing cell damage, increasing antioxidant activity, and inhibiting apoptosis [157]. Kaempferol is associated in different reports to antioxidant and anti-inflammatory properties, helping protect against oxidative stress and inflammation [159,160]. This compound has been shown to have anticancer activity against various types of cancer, as well as neuroprotective effects [161]. It acts also on cardiovascular health as it is a potential free radical scavenger and inhibits lipid peroxidation, helping to reduce the risk of CVD [162].

Chapter IV

CONCLUSIONS

4. Conclusions

The TPC, TFC, and TAC of the investigated samples, determined by UV-Vis showed that the maximum levels were obtained for the purple pitanga (113.7 ± 0.1 mg (GAE)/100 g DW, 30.0 ± 0.3 mg (QE)/100 g DW, and 154.7 ± 0.3 mg TA/100 g DW, respectively). The extracts of pitanga and *Eugenia uniflora* L. leaves had a higher scavenging capacity for the ABTS radical compared to DPPH, with the highest value recorded for ABTS (278.5 ± 3.4 μM (TE)/g DW) for the orange pitanga. The values obtained may be because the phenolic compounds present in the samples have a greater scavenging capacity against ABTS than the DPPH radical. In the ACE inhibition assay, the purple pitanga showed an ability to inhibit this enzyme of 46.9 ± 0.1%, which is a good indicator of its antihypertensive capacity. In the protein denaturation inhibition assay, both varieties showed optimum inhibition of around 82%, indicating good anti-inflammatory capacity.

The validation results indicate that the μ-QuEChERS/UHPLC-PDA methodology effectively extracts and quantifies polyphenols in *Eugenia uniflora* L. fruit varieties and leaves. There were interferences in the retention times at which the analytes of interest are eluted, demonstrating that the approach is selective. Linearity was also observed for all the compounds within the concentration range studied. The coefficients of determination were greater than 0.994, indicating an acceptable fit of the value obtained to the calibration curve. The LODs obtained ranged from 0.011 mg/L (resveratrol) to 0.139 mg/L (gallic acid), while the LOQ values ranged from 0.033 mg/L (resveratrol) to 0.421 mg/L (gallic acid). The results for repeatability and intermediate precision were less than 20%, showing that the approach performed well in these parameters. The accuracy values for the analytes used in this study ranged from 75% (syringaldehyde) to 117% (quercetin), which is within acceptable limits.

Six polyphenols (gallic acid, syringaldehyde, resveratrol, quercetin, cinnamic acid, and kaempferol) were identified in the investigated samples using the μ-QuEChERS/UHPLC-PDA methodology. Across all the samples, gallic acid was the most prevalent component, with the orange pitanga having the greatest content (13.1 ± 0.5 mg/100 g DW). These polyphenols have been acknowledged in the literature for their interesting biological activities, consequently, they provide numerous uses in the pharmaceutical, and food sectors, among others.

The findings of this study shed valuable light on the chemical composition of both orange and purple varieties of *Eugenia uniflora* L., as well as its leaves. The samples demonstrated anti-inflammatory and antihypertensive properties, suggesting potential in the prevention of

CVDs. These promising results underscore the need for further investigation into the phenolic composition of *Eugenia uniflora* L. to deepen our understanding of its health benefits.

4.1. Future work

Based on the obtained results we can propose some activities to deepen and enlarge in a comprehensive way the scientific knowledge behind the *Eugenia uniflora* L. fruits and leaves. In this context, the next steps could be:

- Isolate polyphenols from *Eugenia uniflora* L. varieties.
- Explore the biological properties of polyphenols from *Eugenia uniflora* L. to identify potential industrial applications.
- Carry out an epidemiological study to understand the effect of the consumption of pitanga juice and tea from *Eugenia uniflora* L. leaves on CVDs patients.

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