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Department of Life Sciences

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Laboratory of Plant Molecular Biology and Biotechnology

Potential health promoting effects of fruit and vegetable juices: phenolic compounds, antioxidant capacity and endothelium function.

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List of abbreviations:

12-HHT	Hydroxyheptadecatrienoic acid
AA	Amino acids
AMPK/Klf2	5' adenosine monophosphate-activated protein kinase/ Krüppel-like Factor 2
Ang II	Angiostein 2
C	Catechin
CAT	Catalase
CBDL	Chronic bile duct ligation
COX	Cyclo-oxygenase
CVD	Cardiovascular diseases
Cy	Cyanidin
CyG	Cyanidin -3-*O*-glucoside
CyR	Cyanidin -3-*O*-rutinoside
D	Delphinidin
DG	Delphinidin -3-*O*-glucoside
DR	Delphinidin -3-*O*-rutinoside
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Epicatechin
ECG	Epicatechin gallate
EDH	Endothelium-derived hyperpolarization
EETs	Epoxyeicosatrienoic acids
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
eNOS	Endotelial Nitric Oxide Synthase
Erα	Alpha isoform of the estrogen receptor
ET-1	Endothelin 1
ETC	Electron Transfer Chamber

FR	Free radicals
G6P	Glucose 6 Phosphate
G6PDH	Glucose 6 Phosphate dehydrogenase
GAE	Gallic Acid Equivalent
GC	Gallocatechin
GSH	Reduced Glutathione
GSpx	Glutathione Peroxidase
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
HAT	Hydrogen atom transfer
HDL	High Density Lipoprotein
iNOS	Inducible Nitric Oxide Synthase
Keap1	Kelch-like ECH-associated protein 1
LDL	Low Density Lipoprotein
LOOHs	Lipid hydroperoxides
M	Malvidin
MG	Malvidin -3-*O*-glucoside
MPO	Myeloperoxidase
nNOS	Neural Nitric Oxide Synthase
NOS	Nitric Oxide Synthase
NOX	NADPH-oxidase
NPC1L1	Niemann–Pick C1-like 1
Nrf2	Nuclear reactor factor 2
ORAC	Oxygen Radical Absorbance Capacity
Pel	Pelargonidin
Peo	Peonidin
Pet	Petunidin
PG	Peonidin -3-*O*-glucoside

PGI2	Prostaglandin 2
PMFs	Polymethoxylated flavonoids
PR	Peonidin -3-*O*-rutinoside
Quer	Quercetin
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RS	Reactive Species
SET	Single Electron Transfer
SGLT1	Sodium glucose transporter proteins
Sirt1	Sirtuin 1
SOD	Superoxide Dismutase
Src/Pi-3Kinase/Akt	Src-mediated phosphatidylinositol-3-kinase dependent phosphorylation of Akt
TAX2	Thromboxane A2
XO	Xanthine Oxidase

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SUMMARY

Reactive oxygen species (ROS) are vital for the functioning of the cellular system. However, the excess production of ROS, whether due to endogenous or exogenous factors, can cause damage to biomolecules, possibly leading to the development of cardiovascular or neurodegenerative diseases, or even cancers. The NADPH oxidase enzyme complex (NOX complex) plays an important role in the production of ROS in leukocytes, specifically in neutrophils. The formation of oxidants such as superoxide anion and hydrogen peroxide are attributed to NOX complex with the participation of myeloperoxidase (MPO). These oxidants serve as the basis for the formation of other oxidants such as hypochlorous acid and hypothiocyanous acid, causing inflammatory responses. However, moderate production of ROS allows the expression and activation of the Keap1/Nrf2/ARE pathway leading to the expression of certain antioxidant enzymes.

On the other hand, to counteract the excess of ROS there are both enzymatic and non-enzymatic antioxidants, in the latter group, the great family of polyphenols with antioxidant properties stands out. Polyphenols, which have antioxidant properties, are compounds present in fruits, vegetables, beverages and are commonly consumed in diets. Around the world there are varied diets that contain products rich in polyphenols. The Mediterranean diet composed of a wide range of fruits and vegetables, cereals, bread, pasta, fiber and phytochemicals has been the subject of several studies to date. It has been shown that the Mediterranean diet correlates with the low incidence of several chronic diseases, attributing these effects to products rich in polyphenols. For instance, consuming products rich in polyphenols correlates up to 36% with a reduction in the risk of hypertension.

In this research thesis, we quantified the total polyphenols content (TPC) as well as some polyphenol subclasses (flavan-3-ol, flavonols and anthocyanins) by Ultra-Performance Liquid Chromatography (UPLC), present in several fruit and vegetable juices available on the French, Belgian and German markets. In the same way, we evaluated the antioxidant capacity of these juices using the *in vitro* ORAC (oxygen radical antioxidant capacity) method, which is a method mostly used by the industry to determine the antioxidant capacity of products. However, this method is not really standardized, nor does it have physiological relevance.

Under *in vivo* conditions, increased superoxide anion free radical production results from mitochondrial respiratory chain and endothelial dysfunctions, xanthine oxidase activation, and activation of cellular NADPH complex in neutrophils. The inhibition of superoxide anion production was tested according to the protocol described by Baptista et al. (2012) on PMA activated whole blood (Chemiluminescence Method).

To evaluate the vascular reactivity, rates of vasorelaxation were assayed *ex vivo* on rat aorta segments with or without endothelium in the presence of different dilutions of fruit or vegetable juices.

Results related to total polyphenol content, antioxidant capacity and vasorelaxation were published in two scientific articles. Red fruits clearly exhibited the highest TPC values. For all juices, a significant positive correlation was observed between TPC and the ORAC assay ($r = 0.50$, $p = 0.02$). Blackcurrant and pomegranate juices caused more than 50% inhibition of superoxide anion production in PMA-activated whole blood. The exact mass concentration (µg/mL) of each phenolic compound present in juices in the medium correlated with superoxide anion inhibition only for peonidin -3-*O*-glucoside (PG, $r = 0.87$, $p = 0.010$), epigallocatechin gallate (EGCG, $r = 0.67$, $p = 0.0009$), catechin (C, $r = 0.47$, $p = 0.02$) and quercetin ($r = 0.46$, $p = 0.036$) correlated with superoxide anion inhibition.

Of all juices, blackcurrant ones, known for their high polyphenol content, showed the highest capacity to induce vasorelaxation at 1% v/v, in the presence but not in the absence of endothelium. According to the mass concentration, kaempferol, EGCG, delphinidin -3-*O*-glucoside (DG), delphinidin -3-*O*-rutinoside (DR), Cyanidin -3-*O*-rutinoside (CyR) and PG correlated with the vascular activity of the juices. However, concentrations of DR, CyG and in a less extent of PG required for inducing *ex vivo* vasorelaxation where higher than those detected in blood plasma. More detailed *in vivo* studies are now required to determine if the concentrations found in the blood after ingestion of juices have direct effects on antioxidant capacity and endothelial function.

INTRODUCTION

Chapter 1: Reactive Oxygen Species (ROS), Oxidative Stress and Antioxidants

1. Reactive Oxygen Species

ROS are highly reactive molecules originating from the oxygen present in cellular metabolism. Indeed, the oxygen essential for life, paradoxically is the basis to produce ROS (Figure 1). These molecules are mainly and inevitably formed in mitochondria.

ROS are vital to the normal functioning of cellular systems. ROS are imperative for redox homeostasis, as well as proper function in the cardiovascular system, and immune system (Liemburg-Apers et al. 2015). The body requires a balance in its ROS levels for homeostasis. However, excessive level of ROS either from endogenous or exogenous sources, due to increased production rate or diminished antioxidant defenses, result in the development of oxidative stress (Habtemariam 2019).

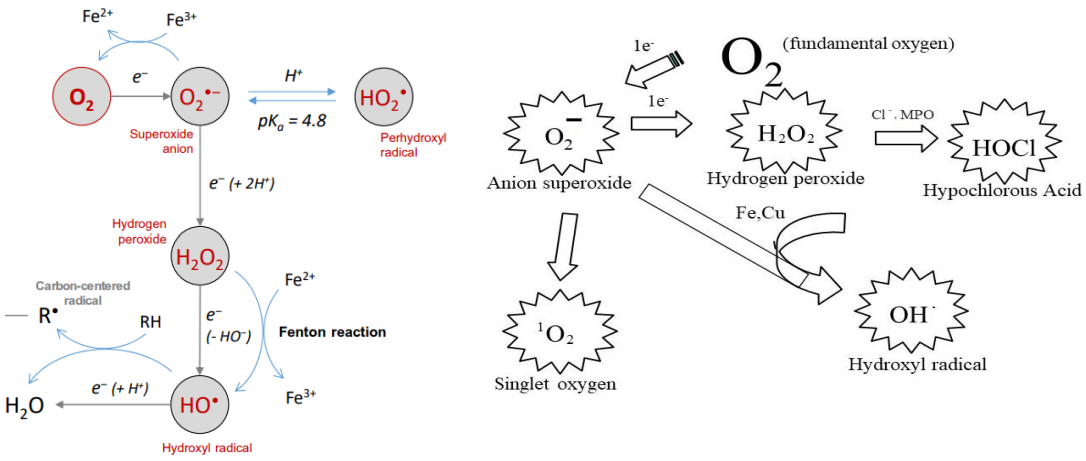


Figure 1. The various reactive oxygen species produced from the fundamental oxygen molecule (O_2) necessary for life (Collin 2019).

1.1. Superoxide Anion

Among the ROS, the free radical superoxide anion ($O_2^{\cdot -}$) is of critical importance, because this molecule is the primary species produced in the cells, and many other reactive species of physiological significance, including H_2O_2, hydroxyl radical ($OH^{\cdot}$), and $ONOO^-$, are derived from $O_2^{\cdot -}$ as products of the downstream reaction cascade (Hayyan, Hashim, and Alnashef 2016).

1.2. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a neutral molecule, which is the least reactive molecule among ROS and is stable under physiological pH and temperature in the absence of metal ions. H_2O_2 could be produced from superoxide anion by superoxide dismutase (SOD) through a dismutation reaction. Amino acid oxidase and xanthine oxidase can also produce H_2O_2 from superoxide anion (Olusegun et al. 2012).

1.3. Hypochlorous acid (HOCl)

HOCl is a highly unstable and highly reactive oxygen-dependent undissociated form of chlorine. As it is one of the strongest hypo halogenated acids, it is also one of the most powerful oxidants among the chlorinated oxoacids and is directly responsible for the bactericidal action of chlorine-derived compounds. Because the concentration of chlorine in plasma is a thousand times higher than that of other halogenated compounds, the H_2O_2 myeloperoxidase system uses chlorine to form HOCl, which is highly reactive.

1.4. Hydroxyl radical

It is a derivative of the superoxide ion. The Fe^{2+} and Cu^{2+} ions act as catalysts in the formation of hydroxyl radicals. In the presence of these ions, hydrogen peroxide (H_2O_2) reacts violently with oxygen molecules, giving rise to hydroxyl radicals (Urbański and Beręsewicz 2000).

1.5. Singlet Oxygen

When energy is supplied to oxygen, it changes to the singlet state $^1O_2$ which represents the activated form. It is a very energetic, highly reactive form that can oxidize many molecules (Min and Boff 2002).

2. Factors influencing ROS production and role of ROS in the inflammatory response.

Not all the oxygen present in the body can generate energy. Around 98% of cellular oxygen in water contributes to the formation of 36-38 molecules of Adenosine tri phosphate (ATP) per oxidized glucose molecule while 2% to the formation of superoxide anion, which is the starting point for the formation of other ROS. The mitochondria are considered the main source of ROS production, however there are several internal and external factors that contribute to the increase of these molecules (Table I). Among the internal factors, hyperglycemia, hypercholesterolemia, endothelial dysfunction, and chronic inflammation stand out. Another aspect to consider in the increase in ROS is the lifestyle of each human being. For example, sedentary lifestyle, intense physical exercise, excessive alcohol consumption, aging, contraceptive pills, poor consumption of fruits and vegetables, high consumption of fatty acids and smoking can be considered as having an important impact on ROS accumulation.

The excess production of ROS (at micromolar level) leads these molecules to react quickly with the biomolecules present in the body. The oxidative damage generated rapidly triggers cardiovascular (lipids oxidation), neurodegenerative diseases (proteins oxidation) and even cancers (DNA oxidation) (Figure 2).

Table I. Factors that contribute to increase ROS

Internal factors	External factors	Lifestyle
Mitochondrial disruption	Atmospheric pollution	Sedentary lifestyle
Hyperglycemia	Exposure to asbestos and nanoparticles	Intense physical exercise
Hypercholesterolemia	Irradiation	Excessive alcohol consumptions
Chronic and hyper inflammation	Surgical operations	Ageing
Endothelial dysfunction		Contraceptive pills
Iron overload		Poor consumption of fruits and vegetables
		High consumption of fatty acids

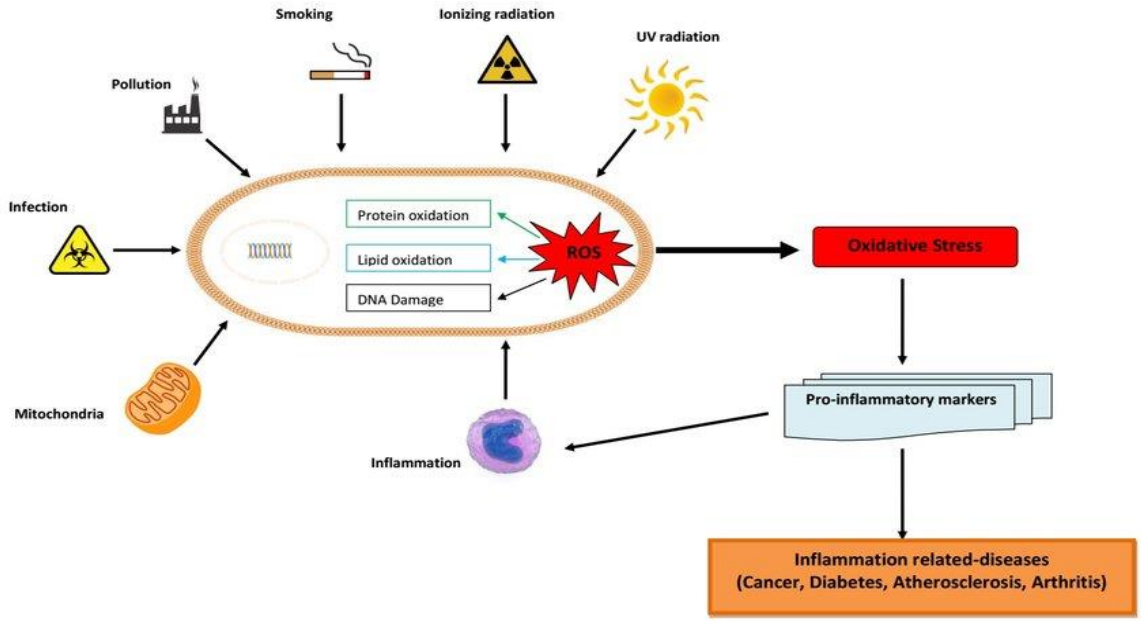


Figure 2. Reactive oxygen species (ROS) can be produced by (1) exogenous sources such as (air pollutions, infection, UV light, radiations, stress, and smoking); or (2) endogenous sources during the oxidation reactions of metabolic pathways in mitochondria, drugs metabolism, and inflammation (Ranneh et al. 2017).

The inflammatory response is a defense mechanism that evolved in higher organisms to protect them from infection and injury. Its purpose is to localize and eliminate the injurious agents and to remove damaged tissue components so that the body can begin to heal. The response consists of changes in blood flow, an increase in permeability of blood vessels, and the migration of fluid, proteins, and white blood cells (leukocytes) from the circulation to the site of tissue damage. An inflammatory response that lasts only a few days is called acute inflammation, while a response of longer duration is referred to as chronic inflammation. Neutrophils are the most abundant leukocytes in the circulation and have been regarded as first line of defense in the innate arm of the immune system. They capture and destroy invading microorganisms, through phagocytosis and intracellular production of ROS and hypochlorous acid (HOCl). However, neutrophils also respond to multiple signals with as consequence the release in the extracellular medium of several cytokines, ROS and other

inflammatory factors that regulate acute and chronic inflammation. ROS production occurs through the action of NADPH oxidase complex (NOX2). NOX2 is a multicomponent electron-transfer complex. This complex is located in the cell membrane. It catalyzes the reduction of oxygen by a single electron, with the electrons shuttling through the complex from NADPH (figure 3). The most relevant oxidants produced by the neutrophil, apart from superoxide and hydrogen peroxide, are those generated by myeloperoxidase (MPO). MPO uses hydrogen peroxide to oxidize a wide range of substrates to reactive products. These include nonradical oxidants, the main ones being hypochlorous (HOCl) and hypothiocyanous acids ($OSCN^-$), as well as radicals produced from organic and inorganic substrates. Which of these reactive species is produced depends on whether MPO is present and the relative availability of its substrates, and this will differ depending on where NOX2 activation occurs (Figure 4) (Winterbourn, et al. 2016).

However, this inflammatory response can get carried away as it does with the SARS - CoV-2 virus. When entering the lungs, the virus triggers an immune response, attracting immune cells to the region to attack the virus, resulting in localised inflammation. But in some patients, excessive or uncontrolled levels of cytokines (cytockines storm) are released which then activate more immune cells, resulting in hyperinflammation with excessive production of ROS leading to lung destruction (Tang et al. 2020).

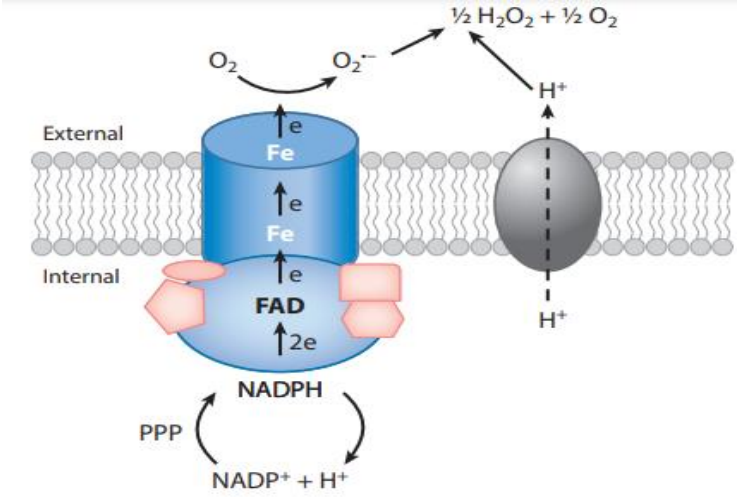


Figure 3. Membrane assembly of NOX2. NOX2 is a multicomponent electron-transfer complex. Electrons are transferred singly to oxygen through a heme relay from FAD, which then undergoes two-electron reduction by NADPH. NADP+ is recycled to NADPH through the pentose phosphate pathway (PPP). The charge is balanced primarily through proton channels. Note that electron flow is directional, from the cytoplasm to the external surface of the membrane (Winterbourn, et al. 2016).

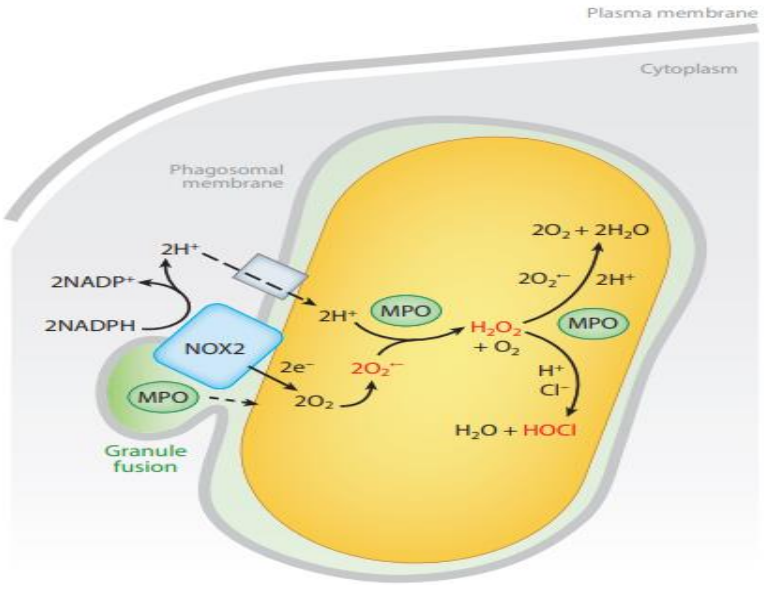


Figure 4. NOX2 and MPO activity in activated neutrophils. The main redox reactions are shown. Superoxide (O_2 •−) is released into the narrow space. The charge is balanced through proton channels. O_2 •− dismutation [mainly catalyzed by myeloperoxidase (MPO)] gives hydrogen peroxide (H_2O_2) as a substrate for MPO, which is released from azurophil granules. Competition between chloride (Cl^-) and O_2 •− regulates hypochlorous acid (HOCl) production (Winterbourn, et al. 2016).

3. Oxidative Stress definitions

Pathological oxidative stress can be defined as an imbalance between oxidant production (ROS, including free radicals, hydrogen peroxide, singlet oxygen, hypochlorous acid) and antioxidants in favor of the former leading to a redox disfunction with irreversible cell damage.

Physiological oxidative stress consists of the imbalance between oxidants and antioxidants but without causing irreversible cell damage, based solely on the breakdown of redox signals (Figure 5).

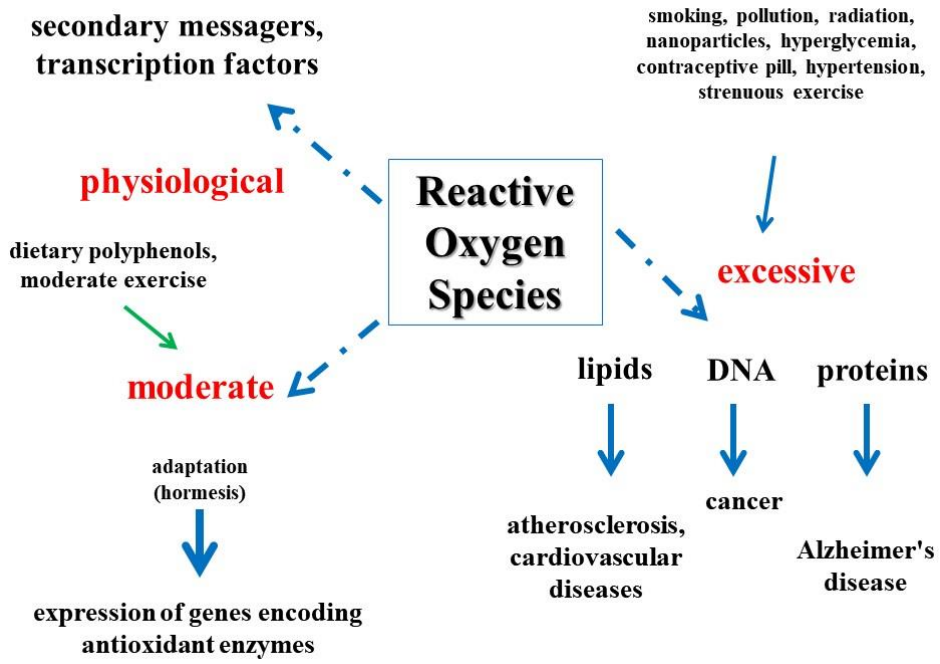


Figure 5. Types of Oxidative Stress (physiological, moderate, and excessive rate) and the consequences on the body according ROS production (Pincemail, 2016)

There is another type of stress considered "adaptive", in this stress the ROS in moderate production (above the physiological concentration) do not cause irreversible damage in the cell but an adaptation in it causing an overexpression and activation of the Keap1/Nrf2/ARE system , leading to the activation of genes encoding antioxidant enzymes (Figure 6). This phenomenon is known as hormesis. It will be developed in point 3.1.7.

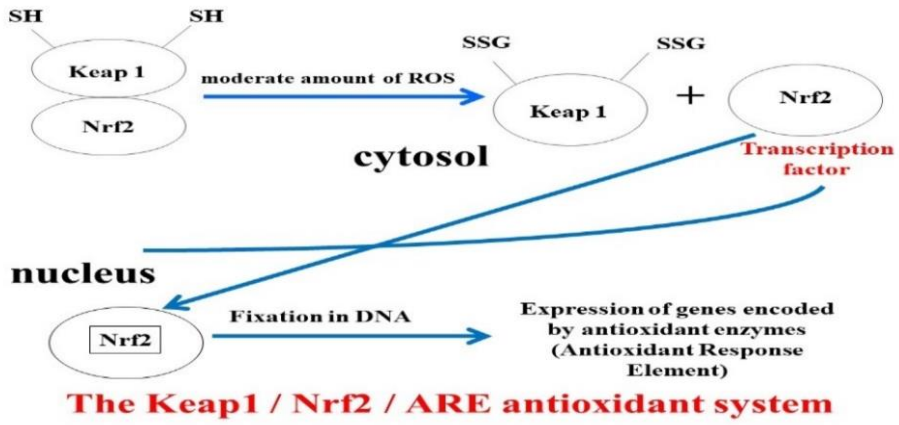


Figure 6. Moderate amounts of ROS make the Keap1 protein located in the cytoplasm detach from Nrf2 factor. Keap1 is metabolically modified by *S*-glutathionylation (SSG). Nrf2 factor migrates to the nucleus to activate the ARE antioxidant defense system. Pincemail (2016).

3.1 Effects on nucleic acids

ROS damage DNA by reacting with nitrogenous bases and deoxyribose. Oxidative damage to DNA is extremely important because damaged nitrogenous bases can generate mutations that in turn can result in carcinogenesis, apoptosis, necrosis, and even inherited diseases (Nimse and Pal 2015). In relation to this, more than 20 types of structural modifications of the bases have been detected. It has been observed that in the presence of ROS, DNA fragments and internucleosomal fragments appear, formed by the breakdown of DNA between nucleosomes, thus causing problems in compaction and coiling of DNA

within the chromatin and with it, alterations in the functional properties of the chromatin itself, which plays an important role in the regulation of gene transcription (Guo et al. 2017).

3.2 Effects on lipids

The main effect of excess ROS on lipids is lipoperoxidation. In this reaction the free radical oxidizes an unsaturated lipid chain, giving a hydroperoxidized lipid and an alkyl radical (Ahmed et al. 2016). The alkyl reacts with an oxygen molecule and regenerates the initial species, constituting a reaction that is repeated. This lipoperoxidation causes alterations in the structure of the membrane, affecting its fluidity and causing damage to its integrity. The peroxidation of lipids generates cytotoxic species such as malondialdehyde and 4-hydroxy-2-nonenal, acting as electrophilic agents capable of interacting with other cellular components, mainly proteins and DNA. It should be mentioned that lipoperoxidation is a process identified in cardiovascular diseases. One of the important processes is the oxidation of low-density lipoproteins, an effect that has been correlated with atherosclerosis (Cheng et al. 2017).

3.3 Effects on proteins

One of the most critical aspects of oxidative stress is the damage caused to proteins, because it can cause loss of catalytic activity of enzymes, damage to the integrity of structural proteins or disrupt the regulation of metabolic pathways (Dean et al. 1997). Unlike nucleic acids, protein repair systems are only limited to methionine residues, so oxidized proteins must be hydrolyzed to prevent their diffusion into the metabolic network or their interaction with other proteins. The effects of ROS on proteins are the oxidation of amino acid residues, the breaking of peptide bonds and the aggregation between proteins. A wide variety of diseases have been linked to the presence of oxidized proteins, some of which are: Alzheimer's disease, rheumatoid arthritis and catarogenesis.

4. Antioxidants

Antioxidants can prevent or slow down the oxidation of a biological substrate, and in some cases reverse the oxidative damage of the affected molecules. Figure 7 shows the network of antioxidants. They can be divided in two enzymatic and non-enzymatic systems.

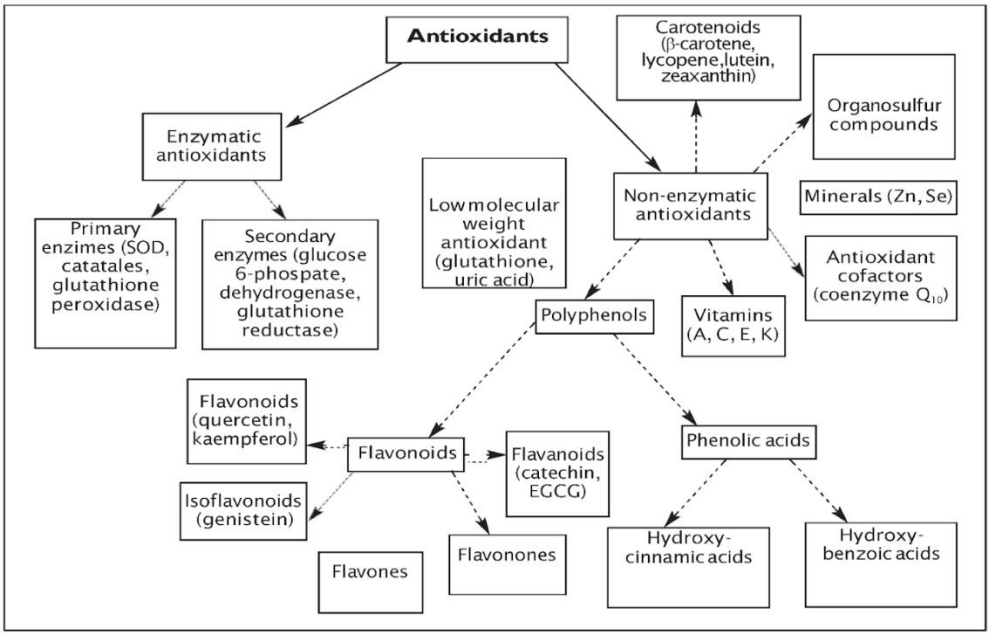


Figure 7 : Antioxidants (Olusegun et al. 2012)

4.1 Types of antioxidants

Although there are several ways to classify antioxidants, from the perspective of their origin and presence in the body, it is possible to distinguish between those antioxidants that are normally bio-synthesized by the body, and those that enter it through the diet. Among the former are:

4.1.1. Enzymatic antioxidants:

Primary antioxidant enzymes include superoxide dismutase (dismutation of superoxide anion), catalase (destruction of hydrogen peroxide) and glutathione peroxidase (destruction of anion peroxide and lipid peroxide). For their antioxidant activities, superoxide dismutase and glutathione peroxidase respectively require trace elements such as copper, zinc, manganese, and selenium. Other enzymes are also involved in antioxidant response, such as glutathione S-transferases, thioredoxin reductases, sulfoxy-methionine reductases, heme oxygenase, etc.

4.1.2. Non-enzymatic antioxidants:

These antioxidants are also called free radical scavengers; in other words, they directly inactivate ROS. Among them, we can cite vitamin C (ascorbic acid), vitamin E (alpha-and gamma-tocopherol), carotenoids, glutathione, ubiquinol (Co-enzyme Q_{10}), lipoic acid, and the great family of polyphenols.

4.2 Antioxidants in diets

The presence of natural antioxidants in food is important, not only because these compounds help to define the organoleptic characteristics and preserve the nutritional quality of the products that contain them, but also because when ingested, they help to preserve the health of the individuals who consume them (Schwingshackl and Hoffmann 2014). Indeed, the recommendation to increase the intake of foods rich in natural antioxidants is currently considered one of the most effective ways to reduce the risk of developing those chronic non-communicable diseases that most limit the quality and life expectancy of patients around the world (Ruxton, Gardner, and Walker 2006; Rodríguez-García, Sánchez-Quesada, and Gaforio 2019).

Regarding antioxidants that enter the body only through the diet, these are essentially classified into:

i) antioxidant vitamins, such as ascorbic acid, alpha-tocopherol, and beta-carotene (or pro-vitamin A),
ii) carotenoids (such as lutein, zeaxanthin, and lycopene),
iii) polyphenols, in their flavonoid and non-flavonoid categories, and
iv) compounds that do not fall into the three previous categories, such as some glucosinolates (e.g., isothiocyanates) and certain organo-sulfur compounds (e.g., diallyl-disulphide).

Among the antioxidants that are most abundant in the diet, it is worth mentioning: ascorbic acid, vitamin E, carotenoids, and polyphenols.

4.2.1 Vitamin C

Ascorbic acid or vitamin C is a water-soluble compound that performs important functions as an antioxidant in the body. As such, it has the potential to protect proteins, lipids, carbohydrates, and nucleic acids against oxidative damage caused by various free radicals and reactive species. From a nutritional point of view, ascorbic acid is an essential nutrient. Vitamin C is necessary for the synthesis of collagen (a structural component of blood vessels and of tendons, ligaments, and bones). It also plays an important role in the synthesis of noradrenaline, carnitine, and possibly in the metabolic conversion of cholesterol to bile acids. Severe vitamin C deficiency can lead to scurvy (Pehlivan 2017).

4.2.2 Vitamin E

The term vitamin E comprises two chemically related types of molecules: tocopherols and tocotrienols. From a structural point of view, both molecules include a hydroxyl group attached to a C-6 of an aromatic ring which is itself attached to an

oxygenated heterocycle. On this said heterocycle (C-2), there is a long hydrocarbon side chain which, in the case of tocopherols, is fully saturated (phytol chain), while in tocotrienols it exhibits three unsaturation. Both tocopherols and tocotrienols occur in the form of alpha, beta, gamma, and delta isomers (Shahidi and De Camargo 2016).

In foods, the concentration of tocopherols is substantially higher than that of tocotrienols. Alpha-tocopherol levels in the blood are approximately ten times higher than gamma-tocopherol (Ahsan et al. 2014). This is due to the presence in the human liver of a tocopherol transfer protein that does not respond to gamma but only to the alpha isomer. This allows storage, incorporation into lipoproteins and subsequent transport and distribution of this to other tissues. Furthermore, relative to the alpha isomer, the other tocopherols are actively bio-transformed (degraded) in the body, which does not allow their accumulation.

4.2.3 Carotenoids

Carotenoids are natural fat-soluble pigments that are synthesized by plants, algae, and photosynthetic bacteria as well as some fungi. In our diet, carotenoids are mostly concentrated (in the form of all-trans isomers) in fruits, vegetables, and cereals, giving them yellow, orange, or red colors (Maoka 2020). Carotenoids are most likely involved in the scavenging of two of the ROS: singlet molecular oxygen, and peroxyl radicals. Further, they are effective deactivators of electronically excited sensitizer molecules which are involved in the generation of radicals and singlet oxygen. The carotenoids interact with singlet oxygen through the transfer of energy from singlet oxygen to the carotenoid molecule. There is formation of oxygen in the fundamental state, and of the carotenoid in its triplet excited state. This last one dissipates its energy by interaction with the surrounding environment, returning to its fundamental state (Stahl and Sies 2003).

From a structural point of view, carotenoids are classified as: carotenes, represented by alpha-carotene, beta-carotene, and lycopene, and xanthophylls, represented for instance by beta-cryptoxanthin, lutein and zeaxanthin. Xanthophylls are carotenoids that include one or more oxygen atoms in their structures (Maoka 2020).

4.2.4 Polyphenols

Polyphenols are compounds bio-synthesized by fungi and by plants (their fruits, leaves, stems, roots, seeds, or other parts). All polyphenols exhibit antioxidant properties. These compounds are present in fruits (41%), vegetables (11%), and certain herbal teas and natural drinks (33%) commonly consumed by the population represent most of the antioxidant activity found as antioxidant defense in the body. From a chemical point of view, all polyphenols exhibit in their structure one or several hydroxylated phenyl rings (Figure 8).

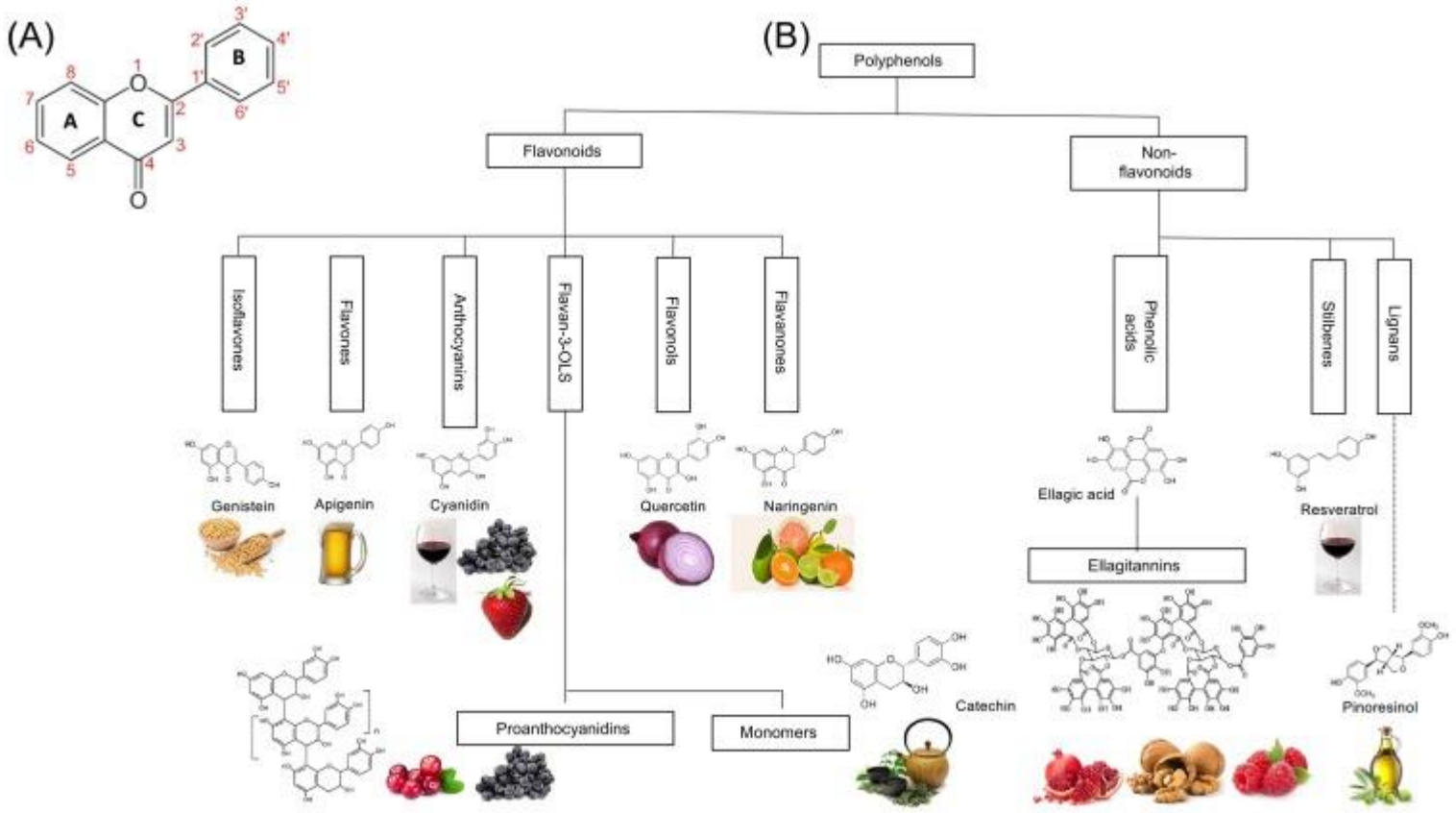


Figure 8. Chemical structures and dietary sources of different groups of polyphenols. Common flavonoid backbone (A) and range of polyphenols found in food (B). Flavonoids are most abundant dietary polyphenols and share a similar basic structure: two phenol rings A and B and a 3-carbon linking chain forming an heterocycle C; numbers on cycles and chain represents carbon atoms that can be hydroxylated. Flavones, flavonols, flavanones, flavanols, isoflavone and anthocyanidins are main subclasses of flavonoids. (Anhê et al. 2019).

4.2.4.1 Phenolic acids

Phenolic acids are a specific class of polyphenols, with phenylalanine being the substrate for the initiation of their synthesis via the phenyl propanoid pathway. They are derived from hydroxycinnamic acid (caffeic acid, ferulic acid, p-coumaric acid, etc.) and hydroxybenzoic acid (syringic acid, vanillic acid, gentisic acid, etc.), having one or more hydroxyl groups and a carboxylic acid function at the benzene ring. The structures of cinnamic and benzoic acids derivatives are presented in Tables II and III. Phenolic acids are plentiful in a stable daily diet that includes enough fruits, vegetables, and whole grains (Călinoiu and Vodnar 2018). The main phenolic acid sources are berries, cherries, apples, citrus fruits, rice bran, passion fruit, mangoes, coffee, kiwis, tea, wheat, corn, and oat flours.

Table II. Phenolic acids and derivates from cinnamic acid (Călinoiu and Vodnar 2018)

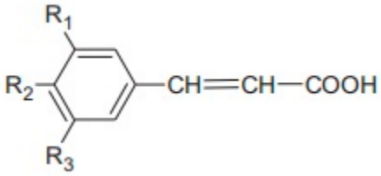
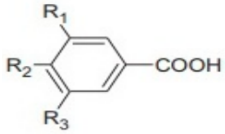
Cinnamic Acid Derivatives	Substitutions		
	**R1**	**R2**	**R3**
Cinnamic acid	H	H	H
p-Coumaric acid	H	OH	H
Caffeic acid	OH	OH	H
Ferulic acid	CH_3O	OH	H
Sinapic acid	CH_3O	OH	CH_3O

Table III. Phenolic acids and derivates from benzoic acid (Călinoiu and Vodnar 2018)

Benzoic Acid Derivatives	Substitutions		
	**R1**	**R2**	**R3**
Benzoic acid	H	H	H
p-Hydroxybenzoic acid	H	OH	H
Protocatechuic acid	H	OH	OH
Vanillic acid	CH_3O	OH	H
Syringic acid	CH_3O	OH	CH_3O
Gallic acid	OH	OH	OH

4.2.4.2 Flavonoids

Flavonoids are polyphenolic compounds that are produced as secondary metabolites throughout the plant kingdom. They are ubiquitously present in foods, and medicines derived from plants, and have attracted much attention because of their possible roles in the prevention of a wide range of metabolic disorders (Farzaei et al. 2019). Most of them are low molecular weight compounds that share a common diphenyl pyran skeleton (C6-C3-C6'), made up of two phenyl rings (A and B) linked through a heterocyclic pyran C ring. The individual carbon atoms of rings A, B, and C are numbered by a system that uses ordinary numbers for rings A and C, and prime numbers for ring B. All flavonoids are hydroxylated structures in their aromatic rings and are therefore polyphenolic structures.

Flavonoids are found mostly as glycosides, but they can also appear in free form (also called flavonoid aglycones). Furthermore, they can be found as sulfates, dimers, or polymers. Glycosides can be found in two forms: as *O*-glycosides with carbohydrates linked through oxygen atoms (hemiacetal bond), or as *C*-glycosides with carbohydrates linked through carbon-carbon bonds. Of all these natural forms, *O*-glycosides are the majority.

There are several subgroups of flavonoids. The classification of these compounds is made according to the oxidation state of the heterocyclic ring (ring C) and the position of ring B. Within each family there are a wide variety of compounds, which differ from each other by number and position of the hydroxyl groups, and by the different functional groups that they can present (methyl, sugars, organic acids). The main sub-classes of dietary flavonoids are flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones and isoflavones (Figure 9).

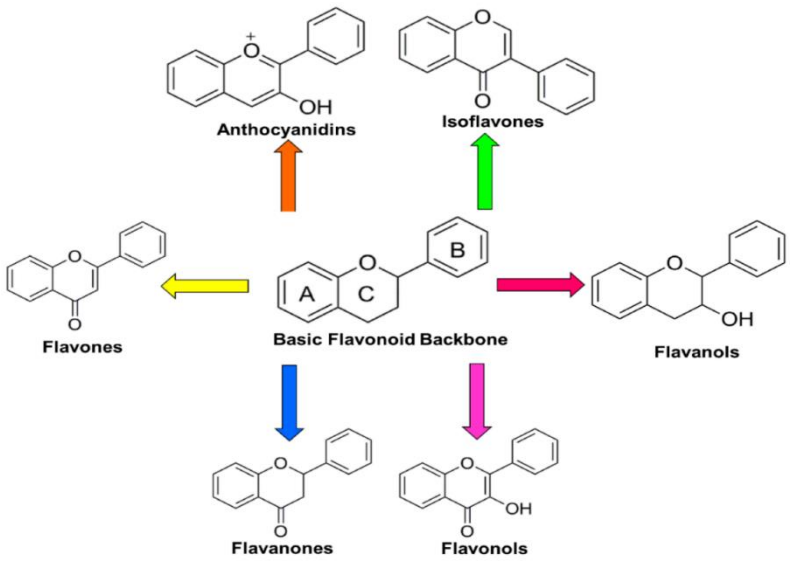


Figure 9. Basic structure of flavonoids and their subclasses. The core structure of flavonoids is a diphenyl propane skeleton (C6-C3- C6), which contains two phenyl rings and one heterocyclic ring. Based on their chemical structure, flavonoids can be further classified further into flavones, flavanones, flavanols, flavonols, isoflavones, and anthocyanins/anthocyanidins (Y. Li, Zhang, and Chen 2018).

4.2.4.3 Flavanols

Flavan-3-ols, generally known as catechins, constitute a complex group of flavonoids with the backbone structure of 2-phenyl-3,4-dihydro-2H-chromen-3-ol. The main dietary sources of catechins are tea, wine, cocoa, apples and fruit juices or jams. Catechins are found abundantly in green and black tea. Catechin comprises catechin (C), (-)-epicatechin (EC), (-)- epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), and gallocatechin (GC) (Figure 10).

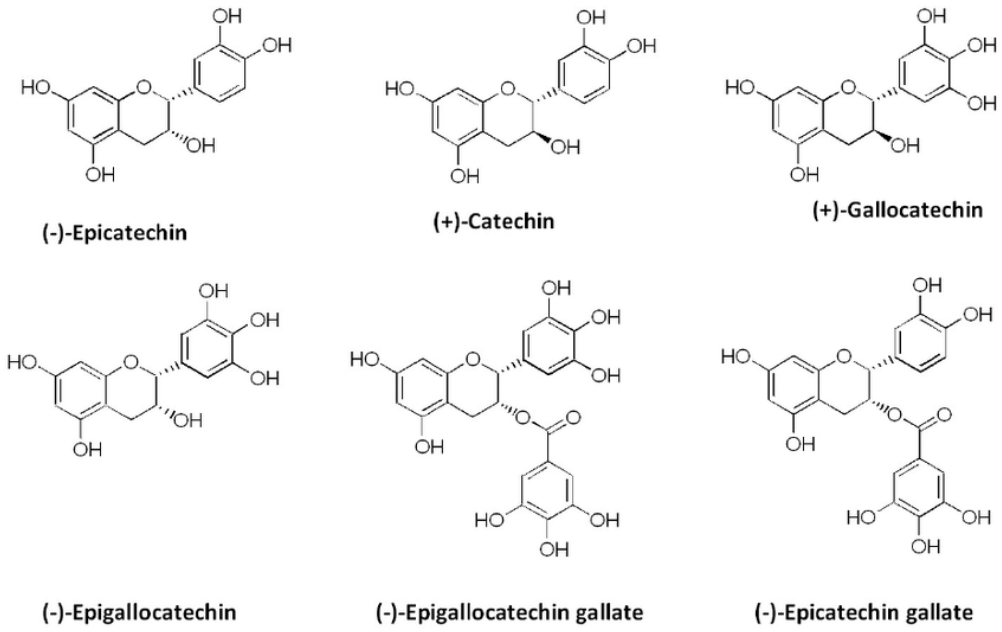


Figure 10. Structures of flavan-3-ols (Nishiumi et al. 2011)

4.2.4.4 Flavonols

Flavonols are a class of flavonoids with a 3-hydroxy-2-phenylchromen-4-one backbone. This class of flavonoids are the most common flavonoids in fruit and vegetables, accumulating mainly in skin and leaves. Onions, leeks, kale, apples, berries, grapes, and grape products are all major food sources of flavonols. Flavonols are represented mainly by quercetin, kaempferol, and myricetin, while the methylated derivative isorhamnetin is also common in the diet. Flavonols exist as two forms: the aglycons and the glycosides (Figure 11). The most common flavonol aglycones are the quercetin and kaempferol, and these compounds have at least 279 and 347 different glycosidic combinations, respectively.

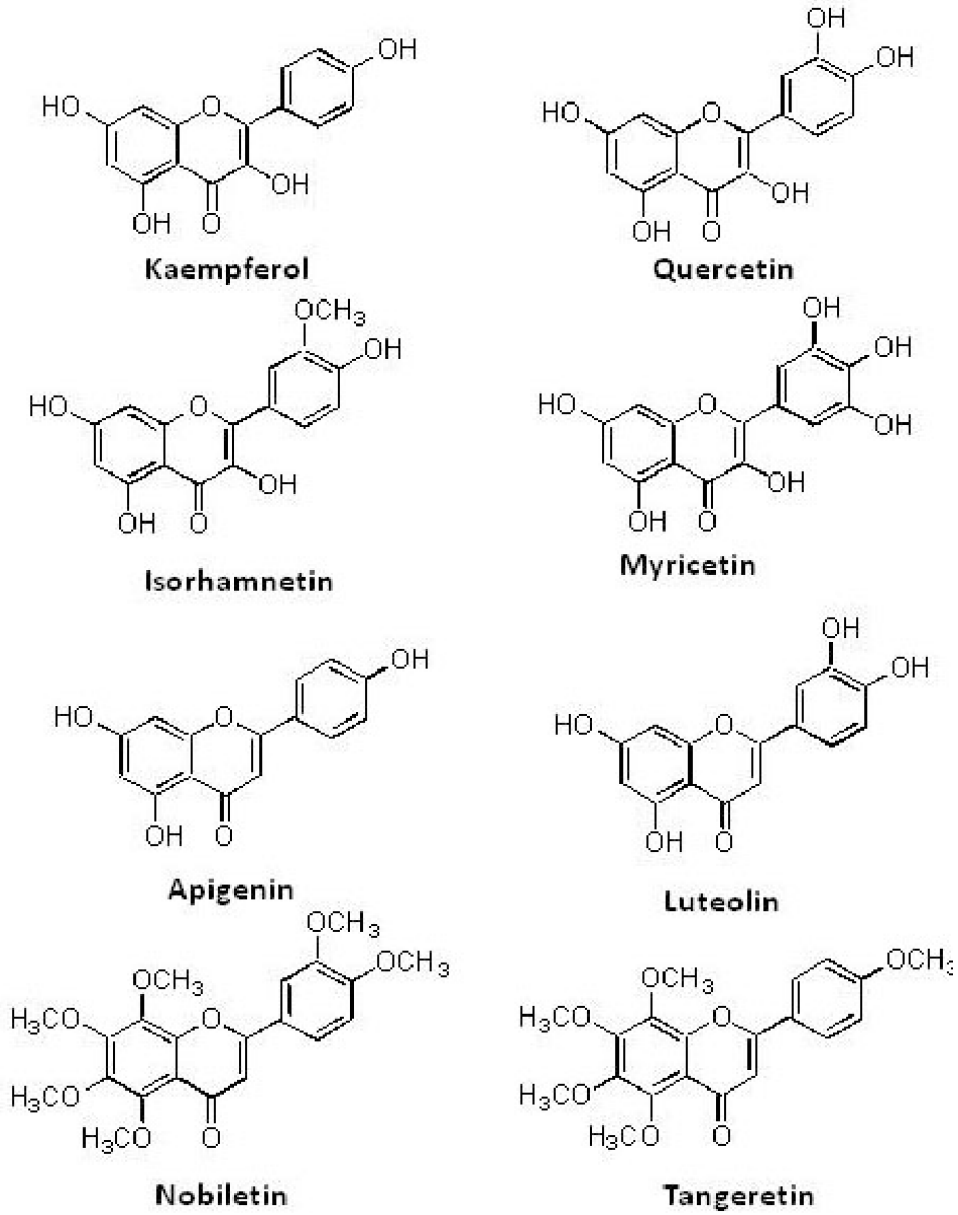


Figure 11. Structures of flavonols. (Nishiumi et al. 2011)

4.2.4.5 Anthocyanins / anthocyanidins

Anthocyanins and anthocyanidins are a group of water-soluble pigments with significant antioxidant activity responsible for the blue, red, and purple colors present in many fruits and vegetables, such as red-skinned grapes, apples, pears, radishes, and red/purple cabbages. Anthocyanidins have the backbone structure of 2-

phenylchromenylium. They are formed by glycosylation (mainly addition of glucose) and other modifications to the main structure of major anthocyanidins (addition of acyl, hydroxycinnamic acid or other moieties) The most common anthocyanidins are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Figure 12). These compounds are invariably found as sugar conjugates that are known as anthocyanins or anthocyanins, which may also be conjugated to hydroxycinnamates and organic acids such as acetic acid. Although glycosylation can take place on carbons 3, 5, 7, 3´ and 5´, it occurs most often on C3 (Crozier et al. 2009) (Figure 9).

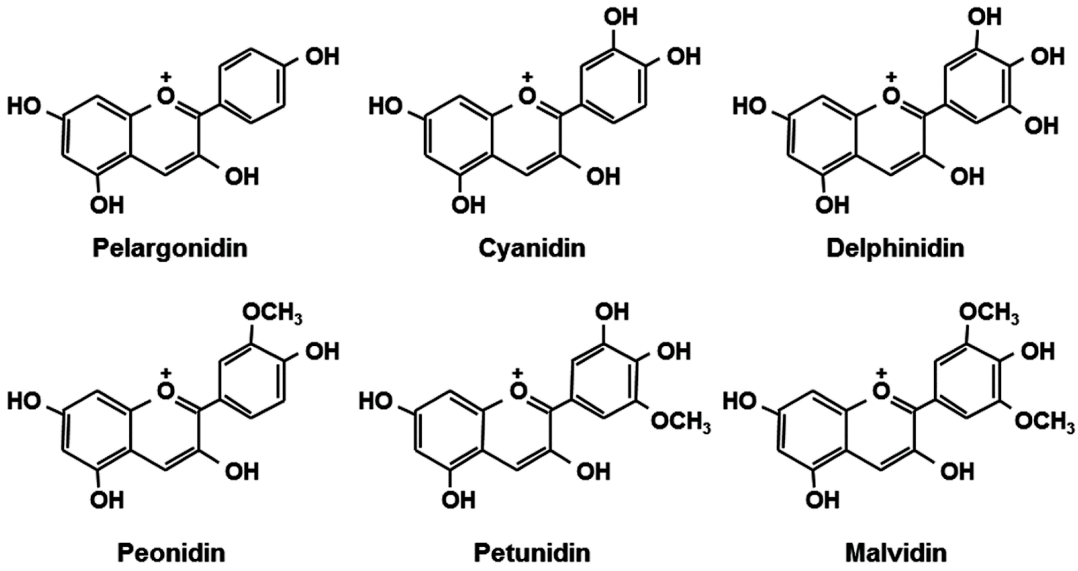


Figure 12. Chemical structure of anthocyanidins (Kato et al. 2015)

4.2.4.6 Isoflavones

Isoflavones differ from other flavonoids in the position of the benzene ring B in C3. Isoflavones are found in leguminous plants, soya and its processed products being the main sources in the human diet. Isoflavones from soybeans include three main molecules: genistein, daidzein and glycitein, which occur mainly as acetyl or malonyl glycosides such as genistin, daidzin, and glycitin (Figure 13).

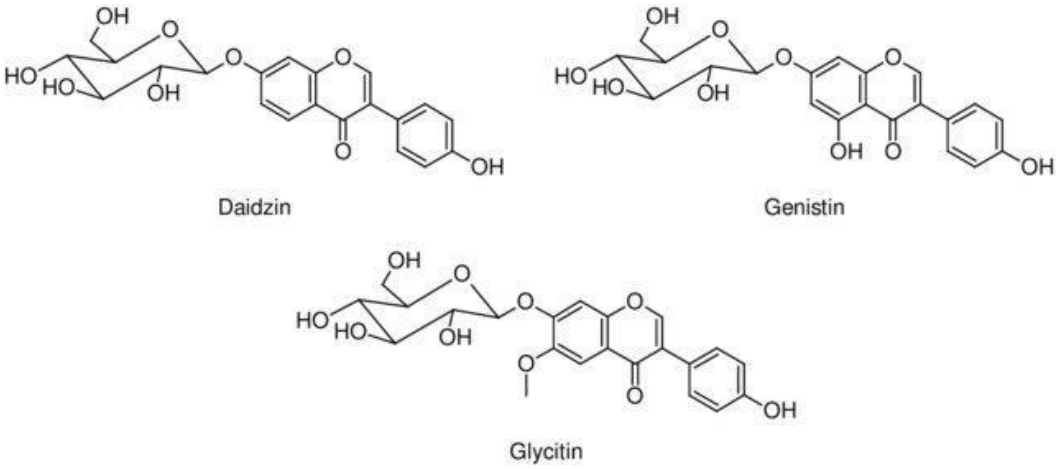


Figure 13. Principal isoflavones (Ulsow, Idenschink, and Elzig 2015)

4.2.4.7 Flavones

Flavones are a group of flavonoids that contain a 2-phenyl-1-benzopyran-4-one skeleton. These types of flavonoids are the most reported in herbs such as parsley and celery. The vast majority of flavones reported in cereal grains exist as O- or C-glycosides of apigenin and luteolin (Figure 14), although free forms are also significant in some grains, such as sorghum.

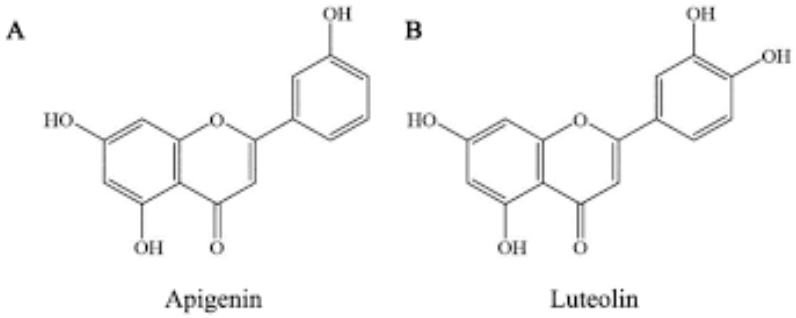


Figure 14. Principal glycosides flavones (Treatment and Addition 2019)

4.2.4.8 Flavanones

Flavanones known as 2-phenyl-chroman-4-one are polyphenolic compounds that include the hesperidin, naringenin, iso-sakuratenin, and heridictyol (Figure 15).

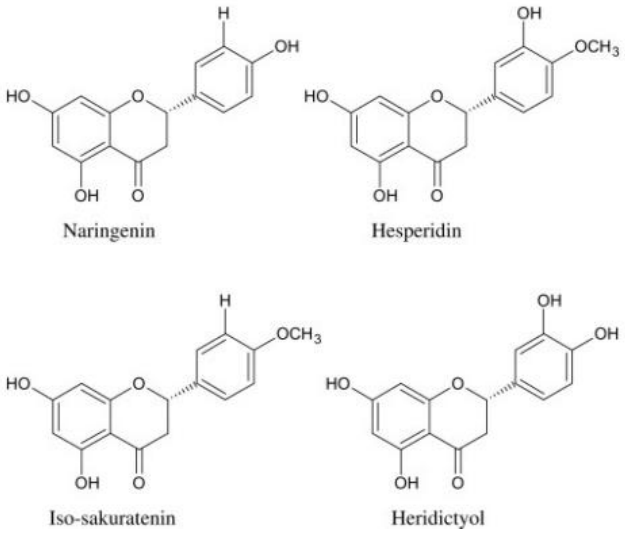


Figure 15. Principal flavanones (Das, Goud, and Das 2019)

Chapter 2: Nutrition and health

Currently, various studies show that dietary pattern is fundamentally important and influences health, just like physical activity. Diets with a low proportion of saturated fats and sugars decrease the risk of cardiovascular diseases (CVD) (Siervo et al. 2015), including high blood pressure (Margerison et al. 2020), and type 2 diabetes (T2D) mellitus (McMacken and Shah 2017). However, a healthy diet must include proteins, fats, and carbohydrates (macronutrients), and the quality of these macronutrients can also have an impact on health (such as type of carbohydrate, some fibers, and carotenoids). A diet rich in carbohydrates can have negative metabolic consequences since it would raise the level of triglycerides and reduce high-density lipoproteins (HDL) (Willett and Stampfer 2013). Lean and active people tolerate a diet rich in carbohydrates better than sedentary and overweight people. And although certain groups of Asian people, for example, do not have overweight problems, presumably due to genetic aspects, these people present high risks of contracting diabetes due to the intake of high sugar content (Res et al. 2018).

On the other hand, one of the causes of the increased risk of CVD is the consumption of large amounts of saturated fat. But serum cholesterol level and more precisely the ratio of HDL on low density lipoproteins (LDL) is a good diagnostic tool of these risks. A diet rich in saturated fats and dietary cholesterol will lower the HDL/LDL ratio, while the consumption of polyunsaturated fats will increase it. Replacing carbohydrates with saturated fats contributes to reducing HDL levels, while replacing monosaturated fats with saturated fats decreases LDL without altering HDL, improving HDL/LDL ratio, and helping to reduce blood sugar and triglycerides in diabetic people (Willett and Stampfer 2013). One of the polyunsaturated fats inversely associated with the risks of CVD is linoleic acid and although there are no specific amounts of consumption, a 5% of energy increment in linolenic acid intake replacing energy from saturated fat intake was associated with a 9% lower risk of Coronary Heart Diseases (CHD) events (Farvid et. al., 2008). Likewise, it has been shown that substituting proteins or monounsaturated fat for carbohydrates, reduces blood pressure and improves blood lipids, in addition to reducing the risks of CHD (Willett and Stampfer 2013). The protein source is more important than the total protein intake, so replacing red meat with a combination of nuts, fish,

poultry, and legumes as the main protein sources, seems to be optimal for overall long-term health. In addition, a diet must have fruits and vegetables in its composition since these have been associated with reducing the risk of non-infectious diseases (Kjøllesdal et al. 2016; X. Wang et al. 2014). In fruits and vegetables there are high contents of dietary fiber, minerals, vitamins, electrolytes, and some phytochemical compounds, especially "antioxidants". These have the property of cleaning free radicals before they cause harmful effects on the health. There is information that the consumption of red wine in moderate quantities could prevent several chronic diseases due to its high resveratrol content, a compound active in the prevention of cardiovascular diseases by neutralizing oxygen free radicals and reactive nitrogen radicals (Snopek et al. 2018).

With all this background information on the effects of the diet on health, we can state that the relationship between food and health is complex. In the United States, for example, for 40% of adults and 48% of adolescents seeking to lose weight, food consumption is stressful. Considering social aspects, the quality of a diet is correlated with the level of education. According to the Healthy Eating Index, in the USA, adults with a college degree have a higher dietary quality compared to those with a high school education (Bleich et al. 2015).

2.1 Definition of healthy diet

A healthy diet can be defined as a pattern of food intake that has beneficial effects on health or at least no harmful effects (Stevenson 2017). Although it has proven difficult to specify the exact nutritional elements that contribute to health, it is necessary to characterize the good intake of various categories of food compounds:

1. Dietary fat and specific fatty acids
2. Carbohydrates
3. Protein
4. Vegetables and fruits

Low quality diets can be characterized by high intakes of processed foods, sugary drinks, trans and saturated fats, little consumption of fruits and vegetables and whole grains. It is difficult to establish specific elements of a healthy diet despite the wide variety of observational studies. In addition, dietary guidelines are presented in various ways. For instance, some countries present the consumption guide in the form of a pyramid (Figure 16). According to a German survey, 28% of consumers adopted their diet from relatives or friends, while 48% adopted it from social networks or the internet (de Ridder et al. 2017).

Figure.16. A generalized healthy diet and lifestyle pyramid (Cena and Calder 2020).

There are lots of information currently available on the different diets, and how each of this influence health. An optimal diet is associated with an increase in life expectancy and a reduction in the risks of suffering from chronic diseases. The adoption of some type of diet varies according to the people and the geographic locations. Diets can vary due to their predominant content of carbohydrates and proteins of both animal and vegetable origin, due to their low total fat content, which includes vegetarian diets that are based mainly on the consumption of plants but including the consumption of dairy products and eggs as well as some seafood. Some diets do not allow the consumption of certain vegetables and fruits due to the types of carbohydrates present. A diet of greater interest is the Mediterranean diet: it greatly limits the consumption of meat while promoting sporadic consumption of seafood.

The characteristic of this diet is the focus on the consumption of olive oil, vegetables, fruits, nuts, and whole grains, adding to this a moderate intake of wine. Likewise, there are balanced diets that are governed by dietary recommendations from health institutes or the World Health Organization. Most diets have in common the limited consumption of refined starches, processed products with incorporated sugars, limited consumption of certain fats, whole grains, fish, poultry, and seafood.

In recognition of the importance of the diet as a determinant of disease risk, the World Health Organization (WHO) Global Action Plan for the Prevention and Control of Noncommunicable Diseases includes strategies for addressing unhealthy diets (Menezes, Lopes, and Nogueira 2016; Maskapai et al. 2013). Their recommendations include balancing energy intake, limiting saturated and trans fats and shifting toward consumption of unsaturated fats, increasing intake of fruits and vegetables, and limiting the intake of sugar and salt. Many of these dietary targets naturally occur in regional diets such as the Mediterranean diet (Bach-Faig et al. 2011).

2.2 The Mediterranean diet and impact on health

The Mediterranean diet is a dietary pattern or model that integrates several variations of the traditional diet of the countries of the Mediterranean coast, and these variations have to do with the heritages and cultures of each country. It is based on cereals such as whole-grain bread, pastas, couscous, and other unrefined grains that are rich in fiber and a variety of fruits and vegetables of different colors and textures that are high in micronutrients, fiber, and phytochemicals (Cena and Calder 2020; Katz and Meller 2014). Some keys to the traditional Mediterranean diet have been the climate, flora, and import deprivation, as well as the high costs of red meat in the past. It also promotes the consumption of dairy products, mainly fermented cheeses (Bach-Faig et al. 2011). In this type of diet, the total lipid intake can be high (≥ 40% of the total energy ingested, as in Greece) or moderate (with 30% of the total energy ingested as in Italy). It should also be noted that both lipids and carbohydrates are the main sources of energy.

There are several studies that correlate the Mediterranean diet with the incidence of various chronic diseases including cancer, diabetes, and neurodegenerative diseases (Yubero-Serrano et al. 2020; Papadaki, et al. 2020). The relationship of this dietary pattern with CVD, such as CHD, ischemia heart disease (IHD), stroke, high blood pressure and rheumatic heart disease (RHD) is of great interest. Indeed, this diet is well known and well-studied as a dietary pattern of health. Several evidences show how this type of diet is a useful tool to control risk factors for CVD such as hypercholesterolemia and hypertension, in addition to presenting inverse relationships with CHD in southern European countries and even in Asian countries such as India where the consumption of some variants of Mediterranean diet confer up to 50% less risk of contracting IHD (Antonia Trichopoulou et al. 2014). There are several variants of the Mediterranean diet, but some common components can be identified: high monounsaturated/saturated fat radio, ethanol consumption at moderate levels mainly in the form of red wine, high consumption of fruits, vegetables and grains, moderate consumption of milk and dairy products and low consumption of red meat and meat products. When we compare it with other "healthy" diets, there are two elements that are unique:

1) abundant consumption of fats from virgin olive oil, tree nuts and fatty fish; and

2) moderate red wine intake during meals. In fact, Mediterranean alcohol consumption patterns appear to be the key to reducing mortality rates.

For UNESCO, the Mediterranean diet constitutes "a set of skills: knowledge, rituals, symbols, and traditions concerning crops, harvesting, fishing, animal husbandry, conservation, processing, cooking, and particulary the sharing and consumption of food.". According to Michel de Lorgeril (Antonia Trichopoulou et al. 2014), cardiologist and nutritionist at the French National Centre for Scientific Research and the School of Medicine at Grenoble University in France, "Mediterranean diet can be considered as a robust and complex scientific concept since by adapting to new geographic areas and populations it becomes a modernized Mediterranean diet. It opens the way to a scientifically

founded protective dietary pattern that could be independent of Mediterranean geography, climate, and cultures".

The health benefits of the Mediterranean diet were first described in 1975 by Ancel Keys, who observed a reduction in CVD risk among populations whose nutritional model was consistent with practices of peoples from the Mediterranean Basin (Altomare et al. 2013). There are countless case controls and clinical studies that have shown some positive effect in the prevention of chronic diseases. For example, MONICA (Monitoring Trends and determinants in cardiovascular disease) project, which followed the eating behavior of 7 million people from 37 countries for 10 years, concluded that the population residing in France had 3 times less mortality from CVD, compared to other industrialized countries. This was partly attributed to regular wine consumption, despite consuming three times more saturated fatty acids (Romagnolo and Selmin 2017). The effect of Mediterranean diet in reducing the incidence of neurodegenerative diseases such as Alzheimer's and Parkinson's have also been demonstrated. Fish oil and moderate wine intake have been linked to slow cognitive decline and reduction of Alzheimer's in observational studies. The Mediterranean diet has also been linked to a lower risk of developing breast and colorectal cancer, inflammatory bowel disease, and aging. According to Sofi et al. 's (2008) meta-analysis of a prospective cohort studies of 1,574,299, a high adherence to the Mediterranean diet is associated with a significant reduction in overall mortality, CVD mortality, cancer incidence or disease and incidence of neurodegenerative diseases (Parkinson and Alzheimer) (9%, 9%, 6% and 13%, respectively) (Romagnolo and Selmin 2017).

Chapter 3: Polyphenols, role, and health effect.

3.1 Polyphenol and health

One of the important characteristics of the Mediterranean diet is the presence of polyphenols (found in fruits, vegetables, tea, olive oil and wine). Their intake correlates up to 36% with the reduction of the risk of hypertension. They also lead to an improvement of inflammatory biomarkers related to atherosclerosis (Potì et al. 2019; Karlsen et al. 2010; Setorki et al. 2009). Two prospective cohort studies and a meta-analysis of 26 cohort studies conducted by Wang showed that eating 3 fruits and 2 vegetables were associated with lower mortality (D. D. Wang et al. 2021). According to the NHS website, 80g of fresh, canned, or frozen fruit and vegetables counts as 1 portion (www.nhs.uk/live-well/eat-well/5-a-day-what-counts/).

Polyphenols are compounds bio-synthesized by plants (in their fruits, leaves, stems, roots, seeds, or other parts). Studies conducted by Pincemail (2016) determined the content of polyphenols in a great variety of fruits, the blackcurrant fruit being the one with the highest content on average (Figure 17). Polyphenols are divided into flavonoids, phenolic compounds, stilbenes, and lignans. The most common phenolic acids are caffeic acid (present in many fruits and vegetables, most often esterified with quinic acid as in chlorogenic acid, which is a major phenolic compound in coffee) and ferulic acid present in cereals which is esterified to hemicelluloses in the cell wall. The best studied stilbene is resveratrol in grapes, grape products, and red wine. The richest source of lignans is linseed present mainly as secoisolariciresinol (up to 3.7 g/Kg dry weight) with low quantities of matairesinol, while minor sources are cereals, lentils, fruits (pears, prunes) and vegetables (garlic, asparagus, carrots) (Y. Kim, et al. 2016). Chemical structures and dietary sources of different groups of polyphenols are shown in Figure 8. All polyphenols exhibit antioxidant properties. These compounds account for most of the antioxidant activity exhibited by fruits, vegetables and certain herbal teas and natural drinks commonly consumed by the population. Although all polyphenols exhibit antioxidant properties, it has been established that some of these compounds also exhibit other activities as anti-inflammatory (Asgary et al. 2014), anti-platelet aggregating (Bijak et al. 2019), anti-bacterial (Bouarab-Chibane

et al. 2019), estrogenic (Cipolletti et al. 2018) and modulating the activity of numerous enzymes, including that of certain digestive enzymes (Sun and Miao 2020).

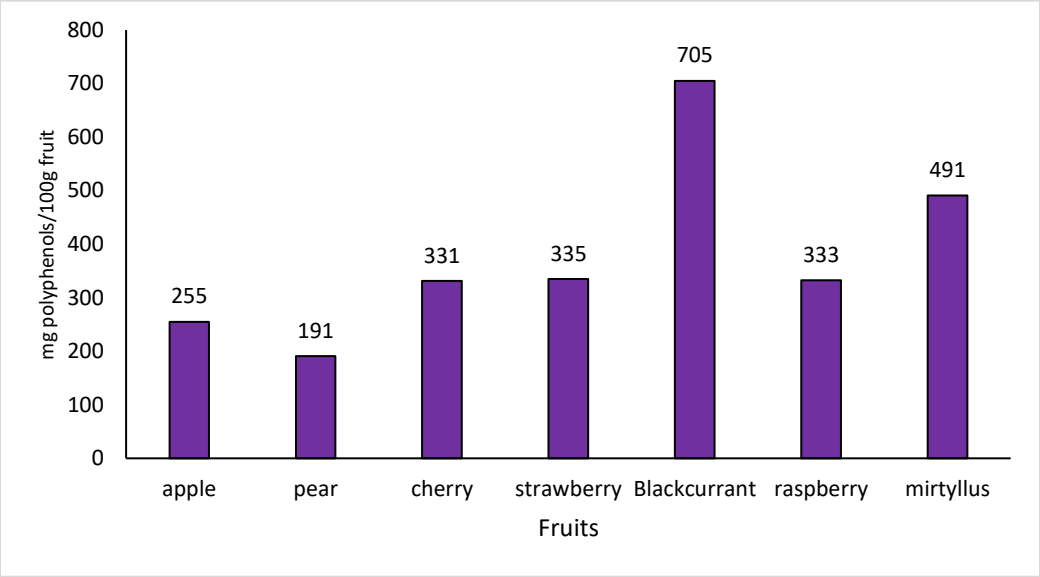


Figure 17. Average polyphenol content of various Belgian fruits. Pincemail (2016).

3.1.1 Cholesterol regulation

The metabolic syndrome is defined as a group of metabolic disorders, which include impaired glucose metabolism, high blood pressure, low HDL levels, dyslipidemia, and abdominal obesity. An study carried out by Ruiz-Roso et al. (2010) shows that consuming foods rich in polyphenols can reduce total cholesterol, LDL cholesterol, LDL/ HDL cholesterol ratio and triglycerides. On the other hand, Castro-Barquero et al. (2020) in their cross-sectional analysis involving 6663 men and women included in the PREDIMED-PLUS study, found an association between the polyphenol consumption (846 mg/day) and high levels of HDL.

3.1.2 Antibacterial properties

The main and most common mechanism of action of polyphenols against bacteria is based on their accumulation on the surfaces of bacteria (Negi 2012). Bouarab-Chibane et al. (2019), demonstrated in their *in vitro* study and using Quantitative Structure-Activity Relationship (QSAR) models, that polyphenols (1g L^{-1}) such as EGCG (flavonoid) and caffeic acid achieve 74.7 and 84% inhibition of *Pseudomonas aeruginosa* growth, respectively. The stilbene resveratrol (1g.L^{-1}) seems to have the highest inhibitory properties for bacterial growth as shown with *Escherichia coli* (100%), *Salmonella enteritidis* (100%), *Staphylococcus aureus* (100%), *Listeria monocytogenes* (100%), *Bacillus subtilis* (75%) and *P. aeruginosa* (75.2%).

3.1.3 Anti-inflammatory actions

Polyphenols from red grapes have been studied as potential anti-inflammatory agents in various inflammatory diseases, such as non-alcoholic fatty liver disease (NAFLD), inflammatory bowel disease, autoimmune diseases, cancer and neurodegeneration (Magrone et al. 2020). According to Singh et al. (2020), polyphenols could modulate inflammatory signaling pathways via antioxidant-based mechanisms. These metabolic pathways are described in figure 18. NADPH oxidase enzymes (NOX complex) are major producers of ROS, generating species such as hydrogen peroxide and superoxide anion. Inhibition of NOX complex by flavonoids depends on additional substitutions on ring B. It is the case of O-methylation (as in 3-O-Methyl-epicatechin, isorhammentin and tamarixerin), of the presence of a 4′-OH group (as in kaempferol and apigenin), and of the addition of an extra OH group (as in epigallocatechin). In addition, it seems that the presence of a OH or methyl group in an aromatic ring also provided inhibitory effects on NADPH oxidase activity as seen for resveratrol, caffeic acid, ferulic acid, for gallic acid and 3-O-caffeoylquinic acid (3-CQA) (Serino and Salazar 2019).

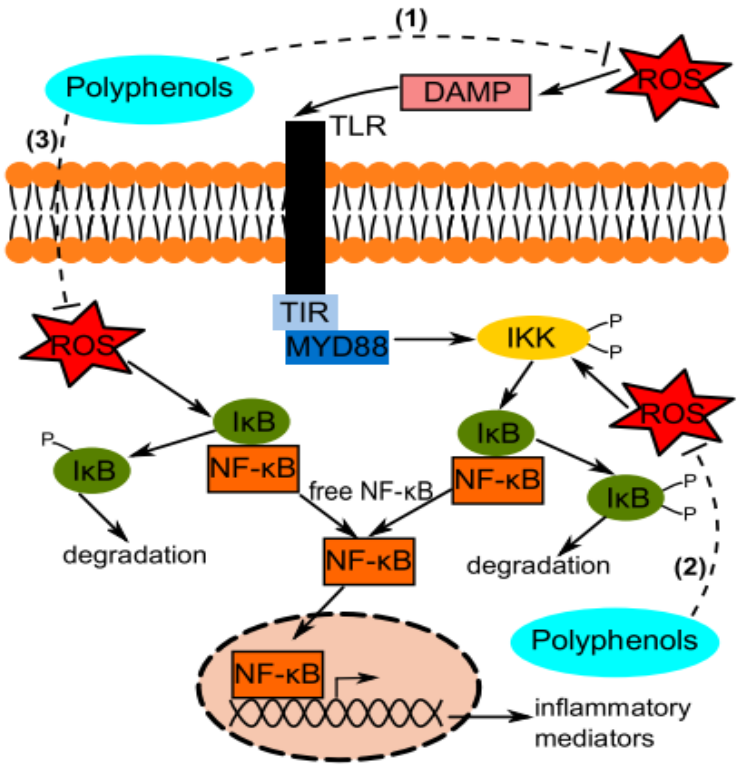


Figure 18. Potential mechanism of action of polyphenols in inflammation inhibition. Polyphenols may target the reactive oxygen species (ROS) to reduce oxidative stress. (1) ROS reduction could reduce the amount of damage-associated molecular patterns (DAMPs); (2) ROS reduction could also arrest phosphorylation of IκB kinase (IKK), which would block the dissociation of IκB from NF-κB; (3) ROS could directly acting in the separation of IκB from NF-κB which could be prevented by polyphenols. These pathways would inhibit nuclear translocation of NF-κB (Singh et al. 2020)

3.1.4 Post-prandial oxidative stress

The gastrointestinal tract is constantly exposed to food, some of it containing oxidized compounds. Consumption of a meal containing oxidized and oxidizable lipids gives rise to an increased plasma concentration of lipid hydroperoxides (figure 19). This is associated with increased susceptibility of LDL to oxidation, apparently due to structural perturbation at the particle surface brought about by lipid oxidation products (Ursini and Sevanian 2002). Lipid oxidation in the stomach was demonstrated by ingesting heated red meat in rats. Red wine polyphenols added to the rat's meat diet prevented lipid peroxidation in the stomach and absorption of malondialdehyde (MDA) in plasma

(Kanner et al. 2012). In humas, postprandial plasma MDA levels rose by 3-fold after a meal of red meat cutlets. MDA derived from meat consumption caused postprandial plasma LDL modification in human. The levels of plasma MDA showed a 75% reduction by consumption of red wine polyphenols during the meat meal (a reference is missing here).

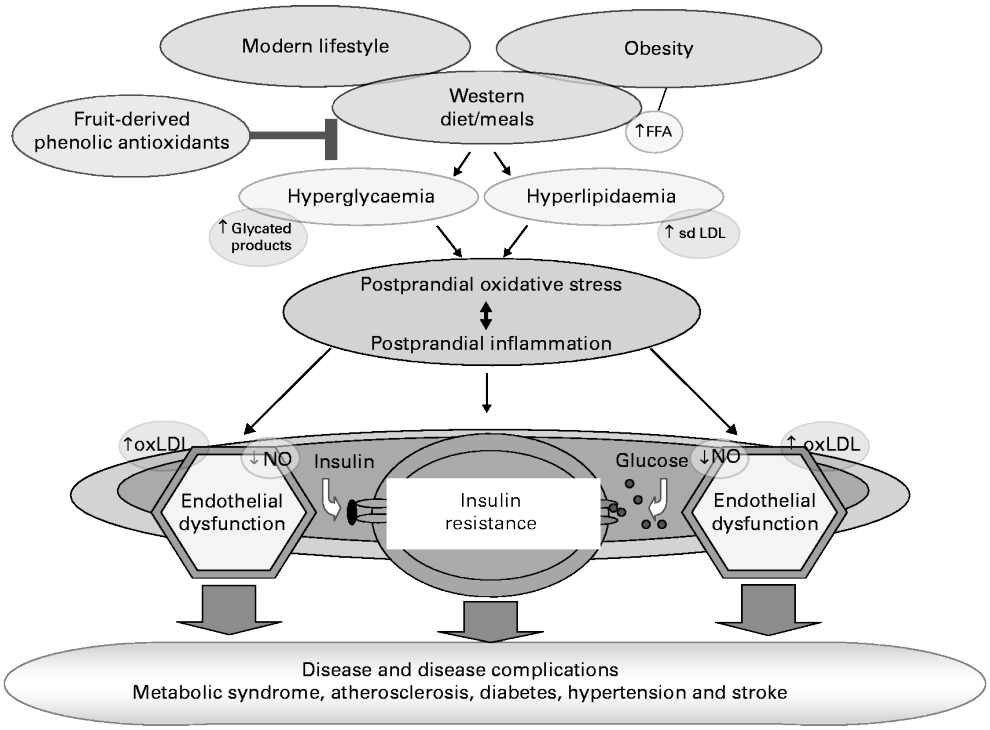


Figure 19. Postprandial dysmetabolic-induced oxidative stress and possible mechanisms for fruit phenolic action. FFA, free fatty acid; sd LDL, small dense LDL; ox LDL, oxidised LDL; NO, nitric oxide (Burton-Freeman 2010).

A recent review by Davis et al. (2020), analyzing diets rich in polyphenols establishes some guidelines to follow to improve the postprandial state. They suggest consumption of fruits such as grapes, strawberries and blackcurrant, and some physical activity such as walking, cycling, climbing, and walking downstairs to acutely improve postprandial dysmetabolism in adults with characteristics of metabolic syndromes. Although there are controlled trials in different regions on the effect of diets rich in polyphenols on postprandial metabolism, a single strategy to control oxidative balance and health cannot be established.

3.1.5 Hormetic mechanisms

Hormesis can be defined as "the process by which exposure to a low dose of a chemical agent or an environmental factor, which is harmful at high doses, induces an adaptive response and/or a beneficial effect on the cell or organism (Figure 20). The most important hormetic agents identified to date are radiation, heat, heavy metals, antibiotics, ethanol, pro-oxidants, exercise, and food restriction. Hormesis was originally described as a dose-response phenomenon characterized by low-dose stimulation and high-dose inhibition. That is, it is a dose-time-response relationship in which there is an initial dose dependent on a toxic response, followed by a compensatory/rebound response. Likewise, the hormetic dose to which a cell or organism responds may vary depending on the individual and their characteristics.

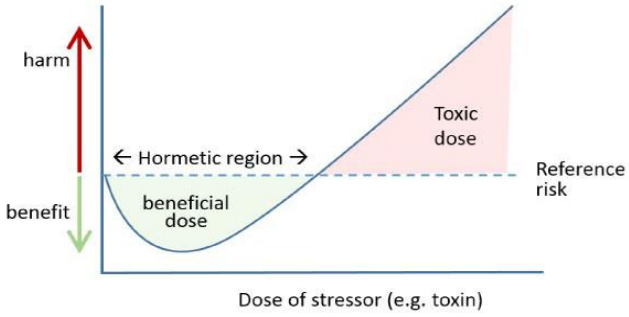


Figure 20. Schematization of hormesis (seven-health.com)

A remarkably interesting example of a dose-response is that observed when the levels of hydrogen peroxide (H_2O_2) increase. At micromolar concentrations, H_2O_2 functions as a signal or second messenger, promoting transient cell cycle arrest and inducing protective adaptive processes by modifying the expression of different genes. At millimolar concentrations or higher, H_2O_2 clearly induces a state of oxidative stress that the cell cannot counteract, thus causing cell death, either by apoptotic or necrotic processes (López-Diazguerrero et al. 2013).

In cells, concentration of polyphenols is very low (about 1uM) due to their poor availability. Therefore, there are unable to act as free radical scavengers. In fact, polyphenols produce free radical species (superoxide anion) by autooxidation (figure 21),

resulting in the activation of Keap1-Nrf2-ARE system as shown in figure 22. Antioxidant Response Elements (ARE) are fond in genes coding a large number of antioxidant enzymes (heme oxygenase, glutathione-S-transferase, NADPH quinone oxi-reductase, glutamate-cysteine ligase, heat shock proteins, proteasome S26, superoxide dismutase, thioredoxin reductase, etc).

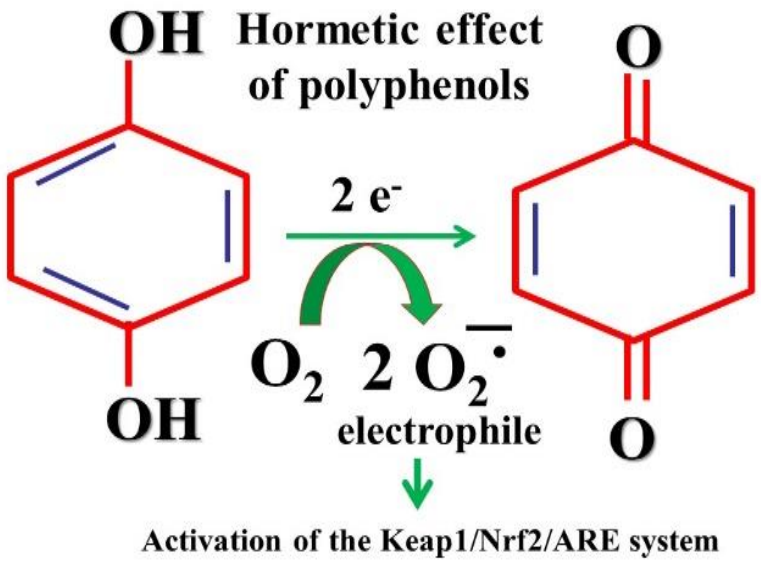


Figure 21. Hormetic effects of polyphenols (Pincemail, 2016).

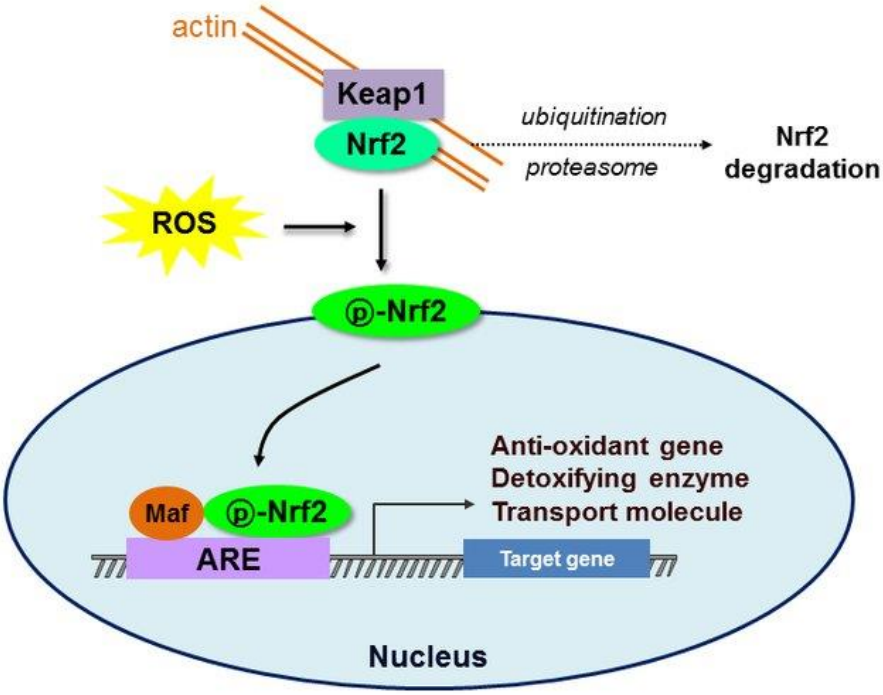


Figure 22. Schematic diagram of the Nrf2-Keap1-ARE signaling pathway. Under normal conditions, nuclear erythroid-2 like factor-2 (Nrf2) is constantly ubiquitinated through Kelch-like ECH-associated protein1 (Keap1) and degraded in the proteasome. After exposure to oxidative stress (ROS), Keap1 is inactivated and Nrf2 becomes phosphorylated. Phosphorylated Nrf2 (p-Nrf2) accumulates in the nucleus and binds to antioxidant response element (ARE) sites, subsequently activating many genes coding enzymes involved in antioxidant production, detoxifying enzymes, and transport molecules (Oh and Jun 2018).

Resveratrol, found in red wine, has shown effects on the normalization of renal expression of Nrf2/Keap1 in diabetic rats (Palsamy and Subramanian 2011). Moreover this stilbene can effectively inhibit endothelial vascular dysfunction, inhibiting ROS/RNS production and related inflammatory responses up to certain point with the Nrf2/HO-1 signaling pathway (J. W. Kim et al. 2010). In addition to this polyphenol, caffeic acid can decrease the expression of Keap1, allowing a more active Nrf2 to trigger a greater expression of antioxidant signals including the synthesis of HO-1 and NAD(P)H quinone

dehydrogenase 1 (NQO1) to prevent induced hepatotoxicity in pathogen-free male ICR mice (Pang et al. 2016).

3.1.6 Regulation of arterial pressure

Polyphenols are able to stimulate the production of nitric oxide (NO) by non-functional endothelial cells (endothelial dysfunction) through the overexpression of their NO synthetase. In over 20,000 people, the EPIC (European Prospective Study of Cancer and Nutrition) study showed that adherence to the polyphenol-rich Mediterranean diet is clearly associated with lower blood pressure. Many studies, always in humans, have also shown that the ingestion of a wide range of foods rich in polyphenols (berries, strawberries, red wine, red grapes, extra virgin olive oil, curry, onions, etc.) is able to improve (or restore) endothelial function within hours of ingestion and thus to lower blood pressure. A recent meta-analysis of 42 studies (1,297 subjects) showed that acute or chronic consumption of dark chocolate very rich in catechins leads to a significant decrease in mean blood pressure of 1.64 mm Hg and diastolic blood pressure of 1.60 mm Hg in hypertensive subjects. Based on several studies, the EFSA (European Food Safety Authority) considered that it had received enough evidence to admit that the intake of 200 mg of cocoa flavanols (provided by 2.5 g of flavanol-rich cocoa powder or 10 g of flavanol-rich dark chocolate) can obtain the following health claim: "The flavanols in cocoa help maintain the elasticity of blood vessels, which contributes to normal blood flow" (Pincemail, et al. 2017).

3.1.7 Prevention of cognitive decline

Other very promising effects of polyphenols are at the cerebral level. Several animal studies have described the neuroprotective actions of extracts of berries (blueberries, grapes, strawberries) or fruits (pomegranate) particularly rich in polyphenols by their ability to protect neurons against oxidative stress or to stimulate neuronal regeneration. On the basis of food habits questionnaires collected between 1994 and 1996 from 2,574 subjects, the

French SUVIMAX study (supplementation with antioxidant vitamins and minerals) shows that subjects (average age of 65.6 years) with a high polyphenol intake (1487 mg/day) showed a much better verbal memory than those with low intakes (863 mg/day). In general, it clearly appears that adherence to the Mediterranean diet very rich in polyphenols is associated with better cognitive function in elderly subjects (Pincemail, et al. 2017).

3.1.8 Polyphenols and epigenetic

Recently, a new line of research has emerged, namely the role of polyphenols in epigenetic regulation. A high level of methylation (hypermethylation) of cytosine to 5-methylcytosine of CpG (cytosine–phosphate–guanine) dinucleotides of DNA by DNA methyltransferase (DNMT) usually leads to inactivating the gene and thus rendering it silent, as is for example the case of the genes responsible for suppressing the development of tumors. On the other hand, low methylation (hypomethylation) most often leads to high gene expression. Generally, hypermethylation of histones under the action of histone methyltransferases (HMT) also results in chromatin closure although this is not always the case. Demethylation is mediated by histone demethylases (HDM). Histones can also be found in an acetylated (action of histone acetyltransferases or HAT) or deacetylated (action of histone deacetylases or HDAC) form. Acetylation on histone lysines leads to chromatin opening, which allows gene transcription. This is where dietary polyphenols come in, which can act as chemoprotective agents in particular by inhibiting DNMT (hypermethylation) and HDAC (deacetylation) enzymes, thus allowing the genes responsible for tumor suppression to remain active (Pincemail, et al 2017).

3.2 Metabolism and bioavailability of polyphenols

Regarding the word "bioavailability", one of the best accepted concepts in the scientific community is "The rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action" enunciated by

the U.S. Food and Drug Administration (Guidance 2002). This statement is perfectly adapted to flavonoids.

Bioavailability is of great importance, given that the most abundant polyphenols are not always the most active in the body, either because they have less intrinsic activity, their absorption in the intestine is low, they are highly metabolized, or they are rapidly excreted. In general, the metabolism of polyphenols occurs through a sequence of reactions common to all of them, which is similar to the metabolic detoxification suffered by many xenobiotics to reduce their potential cytotoxic effect, increase their hydrophilicity and facilitate their urinary or biliary elimination (Manach et al. 2004). A not less important fact to consider is the diversity of polyphenol molecular structures and their ability to conjugate in the body, and therefore their action on specific cells (Suganthy et al. 2016; Yanyan Li, et al. 2018). Among the polyphenols, flavonoids have been shown to have antioxidant (Granato, et al. 2015), hypocholesterolemic (Ei 2005) and anti-inflammatory (Asgary et al. 2014) properties. However, determining the amount of them in the diet, their absorption, and the mode of action in the human body has been the subject of studies in the last decade. In figure 23 we can see an illustration of the metabolism of flavonoids. Ingested flavonoids undergo metabolic transformations in the intestine, and then reach the liver through the hepatic vein. Once in the liver, the metabolites undergo sulphated and methylated conjugations and via the bloodstream target specific cells and tissues.

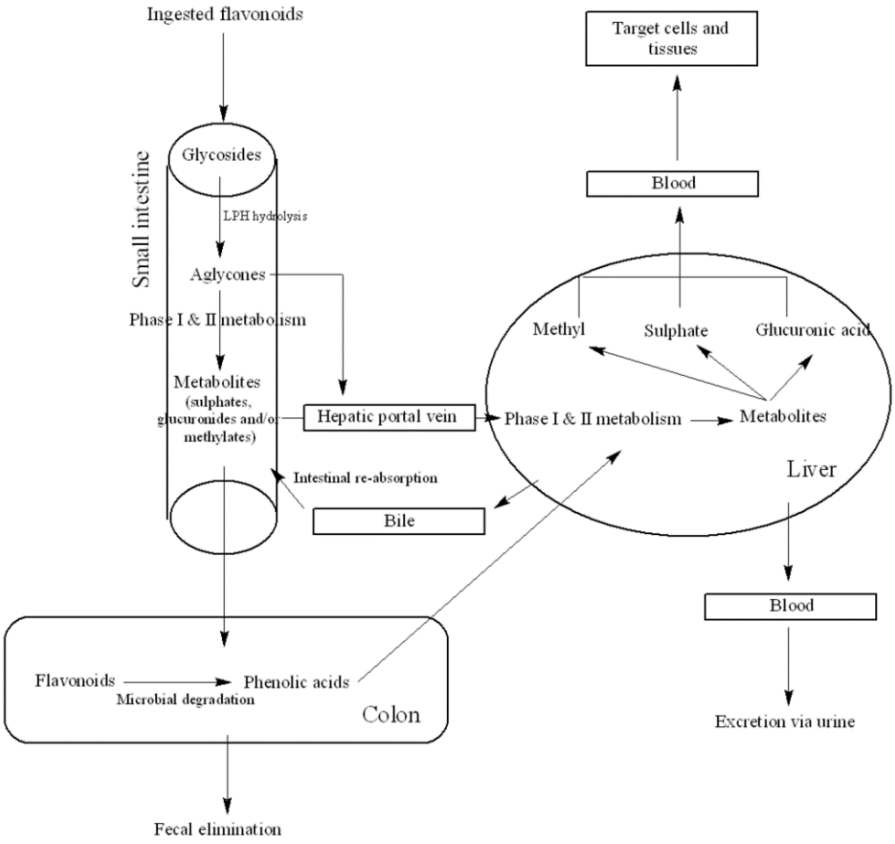


Figure 23. Metabolism of flavonoids (Thilakarathna and Rupasinghe 2013).

There are several factors that affect the bioavailability of ingested flavonoids, among which the following stand out: molecular weight, uptake, transport in the body and excretion. Each of the polyphenol present in the food matrix has different linkages with carbohydrates, organic acids or with each other. The first step of bioaccessibility is digestion and it begins in the oral cavity, where the enzyme amylase is the first to interact and release polyphenols at relatively low rates. The greatest release of polyphenols occurs in the gastric phase, both in the stomach and in the small intestine. The existing pH there causes enzymatic activations in the bile and pancreas (phospholipase, sterol esterase, amylase, carboxypeptidase, trypsinogen, chymotrypsinogen, lipase and bile salts) helping the digestion of non-polar food compounds such as lipids, micronutrients and phytochemicals (Bohn 2019). Low molecular weight polyphenols pass directly into the bloodstream after gastric metabolic transformation. However, those with higher molecular weight are excreted

in the urine after passing through the colon and are catabolized by microorganisms (Williamson and Clifford 2010).

Some flavonoids, due to their structural characteristics, tend to undergo transformation processes before reaching the bloodstream (Williamson, Kay, and Crozier 2018). Gastric absorption of some polyphenols, and their presence in plasma is strongly linked to rapid postprandial onset (Thomas Walle 2003). Once in the bloodstream, polyphenols can be distributed in most tissues and their concentrations in them vary according to the type of cells. Studies carried out to date, suggest that the highest excretion of polyphenols occurs in the kidneys and through bile, either in their free or conjugated forms (Manach et al. 2005).

Chapter 4: Endothelium and polyphenol effect on endothelium dysfunction

4.1. Endothelium structure and functions

Blood vessels are composed of three layers: an intimal monolayer of endothelial cells (tunica interna), a medial vascular smooth muscle layer (tunica media) and the adventitia or tunica externa (made of connective tissue) (Figure 24). The endothelium that forms the inner layer of blood vessels weighs approximately 1 Kg in an average-sized human. It covers an area of between 4,000 and 7,000 m^2, and is made up of approximately 1 to 6 x 10^{13} cells (Cines et al. 1998). Currently, the endothelium is considered as a dynamic, heterogeneous, and disseminated organ that has secretory, synthetic, metabolic, and immunological functions, and whose alteration is decisive in the course of some vascular diseases. Various studies demonstrate that the endothelium participates in a multitude of physiological processes that include the control of transport of molecules between blood and tissues (Yazdani et al. 2019), the regulation of vasomotor tone (Sandoo et al. 2015), the maintenance of blood fluidity and the growth of new blood vessels (vasculogenesis and angiogenesis) (Velazquez 2011).

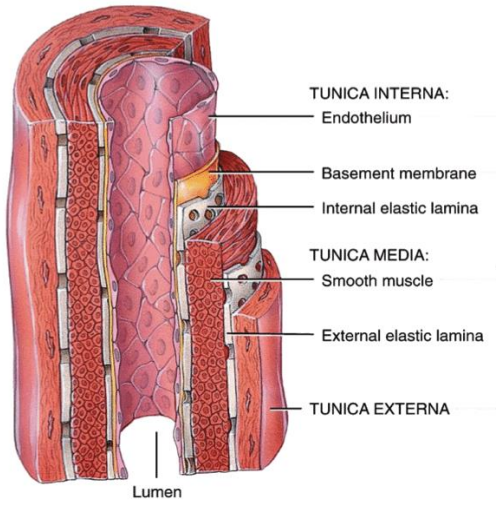


Figure 24. Structure of a medium-size elastic artery (Almeida 2013).

Endothelial cells

An endothelial cell is a type of flattened cell that lines the interior of the blood vessels and especially the capillaries, forming part of its wall. Endothelial cells form the vascular endothelium, which is a simple, flat (single-layer cell) epithelium that lines the inside of the blood vessels and heart. Endothelial cells have various functions in homeostasis, including the following (G. Rubanyi, n.d.):

- They form a smooth surface that facilitates the laminar flow of blood and prevents the adhesion of blood cells.
- They create a permeability barrier for the exchange of nutrients between the plasma and the cell gap, while regulating the transport of substances.
- They regulate angiogenesis and vascular remodeling.
- They contribute to the formation and maintenance of the extracellular matrix.
- They produce growth factors in response to vascular damage, especially influencing the proliferation of vascular smooth muscle.
- They produce substances that regulate platelet aggregation, coagulation, and fibrinolysis.
- They participate in the immune response by generating cytokines that modulate the activity of lymphocytes.

There are several substances secreted by the endothelium showing vasodilatory activity such as nitric oxide (NO), prostacyclin (PGI2) and a group of compounds that provokes endothelium-derived hyperpolarization (EDH). Others show vasocontractile activity such as endothelin-1 (ET-1), angiotensin II (Ang II), reactive oxygen species (ROS), thromboxane A2 (TAX2). NO plays a dominant role in conduit arteries and EDH in resistance vessels. An imbalance between NO and EDH would complicate the maintenance of cardiovascular homeostasis (Godo et al. 2016).

4.2. Vasodilators factors

4.2.1. Nitric Oxide

NO is synthesized by the action of endothelial nitric oxide synthetase (eNOS) by oxidation of L-arginine. There are three forms of NOS: type I or neuronal (nNOS); type II or inducible (iNOS), whose activity is independent of Ca^{2+} concentration, and type III or endothelial (eNOS) that is specific to the endothelium (Förstermann and Sessa 2012) and represent the main source of NO production in the vasculature. Moreover, a new isoform of eNOS in mitochondria (mtNOS) has been considered and it is responsible of the NO production in the mitochondria. It has been demonstrated that the NO-synthesizing capacity of mtNOS is higher than that derived from the combined activity of the all other NOS isoforms. (Forte et al. 2016). The production of NO, which occurs in the cerebral, coronary, systemic, mesenteric, and pulmonary arteries, forms the basis of the relaxation regulation by the endothelium.

In endothelial cells, NO synthesis depends on the intracytoplasmic calcium/calmodulin complex (Ca2+/CaM), phosphatidylinositol-3-kinase-dependent phosphorylation of Akt, adenosine monophosphate-activated protein kinase pathways 5'/ Krüppel-like factor 2 (AMPK/Klf2), and mitogen-activated protein kinases (MAPK) and Sirtuin 1 (Sirt1) (Oak et al. 2018). Another mechanism involved in the production of eNOS-derived NO is the activation of the adrenoreceptors in response to the increase of catecholamines that are produced at high levels in condition of oxidative stress associated with endothelial dysfunction.

Once synthesized, NO diffuses into smooth muscle cells by diffusion, stimulating guanylate cyclase which increases cyclic guanosine monophosphate (cGMP) levels and produces smooth muscle relaxation. Furthermore, the NO diffuses into the lumen of the vessel where it inhibits the adhesion of leukocytes and platelets on the endothelium (Ruano et al. 2016) (Figure 25).

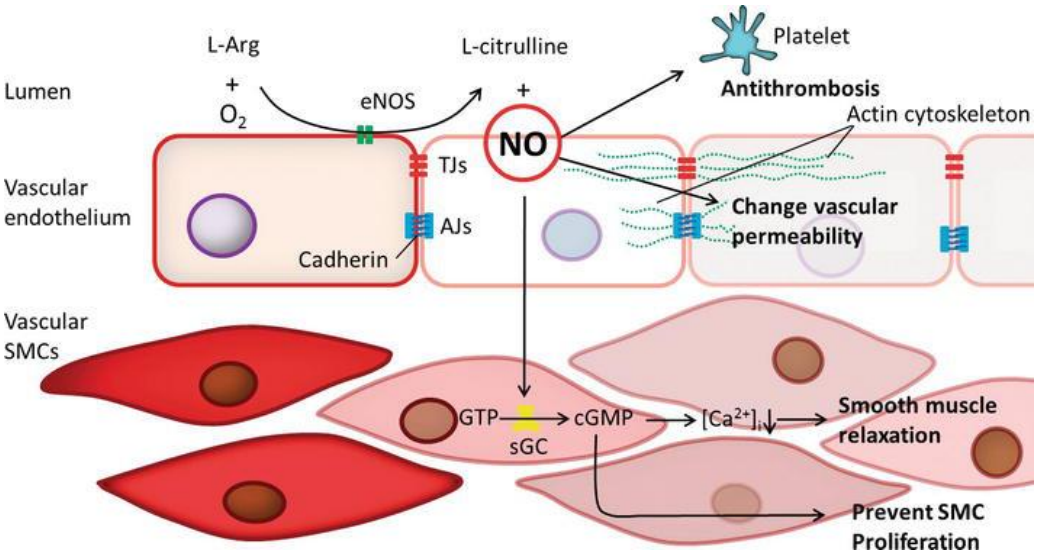


Figure 25. Biosynthesis of NO by eNOS and the biological effects of NO in the vascular system (Ruano et al. 2016).

4.2.2. Prostacyclin

Prostacyclin (PGI2) generated by the vascular wall is a potent vasodilator, and the most potent endogenous inhibitor of platelet aggregation (Hamilos, et al. 2018). Prostacyclin is synthesized from the 20 carbon fatty acid (20:4) arachidonic acid by the concerted actions of cyclo-oxygenase (COX) and prostacyclin synthase (figure 26) (Mitchell et al. 2014). PGI2 is not constitutively synthesized by the endothelium and therefore does not regulate basal tone. It is released at sites of vascular alteration in response to hypoxia. It has a synergistic effect with NO (Camacho et al. 2011). Unlike NO, it produces its vasodilatory effect through specific receptors. They are present in the membrane of the smooth muscles of the vessel. Its action generates an increase in cAMP facilitating the decrease of intracellular Ca^{2+}, inducing muscle relaxation and regulating thus vasoconstriction.

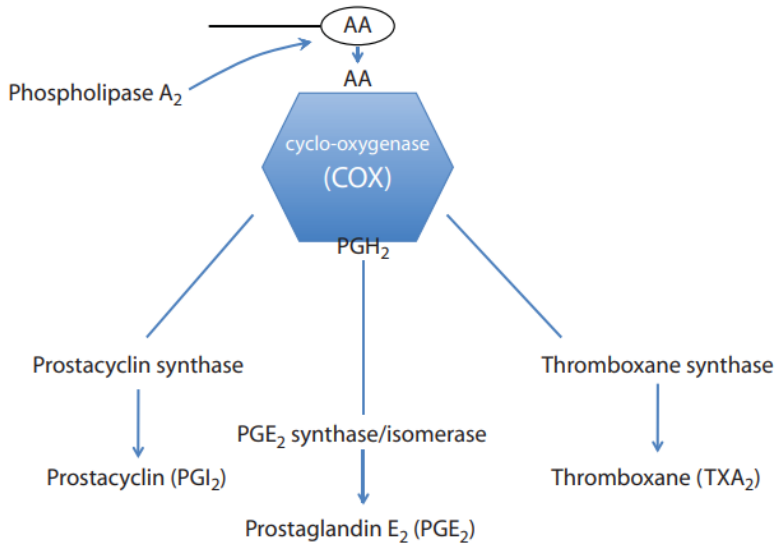


Figure 26. Synthesis of prostacyclin (Mitchell et al. 2014)

4.2.3. (PI-3-kinase)-dependent phosphorylation of Akt

It is well known that polyphenols have antioxidant properties. However, in endothelial cells, it has been possible to show a pro-oxidant effect on endothelium-dependent arterial dilation due to the formation of NO by Src-mediated phosphatidylinositol-3-kinase (PI-3-kinase)-dependent phosphorylation of Akt. Benito et al. (2002) demonstrated that after a consumption of diets containing 35% ($v.w^{-1}$) dealcoholized wines or individual flavonoids such as quercetin or catechin 0.3 % ($v.w^{-1}$) for 10 days, present an endothelium-dependent vasorelaxation in rat aorta rings. This is due to the increase in the NO cyclic GMP pathway. Sometime later, Auger et al. (2010), showed that polyphenols, specifically procyanidins and anthocyanins, present in red wine extracts increase NO synthesis. This occurs through the activation of PI3-Kinase/Akt pathway that synthesize endothelial eNOS, petunidine-O-Coumaroyl-glucoside being the most potent activator. Another *ex vivo* study, carried out by Taguchi et al. (2020), showed that quercetin and morin are potent vasodilators in rats with induced diabetes.

4.2.4. Transient increase in the calcium signal

A study by Martin et al. (2002), demonstrated a Ca^{2+} -dependent release of NO from bovine arteries endothelial cells in the presence of phenolic compounds from red wine extracts containing 16% anthocyanin-enriched fractions (including 36% malvinidin-3-*O*-glucoside). These polyphenols, in addition to causing an increase in NO, increased the cytosolic free calcium, by activating tyrosine kinases.

4.2.5. Activation of estrogen receptors

It has been shown that an alpha isoform of the estrogen receptor (ERα) triggers the vascular protection of polyphenols. A study carried out by Leonetti et al. (2017) in ERα deficiency 8-week-old female mice, and fed daily with diets rich in polyphenols (flavanols: 315; flavonols: 15.10; anthocyanins: 3.89 mg/per gram weight) from red wine extracts for 12 weeks, showed a decrease in NO production, while the same study with wild-type mice showed a significant increase in NO. This information demonstrated that ERα is involved in the increase of NO production induced by polyphenols.

4.2.6. Activation of Sirt1/KLF2 pathway.

It has been shown that certain polyphenols present in grape seed extracts increase the presence of NO through the higher activity of eNOS in human umbilical vessel cells, *in vitro*. This has been attributed to the transcription factor Krüpple 2 (KLF2). 250 mg/Kg per day of this extract are able to activate protein kinase (AMPK) and to increase levels of the protein Sirtuin 1 (SIRT1), responsible for the induction of KLF2 synthesis (Cui et al. 2012).

4.2.6. Endothelium-derived hyperpolarization

Endothelium-derived hyperpolarization (EDH) factors are a group of compounds generated by the endothelium that induce vasorelaxation. Although the chemical structure is not fully established, this function is carried out by epoxyeicosatrienoic acids (EETs), which are amino acid metabolites generated by the enzyme P450 epoxygenase; these EETs act on smooth muscle cells opening the Ca^{2+}-dependent K^+ channels and hyperpolarizing the membrane (Goto and Kitazono 2019) (Figure 27). The importance of this relaxation factor increases as the diameter of the arteries decreases.

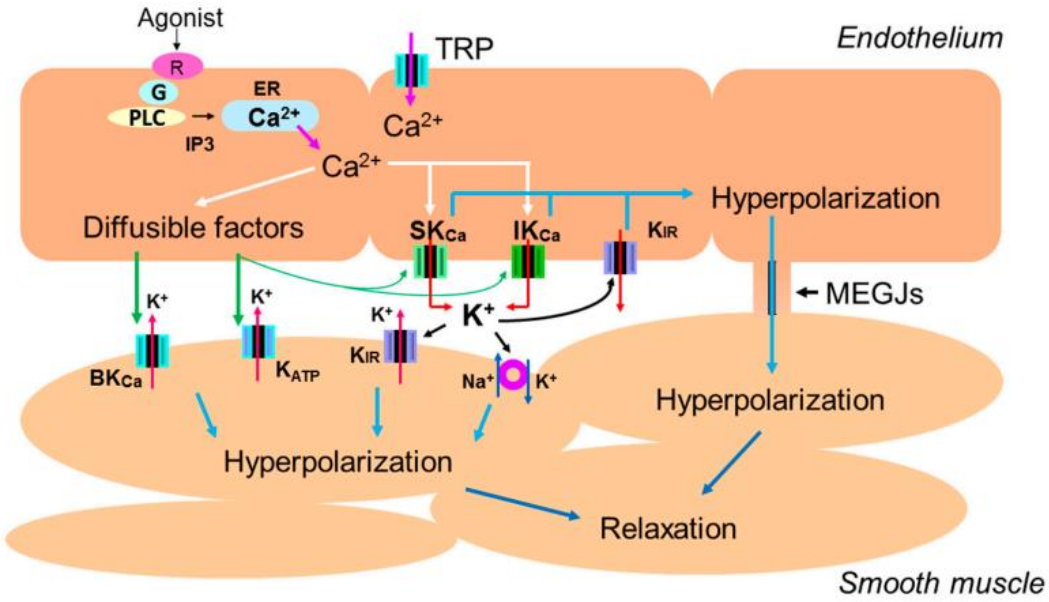


Figure 27. Endothelium-dependent hyperpolarization of vascular smooth muscle cells. Endothelial stimulation with agonists or by shear stress increases the intracellular $Ca2^+$ concentration due to $Ca2^+$ release from the endoplasmic reticulum (ER) and $Ca2^+$ influx through endothelial nonselective cation channels of the transient receptor potential (TRP) family. The rise in the endothelial $Ca2^+$ concentration subsequently activates small (SKCa) and intermediate conductance (IKCa) $Ca2^+$-activated K^+ channels, generating endothelium-dependent hyperpolarization (EDH). The EDH then spreads to adjacent smooth muscle cells via myoendothelial gap junctions (MEGJs), leading to vasorelaxation in a number of vascular beds. In some vascular beds, diffusible factors hyperpolarize vascular smooth muscle cells via the opening of potassium channels and/or activation of Na^+/K^+-ATPase. Diffusible factors also act on endothelial potassium channels to generate or amplify EDH in certain vascular beds in specific conditions (Goto and Kitazono 2019).

4.3. Vasoconstrictors factors

4.3.1. Endothelin- 1

Endothelin-1 (ET1) is a 21–amino acid peptide secreted by vascular endothelial cells in response to different stimuli, including pulsatile stretch, sheer stress, neurohormones, cytokines, growth factors, and thrombin. The secretion of ET1 has been shown to be increased by hypoxemia in humans. The effects of ET1 are mediated by both endothelin A (ETA) and endothelin B (ETB) receptors (Figure 28) (Schneider et al. 2010). ETA is localized on vascular smooth muscle cells, and ETB is expressed on vascular smooth muscle cells, endothelial cells, and fibroblasts. The effects of ET1 include vasoconstriction, hyperplasia, hypertrophy, fibrosis, and increased vascular permeability.

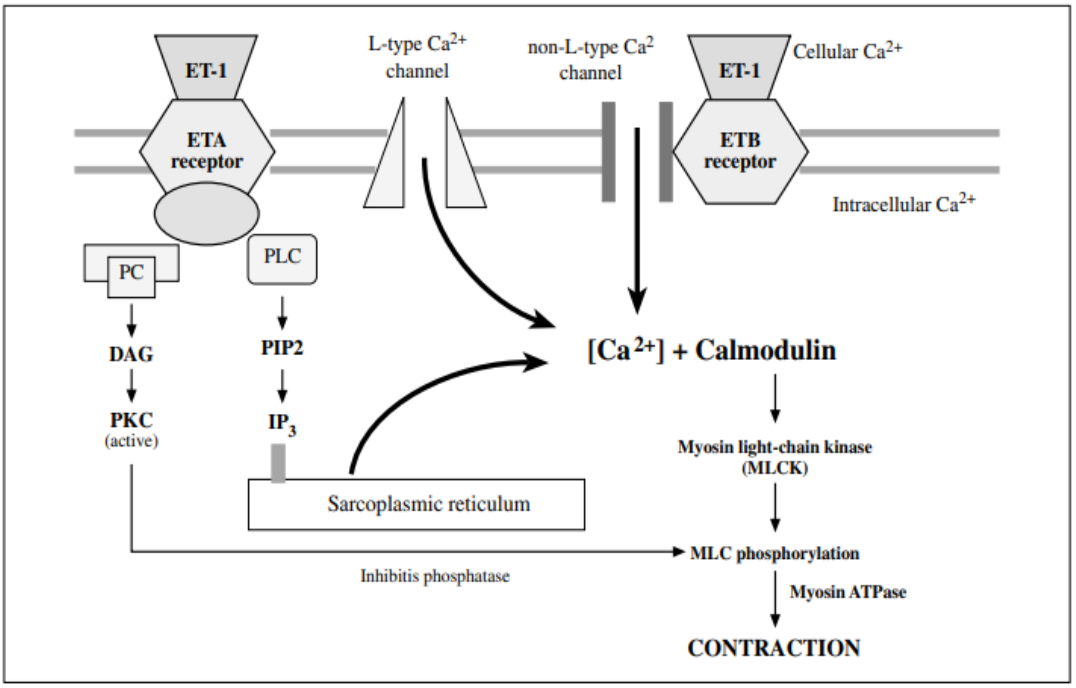


Figure 28. Endothelin receptors, endothelin A (ETA) and B (ETB), and the mechanisms by which ET-1 induces smooth muscle contraction (Akhtar et al. 2008)

4.3.2. Angiotensin II

Angiotensin II (Ang II) is a potent vasoconstrictor whose function, in hypovolemic or hypotensive conditions, is to maintain vascular tone and thus maintain blood pressure at adequate values (Busse et al. 2017). This was the first recognized function of angiotensin II and to which it owes its name. Besides, it has different properties in addition to the vasoconstrictor effect. It induces the secretion of aldosterone in the glomerular zone of the adrenal cortex (Yatabe et al. 2011). In hypotensive situations, this causes retention of water and sodium with a significant increase in the circulating volume that, together with the vasoconstrictor effect, raises or maintains the blood pressure within normal limits. Inappropriate elevated levels of Ang II can cause hypertension through vasoconstriction and retention of water and sodium.

4.3.3. Thromboxane A2

Thromboxane A2 (TXA2) is a potent constrictor of smooth muscle. It was originally described as being released from platelets, but it is now known to be released from other cells, including macrophages and neutrophils (Offermanns 2006). TXA2 is generated from prostaglandin H2 by thromboxane-A synthase in a metabolic reaction which generates approximately equal amounts of 12-Hydroxyheptadecatrienoic acid (12-HHT) (Figure 29). TXA2 has prothrombotic properties, as it stimulates the activation of platelets and platelet aggregation. TXA2 is also a known vasoconstrictor and gets activated during times of tissue injury and inflammation.

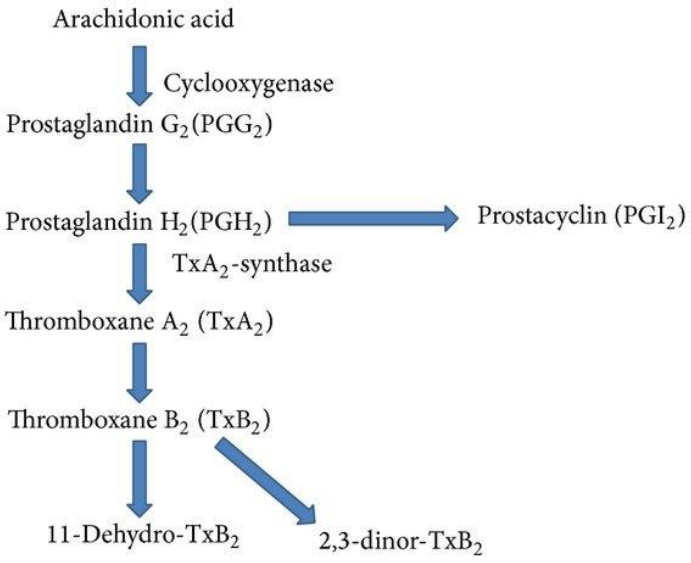


Figure 29. Synthesis of Thromboxane (Ghoshal and Bhattacharyya 2014)

4.4. Endothelial dysfunction

The good balance between dilator and constrictors factors guarantees good arterial function (120/80mmHg) (Figure 30). However, an excess of ROS causes non-functional eNOS and therefore arterial hypertension (140/90mmHg). It was Furchgott and Zawadzki who started the first studies related to endothelial dysfunction in the 1980s. Thereafter the endothelium has been recognized as the most important regulator of vascular homeostasis. Endothelial cells are strategically located between circulating blood cells and fixed blood cells, as well as vascular muscle cells.

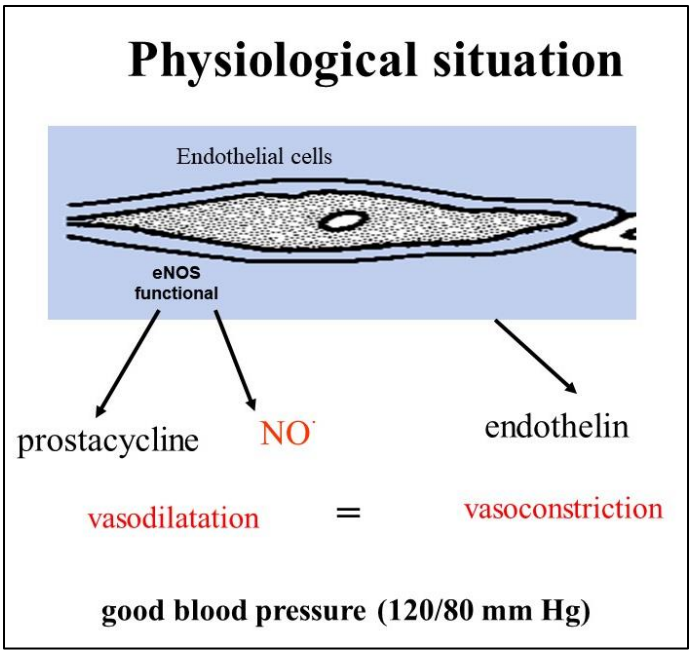


Figure 30. balance between vasodilator and vasoconstrictor factors for proper arterial function (Pincemail, 2016).

The functional integrity of the endothelium is crucial for the maintenance of blood flow and antithrombotic capacity because the endothelium releases humoral factors that control relaxation and contraction, thrombogenesis, and fibrinolysis, as well as platelet activation and inhibition of themselves. Therefore, the endothelium helps control blood pressure, blood flow, and vessel power. It is now clear that endothelial dysfunction contributes substantially to cardiovascular disorders such as atherosclerosis, hypertension, heart failure, which lead to hypoperfusion, vascular occlusion, and terminal damage or destruction of the organ. Hypercholesterolemia, which is increased cholesterol in the blood, inhibits endothelium-dependent relaxation, which ultimately results in atherosclerosis (G. M. Rubanyi 1991). Low density lipoprotein (LDL) appears to be the main determinant of this phenomenon. In hypertension, which is a common condition in which the force exerted by blood against the walls of the arteries, can contribute to increased peripheral vascular resistance. In many models of hypertension, elevated blood pressure is associated with a reduction in endothelial-dependent relaxation (Brandes 2014). Endothelial dysfunction is more prominent in some blood vessels than others and appears to occur as blood pressure

increases; therefore, endothelial dysfunction is more a consequence than a cause of hypertension. Aging meanwhile is a physiological process associated with an increase in cardiovascular morbidity and mortality, even in the absence of known cardiovascular risk factors (Rodgers et al. 2019). This may be related to cellular changes in response to increased oxidative stress or to other factors such as decreased release of vasoactive mediators. Although plasma endothelin levels increase with age, the response to decreased endothelin concentration is presumably due to downregulation of receptors in most vessels (Sena et al. 2018). Furthermore, it has been shown that high glucose levels in patients with diabetes cause endothelial dysfunction (Hadi and Al Suwaidi 2007). The underlying mechanism may involve increased endothelin synthesis and / or L-arginine NO pathway impairment. Vascular dysfunction due to high glucose levels seems to be mediated *in vivo* by vascular endothelial growth factor via the pathway associated with NO synthetase.

In general terms, endothelial dysfunction is characterized by an imbalance in vasodilator and vasoconstrictor factors derived from the endothelium in favor of the latter. In physiological conditions, functional eNOS produced NO in presence of arginine and BH_4. However, in case of arginine and/or BH_4 deficiency, eNOS becomes unfunctional leading to superoxide anion in place of NO (figure 31). This results to a potent reaction between NO and superoxide anion resulting to a less bioavailability of NO and therefore to vasoconstrictive effect.

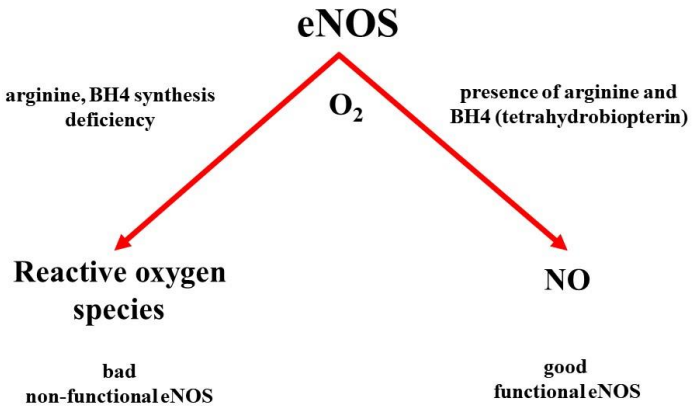


Figure 31. NO production by eNOS depending on arginine concentration (Pincemail, 2016).

It can be the cause or consequence of vascular diseases and is moderately known as a marker of cardiovascular risk factors. It is interesting that endothelial dysfunction precedes alterations in the vascular structure, indicating a protective role of functionally intact endothelium. While some vessels are particularly prone to develop endothelial dysfunction and atherosclerosis (epicardial coronary arteries, large arteries such as the aorta or iliac artery), others appear to be protective (the internal mammary artery, the brachial artery). Under healthy condition, endothelial cells synthesize and release a wide spectrum of vasoactive substances including NO promoting vasodilatation and maintaining endothelial functions. However, these that can be altered by risk factors associated with the progress of endothelial dysfunction such as obesity, hypertension, diabetes, uremia, and heart failure. In these conditions, the endothelial cells turns into a proinflammatory and prothrombotic state, characterized by a reduced bioavailability of NO counterbalanced by an increase in other substances and mediators that are harmful to the arterial wall (Di Pietro et al. 2020). Under oxidative condition, NO may react with O_2^- to form $ONOO^-$; this leads to the decrease of NO bioavailability leading to endothelial dysfunction, enhanced LDL peroxidation, and chronic vascular inflammation. This is associated with lipid accumulation in the arterial wall, an NF-κB activation that in turn triggers the upregulation of vascular adhesion molecules (VCAM-1) and intercellular adhesion molecules (ICAM-1). The increased

VCAM-1 and ICAM-1 membrane exposure leads to increased adhesion and infiltration of monocytes (Figure 32).

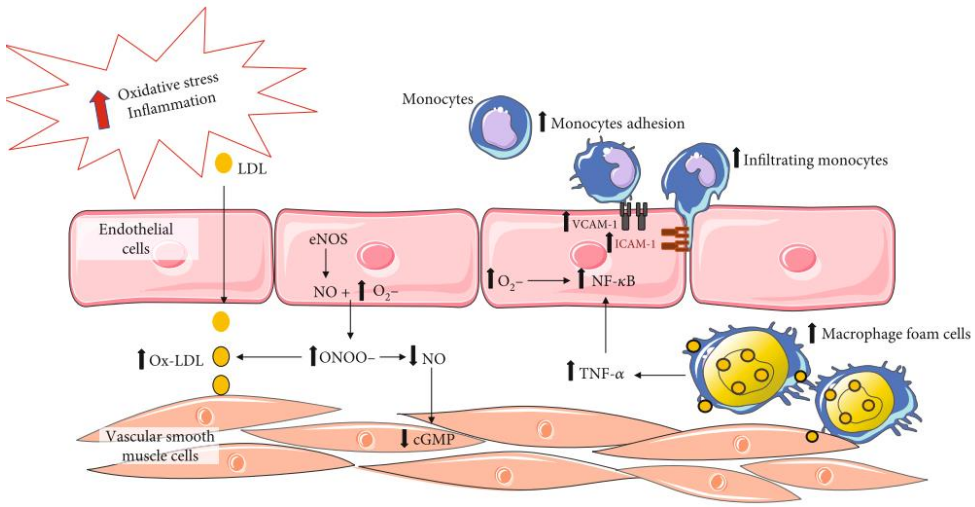


Figure 32: Endothelial dysfunction (Di Pietro et al. 2020)

4.5. Polyphenols and endothelium dysfunction

Countless studies support the beneficial action of flavonoids or products rich in flavonoids on endothelial dysfunction. Some flavonoids have protective effects on endothelial cells that increase the formation of certain protective factors, including NO, essential in delaying vascular aging. The study by Wu et al. (2006) showed that EGCG up-regulates HO-1 expression by activation of the Nrf2/ARE pathway in endothelial cell, conferring resistance against H_2O_2-induced cell death, suggesting a hormetic mechanism of action.

Polyphenols are limiting the effect of oxidative stress, their main property is related on their structure which is characterized by the presence of one or more phenolic groups, capable of reducing ROS, and various organic substrates and minerals (Pandey and Rizvi 2009). NADPH oxidase enzymes are major producers of ROS, generating species such as hydrogen peroxide and superoxide. Excessive ROS production has been linked to endothelial dysfunction leading to the origin of CVD (Scioli et al. 2020). Steffen et al.

(2008) tested the role of several polyphenols in oxidative stress in endothelial cells. They established a set of structural requirements for scavenging of ROS and inhibition of NADPH oxidase function as follows:

- superoxide scavenging activity is mediated by flavonoids lacking additional substitutions in ring B. Polyphenols in this category included catechin, epicatechin, quercetin, luteolin and fisetin.
- inhibition of NADPH oxidase activity requires additional substitutions in ring B. For example, O-methylation in ring B (as in 3-O-methyl-epicatechin, isorhammentin and tamarixerin), the presence of a 4'-OH group in ring B (as in kaempferol and apigenin) and the addition of an extra OH group in ring B (as in EGC).
- hydrogenation of the C2-C3 double bond in ring C, as in dihydrokaempferol, taxifolin and naringenin.
- also, it seems that the presence of a OH or methyl group in an aromatic ring provided inhibitory effects in NADPH oxidase activity as seen for resveratrol, caffeic acid and ferulic acid and for gallic acid and 3-O-caffeoylquinic acid (3-CQA) (Figure 33) (Serino and Salazar 2019).

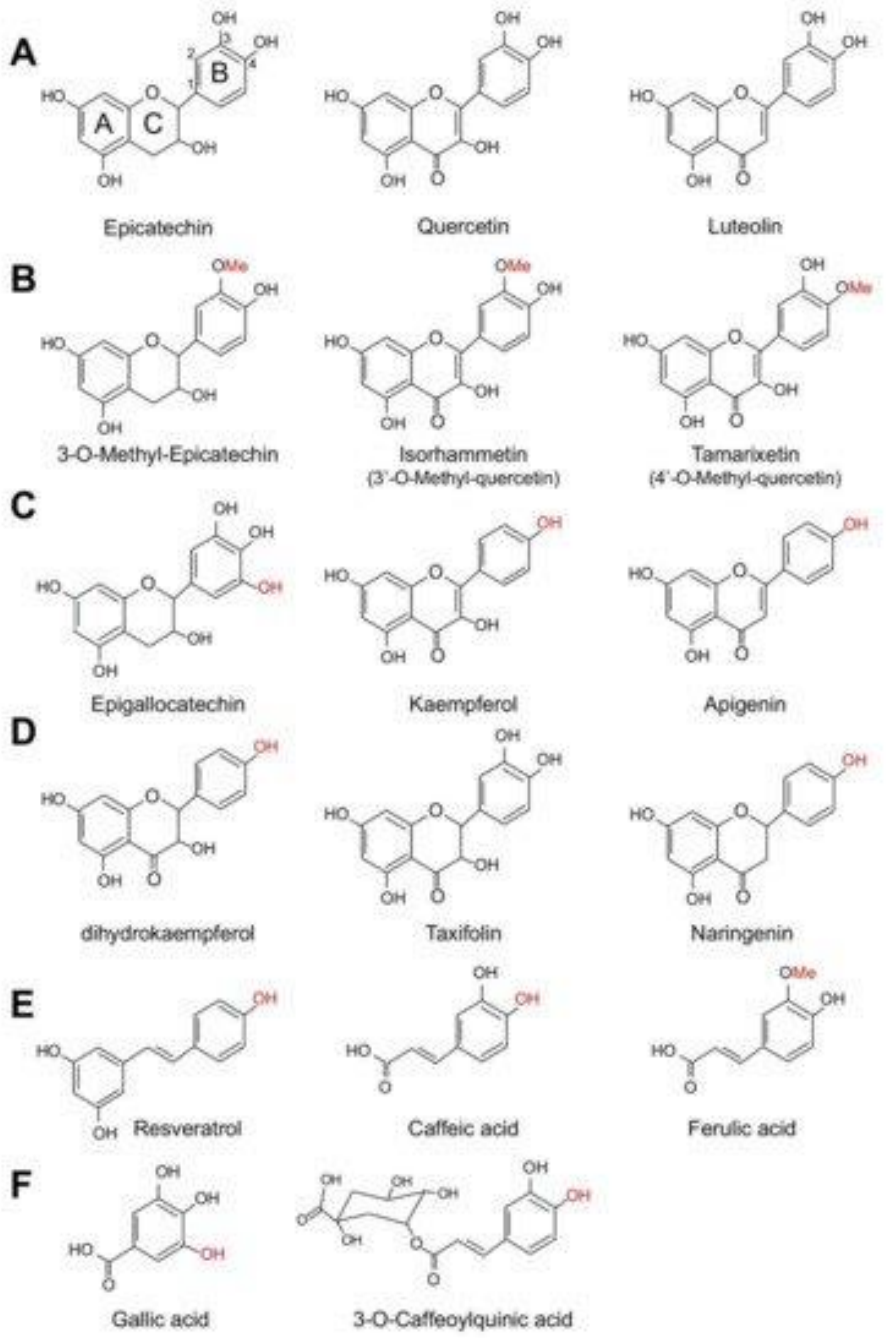


Figure 33. Structure of major polyphenols involved in the regulation of NADPH oxidase activity and expression. (A) Shows the structure of unmodified cathechin; (B–E) show hydroxylated and O-methylated compounds shown to affect NADPH oxidase activity in several cell types and treatment conditions; (F) shows the structure of two phenolic compounds found in blackberry polyphenol extract that according Serino and Salazar (2019) could modulate NADPH oxidase activity and Nox1 expression (Serino and Salazar 2019)

Several polyphenols inhibit the expression and/or activity of signaling pathways involved in senescence. They may inhibit upstream regulators of these molecules. For example, quercetin is associated with downregulation of Nox1, resveratrol reduced Nox4 levels in human umbilical endothelial cells, extracts from blackberry, raspberry and black raspberry reduced NADPH oxidase activity induced by Ang II, and delphidin was associated with upregulation in the expression of nuclear respiratory factor 1 (Nrf1) (Serino and Salazar 2019) (Figure 34).

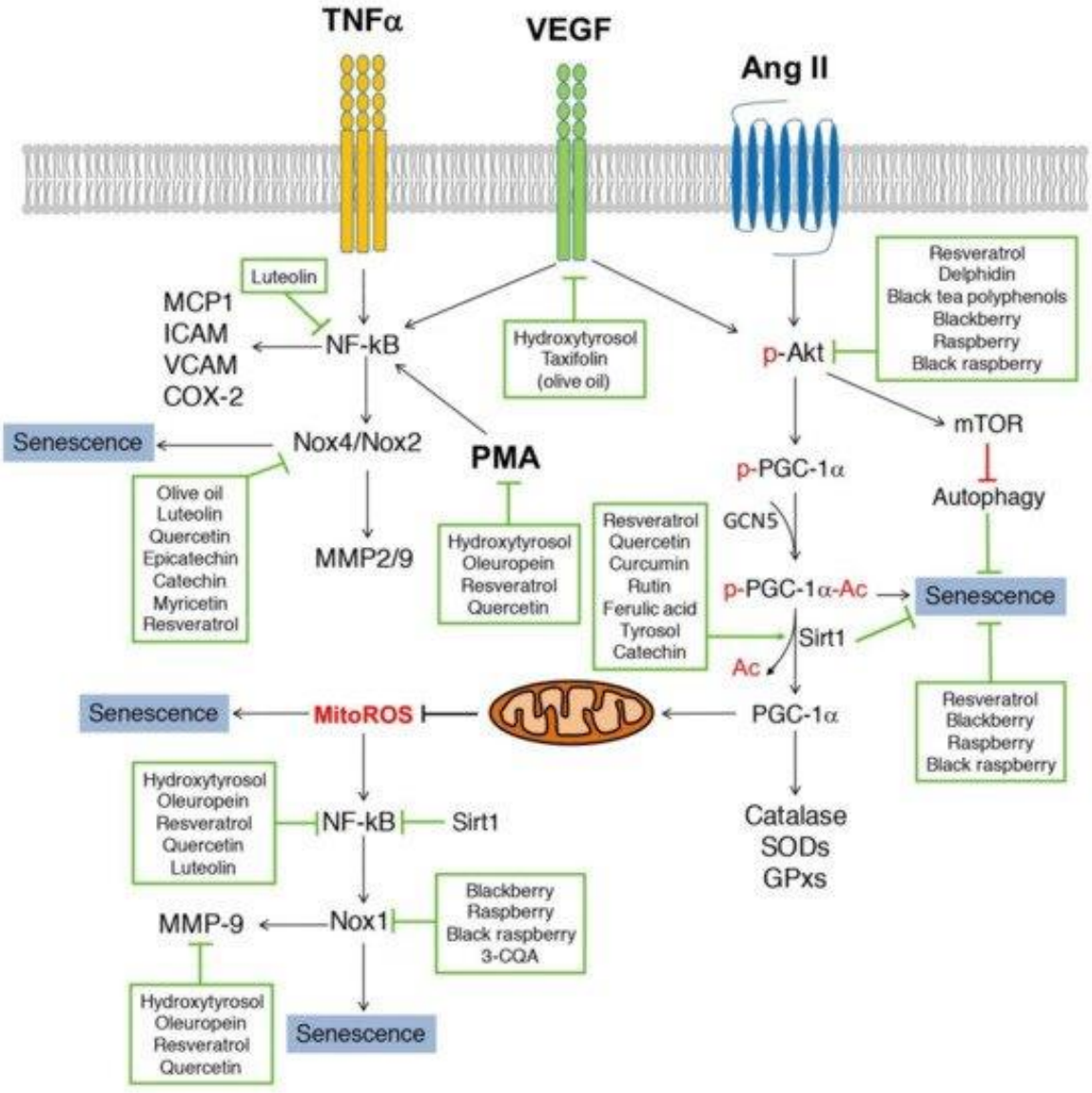


Figure 34. Proposed model by which polyphenols may modulate oxidative stress-induced pathways (Serino and Salazar 2019)

Chapter 5. Critical approach to antioxidant capacity analysis methods.

There are several methods that allow determining the antioxidant capacity of biochemical compounds or of food samples. However, the applicability of one or the other depends mostly on their popularity. The following assays are based on a single electron transfer reaction (SET): DPPH (2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity), FRAP (ferric reducing ability of plasma), CUPRAC (cupric ion reducing antioxidant capacity), and TEAC (Trolox equivalent antioxidant capacity). The following methods are based on a hydrogen atom transfer reaction (HAT): TRAP (total peroxyl radical trapping antioxidant parameter), Crocin bleaching assay, and ORAC (Oxygen Radical Antioxidant Capacity) assay. Among several methods, the latter became an essential marketing argument for industrialists aiming to promote antioxidant properties of foods, and of fruits and vegetables juices particularly. ORAC is a method based on thermal degradation of azo-compounds that produce free radical damage to a fluorescein probe, resulting in a loss of fluorescent intensity over time (Ou et al. 2001). This method, despite being one of the most popular and most used by the industry, has some limitations such as physiological non-relevance, little standardization, and poor reproducibility.

There are several studies carried out to determine the antioxidant capacity of fruit juices using different methods. The results vary from one author to another. Tabart et al. (2009) carried out a comparative study of the different detection methods of the antioxidant capacity of various drinks and their phenolic compounds. In this study marked differences were found in the antioxidant capacity according to the detection method (DPPH, ORAC, TEAC). Among all evaluated drinks (apple, orange, grape, and vegetable juice, ice green tea, and red wine), DPPH method was the least sensitive and the ORAC the most, with grape juice and red wine having the highest antioxidant capacity. When evaluating the antioxidant capacity of the different subclasses of polyphenols present in the same beverages, it was found that the ORAC method presented at least 3 times more activity in comparison to the other methods. One of the aspects to consider when using one or another detection method is the extraction medium (methanol, acetone), since this considerably influences the phenolic compound extraction, as shown by Suárez et al. (2010) in an evaluation of apple juice antioxidant capacity. With extractions in ethanol, the extracts presented

44% less total phenolic compounds and 30% less antioxidant activity (DPPH) with respect to acetonic extractions. Among the limitations of the DPPH method, is the fact that the compounds tend to overlap $DPPH^{\bullet}$ at the same λ of the long-lived nitrogen radical due to its steric inaccessibility (may not react with antioxidants) (Laher 2014).

An evaluation by Khaw, et al. (2016), found significant differences regarding the antioxidant capacity of fresh fruit juices, commercial 100% fruit juices and fruit drinks (apple grape, pomegranate, pineapple, orange), using the DPPH and FRAP methods. The fresh fruit juices presented an average antioxidant capacity of 376 (DPPH) and 384 (FRAP) μmol TE/100 mL, while the commercial 100% fruit juices showed 856 (DPPH) and 909 μmol TE/100 mL. Fruit drinks (generally with high sugar content) showed values of 35 and 37 TE/100 mL for DPPH and FRAP, respectively. Both methods were highly correlated with the total content of polyphenols.

Clinical trials have also been carried out to evaluate the antioxidant capacity of fruit juices in blood plasma. The methods used by Pedersen et al. (2000) to evaluate antioxidant capacity of blueberry and cranberry juice were FRAP and Spin Resonance Spectroscopy (SRE). Consumption of 500 mL of cranberry juice was associated with a significant increase ($P < 0.001$) in antioxidant capacity of plasma, (in nine healthy female volunteers (23 ±41 y)) remaining stable up to 4 hours after consumption. Çam, et al. (2009), using the TEAC method, evaluated the antioxidant capacity of pomegranate juices from different cultivars (n = 8), with an average value of 322.6 TEAC mg/100 mL.

Some characteristics of the methods used for evaluations of antioxidant capacity are detailed in the following table (Table IV), in which we can observe the advantages and disadvantages for their applicability, as well as the calculations and chemical reagents used in each of them.

Due to different free radical generating systems, there is no correlation between the tests described in table IV and also with the polyphenol content of different food matrixes (Tabart et al. 2009). Moreover, a major criticism is that all free radical systems used in the different assays have no physiological meaning. Although the production of superoxide anion and other free radicals have been detected by electron spin resonance (ESR) or the cellular antioxidant activity (CAA), these tests do not have biological relevance. To evaluate the antioxidant capacity of fruit

and vegetable juices, it appears highly desirable to have an experimental assay in which a physiological radical is produced and that the conditions of the assay mimic *in vivo* conditions.

With the previously described background of the importance of NOX in the generation of ROS and specifically the superoxide anion, the method developed by Baptista et al. (2012), which is based on the measurement of superoxide anion production in whole blood cells, is one of the most interesting alternative for measuring the antioxidant capacity of fruit juices in *ex vivo* assays. During the respiratory burst in whole blood stimulated by phorbol 12-myristate 13-acetate (PMA) (a specific activator of protein kinase C thus leading to activation of NADPH oxidase), the superoxide anion is generated not only in leukocytes and polymorphonuclear monocytes but also in lymphocytes. Superoxide anion overproduction is detected by luminol-dependent chemiluminescence. The luminescence intensity is normalized by the leukocyte count. Response of PMA-free whole blood simultaneously incubated is used as control. Percentage of activation of NADPH oxidase (PMA-induced superoxide production x 100/PMA-free superoxide production) is used as an index of PMA-induced overproduction of anion superoxide. Results are obtained within 30 minutes, as opposed to the two hours needed for the ORAC assay. This method has a greater sensitivity in the detection of antioxidant capacity.

Table IV. List of antioxidant assays based on single electron transfer and hydrogen transfer reaction (Pincemail et al. 2014).

Method	Free radical/generator/ oxidant	Standard	Measure/calculations	Advantages	Limitations
TEAC assay	ABTS+ potassium persulfate ($ABTS^{+}$)	Trolox	Decolorization of the ABTS•, at λ=734 nm (Infrared) unit: TEAC (Trolox equivalents)	Simple radical is stable hydrophilic and lipophilic measures automation redox potential: 0,68 V – phenolic compounds	ABTS•+ is not a ROS short duration (4–6 min) – not representative samples can absorb at the same λ.
FRAP assay	FRAP reagent-Fe (III) $(TPTZ)_2Cl_3$	Fe (II) solution	Change of absorbance at λ=593 nm (UV-vis) unit: FRAP (related to Fe (II) solution	Simple, fast automated redox potential: 0,70 V like TEAC (except for the acidic pH)	Short duration (4 min) reaction of Fe (III) with chelators in samples cannot detect thiols, glutathione and proteins does not take in account the quantity of inhibition, no free radicals are introduced in the system.
CUPRAC assay	Phenanthroline Compound	Uric acid	Absorbance at λ=450 nm (UV-vis) related to uric acid	Lower redox potential (more selective) can detect all classes of antioxidants	Limited water solubility variable duration of time (depends on the molecules)
DPPH assay	DPPH•		Fade of color, at λ=515 nm (UV-vis) % DPPH REM antiradical efficiency	Stable nitrogen radical simple, rapid	the compounds tend to overlap DPPH• at the same λ of the long-lived nitrogen radical due to its steric inaccessibility (may not react with antioxidants), DPPH is decolorized by reducing agents.
Crocin bleaching assay	AAPH (peroxyl radicals)	Trolox	Crocin's bleaching λ= 443 nm (UV-vis) (ka/kc) (antioxidant)/ (ka/kc) (Trolox)	Automation	Temperature control is critical, crocin is natural product, so is subject to lot variability interference with other substances.
TRAP assay	ABAP or AAPH (peroxyl radicals)	Trolox	ß-Phycoerythrin's decay in fluorescence (λex = 495 nm and λem= 575 nm) unit: TRAP (Trolox equivalents)	Sensitive to all chain-braking antioxidants, it can use DCFH-DA instead of ß -PE to form DCF which can be monitored both by fluorometric or spectrophotometric ways	Measures duration of time (variable), difficulties to compare results, the apparatus does not measure the las phase considers that lag phase is proportional to AC.
ORAC assay	AAPH (peroxyl radicals)	Trolox	Fluorescein's decay in fluorescence (λex = 485 nm and λem= 538 nm) AUC unit: (Trolox equivalents)	Simple, sensitive, and reliable automation, hydrophilic and lipophilic measures, measures both inhibition time and degree of inhibition, takes reaction into completion	Temperature control is critical, physiological non-relevance, little standardization, poor reproducibility.

OBJECTIVES

Several studies carried out around the world showed the importance of antioxidants for the elimination of excess free radicals. Timely control of these can reduce the damage to lipids, proteins, and nucleic acids. The benefits of diets rich in polyphenols in the human body continue to be a topic of interest. There is a lot of information related to the consumption of products rich in polyphenols and their effect on cell oxidative stress (Cheng et al. 2017) and on vascular function (Schroeter et al. 2006; Schwingshackl and Hoffmann 2014). But there is little information that allows us to identify the individual effect of polyphenols on the vascular endothelium, including knowing if these active concentrations correspond to physiological concentrations.

The aim of this thesis is to show the ability of several phenolic-compound-containing fruit and vegetable juices to mitigate cell oxidative stress and to improve *ex-vivo* endothelial function. Different points will be developed:

a) Characterization of the total content of polyphenols, as well as the individual content of flavonoids present in various commercial juices.

b) Determination of the antioxidant activity by DPPH, ORAC and inhibition of superoxide anion assayed by the chemiluminescence (a physiological method).

c) Determination of the effects of these juices on the vascular reactivity of *ex vivo* rat artery.

Results (Part 1): Antioxidant capacity of fruit and vegetable juices

It has been demonstrated that the consumption of foods rich in polyphenols provides multiple benefits in the prevention of diseases. These benefits are largely attributed to the fact that polyphenols possess antioxidant properties that promote vasodilatory and Vaso protective, as well as antithrombotic (Bojić et al. 2019), antilipemic (W. Wang et al. 2016), antiatherosclerosis (Chou et al. 2019), anti-inflammatory (Schauss 2013) and antiapoptotic (Choi et al. 2003) actions.

We set a study with a new goal: to compare the antioxidant activities of fruit and vegetable juices as measured by *in vitro* assays and with a more physiological assay based on a chemiluminescence methodology. This led to a publication: *Ex Vivo Antioxidant Capacities of Fruit and Vegetable Juices. Potential In Vivo Extrapolation*, (Matute et al. 2021; Antioxidants 2021, 10(5), 770). In this work, a superoxide anion production was performed according to the protocol described by Baptista et al. (2012) on heparin-treated whole blood containing red blood cells, white blood cells, and platelets. The use of an *ex vivo* system combined with luminescence makes it possible to quantify respiratory burst and subsequent anion superoxide production. The use of an *ex vivo* assay led us to extrapolate towards a potential *in vivo* effect. It also allowed us to discuss on the bioavailability of polyphenols and on their plasma concentrations. These concentrations are generally much lower compared to those of other antioxidants such as vitamin C (60 µM) and vitamin E (30 µM), but with multiple preventive and regulatory properties.

antioxidants

MDPI

Article

Ex Vivo Antioxidant Capacities of Fruit and Vegetable Juices. Potential In Vivo Extrapolation

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Abstract: Background: In support of claims that their products have antioxidant properties, the food industry and dietary supplement manufacturers rely solely on the in vitro determination of the ORAC (oxygen radical antioxidant capacity) value, despite its acknowledged lack of any in vivo relevance. It thus appears necessary to use tests exploiting biological materials (blood, white blood cells) capable of producing physiological free radicals, in order to evaluate more adequately the antioxidant capacities of foods such as fruit and vegetable juices. Materials: Two approaches to assessing the antioxidant capacities of 21 commercial fruit and vegetable juices were compared: the ORAC assay and the "PMA–whole blood assay," which uses whole blood stimulated by phorbol myristate acetate to produce the superoxide anion. We described in another paper the total polyphenol contents (TPCs) and individual phenolic compound contents of all the juices were investigated. Results: Ranking of the juices from highest to lowest antioxidant capacity differed considerably according to the test used, so there was no correlation ($r = 0.33$, $p = 0.13$) between the two assays when considering all juices. Although the results of the ORAC assay correlated positively with TPC ($r = 0.50$, $p = 0.02$), a much stronger correlation ($r = 0.70$, $p = 0.004$) emerged between TPC and % superoxide anion inhibition. In the PMA–whole blood assay, peonidin-3-*O*-glucoside, epigallocatechin gallate, catechin, and quercetin present in juices were found to inhibit superoxide anion production at concentrations below 1 µM, with a strong positive correlation. Conclusions: Associated with the determination of total and individual phenolic compounds contained in fruit and vegetable juices, the PMA–whole blood assay appears better than the ORAC assay for evaluating juice antioxidant capacity.

Keywords: fruit and vegetable juices; polyphenols; ORAC assay; ex vivo inhibition of superoxide anion; chemiluminescence assay

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1. Introduction

Many papers have highlighted the beneficial role of polyphenols in preventing several human pathologies (cardiovascular and neurodegenerative disorders, cancer, diabetes [1–5]) where increased oxidative stress and inflammation are observed. The beneficial effects of polyphenols are mainly attributed to anti-inflammatory, antioxidant, and anti-platelet-aggregation properties; stimulation of gene-encoding antioxidant enzymes (hormetic effect); improvement in endothelial function leading to good regulation of blood arterial pressure; a decreased glycemic index; epigenetic regulation; and telomere length preservation [6].

Polyphenols are abundantly found in the human diet, particularly in fruits and vegetables and derived products such as juices. In 2018, consumption of fruit juice and nectar in Europe was 9.2 billion liters, with orange being the preferred flavor (36.5%), followed by

other fruits (21.8%), mixed flavors (19.2%), and apple (15.7%) [7]. Epidemiological studies have shown that fruit and vegetable juice intake may have a cardio-protective effect, but only a small impact on cancer development [8]. Moreover, interventional studies (focusing on acute or chronic conditions) with different juices (apple, orange, mandarin, cranberry grape, pomegranate) have evidenced decreased inflammation, arterial blood pressure, and plasma levels in some oxidative stress biomarkers associated with increased cardiovascular risk (lipid peroxides, oxidized LDL, carbonyl groups) [9–12].

The food industry has largely relied on antioxidant properties to enhance the health effects of its products. The popular in vitro ORAC (oxygen radical antioxidant capacity) assay, among several methods [13,14], has become the essential marketing argument for industrialists aiming to promote antioxidant properties of foods, and of fruits and vegetables juices in particular [15,16]. Yet the ORAC assay was very quickly criticized for many reasons: free radicals produced in vitro but having no physiological relevance, the absence of standardization, variable value expression, a great influence of the analytical procedure (making inter-laboratory comparisons of ORAC values impossible), etc. [17,18]. From a general point of view, the methods to determine the total in vitro antioxidant capacity of a food matric can be divided into two major groups: the methods based on single electron transfer reaction or SET (TEAC (trolox equivalent antioxidant capacity), FRAP (ferric reducing ability of plasma, CUPRAC (cupric ion reducing antioxidant capacity, and DPPH (2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity) assays and the methods based on hydrogen atom transfer reaction or HAT (crocin bleaching assay, TRAP (total peroxyl radical trapping antioxidant parameter)) and assays. Advantages and disadvantages of all these methods have been discussed earlier [14]. However, antioxidant properties and their potential beneficial effects of phenolic compounds can be strongly affected by both their bioaccessibility (digestion and absorption efficiency) and bioavailability (ratio of active ingredient absorbed and detected in the target site to the total amount of orally ingested drug products) [19]. Caco-2 cell models and the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) are two in vitro models allowing both parameters to be determined [20]. Recently, in vivo laboratory methods using the *boulardii* strain have been used to evidence the correlation between the antioxidant activity of phenolic compounds present in high amounts in plants and their bioavailability index [21]. Using a rat model, Gerardi et al. [22] evidenced that the antioxidant capacity of plasma as measured by DPPH and FRAP assays was increased after intake of pomace products in a dose-response effect. Nevertheless, the use of both assays to evidence in vivo antioxidant activity largely remains a matter of debate [23]. In a more accurate way, Curti et al. [24] showed in a mouse model that oral administration of whole brown propolis extract containing galangin is followed by rapid absorption and metabolization of the latter, resulting in adaptations of the antioxidant enzymatic defense system.

To evaluate the antioxidant capacity of fruit and vegetable juices, it appears highly desirable to have an experimental assay in which a physiological radical is produced and/or that mimics in vivo conditions. Under in vivo conditions, increased superoxide anion free radical production (univalent reduction of oxygen) results from mitochondrial respiratory chain [25,26] and endothelial [27] dysfunctions, xanthine oxidase activation [28], and activation of cellular NADPH oxidases (NOX proteins, the prototype of which is the phagocyte NADPH oxidase) [29–31]. With whole blood samples and phorbol myristate acetate (PMA) as activator of NADPH oxidase activity, a respiratory burst occurs in white blood cells, leading to superoxide anion overproduction detectable by luminol-dependent chemiluminescence [32].

In a previous work, we investigated how both the total and individual polyphenol contents of 22 commercial fruit and vegetable juices might regulate the ex vivo vasorelaxation of aorta segments isolated from rats [33]. On the basis of these data, we set in the present study a new goal: to compare the antioxidant activities of fruit and vegetable juices as measured by the ORAC and chemiluminescence assays.

2. Materials and Methods

2.1. Materials

Cyanidin-3-*O*-glucoside (CyG), catechin (C), epicatechin (EC), epicatechin gallate (ECG), gallo catechin (GC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), cyanidin-3-*O*-rutinoside (CyR), delpinidin-3-*O*-glucoside (DG), peonidin-3-*O*-glucoside (PG), malvidin (M), pelargonidin (Pel), peonidin (P), and petunidin (Pet) were purchased from Extrasynthese, Lyon, France. Kaempferol (Kaemp), myricetin (Myr), and quercetin (Quer) were obtained from Merck KGaA, Darmstadt, Germany. Phorbol 12-myristate 13-acetate (PMA), *N*,*N*′-Dimethyl-9,9′-biacridinium dinitrate (lucigenin), and fluorescein sodium salt were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), used as a peroxyl radical generator in the ORAC assay, was ordered from Fluka Chemie GmbH (Buchs, Switzerland).

Twenty-one commercial fruit and vegetable juices were selected from various Belgian and French supermarkets: (1) tomato (Carrefour), (2) tomato (Biotta), (3) carrot (Biotta), (4) orange (Carrefour), (5) pure orange (Vitamont), (6) lemon (Bonneterre), (7) grapefruit (Carrefour), (8) pure grapefruit (Vitamon), (9) grape (Materne), (10) pure grape (Vitamont), (11) pomegranate (Biotta), (12) blackcurrant (Biotta), (13) blackcurrant (Zimmers), (14) blackcurrant (Jacoby bio), (15) blackcurrant (Van Nahmen), (16) blackcurrant (Gut & Günstig), (17) blackcurrant (Albi), (18) pineapple (Carrefour), (19) pineapple juice (De Drie Wilgen), (20) apple (Carrefour), (21) pure apple (Vitamont). All juices were kept at +4 °C until analysis, which was performed within two days after purchase. If necessary, juices were filtrated in order to remove pulp.

2.2. Methods

The total polyphenol content (TPC) in all filtrated juices was evaluated in our previous paper [33] using the Folin–Ciocalteu method [34] directly after opening the bottles (limit of quantification (LOQ): 2 mg gallic acid equivalent (GAE)/L, $0.9 \leq R^2 \leq 1$). The determinations of specific phenolic compound (anthocyanin, flavanol, and flavonol families) amounts were also described in the same publication [33].

2.2.1. Inhibition of Superoxide Anion Production (Chemiluminescence Method)

The respiratory burst test was performed according to the protocol described by Baptista et al. [27] on heparin-treated whole blood containing red blood cells, white blood cells, and platelets. Briefly, a 200 µL blood sample from three subjects, 50 µL lucigenin (10^{-3} M), and 10 µL freshly and filtrated fruit or vegetable juice diluted 10 times in phosphate buffered saline (PBS, pH 7.4) were mixed and incubated for 15 min at 37 °C in microplates. After blood stimulation with PMA (4×10^{-6} M), resulting in increased superoxide anion production, the maximal fluorescence emitted over a 30 min period was measured in quadrupla with a GloMax® Multi Microplate Multimode luminometer (Promega, Madison, WI, USA). The difference between areas under the curves obtained in the presence and absence of PMA was defined as 100% activation. The percentage of superoxide anion inhibition was determined by calculating the area under the curve in the presence of both PMA and a fruit or vegetable juice. The limit of quantification (LOQ) of the assay was determined as LOQ_{SOD} by plotting the inhibition percentage vs. superoxide dismutase (SOD) as a specific inhibitor of superoxide anion, with concentrations in the range of 0 to 1.25 units SOD/mL as final concentration. This allowed us to determine that a cut-off of ≥20% inhibition corresponded to a LOQ_{SOD} of 0.5 U/mL.

Figure 1 shows typical chemiluminescence graphs recorded for juice 18 (pineapple, De Drie Wilgen, Nijlen, Begium) and juice 14 (blackcurrant, Jacobi Bio, Auggen, Germany). The protocol, consisting of drawing blood samples for antioxidant determination, was approved by the institutional ethics committee of Liège University Hospitals (B707201834834; reference 2017/342) and conducted in accordance with the 1964 Declaration of Helsinki and the European guidelines for good clinical practice.

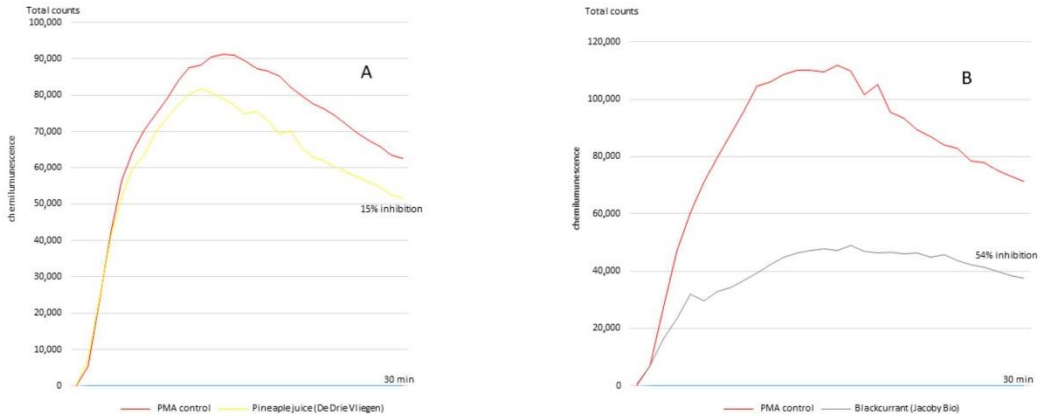

Figure 1. Examples of superoxide anion inhibition by pineapple (De Drie Wilgen) or blackcurrant (Jacoby bio) juice (panels (**A**) and (**B**), respectively) in PMA-activated whole human blood, as determined by chemiluminescence detection (30 min). The area under the curve for each analyzed juice yields the percentage of superoxide anion inhibition in relation to the PMA control.

2.2.2. Oxygen Radical Antioxidant Capacity (ORAC Assay)

AAPH (2,2-azobis [2-amidinopropane] dihydrochloride) was used as a peroxyl radical generator and fluorescein as a fluorescent probe. Filters were used to select an excitation wavelength of 485 nm and an emission wavelength of 535 nm [18]. A total of 175 µL mixture containing fluorescein (3 µM), and AAPH (221 mM) was injected into each well of the microplate. All solutions were prepared in phosphate buffer 1M pH 7.4. Then 25 µL filtrated juice at an appropriate dilution, blank, or Trolox calibration solution (50–200 µM) were added. The fluorescence at 37 °C was recorded every 2 min for 1.5 h with a Victor3 multiplate recorder (Perkin Elmer, Zaventem, Belgium). The final ORAC value was calculated from the net area under the fluorescence decay curve. All assays were performed in triplicate. Results are expressed in micromoles of Trolox equivalents per liter (µM TE) (LOQ: 15 µM TE, $0.9 \leq R^2 \leq 1$).

2.3. Statistical Analyses

Correlations between ORAC values, TPC (expressed in µg GAE/mL), total and individual flavonol, flavanol, and anthocyanin concentrations, as well as percent superoxide anion inhibition, were calculated with Sisvar 5.6 software (University of Lavras, Lavras, Brazil). Pearson correlations were considered significant at $p < 0.05$.

3. Results

As shown in Table 1, already published in [28], the range of TPC values was wide. Juice 1 (tomato, Carrefour, Brussels, Belgium) showed the lowest value (214 µg GAE/mL) and juice 9 (Grape, Materne, Floreffe, Belgium), the highest (1564 µg GAE/mL). The mean TPC for the juices investigated was 852 ± 411 µg GAE/mL. As shown in a previous paper [28], the average flavonol and flavanol concentrations were, respectively, 6.3 ± 4.8 and 108 ± 254 µg/mL. Among the 21 tested juices, only the nine red ones (blackcurrant, grape, pomegranate) contained detectable levels of anthocyanins [28]. The mean total anthocyanin content was 131 ± 123 µg/mL for the nine red juices and 173 ± 132 µg/mL for the six blackcurrant juices considered alone.

Table 1 also shows that all blackcurrant and pomegranate juices caused more than 50% inhibition of superoxide anion production in PMA-activated whole blood. All the other juices showed superoxide anion scavenging activity in the range of 13.3 to 41.2%. The antioxidant profiles obtained with the ORAC assay were different: Only juices 8t

(pure grapefruit, Vitamon, Monflanquain, France), 9 (grape, Materne, Floreffe, Belgium), 12 (blackcurrant, Biotta, Tägerwilen, Swiss), 17 (blackcurrant, Albi, Tournefeuille, France), and 21 (pure apple, Vitamont, Monflanquain, France) exhibited an ORAC value above 10,000 µM TE. A group of 13 juices [4–7,10,11,13–16,18–20], including the red ones, had ORAC values between 6074 (orange d'Espagne, Carrefour, Brussels, France) and 9259 (pure orange, Vitamont, Montflanquain, France) µM TE. Tomato and carrot juices exhibited the lowest values (below 4000 µM TE) of all the tested beverages. Data were strongly different when using the PMA-activated whole blood. For example, juice 9 (grape, Materne, Floreffe, Belgium) had the highest ORAC value (15,603 µM TE) but ranked eighth in terms of its ability to reduce superoxide anion production in the PMA–whole blood assay (41.2%). Conversely, juice 15 (blackcurrant, Van Nahmen, Hamminkeln, Germany) showed the greatest capacity to inhibit superoxide anion production (64.4%), but ranked 11th according to its ORAC value (7681 µM).

Table 1. Total polyphenol content (TPC) [28], Oxygen Radical Capacity (ORAC) values, and % of superoxide anion inhibition by PMA-activated-whole blood assay of 21 commercial fruit and vegetables juices (n = 3 for TPC and ORAC assay; n = 4 for % superoxide anion inhibition). Italic numbers into brackets indicate the ranking from highest to lowest antioxidant capacity of all juices according to the test used.

Number	List of Juices	TPC (mg GAE/L)	ORAC (µM TE)	% Superoxide Anion Inhibition (PMA-Whole Blood)
1	Tomato (Carrefour)	213.6 ± 68.1	2428.7 ± 68.2 *(21)*	18.0 ± 5.9 *(16)*
2	Tomato (Biotta)	358.1 ± 26.0	3117.8 ± 290.1 *(20)*	19.8 ± 19.0 *(15)*
3	Carrot (Biotta)	338.9 ± 5.6	3825.9 ± 840.0 *(19)*	34.7 ± 6.5 *(9)*
4	Orange d'Espagne (Carrefour)	541.6 ± 90.1	6074.3 ± 745.2 *(18)*	14.9 ± 4.6 *(19)*
5	Pure orange (Vitamont)	385.1 ± 155.8	9259.3 ± 288.2 *(6)*	22.6 ± 7.7 *(12)*
6	Lemon (Bonneterre)	1167.5 ± 217.3	6346.3 ± 628.6 *(17)*	29.8 ± 17.4 *(10)*
7	Grapefruit	496.8 ± 13.1	6936.5 ± 1482.1 *(13)*	13.3 ± 3.7 *(20)*
8	Pure grapefruit (Vitamont)	537.6 ± 45.8	13,805.2 ± 591.3 *(2)*	21.5 ± 9.1 *(14)*
9	Grape (Materne)	1564.0 ± 588.3	15,603.6 ± 458.5 *(1)*	41.2 ± 3.3 *(8)*
10	Pure grape (Vitamont)	643.4 ± 56.6	6461.7 ± 808.7 *(15)*	27.1 ± 2.7 *(11)*
11	Pomegranate (Biotta)	1331.9 ± 183.2	9046.1 ± 176.8 *(8)*	51.9 ± 5.4 *(6)*
12	Blackcurrant (Biotta)	1088.8 ± 80.8	11,256.7 ± 380.1 *(4)*	54.3 ± 7.4 *(2)*
13	Blackcurrant (Zimmers)	1392.2 ± 202.7	9219.4 ± 466.0 *(7)*	54.1 ± 4.9 *(3)*
14	Blackcurrant (Jacoby Bio)	1135.6 ± 93.4	9035.1 ± 90.6 *(9)*	51.5 ± 2.5 *(7)*
15	Blackcurrant (Van Nahmen)	1250.9 ± 186.8	7681.7 ± 422.9 *(11)*	64.4 ± 12.4 *(1)*
16	Blackcurrant (Gut & Günstig)	1131.1 ± 210.8	6636.8 ± 577.5 *(14)*	53.2 ± 8.5 *(5)*
17	Blackcurrant (Albi)	1121.6 ± 166.2	12,722.9 ± 622.4 *(3)*	53.5 ± 6.9 *(4)*
18	Pineapple juice (Carrefour)	1018.6 ± 60.8	6363.1 ± 1141.8 *(16)*	17.2 ± 4.5 *(17)*
19	Pineapple juice (De Drie Wilgen)	369.6 ± 21.6	8700.1 ± 213.6 *(10)*	17.2 ± 3.4 *(18)*
20	Apple (Carrefour)	1035.3 ± 8.7	7351.3 ± 2924.7 *(12)*	11.9 ± 6.8 *(21)*
21	Pure apple (Vitamont)	776.4 ± 121.0	10,682.4 ± 1965.4 *(5)*	21.6 ± 2.8 *(13)*

Figure 2 depicts graphically the correlations found between the three assays. All beverages considered, a significant positive correlation was observed between TPC and the ORAC assay (r = 0.50, p = 0.02). A stronger and more significant correlation was observed between TPC and % superoxide anion inhibition (r = 0.70, p = 0.004). It is noteworthy that no correlation was observed between % superoxide anion inhibition and the ORAC assay (r = 0.33, p = 0.13)

Knowing the contents of all the juices in individual phenolic compounds of the flavonol, flavanol, and anthocyanin families (see our paper [28]) and the volume (10 µL) of 10-times-diluted juice added in the whole blood assay (270 µL), it was easy to calculate the exact mass concentration (µg/mL) of each phenolic compound in the reaction medium associated with the % superoxide anion inhibition, as established in Table 1. After the conversion of mass concentrations (µg/mL) to molar concentrations (µM) for each individual phenolic compound and taking into account the whole juices, Table 2 shows that the mean concentrations of Myr, Querc, Kaemp, PG, ECG, C, and EC (potentially associated with decreased superoxide anion production in PMA-stimulated whole blood) were low, ranging from 0.06 ± 0.072 (Myr) to 0.008 ± 0.014 (C) µM. Table 3 shows, for the whole set

of juices, the correlations between concentrations of individual phenolic compounds on the one hand and either the ORAC value or the percentage of superoxide anion inhibition on the other. Only the Myr, ECG, and C concentrations were found to correlate positively and significantly with the ORAC values, whereas very strong correlations were observed between superoxide anion inhibition and PG ($r = 0.87$, $p = 0.010$), EGCG ($r = 0.67$, $p = 0.0009$), and, to a lesser extent, C ($r = 0.47$, $p = 0.02$) and Q ($r = 0.46$, $p = 0.036$).

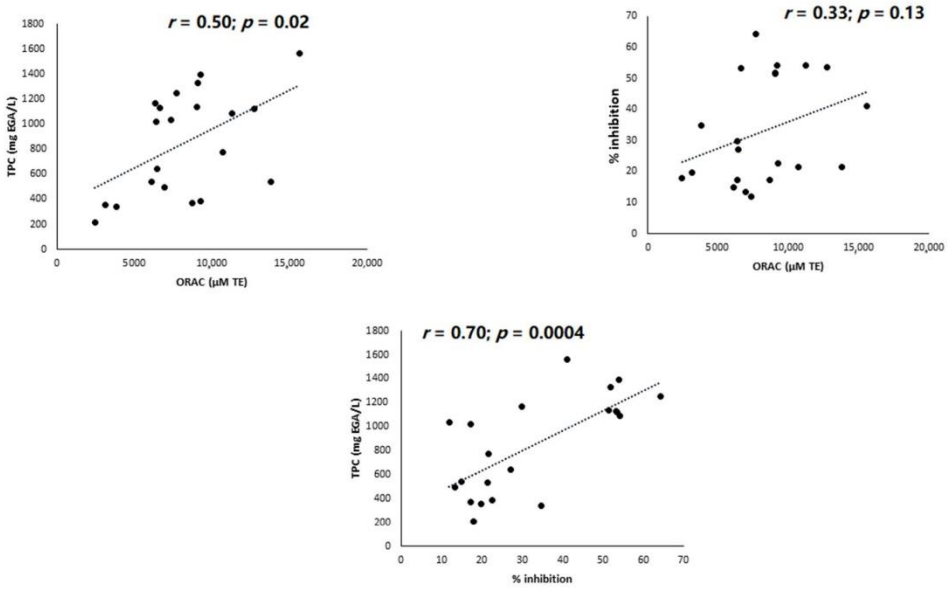

Figure 2. Graphic correlations between total polyphenol content (TPC), Oxygen Radical Antioxidant Capacity (ORAC) values, and % superoxide anion inhibition. EAG: equivalent gallic acid; TE: trolox equivalent.

Table 2. Mean concentrations (µM), established for the whole set of juices, of phenolic compounds present in the PMA–whole blood assay (260 µL) associated with inhibition of superoxide anion production.

	Flavonols (µM)
Myr	0.060 ± 0.072
Quer	0.039 ± 0.019
Kaemp	0.011 ± 0.009
	Flavanols (µM)
EGC	0.949 ± 3.266
EGCG	0.332 ± 0.472
ECG	0.043 ± 0.040
C	0.008 ± 0.014
ECG	0.043 ± 0.033
	Anthocyanins (µM)
DG	0.745 ± 1.531
DR	1.633 ± 3.540
CyG	0.196 ± 0.308
CyR	1.424 ± 2.720
PG	0.005 ± 0.005
GC	0.367 ± 1.141

Table 3. Correlations, established for the whole set of juices, between individual phenolic compounds present in the PMA–whole blood assay and either the ORAC values or the % inhibition of superoxide anion production.

Phenolic Compounds	ORAC		% Inhibition	
	r	*p*-Value	*r*	*p*-Value
DG	0.32	0.40	0.17	0.66
DR	0.37	0.32	0.19	0.63
CyG	0.36	0.34	0.22	0.57
CyR	0.29	0.44	0.18	0.65
PG	0.25	0.52	0.87	0.010
GC	0.04	0.87	0.21	0.33
EGC	0.11	0.62	0.28	0.20
EGCG	0.30	0.19	0.67	0.0009
ECG	0.45	0.043	0.26	0.24
C	0.47	0.032	0.47	0.02
EC	0.28	0.21	0.04	0.99
Myr	0.48	0.027	0.33	0.14
Quer	0.21	0.36	0.46	0.036
Kaemp	0.13	0.57	0.24	0.30

4. Discussion

In order to claim health effects of fruit- and vegetable-derived products such as juices, the food industry has highlighted their antioxidant properties. To do so, manufacturers have relied on the first database of ORAC values published in 2007 by the United States of Department of Agriculture (USDA) [35], containing 277 selected foods at the time and extended to 326 foods in 2010 [36]. Despite the counterarguments of long-time ORAC researcher R. Prior, the USDA decided in 2012 to withdraw its web publication of ORAC values due to lack of scientific evidence that ORAC has in vivo relevance in terms of human health [37]. The USDA also mentioned that ORAC values were misused by food and dietary supplement companies as the sole basis for guiding consumer choices [37]. Even when a strong positive correlation was evidenced between ORAC values and the TPC of a food matrix, the European Food Safety Authority (EFSA) issued guidelines that forbade claiming, on food product labels, an antioxidant benefit on the basis of data obtained from any in vitro assay, including the TRAP, FRAP, TEAC, ORAC, and FOX assays [38,39]. Nevertheless, numerous food companies and marketers continue to promote products having the "highest" ORAC values. One major criticism formulated against the ORAC assay is that the generated free radicals result from the thermal decomposition of a purely chemical compound and that they therefore have no in vivo relevance. Furthermore, the fact that ORAC values are strongly affected by experimental conditions makes inter-laboratory comparisons impossible [18]. In the present paper we therefore suggest using an ex vivo blood test, in better agreement with physiological conditions, to test the antioxidant capacities of fruit and vegetable juices.

In vivo, the superoxide anion is the first physiological ROS resulting from the univalent reduction of oxygen. Among many possible in vivo sources of ROS, the NADPH oxidase family of enzymes (NOX1, NOX2, NOX4, NOX5) has been identified as an important one [29]. NOX2 is found notably in endothelial cells and adventitial fibroblasts, and also in phagocytic cells such as neutrophils (or polymorphonuclear leukocytes (PMNs)), macrophages, and monocytes. In resting PMNs, NOX2 is inactive. Upon exposure to stimuli, increased oxygen consumption (a respiratory burst) occurs in PMNs, leading to excess production of superoxide anion and hydrogen peroxide in the extracellular medium through activation of NOX2. There is strong evidence that excessive NOX2 oxidase activity in PMNs plays an important role in the development of vascular inflammation and cardiovascular disease, including hypertension (via inhibition of eNOS by superoxide anion), atherosclerosis, diabetes, cardiac hypertrophy, and heart failure [30,31]. As early as

1986, Pagonis et al. [40] described that some flavonoids, in their aglycone form (quercetin, kaempferol, morin), can limit ROS production by in vitro stimulated neutrophils. Possible mechanisms underlying ROS inhibition were attributed to NADPH oxidase inhibition through the blocking of phospholipase D (signaling pathway) [41] and, to a lesser extent, to direct superoxide anion scavenging [42]. Using luminol-dependent fluorescence, Zielinska et al. [43] evidenced that the intracellular production of ROS in stimulated neutrophils depends on the number of hydroxyl groups present on rings A and B of flavonoids, in agreement with other studies [44,45]. The antioxidant and anti-inflammatory activities of phenolic compounds, associated with protective effects against major chronic diseases such as cardiovascular disease, have been well described [46,47].

Phorbol myristate acetate, such as protein kinase C agonists, is well known to induce NADPH oxidase activation in neutrophils. In both isolated PMNs and whole blood, different detection techniques (fluorescence, chemiluminescence, accumulation of a dye in cells, electron spin resonance spectroscopy) have been used to evidence superoxide anion production. In the present study, we used the protocol described by Baptista et al. [32] involving PMA stimulation in whole blood and the detection of superoxide anion by chemiluminescence, involving an in-house-automated process using 96-well microplates introduced into a luminometer. Using this method to measure the antioxidant activities of polyphenol-rich fruit and vegetable juices, we achieved results within 30 min of the start of the test, as opposed to two hours with the ORAC assay. In contrast to this last test, Table 1 clearly highlights a common and powerful antioxidant capacity for all blackcurrant juices when using the PMA–whole blood assay. Figure 2 also shows that with this test, a greater and more significant correlation was observed ($r = 0.70$, $p = 0.0004$) between TPC and % superoxide anion inhibition than between TPC and the ORAC test ($r = 0.50$, $p = 0.02$). Of interest is the absence of correlation between the ORAC and PMA–whole blood assays, demonstrating the higher sensitivity of the latter in determining the antioxidant capacities of fruit and vegetable juices.

Unlike the ORAC assay, this ex vivo biological test on blood could allow the potential in vivo relevance of the presence of specific phenolic compounds in juice to be evaluated. Although the polyphenols of most classes in their aglycone forms are sufficiently absorbed through the intestinal barrier, their concentrations in the bloodstream reach only 1 µM, in contrast to other antioxidants such as glutathione (600 µM), vitamin C (60 µM), and vitamin E (10 µM). Manach et al. [48] showed the catechin (C) concentration in human plasma to range from 0.14 to 0.49 µM after ingestion of 0.36 mg/kg pure C. After consumption of flavonol at 110 mg/day for 14 days, the quercetin concentration in human plasma was found to increase from 0.078 µM, observed with a baseline diet, to 0.304 µM [49]. Liu et al. [50] found the plasma concentration of cyanidin-3-*O*-galactoside to peak at 0.0031 µM about two hours after ingestion of 100 g Saskatoon berries containing 123.5 mg of this compound. Giordano et al. [51] reported a plasma cyanidin-3-*O*-glucoside (CyG) level reaching 0.0040 µM one hour after ingestion of 500 mL orange juice containing 12.89 mg CyG. Rechner et al. [52] observed peak plasma concentrations of delphinidin-3-*O*-glucoside (0.006 µM), delphinidin-3-*O*-rutinoside (0.051 µM), cyanidin-3-*O*-glucoside (0.0035 µM), and cyanidin-3-*O*-rutinoside (0.024 µM) about one hour after ingestion of 330 mL blackcurrant juice concentrate containing 1 g total anthocyanins.

In our previous study [33], we determined in each fruit and vegetable juice tested here the precise amounts of phenolic compounds belonging to the flavonol, flavanol, and anthocyanin families. Table 2 shows that, all juices considered, the majority of phenolic compounds liable to inhibit superoxide anion production did so at mean concentrations in the physiological range (<1 µM). PG, an anthocyanin present in blackcurrant juices, appeared particularly effective, with a mean value as low as 0.005 µM. Table 3 shows that only PG, EGCG, C, and Q could be selected as potential in vivo-acting candidates, thanks to their strong and positive correlations with superoxide anion inhibition at potential in vivo concentration. According to previous studies [43,44], PG, Q, C, and EGCG are likely to exert superoxide anion scavenging activity because they are characterized by the presence of

additional –OH groups on ring B. Our observations were, however, opposite to experiments on stimulated human neutrophils showing that pure flavonoids such as quercetin had to be present at levels well above their physiological concentrations, i.e., in the range 1 mM (22.4 % inhibition) to 100 mM (90.7% inhibition), in order to exert "antioxidative" activity [43]. Possibly, therefore, the reduction in superoxide anion production by fruit juices in the blood test might result from synergy between all the phenolic compounds present in the beverages. Mechanistically, recent studies have also highlighted that the protective effects of dietary polyphenols can be attributed to hormetic pathways rather than to a direct antioxidant effect. In cells, polyphenols at low concentration may produce, through moderate autooxidation, small amounts of ROS capable of inducing, via activation of the Keap1/Nrf2/ARE pathway, the expression of gene-encoding antioxidant enzymes (antioxidant response element) and phase-2 enzymes [53–55].

Limitations of the PMA–Whole Blood Assay

Although it yields more precise information than the ORAC assay regarding the potential antioxidant capacities of fruit and vegetable juices and of their phenolic compounds in particular, the PMA–whole blood assay has limitations. Firstly, it is an invasive method requiring a blood test. Secondly, it does not take into account factors such as bioavailability, metabolism, or synergistic or antagonistic effects of phenolic compounds present in the beverages. The forms appearing in the blood are different from those found in foods. The only way to evaluate scientifically the antioxidant capacity of a given polyphenol-rich beverage is to conduct an in vivo human study. Subjects would be asked to consume a determined volume of juice with a well-characterized content of phenolic compounds. One to four hours later, the plasma concentrations of those compounds (and/or of their metabolites, if possible) would be measured and compared with pre-ingestion values (control). Then one could use the ex vivo PMA–whole blood assay, with concentrations of pure phenolic compounds similar to those detected in vivo, to get a better picture of potential in vivo "antioxidant" properties of fruit and vegetable juices.

5. Conclusions

Although used abundantly by food companies to support claims regarding health benefits of products such as fruit juices, the ORAC scores reflecting in vitro antioxidant activity are not adequate for this purpose. This is because this assay uses a non-physiological ROS, is greatly influenced by experimental conditions, and has no in vivo relevance recognized by food safety agencies. It is thus imperative to use more appropriate assays based on biological materials. An example is the ex vivo PMA–whole blood assay, which allows evaluating how some given juice rich in antioxidants, particularly polyphenols, interacts with the superoxide anion, i.e., the first physiological radical produced by univalent reduction of oxygen. Assessing the potential in vivo relevance of this assay, however, will require performing it in parallel with precise determinations of all phenolic compounds present in fruit juices.

Author Contributions: J.D. welcomed to his laboratory A.M., from Quevedo University, Equador, in the context of work towards a Ph.D. in Biochemistry and Molecular Biology from the University of Liège, Belgium. As Head of the Laboratory of Plant Molecular Biology and Biotechnology of University of Liège, J.D. was the main supervisor of A.M.'s work. J.P., A.M., J.T., and C.K. were the main investigators and contributors to drafting the present manuscript. They decided together to submit the paper for publication in *Antioxidants*, with the agreement of all the other authors. J.-P.C.-B. greatly contributed to the good running of experiments. J.-O.D. submitted the notice protocol to the local ethics committee. All authors have read and agreed to the published version of the manuscript.

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Results (Part 2): Phenolic compounds of juices and ex-vivo vascular reactivity

Today, dietary polyphenols are considered as interesting compounds for the prevention of several diseases. Many polyphenols are present in our diets in varying amounts. Of all the polyphenols, the flavonoids presents in fruits, vegetables and their derivate products are the molecules that have been shown through *in vivo, ex vivo* and *in vitro* studies to be responsible for reducing the risks of developing cardiovascular diseases (Habauzit and Morand 2012; Yamagata,et al. 2015; Mendonça et al. 2019). These cardioprotective effects are based on their antioxidant and pro-oxidant capacities, acting on various organs of the human body by decreasing oxidized LDL in blood and improving endothelial function (Khurana et al. 2013). Indeed, polyphenols can increase the bioavailability of nitric oxide through various metabolic pathways such as Sirt1/KLF2, PI-3-kinase-dependent phosphorylation of Akt, or transient increase in the calcium signal and activation of estrogen receptors.

The studies carried out to date suggest that the flavonoids present in commercial fruit and vegetable juices can promote both endothelium-dependent (Tabart et al. 2018) and endothelium-independent vasorelaxation (Kang et al. 2016), depending on the polyphenol plasma concentration. This is because the concentrations in the plasma differ between polyphenols.

There is few information from *in vitro* studies that consider the concentration of polyphenols in the blood, whether in free or conjugated forms. In this article, we sought to determine the concentration of polyphenols present in various commercial fruit and vegetable juices, as well as the different subclasses of flavonoids (anthocyanins, flavanols, flavonols) and their possible vasodilator effects at physiological concentrations on *ex-vivo* endothelial function.

We investigated how both the total and individual polyphenol contents of 22 commercial fruit and vegetable juices could regulate the *ex vivo* vasorelaxation of isolated rat aorta segments. This article was published in the journal Antioxidants: Compared phenolic compound contents of 22 commercial fruit and vegetable juices: Relationship to *ex-vivo* vascular reactivity and potential *in vivo* projection (Matute et al. 2020; Antioxidants 2020, 9, 92; doi: 10.3390 / antiox9020092).

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Compared Phenolic Compound Contents of 22 Commercial Fruit and Vegetable Juices: Relationship to Ex-Vivo Vascular Reactivity and Potential In Vivo Projection

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Abstract: The real impact of polyphenol-rich vegetable and fruit juice intake on cardiovascular health remains a matter of controversy. In the present study, rat aorta segments immersed in an organ bath (OB) were used to explore whether the total polyphenol content and/or individual phenolic compound contents of 22 commercial vegetable ($n = 3$) and fruit juices [(citrus ($n = 5$), berries ($n = 10$), apple ($n = 2$), pineapple ($n = 2$)] might be associated with vascular tone. Red juices (particularly blackcurrant) and lemon juice caused the most marked vasorelaxation, its amplitude being endothelium dependent or not according to the volume ratio of juice to initial OB solution V_{juice}/V_{OBS}). At volume ratios 5% and 10%, both the juice and OB total polyphenol for all juices and total anthocyanin contents for berry juices significantly correlated with aorta vasorelaxation intensity. This was not the case for total or individual flavonols (except kaempferol) or for total or individual flavanols (except epigallocatechin gallate). If one relates our measured concentrations of individual phenolic compounds in OB to what is known about their physiological concentrations, and given our evidenced correlations between compound concentrations and vasorelaxation intensity, kaempferol, epigallocatechin gallate and peonidin-3-*O*-glucoside seem to emerge as the interesting phenolic compounds likely to be responsible for the potent vasorelaxation observed with fruit juices, and more particularly blackcurrant ones. Clinical investigation is required, however, to confirm our observations.

Keywords: fruit and vegetable juices; phenolic compounds; vasorelaxation effect; rat aorta

1. Introduction

Long considered anecdotal, the role played by antioxidants, and particularly polyphenols, is now viewed as pivotal in human disease prevention [1–5]. According to their chemical structure, natural polyphenols can be divided into lignans, lignins, stilbenes, styrylpyrones, arylpyrones, coumarins, tannins, phenolic acids (i.e., benzoic and cinnamic acid derivatives), and flavonoids (i.e., flavonols,

flavanones, flavones, flavanols or catechins, anthocyans, and isoflavones). Flavonoids are particularly abundant in fruits, vegetables, and plant-derived products such as fruit juices, dark chocolate, green tea, red wine, coffee, and olive oil. Zutphen's study [6] was the first to evidence, in an elderly Dutch population, a strong inverse correlation between flavonoid consumption and the risk of developing cardiovascular disease. Using a food frequency questionnaire and the Phenol-Explorer database, Mendoça et al. [7] recently described the same relationship in a prospective cohort of 17,064 Spanish middle-aged university graduates (the "Seguimiento Universidad de Navarra" or SUN study). After adjusting for potential confounders, participants with a higher flavonoid intake (fifth quintile) showed a 47% lower incidence of cardiovascular events than those in the lowest quintile.

Polyphenols exert various actions likely to explain their cardioprotective effect. They notably have platelet aggregation and anti-inflammatory properties as well as ability to increase the high density lipoprotein (HDL)/low density lipoprotein (LDL) cholesterol ratio. They can also act as direct free radical scavengers but only at the stomach level. Despite low oral bioavailability and rapid metabolism, polyphenols may, however, at cellular level produce—through moderate auto-oxidation—small amounts of reactive oxygen species (ROS), which induces an antioxidant adaptive response in cells through the activation of the Keap1/Nrf2/ARE pathway. This results to over-expression of genes coding for antioxidant enzymes (Antioxidant Response Element) [8]. The physiological mechanism most often put forward to explain cardiovascular protection due to polyphenols is their ability to regulate arterial blood pressure by maintaining or improving endothelial function [9,10]. Indeed, polyphenols can increase the bioavailability of nitric oxide (NO), which has potent vasodilator properties. Many papers have evidenced an inverse relationship between endothelial dysfunction, as evaluated on the basis of flow-mediated dilation (FMD), and the risk of developing cardiovascular disease [11–13]. In contrast, important meta-analyses have clearly shown that a Mediterranean diet [14] or regular consumption of fruits, vegetables, or plant-derived products (dark chocolate, green tea, olive oil) can improve endothelium function and hence reduce both systolic and diastolic arterial pressure [15].

The aim of the present study was to examine the ability of several popular phenolic-compound-containing fruit and vegetable juices to improve ex-vivo endothelial function. Another objective was to identify the most effective subclasses of polyphenols and to see more generally whether a vasodilator effect could be observed in the presence of polyphenols at physiological concentrations (μM) [16].

2. Materials and Methods

2.1. Materials

The following reagents were purchased from Merck KGaA, Darmstadt, Germany: cyanidin-3-*O*-glucoside (CyG), catechin (C), picatechin (EC), epicatechin gallate (ECG), gallocatechin (GC), epigallocatechin (EGC), and epigallocatechin gallate (EGCG). Cyanidin-3-*O*-rutinoside (CyR), delpinidin-3-*O*-glucoside (DG), delphinidin-3-*O*-rutoside (DR), peonidin-3-*O*-glucoside (PG), malvidin-3-*O*-glucoside (MG), delphinidin (D), cyanidin (Cy), petunidin (Pet), pelargonidin (Pel), peonidin (Peo), malvidin (M), kaempferol, myricetin, and quercetin were obtained from Extrasynthese Lyon, France.

Further, twenty-two commercial juices were bought in Belgian, French and German supermarkets and classified as vegetable or fruit juices. These were: (1) Tomato (*Carrefour*), (2) Tomato (*Biotta*), (3) Carrot (*Biotta*), (4) Orange (*Carrefour*), (5) Pure Orange (*Vitamont*), (6) Lemon (*Bonneterre*), (7) Grapefruit (*Carrefour*), (8) Pure Grapefruit (*Vitamont*), (9) Grape (*Materne*), (10) Pure Grape (*Vitamont*), (11) Pomegranate (*Biotta*), (12) Blackcurrant (*Biotta*), (13) Blackcurrant (*Natreen*), (14) Blackcurrant (*Jacoby bio*), (15) Blackcurrant (*Van Nahmen*), (16) Blackcurrant (*Schlör Nectar*), (17) Blackcurrant (*Gut and Gunstig*), (18) Blackcurrant (*Jacoby*), (19) Pineapple (*Carrefour*), (20) Pineapple Juice (*De Drie Wilgen*), (21) Apple *(Carrefour*), and (22) Pure Apple (*Vitamont*) (Figure 2). All beverages were 100% pure vegetable or fruit juices except the blackcurrant ones that were nectar.

2.2. Determination of Total Phenolic Content

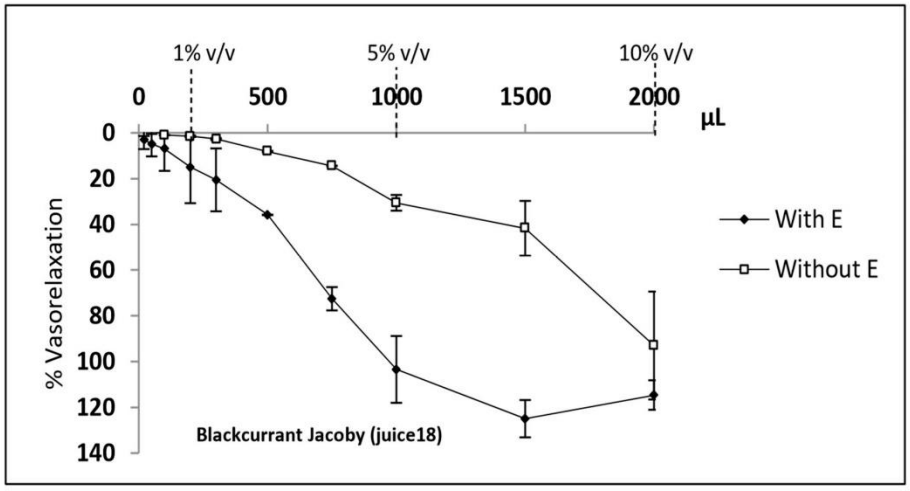
The total polyphenol content of each juice (TPC-J) was determined according to the Folin–Ciocalteu (F–C) method [17]. In a 96-well microplate, 20 µL of an appropriate juice dilution or standard solution (0–50 mg/L gallic acid) was mixed with 100 µL of 10% F–C reagent. After a 3-min incubation, 80 µL Na_2CO_3 solution (7.5% weight/volume) was added. The plate was then incubated at 30 °C for 1 h. The absorbance at 750 nm was measured with a microplate reader (Multiskan Ascent, Thermo Labsystems, Helsinki, Finland). Results are expressed in µg gallic acid equivalents/mL juice (µg GAE/mL, GAE: gallic acid equivalents).

2.3. Specific Flavonoid Contents

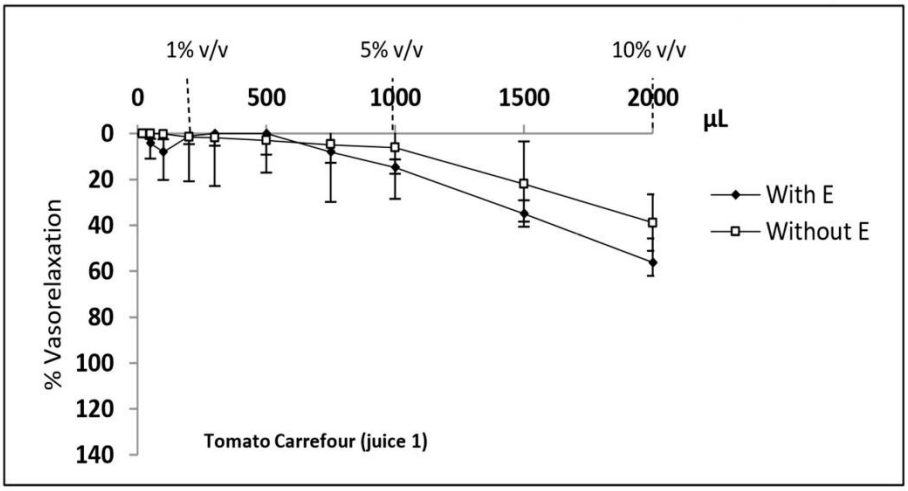
Separation and measurement of flavonols and anthocyanins were done by Ultra-Performance Liquid Chromatography (UPLC, Waters Corporation, Milford, MA, USA), according to protocols previously described by us [18]. The total flavonol content, expressed in µg/mL, was the sum of the quercetin, myricetin, and kaempferol contents. The total anthocyanin content, expressed in µg/mL, was the sum of D, DG, DR, Cy, CyG, CyR, PG, M, MG, Pet, Pel, and P. For flavanols, we used an HSS T3 steel cartridge (Waters), 2.1 mm × 100 mm, filled with 1.8 µm particles and kept at 40 °C. The mobile phase (flow rate: 0.2 mL/min) consisted of water/acetonitrile/formic acid 91/4/5 (*v*/*v*/*v*) at time 0 and 75/20/5 after 4 min, followed by a steady state for 2 min. For GC, ECG, EGC, and EGCG, the absorbance was recorded at 280 nm. C and EC were analyzed by fluorescence spectroscopy (recording at 310 nm after an excitation at 280 nm). The total flavanol content, expressed in µg/mL, was the sum of C, EC, GC, ECG, EGC, and EGCG.

2.4. Vascular Reactivity

Six-week-old male Wistar rats were obtained from the Central Animal Facility of the University Hospital Center of Liège and handled as recommended by the Ethics Committee for Animal Use of the University of Liege, Belgium (file 857, accepted in 2016). After anesthesia (pentobarbital, 60 mg/mL/kg), the thoracic aorta was removed carefully, cleaned of adhering fat and connective tissue, and cut into rings (2–3 mm long). The rings were then mounted and immersed in a 20 mL organ bath solution (OBS) at 40 °C, consisting of Krebs liquid (118 mM NaCl, 25 mM $NaHCO_3$, 5.5 mM D-glucose, 4.7 mM KCl, 1.18 mM KH_2PO_4, 2.4 mM $MgSO_4$, and 3.3 mM $CaCl_2$, pH 7.8) continuously bubbled with 95% O_2 and 5% CO_2. The rings were equilibrated for 2 h before initiating the experimental protocols. To test the reactivity of the endothelium, contraction was induced with KCl (80 mM). When contraction reached a plateau, the OB chamber was washed thrice with Krebs solution. The functional endothelium was then tested by adding first 0.5% noradrenaline (to 0.1 µM), to induce contraction, and then acetylcholine (to 10 µM), to induce at least 80% vasorelaxation. After equilibration, the rings were contracted with phenylephrine (0.5 µM) until contraction reached a plateau. Cumulative volumes of a fruit or vegetable juice (V_{juice}) were then added in OBS (V_{OBS}) to reach a final volume of 2000 µL (V_{juice}/V_{OBS} 10%) over a period of 1 h and a concentration-relaxation curve was constructed. Three to four independent assays were run for each concentration. Figure 1 depicts typical vasorelaxation graphs obtained with juices 1 and 18 (Tomato Carrefour and Blackcurrant Jacoby). Four-parameter sigmoidal dose–response curves were fitted to the data with GraphPad Prism (version 6.0, GraphPad Software, Inc., San Diego, CA, USA). E^+ represents the mean percentage of vasorelaxation determined at three different ratios (expressed as percentages) of juice volume to initial volume of OB solution (V_{juice}/V_{OBS} ratios): 1%, 5%, and 10%. Relaxation of 100% was considered as high interest. The whole protocol was then repeated after careful removal of the endothelium to determine vasorelaxation in absence of endothelium (E^-).



(**A**)



(**B**)

Figure 1. Examples of endothelial vasorelaxation activity in segments of rat aorta according to the volume of either blackcurrant (Jacoby) and Tomato (Carrefour) juices (respectively panels **A** and **B**) added to 20 mL initial organ bath solution (OBS). The following V_{juice}/V_{OBS} ratios were selected to evaluate the vasorelaxant effect as a function of total or individual concentrations of specific phenolic compounds: 1%, 5%, and 10%. Cumulative volumes of each fruit or vegetable juice were then added to reach a final volume of 2000 µL over a period of 1 h. E = endothelium.

2.5. Statistical Analyses

Correlations between E^+ and TPC-J (expressed in µg GAE/mL), total polyphenols in the juice-containing OB (TPC-OB, expressed in µg GAE/mL), and total and individual flavonols,

flavanols, and anthocyanins in the juice-containing OB (expressed in μg/mL) were calculated with Sisvar 5.6 software. Pearson correlations were considered significant at $p < 0.05$.

3. Results

As shown in Figure 2, the range of TPC-J values was wide. Juice 1 (Tomato, Carrefour) showed the lowest value (214 μg GAE/mL) and juice 9 (Grape, Materne), the highest (1564 μg GAE/mL). The mean TPC-J for the juices investigated was 843 ± 396 μg GAE/mL. The juices also differed greatly in total flavonol and total flavanol contents, as shown in Table 1. Juice 3 (Carrot, Biotta) had the lowest flavonol content (0.8 μg/mL) and juice 12 (Blackcurrant Biotta), the highest (17.7 μg/mL). For flavanols, the lowest (2.62 μg/mL) and highest values (236 μg/mL) were observed, respectively, for juice 6 (Lemon, Bonneterre) and juice 18 (Blackcurrant, Jacoby). The average flavonol and flavanol concentrations were respectively 6.3 ± 4.7 and 109 ± 249 μg/mL. If one excludes juice 11, which reached 1194 μg/mL, the average flavanol concentration was 57.7 ± 58.2 μg/mL.

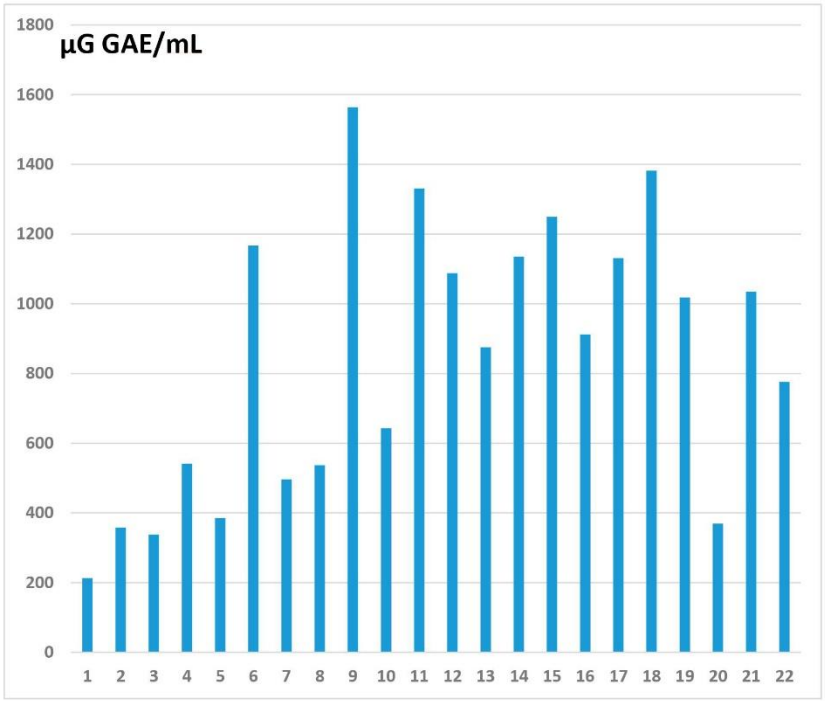


Figure 2. Total polyphenol contents (TPC-J), expressed in μg gallic acid equivalents per milliliter (μg GAE/mL), of 22 commercial vegetable and fruit juices found in Belgian and French markets. GAE: gallic acid equivalents. 1: Tomato (Carrefour), 2: Tomato (Biotta), 3: Carrot (Biotta), 4: Orange d'Espagne (Carrefour), 5: Pure Orange (Vitamont), 6: Lemon (Bonneterre), 7: Grapefruit, 8: Pure Grapefruit (Vitamont), 9: Grape (Materne), 10: Pure Grape (Vitamont), 11: Pomegranate (Biotta), 12: Blackcurrant (Biotta), 13: Blackcurrant (Natreen), 14: Blackcurrant (Jacoby Bio), 15: Blackcurrant (Van Nahmen), 16: Blackcurrant (Schörl Nectar), 17: Blackcurrant (Gut and Günstig), 18: Blackcurrant (Jacoby), 19: Pineapple Juice (Carrefour), 20: Pineapple Juice (De Drie Wilgen), 21: Apple (Carrefour), and 22: Pure Apple (Vitamont).

Table 1. Individual and total flavonol and flavanol contents (µg/mL) of 22 commercial vegetable and fruit juices.

number	List of Juices	Myricetin	Quercetin	Kaempferol	Total Flavonols	EGC	EGCG	ECG	GC	C	EC	Total Flavanols
1	Tomato (Carrefour)	0.3	2.7	0.1	3.1	2.74	8.86	1.64	53.55	0.05	4.34	71.19
2	Tomato (Biotta)	0.1	1.6	0.2	1.9	14.40	8.34	3.89	26.70	0.06	2.67	56.06
3	Carrot (Biotta)	0.1	0.5	0.2	0.8	0.71	29.64	1.58	0.00	0.32	0.85	33.09
4	Orange d'Espagne (Carrefour)	0.5	0.9	0.1	1.5	20.47	7.91	1.57	0.60	0.21	0.22	30.97
5	Pure Orange (Vitamont)	1.3	0.8	0.7	2.8	7.69	2.67	2.19	1.53	0.09	1.09	15.25
6	Lemon (Bonneterre)	0.7	1.3	1.9	4.0	0.52	0.86	0.42	0.11	0.03	0.68	2.62
7	Grapefruit	1.8	2.0	1.1	4.9	4.18	1.48	0.85	0.24	0.14	3.15	10.03
8	Pure Grapefruit (Vitamont)	0.6	0.9	0.5	2.0	9.67	1.99	1.38	0.03	0.04	4.66	17.76
9	Grape Materne (Materne)	13.0	2.7	0.4	16.0	86.68	41.37	14.43	3.89	1.11	7.51	154.99
10	Pure Grape (Vitamont)	7.6	3.3	0.9	11.9	5.63	11.79	8.85	0.27	0.36	1.81	28.71
11	Pomegranate (Biotta)	8.5	4.0	0.2	12.7	849.59	45.95	3.00	294.60	0.09	0.77	1194
12	Blackcurrant (Biotta)	14.4	2.5	0.7	17.7	13.85	37.80	7.11	2.00	0.02	0.63	61.41
13	Blackcurrant (Natreen)	4.8	3.5	0.0	8.3	23.00	18.42	2.10	1.20	0.10	1.39	46.22
14	Blackcurrant (Jacoby Bio)	1.6	2.2	0.3	4.1	21.81	33.17	1.73	4.29	0.26	2.17	63.43
15	Blackcurrant (Van Nahmen)	1.8	2.7	0.5	4.9	22.60	66.02	2.78	10.76	0.33	1.71	104.20
16	Blackcurrant (Schlör nectar)	2.0	1.9	0.3	4.2	22.68	41.27	1.96	6.41	0.22	0.84	73.39
17	Blackcurrant (Cut and Günstig)	1.9	2.7	0.6	5.1	21.90	95.06	4.46	7.77	0.35	2.31	131.85
18	Blackcurrant (Jacoby)	3.2	3.3	0.7	7.2	44.05	181.42	3.25	4.65	0.60	2.07	236.02
19	Pineapple juice (Carrefour)	7.7	1.6	1.2	10.5	1.74	4.66	1.12	0.32	0.12	3.59	11.55
20	Pineapple juice (De Drie Wilgen)	1.6	0.9	0.4	2.9	8.32	14.06	4.94	18.94	0.02	2.40	48.67
21	Apple (Carrefour)	1.3	2.3	1.0	4.5	1.26	3.17	1.37	0.03	0.11	0.45	6.39
22	Pure Apple (Vitamont)	3.5	4.0	0.3	7.9	1.32	2.87	2.80	0.04	0.14	0.74	7.91

Among the 22 tested juices, only the 10 red ones (blackcurrant, grape, pomegranate) contained detectable levels of anthocyanins (Figure 3). Among these, the total anthocyanin content varied widely, from 16 µg/mL (Pure Grape, Vitamont) to 338 µg/mL (Blackcurrant, Jacoby Bio). We calculated mean total anthocyanin contents of 125 ± 99 µg/mL for the 10 red juices and 159 ± 99 µg/mL for the seven blackcurrant juices considered alone.

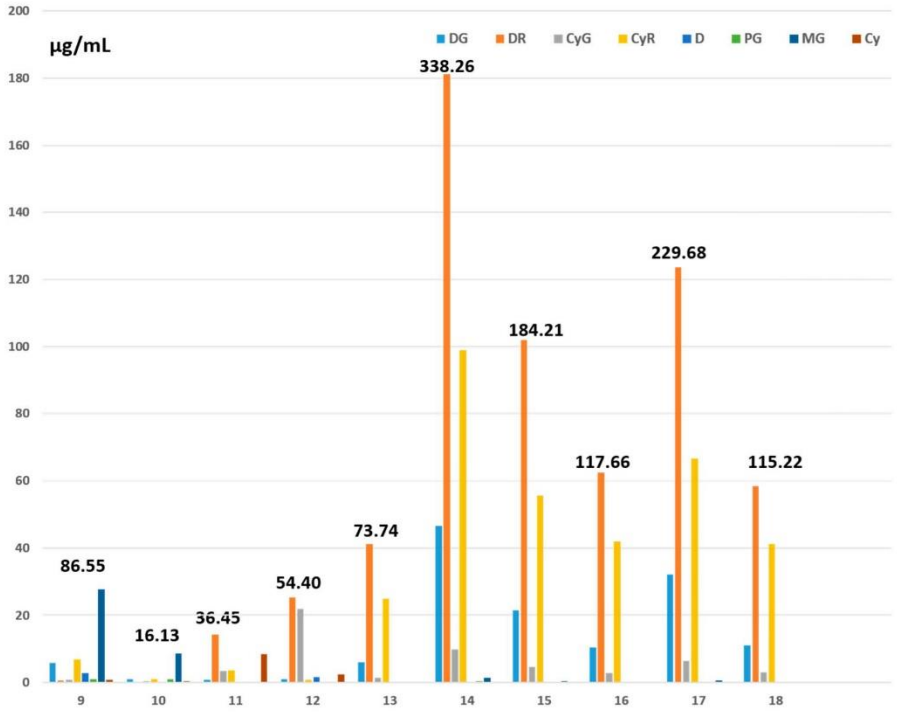


Figure 3. Concentrations of individual anthocyanins (µg/mL) found in 10 commercial red fruit juices. Numbers in bold indicate total anthocyanin concentrations (µg/mL) in the juices. Pet, Pel, P, and M are not represented because of their low to zero concentrations in the juices. 9. Grape Materne (Carrefour), 10. Pure Grape (Vitamont), 11. Pomegranate (Biotta), 12. Blackcurrant (Biotta), 13. Blackcurrant (Natreen), 14. Blackcurrant (Jacoby Bio), 15. Blackcurrant (Van Nahmen), 16. Blackcurrant (Schörl Nectar), 17. Blackcurrant (Gut and Günstig), and 18. Blackcurrant (Jacoby).

Table 2 shows the percentages of vasorelaxation E^+ and E^- elicited by the 22 individual fruit and vegetable juices at three V_{juice}/V_{OBS} ratios (henceforth called "volume ratios"): 1%, 5%, and 10%. Juices issued from vegetables (tomato and carrot) gave rise to a very low E^+ when added at volume ratio 1% or 5%. At volume ratio 10%, however, the E^+ reached 56% for juice Tomato, Carrefour, which caused similar vasorelaxation (38.83%) even after removal of the endothelium. All but one of the citrus juices exerted no vasorelaxant effect at volume ratio 1%, whether the endothelium was present or not. The exception was lemon juice, which elicited a very high percentage of vasorelaxation (81.07%) at this volume ratio, in the presence and absence of endothelium. At volume ratio 10%, the vasorelaxation caused by the other citrus juices increased up to 61.7–120%, both in the presence and absence of endothelium. Among the berry juices, those issued from blackcurrant clearly exerted the greatest vasorelaxant effect. At volume ratio 1%, all of these except juices 13 and 14 gave rise to a substantial E^+, the highest values being observed with juices 12 (44.75%) and 18 (56.76%). No significant vasorelaxant

effect was observed in the absence of endothelium. At volume ratio 5%, all the blackcurrant juices elicited an E^+ close or equal to 100%, but vasorelaxant effects were also evidenced without endothelium, albeit to a lesser extent: 41 to 67%. At volume ratio 10%, each blackcurrant juice gave rise to both E^+ and E^- values equal or superior to 100%.

Table 2. Ability of 22 commercial vegetable and fruit juices to induce vasorelaxation in segments of rat aorta. E^+ and E^- represent percentages of vasorelaxation observed at three V_{juice}/V_{OBS} ratios (1%, 5%, and 10%), respectively in the presence (+) and absence (−) of endothelium.

Number	Juices Origin	With Endothelium			Without Endothelium		
		E^+ (1% *v/v*)	E^+ (5 % *v/v*)	E^+ (10 % *v/v*)	E^- (1% *v/v*)	E^- (5% *v/v*)	E^- (10% *v/v*)
1	Tomato (Carrefour)	1.19 ± 22.87	14.73 ± 3.44	56.19 ± 10.44	1.51 ± 3.1	6.07 ± 11.49	38.83 ± 12.3
2	Tomato (Biotta)		6.9 ± 7.73	28.82 ± 9.63		30.58 ± 5.85	79.82 ± 7.50
3	Carrot (Biotta)	4.28 ± 6.7	7.72 ± 6.39	44.94 ± 18.26	8.52 ± 1.84	35.24 ± 9.45	63.41 ± 22.19
4	Orange d'Espagne (Carrefour)		27.86 ± 27.04	61.70 ± 30.32	6.1 ± 14.35	25.79 ± 16.05	49.68 ± 24.24
5	Pure Orange (Vitamont)		40.63 ± 30.89	83.27 ± 21.00	4.11 ± 4.75	122.9 ± 66.61	173.14 ± 89.22
6	Lemon (Bonneterre)	81.07 ± 9.9	95.06 ± 13.4	87.32 ± 10.97	79.35 ± 1.49	102.04 ± 1.25	98.48 ± 1.11
7	Grapefruit		16.64 ± 17.04	74.39 ± 1.34		36.17 ± 10.46	89.68 ± 2.70
8	Pure Grapefruit (Vitamont)	15.01 ± 0.00	106.56 ± 6.86	120.43 ± 7.10		102.49 ± 6.86	105.41 ± 7.10
9	Grape Materne (Materne)	8.81 ± 0.80	22.98 ± 5.50	45.11 ± 9.58		16.77 ± 4.51	40.49 ± 4.12
10	Pure Grape (Vitamont)	15.01 ± 0.23	20.06 ± 12.54	56.19 ±11.05	0.18 ± 17.56	25.76 ± 22.59	38.83 ± 1.92
11	Pomegranate (Biotta)		93.13 ± 8.42	106.78 ± 5.67	2.09 ± 1.91	59.35 ± 17.94	92.34 ± 19.73
12	Blackcurrant (Biotta)	44.75 ± 22.76	97.29 ± 26.8	110.15 ± 15.87	4.56 ± 7.66	40.56 ± 22.31	94.35 ± 27.06
13	Blackcurrant (Natreen)	9.47 ± 16.59	106.55 ± 4.19	114.65 ± 13.14		42.59 ± 31.06	66.56 ± 30.63
14	Blackcurrant (Jacoby Bio)	5.12± 0.92	96.59 ± 12.4	191.68 ± 3.81		44.21 ± 12.37	91.57 ± 18.88
15	Blackcurrant (Van Nahmen)	19.9 ± 0.97	100.53 ± 5.08	109.73 ± 7.52	2.76 ± 42.66	64.48 ± 25.82	127.59 ± 18.8
16	Blackcurrant (Schörl nectar)	23.75 ± 3.59	85.57 ± 20.41	117.41 ± 8.91	1.64 ± 0.14	42.07 ± 20.44	87.59 ± 26.57
17	Blackcurrant (Gut & Günstig)	19.99 ± 14.51	85.88 ± 8.10	107.18 ± 2.61		52.29 ± 9.00	110.93 ± 7.56
18	Blackcurrant (Jacoby)	56.76 ± 24.49	103.82 ± 5.07	106.1 ± 7.97	0.87 ± 1.23	67.53 ± 15.57	103.43 ± 4.16
19	Pineapple juice (Carrefour)	5.66 ± 14.96	13.04 ± 6.9	35.46 ± 2.12		9.1 ± 7.54	38.19 ± 20.4
20	Pineapple juice (De Drie Wilgen)		12.95 ± 2.19	92.05 ± 0.72		5.88 ± 1.58	34.15 ± 5.00
21	Apple (Carrefour)			29.23 ± 6.97		0.98 ± 3.23	17.83 ± 1.34
22	Pure Apple (Viamont	15.28 ± 4.75	64.01 ± 4.29	86.64 ± 11.96		32.38 ± 19.4	68.68 ± 27.3

From the data in Figures 2 and 3 and Table 1, it was possible to calculate for each OB the concentrations (in µg/mL) of total polyphenols (TPC-OB) and total and individual flavonols, flavanols, and anthocyanins and to establish correlations suggesting which compounds might be responsible for observed vasorelaxant effects. As depicted in Table 3, TPC-OB correlated significantly with E^+ at volume ratios 5% ($r = 0.58$, $p = 0.04$) and 10% ($r = 0.55$, $p = 0.007$). No correlation was found between E^+ and total flavonols or flavanols present in the OB. A strong, significant correlation was found between E^+ and total anthocyanins issued from berry juices added at volume ratio 10% ($r = 0.75$, $p = 0.05$). For the flavonol family, the only correlation evidenced was between kaempferol and the E^+ at ratio 1% ($r = 0.56$, $p = 0.009$). For the flavanol compounds, the only correlation observed was between EGCG and the E^+ at volume ratios 1% ($r = 0.41$, $p = 0.05$) and 5% ($r = 0.45$, $p = 0.03$). For the anthocyanin family, no correlation was evidenced at ratio 1% between the E^+ and the concentration of any individual compound in the OB. At ratio 5%, MG and especially PG from red juices (especially blackcurrant) correlated significantly with the E^+ (respectively $r = 0.8$, $p < 0.05$ and $r = 0.96$, $p < 0.00001$). At ratio 10%, the concentrations of DG, DR, and CyR correlated significantly with the E^+.

Table 3. Correlations between E^+ and TPC-J, TPC-OB, and total and individual OB concentrations of juice flavonols, flavanols, and anthocyanins, according to the volume of juice added to 20 mL initial OB solution (V_{juice}/V_{OBS} ratios 1%, 5%, and 10%). E^+ represent percentages of vasorelaxation observed at three V_{juice}/V_{OBS} ratios in the presence of endothelium.

Compounds	E^+ (1% *v/v*)		E^+ (5% *v/v*)		E^+ (10% *v/v*)	
	r	***p*-Value**	***r***	***p*-Value**	***r***	***p*-Value**
TPC-J	0.32	0.14	0.58	0.04	0.55	0.007
TPC-OB	0.32	0.14	0.58	0.04	0.55	0.007
Flavonols-OB	0.19	0.39	0.17	0.44	0.02	0.99
Flavanols-OB	0.08	0.69	0.27	0.2	0.17	0.44
Anthocyanins-OB	0.54	0.2	0.28	0.5	0.75	**0.05**
Myricetin	0.1	0.6	0.06	0.7	0.07	0.7
Quercetin	0.11	0.61	0.35	0.1	0.19	0.38
Kaempferol	0.56	0.009	0.11	0.96	0.19	0.4
EGC	0.14	0.51	0.22	0.31	0.13	0.54
EGCG	0.41	0.05	0.45	0.03	0.31	0.15
ECG	0.004	0.98	0.13	0.5	0.17	0.44
GC	0.19	0.3	0.14	0.5	0.09	0.68
C	0.07	0.75	0.03	0.87	0.09	0.67
EC	0.19	0.37	0.2	0.35	0.16	0.45
DG	0.17	0.6	0.3	0.39	0.70	0.02
DR	0.06	0.85	0.49	0.14	0.81	0.004
CyG	0.38	0.26	0.37	0.28	0.39	0.25
CyR	0.06	0.86	0.44	0.2	0.76	0.01
PG	0.49	0.28	**0.96**	0.00001	0.61	0.1
MG	0.36	0.2	0.8	0.05	0.61	0.11

As shown on Table 4, the mass concentrations of phenolic compounds in each OB were converted to molar concentrations. For each volume ratio, these concentrations were averaged over all the juice-containing organ baths. The results show that an E+ was observed at all three ratios at average quercetin, kaempferol, ECG, C, EC, CyG, and PG concentrations below 1 µM. This was also the case for myricetin but only at ratios 1% and 5%. The average concentrations of EGC, EGCG, GC, DG, DR, CyR, and MG were above 1 µM.

Table 4. Conversion of individual flavonol, flavanol, and anthocyanin mass concentrations to molarities (μM). The indicated values are the means ± SD calculated for the whole set of juices. * For EGC and GC, values related to juice 11 (pomegranate) were removed for the three ratios because being aberrant.

Compounds	Title		
	V_{juice}/V_{OBS} 1%	V_{juice}/V_{OBS} 5%	V_{juice}/V_{OBS} 10%
	flavonols (μM)		
myricetin	0.12 ± 0.13 (0.005–0.11)	0.56 ± 0.62 (0.022–2.60)	1.06 ± 1.18 (0.042–4.12)
quercetin	0.07 ± 0.03 (0.17–0.62)	0.34 ± 0.16 (0.080–0.623)	0.66 ± 0.31 (0.15–1.2)
kaempferol	0.02 ± 0.02 (0.006–0.67)	0.09 ± 0.07 (0.024–0.32)	0.18 ± 0.14(0.046–0.615)
	flavanols (μM)		
EGC	0.51 ± 0.60 (0.02–2.8)*	2.5 ± 3.0 (0.08–6.8)*	4.8 ± 5.9 (0.21–6.8)*
EGCG	0.6 ± 0.9 (0.02–3.9)	3.10 ± 4.4 (0.09–19)	6.0 ± 8.3 (0.17–36)
ECG	0.1 ± 0.1 (0.01–0.32)	0.4 ± 0.4 (0.05–1.60)	0.7 ± 0.7 (0.09–3)
GC	0.23 ± 0.4 (0.01–1.7)*	1.1 ± (0.01–8.3)*	2.03 ± 3.8 (0.01–16)*
C	0.01 ± 0.01 (0.002–0.38)	0.04 ± 0.04 (0.004–0.18)	0.1 ± 0.1 (0.007–0.35)
EC	0.1 ± 0.1 (0.02–0.26)	0.3 ± 0.3 (0.04–1.20)	0.7 ± 0.6 (0.07–2.40)
	anthocyanins (μM)		
DG	0.29 ± 0.3 (0.02-0.99)	1.39 ± 1.6 (0.08-4.76)	2.66 ± 3.0 (0.14-9.10)
DR	1.97 ± 1.9 (0.01–5.90)	9.48 ± 9.1 (0.03–28)	18.22 ± 17.6 (0.06–54)
CyG	0.12 ± 0.10 (0.01–0.48)	0.57 ± 0.7 (0.05–2.30)	1.10 ± 1.3 (0.09–4.42)
CyR	0.45 ± 0.40 (0.01–1.30)	2.15 ± 2.10 (0.05–6.20)	4.11 ± 4.0 (0.10–12)
PG	0.01 ± 0.01 (0.01–0.02)	0.04 ± 0.04 (0.02–0.11)	0.08 ± 0.07 (0.04–0.21)
MG	>10	>20	>20

4. Discussion

Many studies have evidenced that the intake of fruits and vegetables exerts potent protection against cardiovascular diseases and cancers [19–22]. All these effects have been attributed to the presence, in these foods, of antioxidants, namely polyphenols. Great attention has been paid to the cardioprotective effect of polyphenols, linked to their ability to prevent endothelial dysfunction thanks to restoration of vasodilator nitric oxide (NO) production through expression of endothelial nitric oxide synthase (eNOS) or through activation of endothelium hyperpolarization [23]. However, whether a similar link exists between cardioprotection and vegetable or fruit juice consumption is not so clear, notably because juices contain less fiber than fruits and vegetables. In 2005, Ruxton et al. [24] published a review of epidemiological and small clinical studies showing only a minor impact of fruit and vegetable juices on cancers, as compared to their greater impact on cardiovascular diseases. More recently, Hyson [25] confirmed these cancer data in a critical analysis of the scientific literature related to fruit juices and human health. The same author, however, highlighted that interventional studies with acute or chronic intake of different juices (apple, orange, mandarin, grape, cranberry, pomegranate) resulted in decreased levels of some oxidative stress biomarkers associated with increased cardiovascular risk (lipid peroxides, oxidized LDL, carbonyl groups). Khan et al. [26] have described similar observations with polyphenol-rich blackcurrant juice. To better understand the potential cardioprotective effect of fruit and vegetable juices, it is thus necessary to examine in detail how they differ in polyphenol content and composition. Surprisingly, little information is available on this topic.

Among our 22 tested juices, those prepared from lemon (juice 6), pineapple (juice 19), apple (juice 21), and especially red fruits (juices 9 to 18) clearly exhibited the highest TPC-J values. Using home-made preparations, Tzulker et al. [27] found antioxidant activity, as evidenced in DPPH assays, to correlate significantly with the TPC-J in 29 different pomegranate accessions. They gave further details about levels of total anthocyanins and four major hydrolyzable tannins. Using HPLC-PDA-MS (high performance liquid chromatography–photo diode array–mass spectrometry), Borges et al. [28] later analyzed the TPC-J values of 26 commercial pomegranate juices produced in Europe, identifying ellagitannins as the major antioxidants in these juices. Nowak et al. [29] compared the TPC-J values of

commercial fruit juices and found only berry products characterized by a red-blue color (blackcurrant, grape, blueberry, cranberry, aronia) to induce marked endothelium-dependent vasorelaxation of isolated pig coronary arteries. These authors concluded, but did not formally prove, that the vasorelaxant effect of juices (E^+ at volume ratio 1%) depends on their polyphenol composition rather than on their total polyphenol content. In a previous study [18], we confirmed in the same animal model the absence of correlation between the TPC-J and the capacity of blackcurrant juices to induce vasorelaxation. In our rat aorta model (Table 3), we have observed no correlation between E^+ and TPC-J at the volume ratio used by Auger et al. [34]. At volume ratios 5% and 10%, in contrast, we have evidenced significant correlations: 0.58 ($p = 0.04$) and 0.55 ($p = 0.007$), respectively. The results in Table 3 lead to similar conclusions regarding the TPC-OB, use of this value being a more realistic approach.

Several previously published studies by our group as well as by others on the effect of polyphenol-rich products on the vascular system have indicated that they can induce endothelium-dependent relaxations in isolated arteries, and vasodilatation in vivo in several experimental models and in humans mainly due to the activation of both NO and endothelium-dependent hyperpolarization (EDH) pathways. Further characterization of the transduction pathways indicated that the activation of the NO pathway in response to polyphenol-rich products (red wine extract, blackcurrant, aronia, green tea, etc.) is due, at least in part, to the redox-sensitive activation of the Src/Akt/PI3-kinase pathway [23].Regarding the endothelium-independent relaxations, natural products have been shown to induce endothelium-dependent relaxations through several mechanisms including interaction with the calcium signaling pathway and an activation of the cyclic AMP and cyclic GMP relaxing pathways, in part, due to phosphodiesterase (PDEs) inhibition [40].

In our study of 22 vegetable and fruit juices, we confirm very contrasting ex vivo vasorelaxant effects between juice categories and even between juices of the same category (Table 2). In our model, the vasorelaxant activities of vegetable and citrus juices (Lemon, Bonneterre) do not appear endothelium dependent, as evidenced by similar E^+ and E^- values at all volume ratios. In contrast, and in accordance with previous findings of ours [18], we find blackcurrant juices, known for their high polyphenol content, to induce substantial vasorelaxation at volume ratio 1%, in the presence but not in the absence of endothelium (exceptions: juices 13 and 14). Yet this observation appears to be influenced by the experimental conditions, since at volume ratio 10%, vasorelaxation percentages could reach 100% whether endothelium was present or not.

Of great interest is the question: among the phenolic compounds present in vegetable and fruit juices, which are the ones that favor vasorelaxation? The data in Table 3 reveal no correlation, at any volume ratio, between E^+ and total flavonols or flavanols (mass concentrations) in the OB. At ratio 1%, however, strong correlations appear between E+ and OB concentrations of kaempferol ($r = 0.56$, $p = 0.009$) and EGCG ($r = 0.41$, $p = 0.05$). This was particularly surprising for kaempferol, given its presence in very small amount (0.1–1.2 µg/mL) in all the tested juices (Table 1). Total anthocyanins in OB, particularly those of blackcurrant juices, correlated strongly ($r = 0.75$, $p = 0.05$) with E^+ at volume ratio 10%. Amounts of DG, DR, and CyR also correlated with E^+ at this volume ratio. MG and especially PG ($r = 0.96$, $P = 0.00001$) correlated strongly with E^+, but at volume ratio 5%. At volume ratio 1% we found no correlation. This last observation on our rat model contrasts with our previous findings on porcine artery rings [18]. In the latter case, the potency of blackcurrant juices to induce vasorelaxation at volume ratio 1% correlated significantly with their total anthocyanin content and with their DG, DR, CyR, and MG contents. Such differences in vascular reactivity according to the volume ratio might also be related to human data indicating that flavonoid bioactivity, as reflected by in vivo endothelial function, does not follow a classical linear dose–response curve [41]. It is also possible that the rat aorta and porcine coronary artery differ markedly as regards their vascular reactivity.

What is the in vivo relevance of ex vivo data on vasorelaxation induced by phenolic compounds present in vegetable and fruit juices? This is an important question. Papers describing consistent data related to vasorelaxation induced by pure compounds are scarce. Mahobiya et al. [42] found

kaempferol to induce concentration-dependent relaxation in rat pulmonary artery rings pre-contracted with phenylephrine, when present at 0.01 μM (0% vasorelaxation) to 10 μM (100% vasorelaxation). Xu et al. [43] likewise evidenced that kaempferol at 3 μM can significantly enhance bradykinin-induced relaxation of porcine coronary artery rings, through both endothelium-derived NO production and endothelium-dependent hyperpolarization. Aggio et al. [44] report 13% to 50% vasorelaxation of rat aorta in response to 1 to 100 μM EGCG. Thilavech et al. [45] report that 25 μM cyanidin-3-*O*-rutinoside (CyR) can induce 50% vasorelaxation of rat aorta. In vivo, investigators found plasma concentrations of phenolic compounds to be very low in fasted volunteers, notably because of low bioavailability. When compared to a baseline diet, human plasma quercetin concentration increased form 0.078 μM up to 0.304 μM after consumption of 110 mg/day flavonol diet during 14 days. [46]. Manach et al. [16] showed the human plasma C concentration to range between 0.14 and 0.49 μM after ingestion of 0.36 mg/kg pure C. Giordano et al. [47] report a plasma cyanidin-3-*O*-glucoside (CyG) level reaching 0.0040 μM 1h after ingestion of 500 mL orange juice containing 12.89 mg CyG. Liu et al. [48] likewise found the plasma concentration of cyanidin-3-*O*-galactoside to peak at 0.0031 μM around 2 h after ingestion of 100 g Saskatoon berries containing 123.5 mg of this compound. Rechner et al. [37] observed peak plasma concentrations of delphinidin-3-*O*-glucoside (0.006 μM), delphinidin-3-*O*-rutinoside (0.051 μM), cyanidin-3-*O*-glucoside (0.0035 μM), and cyanidin-3-*O*-rutinoside (0.024 μM) about 1 h after ingestion of 330 mL blackcurrant juice concentrate containing 1 g total anthocyanins. At least, Kay et al. [49] reported that intake of 20 g chokeberry extract containing cyanidin-3-glycosides as high as 1.3 g resulted in an average peak plasma concentration in total anthocyanins and anthocyanins metabolites of 5.1 μM within 2 h post-consumption.

After conversion of mass concentrations to molarities (Table 4), our in vitro study shows that the quercetin concentration (0.07 to 0.66 μM) able to induce vaso-relaxation in OBS at the three V_{juice}/V_{OBS} ratios was in the physiological range according to data above. Unfortunately, any correlation was found between its concentration in OBS and E^+ (Table 3). This was not the case for kaempferol whose concentration was close to or less than 0.1 μM in the vasorelaxation-inducing organ bath and significantly correlated with the E^+ (Table 3). With respect to the flavanol family, all compounds exhibited at V_{juice}/V_{OBS} 1% vasorelaxant activity below1 μM concentration in agreement with in vivo data of Manach [16]. However, only EGCG concentration correlated with E^+ at V_{juice}/V_{OBS} 1% and 5% (Table 3). Concentrations of DG, DR and CyR from the anthocyanin family correlated with E^+ but at a level largely higher than those found in plasma. Of interest was the very strong and significant correlation ($r = 0.96$, $p < 0.00001$) found between PG at a concentration as low as 0.04 μM in OBS and E^+ at V_{juice}/V_{OBS} 5%.

Our study highlights some phenolic compounds (kaempferol, EGCG, DG, DR, CyR and PG) in relation to others as to their potential capacity to induce vascular activity. However, concentrations of DR, CyG and in a less extent of PG required for inducing ex vivo vasorelaxation remain, although largely higher than those detected in plasma. Such a difference might be explained, at least in part, by the fact that the bioavailability of phenolic compounds is often assessed in protein free-plasma, and, hence, is unable to detect natural molecules carried by proteins in blood and/or at the cell membrane. Hence, it might not be relevant to compare our data with plasma values since we have observed in preliminary experiments on rats that anthocyanins can rapidly accumulate into endothelial cells and, hence, most likely can reach much higher levels in intracellular cell compartments than in plasma. However, we also have to keep in mind the important hormetic effect of polyphenols, since they are able, at low concentrations, to stimulate the antioxydant Keap1/Nrf2/ARE system (including e-NOS) through moderate production of ROS [8,50]. Whatever the mechanism, endothelium-dependent vasodilation in both experimental models and humans has been observed after the intake of anthocyanin-rich fruit juices, indicating the ability of natural products to reach the endothelium [23]. Recently, we have shown that the intake of 400 mL of blackcurrant juice Biotta (juice 12) containing 536 mg of polyphenols (including 21.76 mg of total anthocyanins) resulted 1 h after ingestion in a significant increase (35.8%) of human endothelial function as evaluated by measurement of the Reactive Hyperemia Index (RIH) [51].

5. Conclusions

As pertinently noted by Habauzit and Morand [15], only a few publications report in vitro studies using polyphenols at concentrations (μM) nutritionally achievable in aglycone or conjugated form after consumption of polyphenol-containing foods. In the present paper, we have tried to show how to adequately manage and interpret information about vasorelaxation effects induced by vegetable and fruit juices under ex-vivo experimental conditions. In addition to determining TPC-J values, our first step was to assay total subclass (anthocyanins, flavanols, flavonols) and individual levels of polyphenols in the juices studied. High priority was also given to analyzing relationships between vasorelaxant intensity and total and individual phenolic compound concentrations in the organ baths rather than the juices. This enabled us to highlight that kaempferol, EGCG and peonidin-3-*O*-glucoside (PG) present in fruit juices, and more particularly the blackcurrant ones, might have some in vivo relevance, as their OB concentrations were compatible with physiological levels and correlated strongly and significantly with the E+. Yet great caution is required in interpreting data which seem to depend on experimental conditions. A simple example is the non-correlation between TPC-J or TPC-OB and vasorelaxant intensity at V_{juice}/V_{OBS} ratio 1%, in contrast to the correlations observed at 5% or 10%.

Even if endothelium-dependent vasodilation in both experimental models and humans have been reported after the intake of anthocyanin-rich fruit juices [23], large-scale clinical studies are needed to further and better explore in humans the beneficial endothelium-dependent cardiovascular effects of vegetable and fruit juice intake [52] and hence of polyphenols present in these foods [41]. This requires evaluating in vivo endothelial function in three with the measurement of plasma concentrations of both polyphenol metabolites by HPLC-MS/MS [48] and NO. On the basis of results from such an effective battery of tests, the European Food Safety Authority has authorized, under article 13(5) of Regulation (EC) No 1924/2006, a health claim related to cocoa flavanols and maintenance of normal endothelium-dependent vasodilation. By contrast, the PRISMA-compliant meta-analysis recently performed by Zhu et al. [53] does not favor any blood pressure-improving clinical efficacy of anthocyanin supplementation.

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DISCUSSION AND CONCLUSIONS

Fruits and vegetables are essential to meet the nutritional requirements of our body due to their high content of fiber, vitamins, minerals, water, and antioxidants. This is recognized by the World Health Organization when it recommends increasing the consumption to five servings fruits and vegetables (1serving = 80g) to reduce the risk of diseases (Owen and Reid 2012). Even though the consumption of fruits and vegetables have positive effects on human health, due to their high content in antioxidant (more particularly polyphenols), there is little information on the benefits of their juices on human health. Hyson's excellent review (2015) shows effects of various fruit juices and their relationship with some factors that induce some type of disease such as: vascular reactivity, inflammation, lipid-related metabolism, cognitive function, urinary tract infection, platelet reactivity, besides cancer. As all these effects are potentially associated with antioxidant activity, the food industry has understood this very well by offering the public many foods rich in antioxidants, and especially in polyphenols. However, the word antioxidant is misused by various companies who are taking advantage of this word in their marketing procedures. Determining a global antioxidant capacity of food and beverages using ABTS, DPPH and overall ORAC assays and calling it a source of antioxidants is deceptive and misleading for consumers. Especially since such *in vitro* tests are well known to have no relevance *in vivo*, notably due to the use of non-physiological radicals (Schaich, et al.2015). So, the early need to merely identify which natural materials had strong antioxidant efficacy must be replaced by the need to elucidate the action of individual antioxidants, and more particularly polyphenols, present in food matrix such as fruit juices.

With this in mind, our work has focused on several axes: determination as complete as possible of the various phenolic compounds belonging to the subclasses of polyphenols in 21 commercial fruit and vegetable juices, use of an *ex vivo* test for determining antioxidant capacity more in connection with an *in vivo* reality and demonstration of the effects of vasodilation on segments of rat aorta. We also tried to determine whether these effects could occur at individual concentrations of phenolic compounds close to those observed *in vivo*.

In vitro, superoxide anion can be produced by the xanthine/xanthine oxidase system and its detection assayed by reduction of nitro-blue tetrazolium (NBT) or cytochrome C. Phorbol

myristate acetate (PMA), such as protein kinase C agonists, is well known to induce NADPH oxidase activation in neutrophils. In both isolated PMNs and whole blood, different detection techniques (fluorescence, chemiluminescence, accumulation of a dye in cells, electron spin resonance spectroscopy) have been used to evidence superoxide anion. Yunbo Li et al. (1998) well validated lucigenin (Bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems such as phagocytic NADPH oxidase systems. Using PMA activated whole blood and lucigenin, Baptista et al. (2012) evidenced that physical frailty in elderly people was associated with superoxide anion overproduction by NADPH oxidase. We were largely inspired by this protocol for our experiments on fruit and vegetable juices. However, in order to standardize the method as much as possible, we showed that it was required to use EDTA-treated blood samples, to homogenize slowly the sample during 1 hour before experimentation, to use Eppendorf-Multipette for blood distribution in 96 well white plate, to use a final concentration of 4.10^{-6} M PMA and to put, as expected, the luminometer in a total dark place. In these conditions, the intra-assay coefficient variation (CV) was less than 10% which is acceptable. We also checked the specificity of the *ex vivo* system to produce superoxide anion by using superoxide dismutase (SOD) which is well known to accelerate the superoxide anion dismutation into hydrogen peroxide. Figure 35 well shows the existence of a dose response curve.

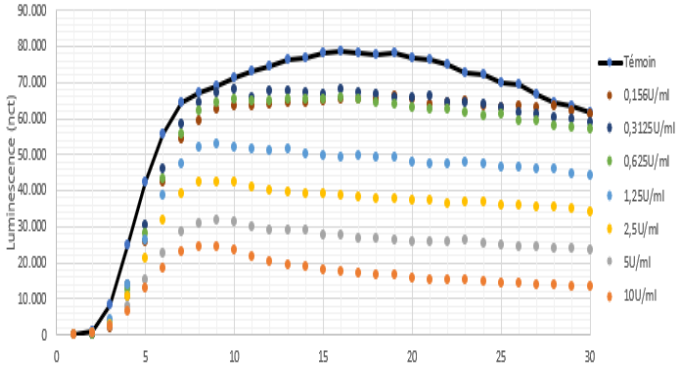

Figure 35: Inhibition of anion superoxide production in whole blood incubation with increasing SOD concentration (U/mL)

As shown in our first article, the whole blood system allowed us to evidence that juices made from red fruits have by far the greatest capacity to inhibit the superoxide anion which is a physiological free radical. Using DPPH assay, Jakobek et al. (2007) reported similar findings. The antioxidant activity of red fruits juices can be explained by a direct scavenging effect of ROS and/or inhibition of neutrophils NADPH oxidase activated by PMA. Interestingly, we showed that the data obtained with the *ex vivo* test were much better correlated ($r = 0.70$, $p = 0.0004$) with the total polyphenol content (TPC) than those with the ORAC test ($r = 0,50$, $P = 0.02$; figure 2, article 1). In our opinion, this demonstrates the higher sensitivity of the *ex vivo* assay in determining the antioxidant capacities of fruit and vegetable juices.

Despite these interesting data, the question remains: what are their potential *in vivo* extrapolations? One of the great advantages of the whole blood system is the possibility to determine the exact concentration of individual phenolic compounds (and more particularly those issued from red juices) knowing that the same quantity of juices was added in the incubation medium. For that, it is required to get in our hands the detailed phenolic composition of all juices as we did it (Table I, paper 2).

Considering the whole set of juices, Table II (paper 2) summarizes the mean concentration (µM) of individual compounds of the flavonols, flavanols and anthocyanins families associated with inhibition of superoxide anion production. Due to their poor availability, it is admitted that the plasma polyphenol concentration does not exceed 1-5 µM. In the *ex vivo* assay, we found that the large majority of individual phenolic compounds show antioxidant activity of at concentrations below 1 µM. However, our data showed a strong correlation between the superoxide anion inhibition and the concentration of only PG, EGCG, C and Q present in the PMA-whole blood assay (Table III, paper 1). As earlier as 1996, Rice-Evans and Miller showed that *in vitro* antioxidant activities (ABTS assay) of aglycon flavonoids were correlated to the numbers of free OH-substituents. EGCG with nine -OH groups had the highest antioxidant activity (4.9 mM) followed by Q (5 -OH groups, 4.7mM), catechin (5 -OH groups 2.4 mM), and P (4 -OH group 2. 22 mM). However, the substitution by glycosides appeared to decrease the antioxidant activity. If focusing on anthocyanins, PG only present in the 10 investigated red juices appears to be particularly active at very low concentration (0.005 µM). Moreover, it is strongly correlated with % superoxide anion inhibition ($r = 0.87$, $p = 0.010$) while it was not the

case using the ORAC assay. Such a concentration in PG could be related to data from Rechner et. al. (2002). Indeed, these authors evidenced plasma concentrations of other anthocyanins such as DG (0.006 µM), DR (0.051 µM), CG (0.0035 µM), and CR (0.024 µM) in the same concentration range about one hour after ingestion of 330 mL blackcurrant juice concentrate containing 1 g total anthocyanins.

Arterial hypertension is one of the most prevalent risk factors for cardiovascular diseases and is the main cause of deaths worldwide. Current research established that dietary polyphenols may help to lower blood pressure thus contributing to the reduction of cardiovascular complications (Alves et al. 2016). This being linked, as explained in detail in the introduction, to their close interaction with the endothelium leading to increased production of nitric oxide (NO) with as final consequence vasorelaxation effects in the blood vessels. Ruxton et. al' (2006) demonstrated that the juices defined as "pure juices" (100% fruit juice) retain most of the nutrients and phytochemicals of a whole fruit and can have important benefits to human health. Those authors reported that polyphenol-rich fruit and vegetable juices had a great impact on cardiovascular diseases through the improvement of flow-mediated dilatation (FMD). Using an *ex vivo* model on isolated pigs coronary arteries, Auger et al. (2011 & 2015) classified 51 juices into subgroups of grape (n = 12), blackcurrant (n = 7), cranberry (n = 5), apple (n = 6), orange (n = 5), red fruits and berries (n = 6), berries (n = 6), and not red (n = 4). Of all of them, only 2 of grapes, 3 of blackcurrant, 4 of cranberry, 1 of apple, 2 of red fruits, and 3 of berries of red fruits in a range of 0.31 to 1.86 g GAE/L, were able to generate up to 50% vasorelaxation at a concentration of 1% (v/v).

Using a model of rat's aorta rings immersed in an organ bath (see more detail in material and methods), we found, in agreement with Auger et al., that some blackcurrant juices (number 12, 15, 16, 17, 18) were able to induce substantial vasorelaxation at 1% Volume juice/Volume organ bath in the presence but not in the absence of endothelium (Table II). Data were more contrasting at higher Volumes juice/Volumes organ bath for unexpected reasons. Considering individual phenolic compounds present in the whole set of juices, kaempferol ($r = 0.56$, $p = 0.009$) and EGCG ($r = 0.41$, $p = 0.004$) correlated with % vasorelaxation at 1% Volume juice/Volume organ bath. At a higher volume ratio (5%), results indicated the % vasorelaxation correlated with TPC in juices ($r = 0.58$, $P = 0.04$) or added in the organ bath (0.58, $p = 0.04$) and

with organ bath amounts of EGCG (0.45, p = 0.03), and, in case of red juices, of MG (0.8, p = 0.05) and overall PG (r = 0.96, p =0.00001). Similar correlations were also observed for DG (r = 0.70, p = 0.02), CyR (r = 0.76, p = 0.02) and DR (r = 0.81, p = 0.004) but only at the highest volume ratio (10%). Such difference in the vasoreactivity of compounds from anthocyanin family could be explained by the fact that phenolic compounds may induce profound vasorelaxation through a direct action on vascular smooth muscle, mediated by an array of mechanisms. They include the inhibitory action on protein kinase C, on cAMP or cGMP phosphodiesterases, and/or on Ca^{2+} influx through voltage sensitive Ca^{2+} channels. It would be particularly interesting to evaluate the effects of pure molecules (PG, EGCG, C, Q) on vasorelaxation activity at the concentrations found in the juices.

In a review on the structure-activity relationship of dietary flavonoids for protecting vascular endothelial function, Zhang et al. (2018) concluded that all tested flavonoids exhibited similar efficacies in improving human endothelial function in healthy people and patients. Flavonols and flavones could regulate the release of NO from endothelial cells resulting in endothelium-dependent vasorelaxant actions. It appears that double bond C2=C3 at C-ring, which is a typical moiety, confers the NO up-regulated activity of flavonols and flavones. In addition, the extent of hydroxylation of rings B and C does not seem to affect the potency of NO synthesis and vasorelaxant activities of flavonols and flavones, as observed for quercetin, myricetin, fisetin, and isoquercitrin

As for antioxidant activity, the question is: what is the potential *in vivo* extrapolation from these *ex vivo* data? As shown in Table IV (paper 2), our study evidenced that only PG present in blackcurrant juices could potentially exert a vasorelaxation effect at a low concentration (0.01 – 0.08 µM) which agrees within a physiological range (0.01 to 0.1 µM). For that, we refer to plasmatic concentration in anthocyanin compounds observed after intake of blackcurrant concentrate juice (see above).

This advanced comparison of plasma concentrations must, however, be taken with some caution. Human serum albumin (HSA), accounts for about 60% of total plasma protein is an important transporter of compounds in the blood. A study conducted by Bi et al. (2004) with fluorescence techniques found strong binding of flavonoids (quercetin) to HSA. The flavonoid

molecule contains groups such as C(3)–OH, C(4)=O or C(5)–OH, C(4) y O or C(3')–OH, C(4)–OH that can bind to the serum albumin. However temperature and pH can influence these binding constants (Cahyana and Gordon 2013). For the first time, Chaker et al. (2021) indeed evidenced in old rat experiments that an important vascular accumulation of anthocyanins can occur in the thoracic aorta and aortic arch at sites of branching after intake of anthocyanins rich blackcurrant juice (ARB, 60 and 120 mg GAE/kg/day) in drinking water for two weeks. The used doses were in the range with those reported in different preclinical studies ranging from 50 to 200 mg/kg/day (Nemes et al. 2019). Such an accumulation was associated with an increased NO production, improved endothelial function and a normalization of vascular oxidative stress in the thoracic aorta. Furthermore, a significantly reduced systolic blood pressure by about 5.4 and 7.6 mmHg for the 60 and 120 mg/kg/day treatment was also observed.

If the organ model using rat aorta segments may refine our understanding about the vasorelaxing effect of red juices, and more particularly the blackcurrant ones, *in vivo* studies are required to evaluate the endothelium function using the rapid, non-invasive and totally non-operator-dependent Endo-PAT 2000 device system (Axtell et al. 2010, Moerland et al. 2012). The device records endothelium-mediated changes in the digital pulse waveform known as the PAT (Peripheral Arterial Tone) signal, measured with a pair of novel modified plethysmographic probes situated on the finger index of each hand (Figure 36). Endothelium-mediated changes in the PAT signal are elicited by creating a downstream hyperemic response. Hyperemia is induced by occluding blood flow through the brachial artery for 5 minutes using an inflatable cuff on one hand. The response to reactive hyperemia is calculated automatically by the system. A PAT ratio is created using the post- and pre-occlusion values. These values are normalized to measurements from the contra-lateral arm, which serves as control for non-endothelial dependent systemic effects.

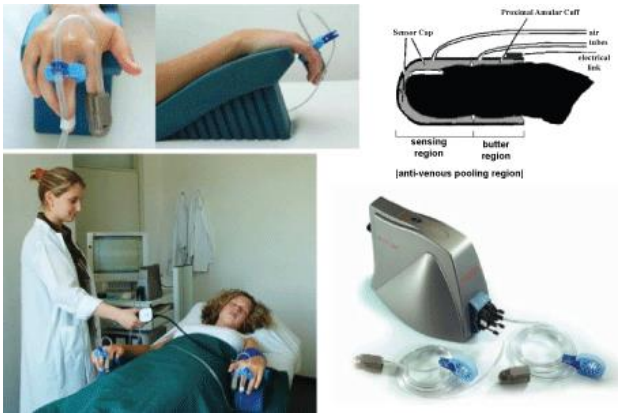

Figure 36. Picture of Endo-Pat 2000 system.

In preliminary experiments, we evidenced using the Endo-Pat 2000 system that 400 mL blackcurrant juice (134 mg polyphenols/100 mL, Biotta, number 12) improved by 35.8% (p = 0.039) the endothelium function as measured by the RHI (Reactive Hyperemia Index) one hour after intake when compared to pre–ingestion (Figure 37). Some authors reported a similar effect (Khan et al. 2014); others not (Jin et al. 2011).

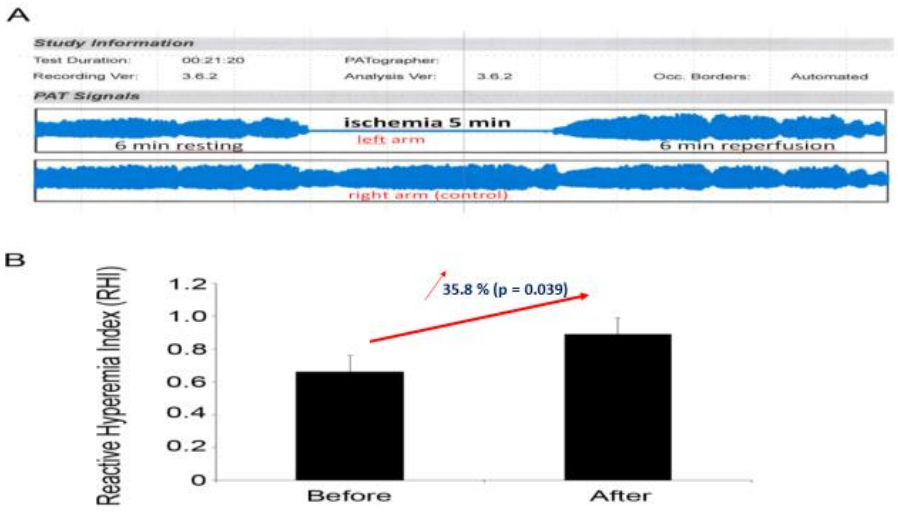

Figure 37: Improvement of endothelial function by intake of 400 mL biota blackcurrant juice (number 12) in human volunteers (n=11).

In conclusion, these two publications provided us interesting information, since by working on *ex vivo* and *in vitro* assays we were able to identify molecules with apparently direct effects on antioxidant capacity and endothelial function. It is imperative to corroborate these behaviors in *in vivo* tests. As we already know the total polyphenol content of the main red juices,

and particularly those made of blackcurrant, evaluating their consumption, and identifying the concentrations of these flavonoids (PG, EGCG, C, Q) in the blood plasma is the new challenge. It will be necessary to base these future studies on mass spectrometry and determine if the concentrations found in the blood after ingestion have direct effects on antioxidant capacity and endothelial function.

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