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Flavonoids

A Coloring Model for Cheering up Life

Edited by Farid A. Badria and Anthony Ananga

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Professor Farid Badria has a PhD (Microbial Transformation, University of Mississippi, USA), and two MScs (Mansoura and Minnesota). The TWAS Prize for Public Understanding of Science (2013), WIPO Gold Medal (2011) (the best inventor in Egypt), State Outstanding Award in Medicine, Egypt (2001), Outstanding Arab Scholar, Kuwait (2000), and Khawrazmi International Award, Iran (2000) are just some of the awards he has received. He has also been a scholar of the Arab Development Fund, Kuwait (2000), ICRO-UNESCO International, Chile (1999), and UNESCO Biotechnology, France (1994). Professor Badria has submitted 46 patents of which 19 have been granted final certificates with intellectual protection for 20 years. With more than 200 publications and over nine books, he continues to lead research projects on developing new therapies for liver and skin disorders and cancer.

Dr. Anthony Ananga is an assistant professor in food science at Florida A&M University in Tallahassee Florida, USA. He has 10 years of teaching and 15 years of research experience. Dr. Ananga's research program focuses on grape vinification and bioprocessing, with concentration on the analysis of antioxidant capacity of phytochemicals in muscadine grapes. His lab investigates the bioavailability and efficacy of these phytochemicals in preventing chronic diseases and understanding their effect in fighting obesity. He also uses the functional genomics platform to improve nutraceutical compounds in muscadine grape cells for human health benefits. In addition to that, Dr. Ananga's lab is focused on food safety with an emphasis on fruit and peanut allergens. He strives to understand, characterize, purify, and investigate major food allergens in grapes and peanuts.

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Scope of the Series

Biochemistry, the study of chemical transformations occurring within living organisms, impacts all of life sciences, from molecular crystallography and genetics, to ecology, medicine and population biology. Biochemistry studies macromolecules - proteins, nucleic acids, carbohydrates and lipids –their building blocks, structures, functions and interactions. Much of biochemistry is devoted to enzymes, proteins that catalyze chemical reactions, enzyme structures, mechanisms of action and their roles within cells. Biochemistry also studies small signaling molecules, co-enzymes, inhibitors, vitamins and hormones, which play roles in the life process. Biochemical experimentation, besides coopting the methods of classical chemistry, e.g., chromatography, adopted new techniques, e.g., X-ray diffraction, electron microscopy, NMR, radioisotopes, and developed sophisticated microbial genetic tools, e.g., auxotroph mutants and their revertants, fermentation etc. More recently, biochemistry embraced the 'big data' omics systems.

Initial biochemical studies have been exclusively analytic: dissecting, purifying and examining individual components of a biological system; in exemplary words of Efraim Racker, (1913 –1991) "Don't waste clean thinking on dirty enzymes." Today however, biochemistry is becoming more agglomerative and comprehensive, setting out to integrate and describe fully a particular biological system. The 'big data' metabolomics can define the complement of small molecules, e.g., in a soil or biofilm sample; proteomics can distinguish all the proteins comprising e.g., serum; metagenomics can identify all the genes in a complex environment e.g., bovine rumen. This Biochemistry Series will address both the current research on biomolecules, and the emerging trends with great promise.

Contents

Preface

The colored pigments of berries, blackcurrants, black carrot, red cabbage, purple potato, and other types of colored foods are potential functional foods that have been consumed as chemopreventives and as strong dietary antioxidants for many diseases. Flavonoids are the most commonly and abundantly available natural coloring compounds in many foods, herbs, and plants.

This book discusses the nature and role of these compounds by studying the molecular mechanism of flavonoids using fluorescence spectroscopy and computational tools. This book also addresses natural vs. synthetic colors from both chemical and biological points of view. More importantly, several chapters are designed to explain in full detail the usefulness of these natural coloring properties that provide a safe, efficient, and economic therapy and/or prophylaxis for many health problems, e.g. obesity and cardiovascular disorders.

The book poses a balance between developments in scientific research and the idea that researchers must be able to absorb and link scientific advances with clinical practice so that the management of diseases can be based on sound physiological concepts.

Several controversial issues regarding these interesting compounds with fascinating pharmacological and therapeutic effects are addressed in three sections. Each chapter has been reviewed and revised, and new authors have provided up-to-date research to make the book more informative, illustrative, and easy to read.

Section 1: Natural Sources and Traditional Uses of Anthocyanins and Flavonoids:

- Anthocyanins: Natural Sources and Traditional Therapeutic Uses
- Anthocyanins in Apple Fruit and Their Regulation for Health Benefits

Section 2: Molecular Mechanism of Flavonoids:

- Molecular Mechanism of Flavonoids using Fluorescence Spectroscopy and Computational Tools
- Flavonoids as Modulators of Synaptic Plasticity: Implications for the Development of Novel Therapeutic Strategies for Healthy Lifestyle

Section 3: New Trends in Therapeutic and Industrial Applications of Anthocyanins and Flavonoids:

- Anthocyanins: Novel Antioxidants in Disease Prevention and Human Health
- Flavonoids: A Promising Therapy for Obesity Due to the High-Fat Diet
- Natural vs. Synthetic Colors

We hope this book will be useful to a wide range of people, from students first learning about natural coloring compounds, to advanced clinicians, nutrition specialists, and researchers who are looking for a review of current treatments and conceptualizations for various conditions.

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Section 1

Natural Sources and Traditional Uses of Anthocyanins and Flavonoids

Chapter 1

Anthocyanins: Natural Sources and Traditional Therapeutic Uses

Yogini S. Jaiswal, Yifu Guan, Ki Hwan Moon and Leonard L. Williams

Abstract

Anthocyanins are water-soluble naturally occurring pigments that are therapeutically beneficial and that have gained considerable interests by researchers in the field of phytopharmaceuticals and pharmacology. The evidence based scientific reports on the potential and efficacy of anthocyanins has caused an upsurge in their testing in clinical trials and formulation of herbal drug supplements since the past few decades. Their structural attributes enable them to be absorbed and react with various biomolecules in the human body, to provide beneficial physiological benefits. The anthocyanins are 2-phenylbenzopyrylium derivatives of dietary phenolics and exhibit antioxidant, anti-inflammatory and protective effects against metabolic and cardiovascular conditions. The metabolism of anthocyanins and their stability *in-vivo* in human body and during post-harvest storage still needs extensive investigation to fully explore their benefits. In the present chapter, we discuss the chemistry, medicinal uses in folklore/traditional medicine and the natural sources of their occurrence. The pre-clinical, clinical and pharmaceutical applications are also discussed, to emphasize the consumer demands and medicinal value of anthocyanins.

Keywords: anthocyanins, bioavailability, stability, metabolic disorders, herbal supplements

1. Introduction

Since several decades, flavonoids have captured the attention of scientists worldwide. The popularity of flavonoids in the scientific world is due to their versatile applications, therapeutic uses and environmental significance. They are reported to possess several beneficial pharmacological effects. Based on published reports, flavonoids exist in eight different classes and they are more than 9000 in number. These classes include anthocyanins, anthocyanidins, lipophilic flavones and flavonols, flavone and flavonol glycosides, chalcones, dihydrochalcones and aurones, flavanones and dihydroflavonols [1, 2]. Anthocyanins occur in various parts of plants including flower petals, stems, leaves and fruits. They are polyphenolic pigments that range in colors from red to purple. Literature survey reveals the presence of more than 700 anthocyanin compounds [3, 4]. Anthocyanins have several phenyl groups in their structure and mostly occur in glycosylated form. When more than one sugar group is attached in the C-ring, they are classified as "anthocyanidins" [5]. The anthocyanidins are reported to be less stable than

anthocyanins [5]. The structural diversity of anthocyanins provides advantage to the chemical modifications that can be carried out. The most reported structural modifications on anthocyanins include acylation. Some studies report the bioavailability of anthocyanins to be low, except for cyanidin-3-glucoside which exhibits a bioavailability of about 12% [6]. Routine consumption of anthocyanins is reported to be beneficial in preventing cardiovascular, neurological and metabolic disorders [7]. They are not essential constituents of diet but can be easily supplemented through intake of fruits and vegetables. There are no dietary intake reference levels established for anthocyanins till date. However, institutes worldwide recommend the use of anthocyanins for promoting good health. Publications such as *Dietary Guidelines for Americans* have been published to create awareness among consumers about their health benefits [8]. A report published on the dietary intake of anthocyanins states that, on an average the female population has a higher intake of anthocyanins compared to males. There is variation also observed among individuals of various races/ethnicities in consumptions of anthocyanins. A report indicates that the intake of anthocyanins was found to be higher in white population compared to Hispanic and non-Hispanic other populations in the USA [9]. Traditionally, fruits especially berries have been recommended as rich sources of anthocyanins. Despite their long use in traditional medicine, the use of anthocyanins in western medicine is still awaited. With their increasing popularity, the application of anthocyanins as a substitute to synthetic colors in food products is gaining acceptance. In the current chapter, the chemistry, pre-clinical, clinical and pharmaceutical uses are discussed in detail. The natural sources of anthocyanins and their traditional medicinal uses are also discussed in detail.

2. Natural sources of anthocyanins

The natural sources of anthocyanins include fruits, flowers, leaves and roots of plants. The most widely consumed anthocyanin natural products include cherries, berries and cereals. Scientific reports reveal several anthocyanins and their derivatives isolated from natural sources. A comprehensive list of some sources with specific anthocyanins is provided in **Table 1**. Anthocyanins occurring in flowers are observed to be more stable than the anthocyanins found in grapes. Studies

Source (common names)	Plant parts	Anthocyanins and derivatives
Chica	Leaves	6,7,3′,4′-Tetrahydroxy-5-methoxyflavylium [10, 11]
Onion	Scales	5-CarboxypyranoCy 3-glucoside [12, 13]
Peruvian lily	Flowers	6-OH Cy 3-(6-malonylglucoside) [14]
Palm-leaf fern	Fronds	Luteolinidin 5-(3-glucosyl-2-acetylglucoside) [15]
Black currant	Seeds	Pyranocyanins C and D [16, 17]
Spanish marigold	Flowers	Dp 3-[2-(2-caffeoylglucosyl)-6-malonylgalactoside]-7-(6-caffeoylglucoside)-3′-glucuronide [18, 19] Cy 3-[2-(2-caffeoylglucosyl)-6-(2-tartarylmalonyl)galactoside]-7-(6-caffeoylglucoside)-3′-glucuronide [18, 19]
White water lily	Leaves	Cy 3-(6-acetylgalactoside) [20]
Thale cress	Leaves and stems	Cy 3-[2-(2-sinapoylxylosyl)-6-(4-glucosyl-p-coumaroyl)glucoside]-5-(6-malonylglucoside) [21, 22]

Source (common names)	Plant parts	Anthocyanins and derivatives
Asian pigeonwings	Flowers	Dp 3-(2-rhamnosyl-6-malonylglucoside) [23]
Blue poppy	Flowers	Cy 3-(2-xylosyl-6-malonylglucoside)-7-glucoside [24, 25]
Blue bugle	Flowers	Dp 3-[2-(6-feruloylglucosyl)-6-p-coumaroylglucoside]-5-(6-malonylglucoside) [26, 27]
Tea tree	Flowers and leaves	Dp 3-(6-p-coumaroylgalactoside) [28, 29]
Ginger-leaf morning-glory	Flowers	Cy 3-[2-(6-caffeoylglucosyl)-6-{4-(6-3,5-dihydroxycinnamoylglucosyl)}caffeoylglucoside]-5-glucoside [30]
Potatoes	Tubers and sprouts	Pn 3-[6-(4-caffeoylrhamnosyl)glucoside]-5-glucoside [31–33]
Grape hyacinth	Flowers	Dp 3-(6-p-coumaroylglucoside)-5-(4-rhamnosyl-6-malonylglucoside) (muscarinin A) [34]
Garden petunia	Flowers	Mv 3-[6-(4-{4-(6-feruloylglucosyl)-p-coumaroyl}rhamnosyl) glucoside]-5-glucoside [35–37]
Purple maize	Cob	Catechin-(4,8)-cyanidin 3,5-diglucoside, cyanidin 3-malonylglucoside, cyanidin 3-succinylglucoside, cyanidin 3-dimalonylglucoside [38]
Black-purple rice	Whole grain	Cyanidin 3,5-diglucoside, pelargonidin 3-glucoside,cyanidin 3-arabidoside [39]
Black rice	Bran, whole grain	Cyanidin 3-glucoside, cyanidin 3-rutinoside [40]
Blue wheat	Whole grain	Delphinidin 3-glucoside, cyanidin 3-glucoside [41]
Winter blue barley	Whole grain	Cyanidin 3-malonylglucoside [42]
Sorghum	Whole grain	Apigeninidin, 7-O-methyl apigeninidin [43]
Rye	Pericarp	Acylated peonidin 3-glucoside [44]

Table 1.
List of sources of anthocyanins found in flowering plants, fruits and cereals.

that investigated the chemistry behind intensification and stability of grape wine color upon storage reveal that a cycloaddition reaction leads to the formation of 4-substituted anthocyanins. These anthocyanins are suggested to be the possible reason for stability of wine color upon maturation. The grape varieties that contain 3,5-diglycosides produce wine of an inferior quality compared with the varieties that contain high levels of 3-monoglycosides [45, 46]. The cycloaddition reaction leading to formation of 4-substituted anthocyanins was also observed in seeds of red currant [1]. Cereals form an important part of human diet and they are available in various colors ranging from red, yellow, purple and black. These colored cereals are a rich source of anthocyanins and include common cereals such as wheat, barley, sorghum, rice, maize, rye, oats, etc. [38, 44, 47–50]. Structures of some representative anthocyanins found in cereals, and fruits and flowers are represented in **Figures 1** and **2**, respectively.

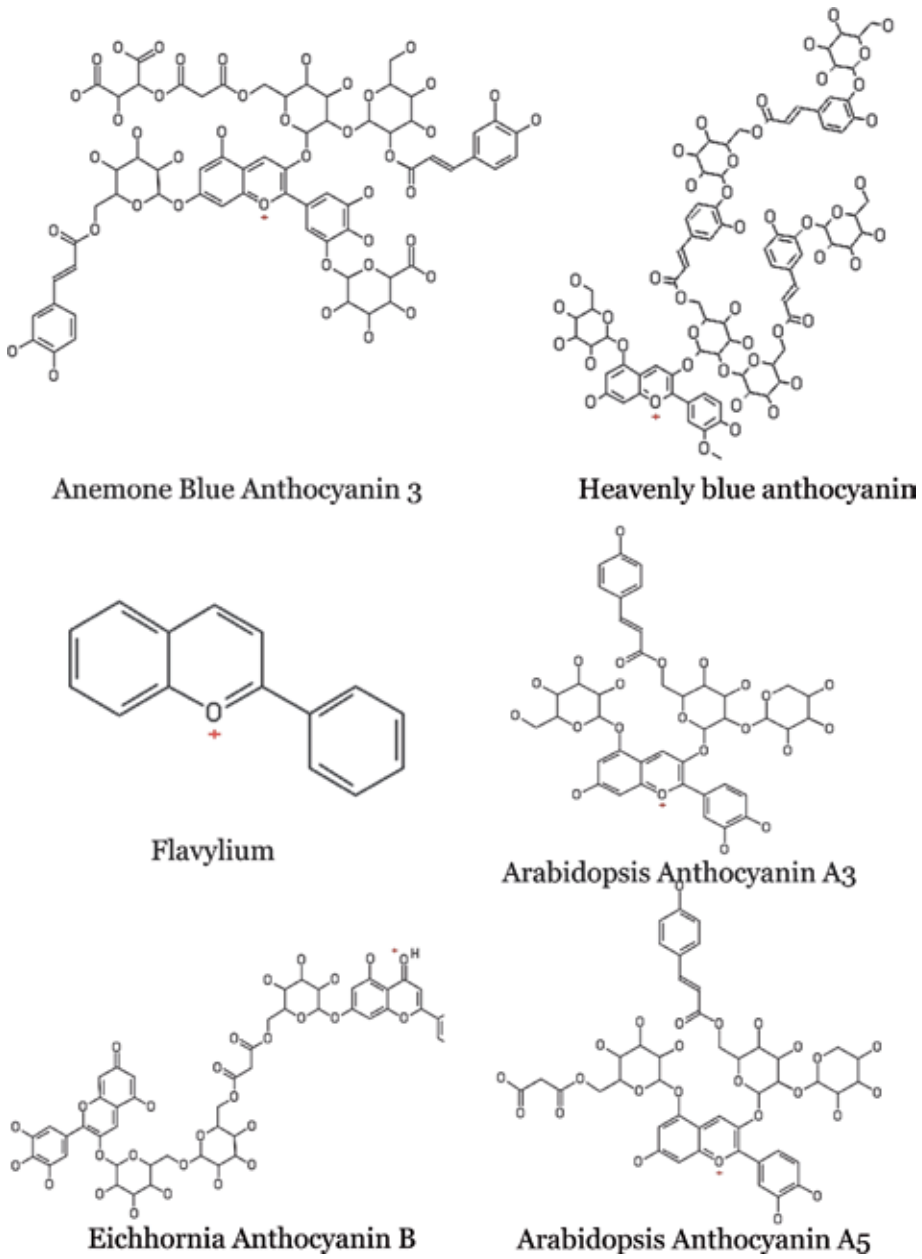

Figure 1.
Structures of representative anthocyanins and their derivatives found in cereals.

3. Chemistry

Anthocyanins are naturally occurring pigments. They are phenolic compounds that are mostly hydroxy derivatives of flavylium salts or glycosides of methoxy derivatives. Anthocyanins vary in forms based on the attachment of acids and hydroxyl groups to the sugar moieties within their structure. The anthocyanins found in plants include: cyanidin, delphinidin, pelargonidin, malvidin, peonidin and petunidin. Of these, cyanidin 3-glucoside is the most widely found anthocyanin [51]. The anthocyanins have an ionic structure and thus their color in solution is pH dependent [52]. They possess colors in shades of blue as the solutions

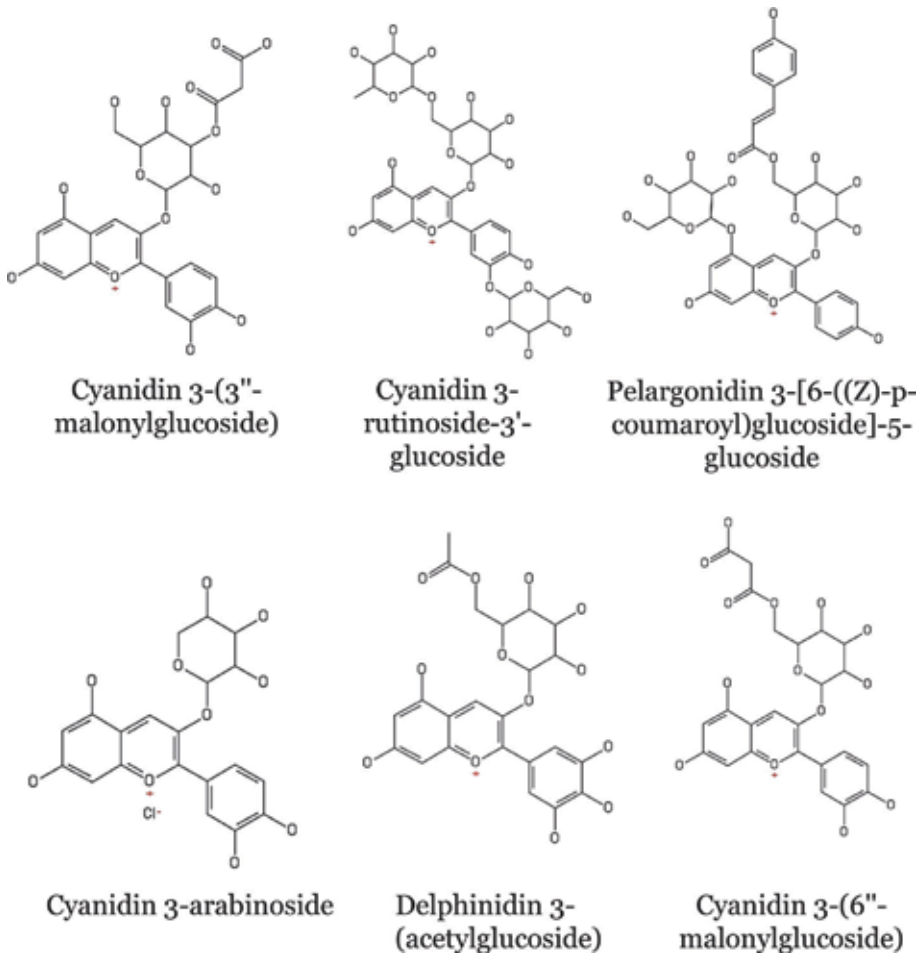

Figure 2.
Structures of representative anthocyanins obtained from fruits and flowers.

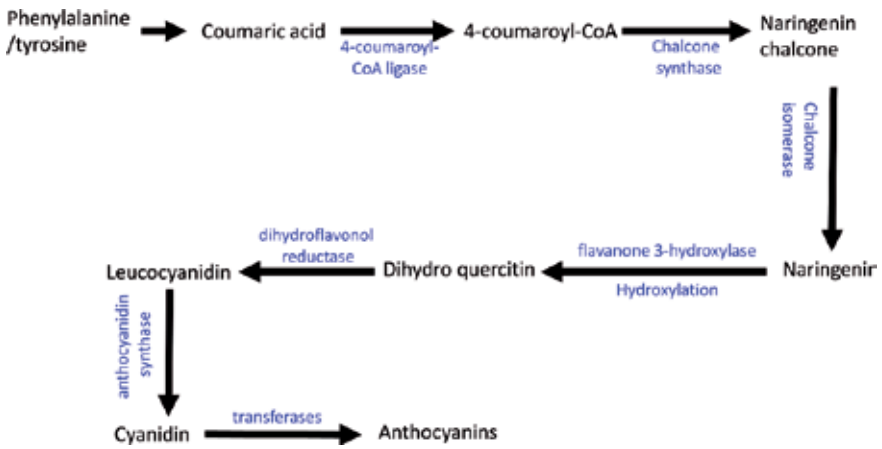

Figure 3.
Biosynthesis of anthocyanins in plants.

approach a neutral pH and a red color shade as the solutions are made acidic. Lower pH values provide higher stability to anthocyanins. The flavylium cations increases the solubility of colored pigments in water at low pH. With increase in protonation

caused due to increase in pH, the concentration and stability of pigments reduces. Polymerization reactions are also reported to increase their color stability. Purple-colored stable quinonoid anions are formed at neutral pH [53].

Plants synthesize anthocyanins and store them in vacuoles. The colors of anthocyanins in the vacuoles will vary depending on the existing pH conditions. A general flavonoid pathway is used by plants for their synthesis. 4-coumaroyl-CoA is formed from phenylalanine or tyrosine and further condensed with malonyl-CoA to produce naringenin chalcone. Chalcone isomerases convert naringenin chalcone to naringenin [54]. Naringenin then undergoes several hydroxylation steps to form anthocyanins. A schematic representation of the anthocyanin formation pathway is provided in **Figure 3**.

4. Traditional and pharmaceutical uses of anthocyanins

Traditionally anthocyanins from plants have been used for treatment of hepatobiliary issues such as hyperbilirubinemia, obstructed bile ducts and treatment of lack of appetite [55]. In this section several therapeutic uses of anthocyanins reported in literature are discussed. A list of the effect of anthocyanin treatment in disease conditions and the tested pathological markers is provided in **Table 2**.

4.1 Antioxidant effects

Anthocyanins act as good free radical scavengers due to the keto group with a conjugated double bond. The high reactivity and instability of aglycones in their structure provides an advantage for anthocyanins in acting as antioxidant agents [61]. Glycosylation reactions reduce and diacylation reactions increase their free radical scavenging activity. Anthocyanins have been investigated in various systems and models for their antioxidant effect. In a study investigating the formation of malondialdehyde after UVB irradiation, delphinidin-3-glucoside and pelargonidin-3-glucoside exhibited significant antioxidant effects [62]. Pelargonidin acts as an excellent hydroxyl radical scavenger, and delphinidin acts as a good oxygen radical scavenger. Studies also report cyanidin and cyanidin-3-glucoside to possess inhibitory effects against oxidation of low-density lipoproteins (LDL) [63].

4.2 Effects on angiogenesis

Angiogenesis is the process of development of new blood vessels with the help of endothelial cells. Chemical mediators within the body (angiogenic and anti-angiogenic factors) maintain the environment required for normal angiogenesis. Angiogenic factors include growth factors such as vascular endothelial growth

Pathological condition	Effect(s) of treatment with anthocyanin extracts
Inflammation	Anti-inflammatory effect and reduction in muscular pain [56, 57]
Hyperglycemia	Reduction in glycated hemoglobin levels. Reduction in triglyceride and very low density lipids (VLDL) contents [58]
Cardiovascular diseases	Reduction in plasminogen activator inhibitor-type-1 and regulation of blood pressure [59]
Brain disorders	Antianxiety effect. Improvement of memory and cognition [60]

Table 2.
Effect of anthocyanin treatment in disease conditions and the tested pathological markers.

factor (VEGF), angiopoietin and fibroblast growth factor. Antiangiogenic factors include factors such as thrombospondins. Disruption in the balance between these factors can lead to complications in disorders such as diabetes and cancer.

Reports published on evaluation of expression of VEGF reveal that colored berries due to their anthocyanin contents, reduce expression of VEGF and VEGF induced tube formation in *ex-vivo* models of human cell lines, under oxidative stress [64]. When high glucose concentrations were induced in human endothelial cells, anthocyanins from purple corn were found to inhibit expression of angiogenic factors [65].

4.3 Antitumor activity

Angiogenesis play a very critical role in proliferation of cancer cells. Reports suggest that anthocyanins extracted from berries have antiangiogenic effects in various cancer cell lines. Blue berry, bilberry and black rice anthocyanins are reported to exhibit anti-invasive properties in breast cancer cells and in *in-vivo* animal models by reducing the expression of cyclooxygenase-2 gene [65]. Other mechanisms of action reported for anticancer effect of black rice include suppression of activation of mitogen-activated protein kinase (MEK), and decreased expression of matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9) [66]. A study of purple potato anthocyanins in CF-1 mice model reports the antitumor effect of its extracts in colon cancer by induction of cell-cycle arrest [66].

4.4 Antidiabetic effects

Anthocyanins from Cornus fruits are reported to induce insulin secretion in *ex-vivo* rodent beta cells. Cornus fruits are used in Traditional Chinese Medicine as antidiabetic agents. It is reported that the property of inducing insulin secretion in anthocyanins may be due to the hydroxyl groups in their B-ring [67]. Anthocyanins reported to induce insulin secretion include delphinidin-3-glucoside and pelargonidin-3-galactoside. Seoritae extract is reported to inhibit diabetic nephropathy by suppressing renal lipid deposition [68]. Anthocyanins from Bilberries act as antihyperglycemic agents by stimulating the adenosine monophosphate-activated protein kinase (AMPK). Bilberry extracts are also reported to improve visual function in patients suffering from diabetic neuropathy and glaucoma [69].

4.5 Neuroprotection

Neuroprotection is achieved by reduction in toxic effects and injuries caused to neurons due to oxidative reactions. Cyanidin glucosides are reported to inhibit DNA fragmentation and oxidative stress caused due to hydrogen peroxide formed *in-vivo* in human neuronal cells [70]. Tart cherry anthocyanins, when tested for neuroprotective activity in mice brain under oxidative stress inhibited generation of apoptosis-inducing factor [71]. Cyanidin-3-O-β-D-glucopyranoside and petunidin-3-glucoside isolated from mulberry and black soybeans, are reported to exhibit neuroprotective activity in *ex-vivo* models. They inhibit cerebral ischemia and cell death caused due to hydrogen peroxide induced oxidative stress [72, 73].

5. Anthocyanins in food products and pharmaceuticals

Incorporation of anthocyanins as an ingredient in foods and pharmaceutical has undergone several challenges. The prime issue with their use, is their stability

and hence their effectiveness as a beneficial component in food and pharmaceutical. Anthocyanins are used as colorants in pharmaceuticals and food products as a substitute to synthetic colors. Studies have compared the stability of anthocyanins as colors with synthetic colors and have found that the synthetic colors have better stability than anthocyanins. In a study, stability of synthetic colors such as Carmoisine, Allura red and Ponceau were compared with natural anthocyanin colors, viz. enocyanin, dark carrot and cochineal. The results of the study indicate that at higher temperatures and higher pH conditions the stability of natural anthocyanins was much lower that the synthetic colors [74]. However, the beneficial effects of anthocyanins cannot be ignored due to their stability issue.

In another study, the stability of freeze-dried powder of anthocyanin extract of wild blueberry was compared at various temperature values over a storage period of more than a month. It was observed that the total anthocyanin content and the content of individual anthocyanins was most retained at 25°C and reduced significantly at higher temperatures [75]. A combination of ascorbic acid with anthocyanins from plant extracts was evaluated for stability under varying humidity, temperature and pH conditions in both freeze dried and solution form. It was observed that in solution form the stability of the combination reduced with increase in pH and temperature. In freeze dried form the stability of the combination decreased with increase in humidity [76]. Commercially several yoghurt variants are available with a variety of fruits. Some studies carried out on extracts of blackcurrant and wine grape extracts in yoghurt indicate that the fermentation process and addition of some probiotics reduce the stability of anthocyanins [77–79].

Microencapsulation is another technique tested for anthocyanin release and stability. In a cell line study carried out microencapsulated glycons and aglycons of some anthocyanins, glycons of anthocyanins were found to be more stable than the aglycon counterparts [80]. The conventional extraction methods are cost intensive and employ application of heat for extraction. Ground breaking research has been carried out in metabolic engineering of microbes for production of anthocyanins. Cyanidin 3-O-glucoside has been successfully produced by *E. coli*. However, its production at commercial scale poses challenges due to imbalances in the expression of other genes and lack of optimized transporters for extracellular secretion [81]. The merger of metabolic engineering with plant chemistry holds a promising future for anthocyanins and their application in food and drug industry.

6. Conclusions

The evidences provided through scientific studies till date, demonstrate the therapeutic benefits of anthocyanins. The anthocyanins are excellent natural sources of bioactive compounds that can help in treatment and prevention of many disease conditions. With the multidisciplinary research carried out in the last few years, anthocyanins have a promising future for their commercialization and application in the pharmaceutical and health foods sector.

Conflict of interest

The authors declare that they have no conflict of interests.

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Chapter 2

Anthocyanins in Apple Fruit and Their Regulation for Health Benefits

Kaori Matsuoka

Abstract

In most industrialized countries, cancer and cardiovascular disease have been linked to lifestyle choices, the most important of which is diet. Antioxidants show protective effects against both of these diseases. Anthocyanins contribute greatly to the antioxidant properties of certain colorful foods, such as apples. Apples are rich in anthocyanins in the peel, followed by the whole fruit and then the flesh. From a nutritional point of view, therefore, regular consumption of apples with peel is recommended to enhance the dietary intake of antioxidant compounds. The anthocyanin concentration of apple peel changes according to internal plant conditions (e.g., fruit maturity and plant hormones), environmental factors (e.g., light, temperature, and nutrients), and the cultivar. The combination of cultivar variation and responsiveness to specific environmental conditions could create opportunities for the production and processing of apples with improved anthocyanins and antioxidant properties.

Keywords: anthocyanin, antioxidant activity, apple fruit, environmental factor, fruit maturation, global climate change, human health, plant hormone

1. Introduction

The apple (*Malus domestica* Borkh.) is produced globally; about 89 million tonnes was produced in 2016, ranking it third in worldwide fruit production [1]. In Poland, most people across all age groups eat from one to six apples a week. Most Polish customers consider flavor, taste, and firmness at the top of the list and give less consideration to nutritional value, peel color, and size [2]. Nevertheless, Polish customers of all age groups prefer more redness over greenness [2]. In a study of Estonian customers from 2007 to 2012, the most important consumer preferences were apple taste, followed by appearance (e.g., color) and health benefits, which were rated of equal importance, and finally price; consumer preferences in taste and color remained unchanged, whereas preference for domestic and organic apples decreased, over the 5-year period survey [3]. In a UK study, consumers associated red apples with sweet sensory descriptors and green apples with grassy, astringent and drying, acidic, sour sensory, or unripe descriptors [4]. Therefore, the color of apple peel is important for market value, and well-reddened fruit has an increased price rating in Japan [5].

The redness of apple peel is due to the accumulation of anthocyanins, which are water-soluble plant pigments responsible for the blue, purple, and red in many

plant tissues of fruits, flowers, and vegetables. Willett [6] showed that approximately 32% of cancers could be avoidable by changes in diet. People with low fruit and vegetable intake have about twice the risk of most cancers as those with high intake [7]. In addition, a case-control study of a group of 33 women recently diagnosed with breast cancer and a control group of 33 healthy women demonstrated that regular ingestion of apples, watermelons, and tomatoes was associated with protection against breast cancer [8]. Increased intake of apple fruits has been associated with reduced risk of disease, because apple fruits—especially their peel—have strong antioxidant activity [9], which benefits human nutritional health.

This review provides a general overview of the nutritional impacts of polyphenols, flavonoids, and anthocyanins, which serve as antioxidants and counteract the prooxidant load of the human body [10]. The review focuses on anthocyanin in apple fruit peel and its health benefits and the means by which this anthocyanin concentration could be improved by regulating cultivation management and breeding cultivars with high anthocyanin levels. The literature on anthocyanin synthesis in red-fleshed apple fruits is not reviewed here; the details of genetic, environmental, and plant hormonal factors involved in anthocyanin synthesis have been reported by Honda and Moriya [11].

2. Polyphenols, flavonoids, and anthocyanins

2.1 Concentrations of polyphenols, flavonoids, and anthocyanins and antioxidant activity in common foods

Polyphenols are among the most numerous products of the secondary metabolism of plants and are an integral part of the human diet [12]. They constitute more than 8000 phenolic structures and can be divided into at least ten different classes, depending on their basic chemical structure. The recent interest in food phenolics has increased greatly owing to their possible benefits to human health, such as prevention of cancer, cardiovascular disease, and other pathologies. The polyphenolic concentration of plant foods can vary by several orders of magnitude [12], with the cereals barley and sorghum having considerably higher levels than those found in some more commonly consumed foods (e.g., fruits, nuts, and vegetables). In the fruits mentioned in the review [12], blackcurrant has the highest polyphenolic concentration, followed by grape, raspberry, and apple. Therefore, apples can serve as an important source of polyphenols as dietary foods.

Plant flavonoids constitute one of the largest groups of naturally occurring phenolics, and they possess an ideal chemical structure for antioxidant activity. They commonly contain diphenylpropanes with a 15-carbon skeleton (C_6—C_3—C_6), which comprises two aromatic rings linked through three carbons that usually form an oxygenated heterocycle. The flavonoids in foods comprise 13 types of basic structure, one of which is anthocyanidin [12]. The glycosides of anthocyanidin are referred to as anthocyanins.

Anthocyanins vary in the number and position of hydroxyl and methoxyl groups on the basic anthocyanidin skeleton. There are over 600 naturally occurring anthocyanins. Approximately 17 anthocyanidins are found in nature, and 6 of them—cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin—are ubiquitously distributed in nature.

Antioxidants are present at high levels in apples [13], and the antioxidant activity of apples has significant positive relationships with total phenolic and flavonoid concentrations [10]. When compared with many other commonly consumed fruits, apples have the second-highest level of antioxidant activity [13].

Antioxidant activity provides a measure of protection that slows the process of oxidative damage, which is an important cause of disease initiation and progression in the human body [14].

2.2 Concentrations of polyphenols, flavonoids, and anthocyanins and antioxidant activity in apple fruits

Many studies have shown that polyphenol, flavonoid, and anthocyanin concentrations and antioxidant activity in apples differ between the edible parts of the fruit. In a study of four cultivars, "Rome Beauty," "Idared," and "Cortland" (all three red-skinned), and "Golden Delicious" (golden-skinned), total phenolic and flavonoid concentrations were highest in the peel, followed by the whole fruit and then the flesh [9]. The peels of these apple cultivars also had significantly higher total antioxidant activities than the whole fruit or the flesh. In a study of ten different cultivars in New Zealand, on average, 46% of polyphenols in whole apples were in the peel, and essentially all of the flavonols (quercetin derivatives) were found to be present in the peel [15]. In a more recent study of "Fuji" (bright red skin with green patches) and "Epagri COOP24" and "Epagri F5P283" (both deep red skin) cultivars, the total phenolic and total flavanol concentrations and total antioxidant activity were also highest in the peel, followed by the whole fruit and then the flesh [16]. The peel and whole fruit possessed 1.1–4.1 times the total phenolic concentration, 1.2–4.9 times the total flavanol concentration, and 1.1–3.9 times the total antioxidant activity of the flesh. The total phenolic concentrations of the flesh, the whole fruit, and the peel were all positively associated with total antioxidant activity [16]. From a nutritional point of view, the above findings suggest that regular consumption of apples with peel is recommended to enhance the dietary intake of antioxidant compounds.

2.3 Anthocyanin concentration in apple peel and its relation to colorimetric values

In apples, pigmentation is controlled by the relative concentrations of anthocyanin pigments; the main pigment responsible for the red is cyanidin-3-galactoside (idaein). The anthocyanin concentration of the peels of mature apples of eight "Gala" strains (bright red over yellow background) provided regression coefficients of determination (R^2) of 0.73 and 0.74 and $P \leq 0.05$ for the colorimetric values of a*/b* (greater value means higher redness) and hue angle (lower value means higher redness), respectively, as measured with a tristimulus colorimeter [17]. This strong relationship between anthocyanin concentration and chromaticity parameters in apple peel shows good prediction of anthocyanin concentration based on chromaticity values and offers the possibility of using a portable colorimeter for nondestructive estimations of fruit anthocyanin concentration in situ.

3. Apple fruits and their health benefits for humans

Regular consumption of apples is inversely associated with breast cancer occurrence and affords some degree of protection against the development of the disease [8]. Wolfe et al. [9] investigated the effects of the apple peel, the whole fruit, and the flesh in inhibiting the growth of $HepG_2$ human liver cancer cells in vitro by estimating the median effective concentration (EC_{50}; expressed as concentration of apple component (mg mL^{-1})) as an indicator of antiproliferative activity. Phytochemical extracts of the apple peels of each cultivar tested ("Rome Beauty,"

"Idared," "Cortland," and "Golden Delicious") inhibited the growth of $HepG_2$ cells more effectively than extracts of the flesh or the whole fruit. The peels also had a lower EC_{50} than the flesh and the whole fruit components, representing higher anti-proliferative activity. The inhibitory effects of the cancer cell proliferation varied widely depending on the apple cultivar, with "Rome Beauty" apple peels showing the greatest bioactivity.

4. Anthocyanin accumulation during fruit maturation

Anthocyanin production depends mainly on carbohydrates (especially galactose), which are formed during photosynthesis and glucose metabolism in apple leaves and are later transported to the fruit [18]. Therefore, anthocyanin synthesis is closely associated with plant metabolism and depends on the stage of fruit development. For instance, during fruit growth, anthocyanin concentrations in "Gala" apple peel are low and fluctuating, and they then increase markedly on the blush side near maturation [19]. Similarly, Chalmers et al. [20] found that anthocyanin accumulation in red apple cultivars occurs rapidly during the transition from the immature to mature stages and precedes the normal harvest date by about 2–3 weeks. Honda et al. [21] also reported that anthocyanin production proceeded rapidly in the fruit peel of potted trees of red-skinned cultivar "Misuzu Tsugaru" during the last week before harvest, not only under control conditions (25°C 12 h/15°C 12 h) but also under hotter temperature conditions (29°C 12 h/19°C 12 h) for 5 weeks. A continuous increase in anthocyanin concentration, especially in the last 2 weeks before harvest, on both the blushed and shaded sides of the apple fruit was also found in a superior redness sport of "Shotwell Delicious" called "Topred Delicious" [18]; later, the authors reported that the largest increase in anthocyanin concentration and color development in the eight "Gala" apple strains occurred from 2 weeks before the commercial harvest date, and the increase continued for up to 1 week after the harvest date [17]. These findings show the importance of the ripening period for anthocyanin accumulation in apple fruit peel.

5. Regulation of anthocyanin synthesis in apple fruits

Anthocyanin synthesis in apple fruits is affected by environmental factors and internal plant conditions, including biotic and abiotic factors such as light, temperature, nutrients, and plant hormones, and by the type of cultivar. The use of special cultivation methods and cultivars that produce high levels of anthocyanin has been proposed for improving anthocyanin concentration and redness of apple peel.

5.1 Light irradiation and its effective use for enhancing anthocyanin accumulation

Development of redness in apple fruit peel requires light [22, 23], and the amount of anthocyanin synthesized is correlated with the light intensity received [24]. Therefore, redness development in apple fruit peel is higher on the side that receives more exposure to light (the blushed side) than on the shaded side (**Figure 1**). Hamadziripi et al. [25] revealed the effects of microclimate at different positions in the apple tree canopy "Starking" on peel redness and anthocyanin concentration. They found that the outer-canopy fruits were redder and contained more anthocyanins than the inner-canopy fruits. This seemed to be related to microclimatic differences in irradiance, because outer-canopy fruits were exposed to almost 30 times the irradiance

Figure 1.
Occurrence of anthocyanins in "Fuji" apple peel on the blushed side (the right side in this photo), which receives more exposure to sunlight.

as fruits in the innermost portion of the canopy, and the average peel temperature of outer-canopy fruits was about 10°C higher than that of the inner-canopy fruits. In addition, among the eight "Gala" strains mentioned in Sections 2.3 and 4, the anthocyanin concentration on the blushed side was about three to five times that on the opposite shaded side at commercial harvest and after the harvest in low- and medium-high-coloring "Gala" strains [17]. Apparent differences in anthocyanin concentrations between the blushed and shaded sides of "Gala" apple peel occur near maturation (125 days from flowering), when the anthocyanin concentration increases markedly only on the blushed side [19].

To enhance peel color, cultivation techniques that are effective in promoting anthocyanin synthesis and improving color in apple peels have been developed. In red-skinned cultivars, to allow the apple skin to receive more exposure to light and thereby promote redness, the leaves covering fruits are removed before harvest [5]. Rotating the fruit and branch management such as pruning and putting support posts beneath the canopy can also improve the redness of apple peel [5]. Placing reflective films on the ground also effectively increases the intensity of light entering the apple tree canopy (**Figure 2**) [26, 27]. Ju et al. [27] reported that, after film application, the light intensity inside the tree canopy "Fuji" increased from 30% of daylight to 50–68% of daylight for foil film (crinkled aluminum foil bonded onto cloth) and metalized film (aluminum metalized polypropylene film); the anthocyanin concentration and percent redness of the fruit peel at harvest were also increased.

Anthocyanin synthesis in apple fruit is highly dependent on light quality, such as ultraviolet-B (UVB) and visible light (e.g., red, blue, and white fluorescent light). UVB and white fluorescent light are essential for anthocyanin accumulation, regardless of the temperature, but white fluorescent light alone does not increase anthocyanin concentration [23]. Anthocyanin synthesis is stimulated more by irradiation with UVB with an emission peak at a wavelength of 312 nm (UVB312) than by UVB353 [28]. UVB312 is more effective in stimulating anthocyanin production

Figure 2.
Reflective material laid on the soil surface between tree rows in a "Fuji" apple orchard to enhance the trees' interception of sunlight.

than red or blue light. Simultaneous irradiation with UVB312 and red light [28] or UVB312 and white fluorescent light [29] produces much higher anthocyanin than irradiation with either light alone. This synergism seems to have an important role in development of the desirable red peel under field light conditions.

5.2 Effect of temperature on anthocyanin synthesis

Temperature has a major effect on anthocyanin synthesis in apple plants. As a result of recent global warming, there are increasing concerns that global warming may be detrimental to fruit reddening.

In general, apples redden best in climates with clear bright days and cool nights during the preharvest period [26]. Night temperatures below 18°C enhance reddening in "Fuji" apples [26]. Reay [23] reported that a cool temperature of 4°C in the dark period followed by 20°C with UVB-visible irradiation was the most effective condition for anthocyanin accumulation in apple peel of "Granny Smith." This is the equivalent of cool nights followed by warm clear days. With continuous 20°C temperature in both dark and UVB-visible irradiation periods, the anthocyanin accumulated was less than half that with the former temperature condition. This result shows the importance of a low-temperature period in the development of the red blush on apple peel.

Generally, high temperatures increase the respiration rate and carbohydrate consumption of apple trees, thus reducing anthocyanin synthesis in the fruit. Wang et al. [30] reported that anthocyanin accumulation during the ripening period in the peel of "Royal Gala" red-skinned cultivar grown in hot climates (17–35°C, using artificial heating of fruit on the tree over two 7-day periods) caused a dramatic reduction in peel anthocyanin concentration and decrease in redness compared with those in unheated control fruit (7–22°C for unheated apples). Proctor [24] had earlier found that redness cultivars of "McIntosh," "Northern Spy," and "Cortland" did not color with supplementary light when the 48-h average temperatures were greater than 20°C, suggesting that anthocyanin accumulation requires temperatures lower than 20°C. Consistent with this notion, Honda et al. [21] showed that 15°C was generally the optimal temperature for anthocyanin accumulation during fruit ripening of redness cultivars, "Tsugaru," "Tsugaru Hime," and "Akibae," whereas anthocyanin accumulation was repressed under 30°C.

Iglesias et al. [18] have noted that poor fruit coloration has already become a problem owing to frequent periods of high temperatures (>30°C) with very low rainfall in summer in many apple-producing areas in Spain. Seasonal differences in temperature and rainfall in the period before harvest often cause harvesting to be delayed in order

to attain a certain degree of color, a practice that has negative effects on quality—especially firmness and flavor. This lack of color is an important cause of reduction in grade and is generally associated with poor consumer acceptance. To overcome this problem, Iglesias et al. [18] applied overtree microsprinkler irrigation for 25–30 days preceding the harvest to improve fruit redness and increase anthocyanin concentration in "Topred Delicious" cultivars. Similarly, the warm climate of California can delay harvest owing to inadequate reddening of "Fuji" apples; this has been associated with physiological problems such as skin cracking and internal browning [26].

On the basis of 30–40 years of climate records, Sugiura et al. [31] pointed out that the annual mean air temperature in Japan has risen by 0.31–0.34°C per decade. They proposed that since such climate warming brings earlier blooming and higher temperatures during the maturation period, it is likely to change the taste (acidity and soluble solids) and texture (fruit firmness and watercore development) of apple fruits. They suggest that the slower advance toward the benchmark blush rate caused by warming during the maturation period will offset the advance in fruit maturity induced by the earlier blooming. Honda and Moriya [11] demonstrated that the temperature during fruit ripening of apple trees is an important factor in anthocyanin synthesis in both apple fruit peel and fruit flesh. They observed that the anthocyanin concentrations in the flesh of mature "Pink Pearl" (normal fruit flesh color, pale pink or red; fruit color, red and orange flush) harvested in 2016 were considerably lower than the typical level, probably because of the high temperatures recorded during the ripening period in that year. This suggests that the higher temperatures caused during fruit ripening by recent climate warming may be detrimental to fruit reddening.

Many reports about the effects of treatment-induced changes (e.g., light, temperature) on the characteristics of fruit peel color in apples indicate that production areas for apple fruits will likely increasingly suffer from inadequate reddening owing to ongoing climate warming. Therefore, further research is needed to clarify the effects of ongoing climate warming on the rate of anthocyanin synthesis in apple peel at each growth stage.

5.3 Enhancing anthocyanin accumulation through the choice of the nutrient and nutrient level

Fruit nutrient composition has a strong association with fruit color. In general, nitrogen (N) negatively affects apple fruit redness [32, 33]. The percentages of well-colored fruit of "Jonathan" apples were higher in trees given ammonium nitrate fertilizer at 0–5 g N m^{-2} $year^{-1}$ than in trees given the same fertilizer at higher rates of up to 10–20 g N m^{-2} $year^{-1}$ from 1978 to 1983 (**Figure 3**) [32, 34]. The excessive nitrogen application decreased the redness of the apple peel. Similarly, in "Elshof" (bright red with yellow background color), the anthocyanin concentration and percentage of blush are generally decreased by increasing the amount of N fertilizer [35]. Foliar application of nitrogen by using urea spray lessens the increase in anthocyanin concentration in the blush-side peel of "Gala" apples at maturity [19]. Furthermore, Awad and Jager [35] reported that the anthocyanin concentration in the peel of "Elshof" and "Elstar" (mostly red with yellow showing) apples at maturity is negatively correlated with the concentrations of N and magnesium (Mg) and with the N to calcium (Ca) ratio in the fruit at maturity, but the most important nutrient factor associated with anthocyanin concentration at maturity is the N concentration in the fruit during growth and at maturity. The result obtained from Awad and Jager [35] suggests that the maximum level of anthocyanin concentration in apple fruit peel could be achieved if the N concentration could be maintained at marginal N fertility levels to minimize the N concentration in the fruit.

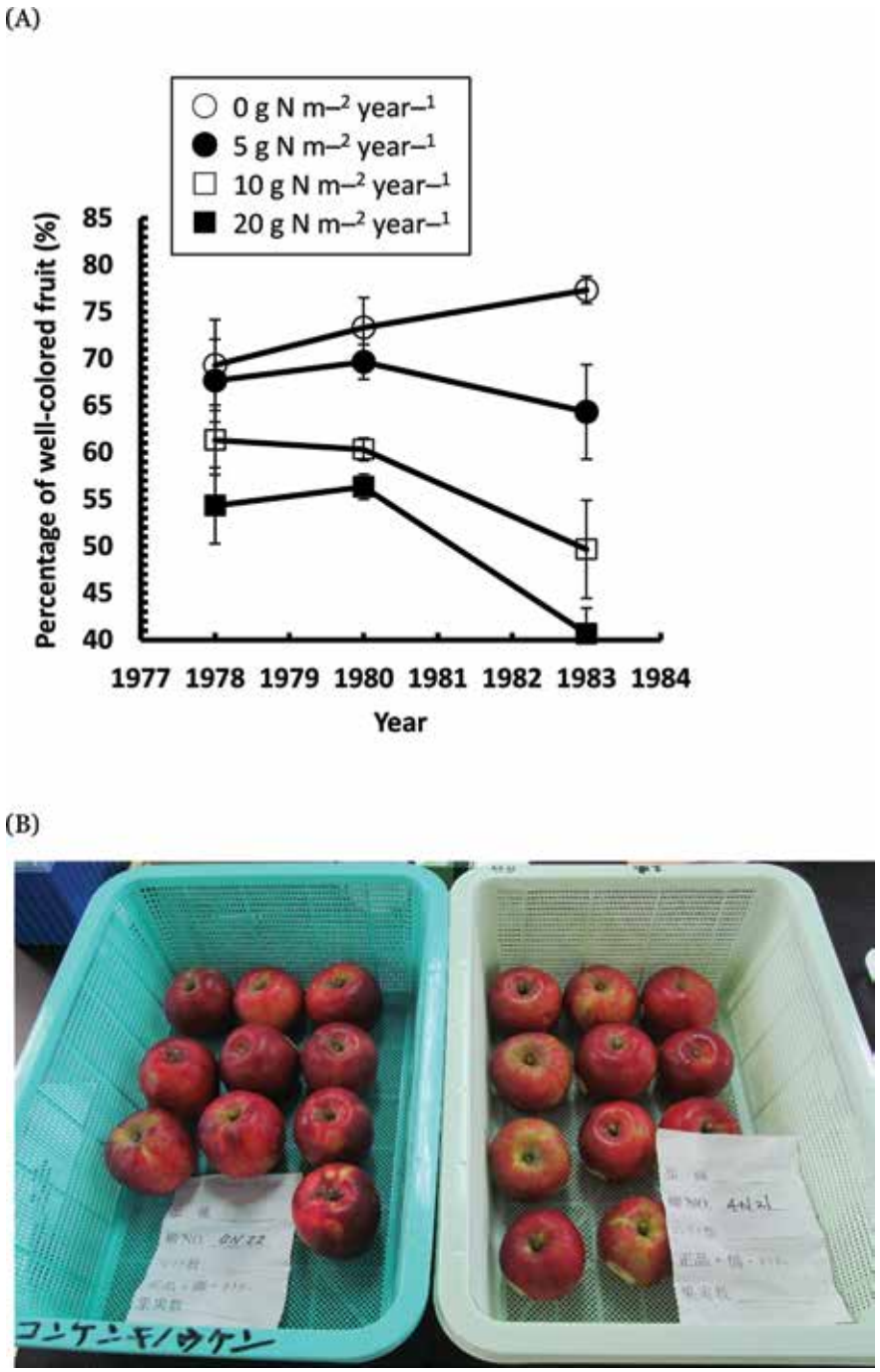

Figure 3.
Effects of N application as ammonium nitrate on the percentage of well-colored "Jonathan" fruit. (A) Percentages of well-colored fruits from 1978 to 1983 in a long-term N fertilization field, Japan. A well-colored fruit is designated as one in which over 80% of the peel is colored. Data are running averages from 3 years ±SD, n = 3. Data are from [34]. (B) Representative fruit from an unfertilized tree (0 g N m^{-2} $year^{-1}$; left picture) and a fertilized tree (up to 20 g N m^{-2} $year^{-1}$ as application of ammonium nitrate fertilizer; right picture); the peel of apples from the unfertilized tree is a superior red.

Anthocyanin synthesis in apple fruit is associated with not only N but also phosphorus (P) and Ca nutrients. P-Ca mixtures with mineral ions, such as Phostrade and Seniphos, have been used widely to enhance fruit coloration. Superior redness

of fruit and high anthocyanin concentrations could be achieved in "Braeburn" apple fruits with foliar application of Phostrade Ca (23.6% P_2O_5, 4.3% CaO, and 3% N); this effect was due to the P and Ca, not the N [36]. They showed that the anthocyanin concentration in the peel of treated apples at harvest was 20 times, whereas the concentration in the untreated apple peels (control) at harvest was only 9 times those at 5 weeks earlier of the harvest. Another P-Ca mixture, Seniphos (310 g L^{-1} P_2O_5, 56 g L^{-1} CaO, and 30 g L^{-1} total N: 1% NO_3 and 2% NH_3, the Phosyn Ltd. Company (Phosyn PLC, York, UK)) is also used commercially to improve peel color in apples. The positive influence of Seniphos on apple redness has been reported in various cultivars, such as "Starking Delicious" [37, 38] and "Fuji" [39], but it is not effective in "Jonagold" [40]. Gómez-Cordovés [37] showed that application of Seniphos increased the anthocyanin concentration in "Starking Delicious" apple peel over the ripening period and also altered the percentage composition of anthocyanins.

Sucrose can promote reddening of apple peel. Anthocyanin concentrations of peel disks of wax apple fruit (light to dark red, sometimes green) increase in a linear fashion with concentration of sucrose in the culture solution [41].

5.4 Plant hormones and their application to enhance anthocyanin accumulation

Ethylene, a hormone involved in maturation processes, plays an important role in regulating anthocyanin synthesis in apples. A close relationship between internal ethylene accumulation and anthocyanin accumulation in "Fuji" apple fruits [27] and in "Jonathan" apple fruits [42] has been reported. Therefore, ethylene can be used commercially to increase anthocyanin accumulation in red apples. Ethephon (2-chloroethyl phosphonic acid), an ethylene releasing compound that acts as a growth regulator, effectively promotes anthocyanin synthesis in "Starking Delicious" [37, 38] and "Fuji" [39] when light and temperature requirements for reddening are met [26]. Awad and Jager [40] compared the effects of the following seven growth regulators on the accumulation of anthocyanin in "Jonagold" apple peel: ethephon, CCC (2-chloroethyltrimethyl ammonium chloride), prohexadione-Ca (3-oxido-4-propionyl-5-oxo-3-cyclohexane-carboxylate), GA_{4+7}, plantacur-E (a vitamin E formulation containing 25% alpha-tocopherol), shikimic acid, and galactose. Among these growth regulators, ethephon resulted in the highest anthocyanin concentration in fruit peel at commercial harvest.

The above results differ from reports that did not find a positive relationship between ethylene synthesis and anthocyanin accumulation in apple fruits that were irradiated with UV for "Starking Delicious," "Jonathan," "McIntosh," "Fuji," "Ralls Janet," "Tsugaru," "Jonagold," "Mutsu," and "Golden Delicious" cultivars [29] or subjected to hot climatic conditions [21]. For instance, apple trees of "Misuzu Tsugaru" subjected to hot climatic conditions (29°C 12 h/19°C 12 h) had lower anthocyanin accumulation in fruit at harvest but 9 times the ethylene production of those grown under the control (25°C 12 h/15°C 12 h) conditions [21]. These findings suggest that ethylene does not always play a role in regulating anthocyanin synthesis in apple fruits when the trees were grown under such light and hot temperature conditions.

In addition to ethephon, the plant hormone methyl jasmonate can alter anthocyanin accumulation. Treatment of apple peel disks of "Tsugaru" (yellow-green background covered in red-pink blush with occasional striations) with methyl jasmonate stimulates anthocyanin accumulation regardless of the fruit growth stage [43]. "Fuji" apple fruit after harvest that was treated with increasing concentrations of methyl jasmonate shows decreasing hue angles (Δh°) (redness) of the peel [44], and field application of methyl jasmonate to apple fruit can also decrease the hue angle [45]. Therefore, the application of methyl jasmonate can enhance apple fruit

anthocyanin formation and peel reddening. Daminozide, paclobutrazol, auxins, and auxin analogs, which are growth regulators, can also promote reddening [44].

5.5 Cultivars

Fruit color development of apple peel varies greatly with the cultivar. The most colored strains of "Royal Beaut" and "Buckeye Gala" (both semistriped) and "Ruby Gala" (blushed) redden on both fruit sides, with a greater average fruit surface colored, whereas the less colored strains of "Galaxy" and "Mondial Gala" (both striped) exhibit different colorations between sides, more bicolor fruits, and lower average fruit surface colored [17]. Furthermore, the reddest strains ("Royal Beaut," "Buckeye Gala," "Ruby Gala," etc.) have high coloring potential even at the early stages of fruit development or under environmental conditions associated with hot temperatures or with low-light conditions such as the shaded parts of tree canopy. However, in medium or poorly colored strains ("Galaxy," "Mondial Gala," etc.), color development depends mainly on fruit maturity. The genotype is therefore one of the main factors determining the degree of redness in apple fruits, and this provides important information on how to make the best use of apple in breeding.

6. Conclusions and perspectives

Consumption of apple contributes to improved health and well-being by helping protection from diseases, including cancer and cardiovascular disease. Increasing the consumption of apple antioxidants requires the development of apples with high antioxidant content without sacrificing taste or storability. One of the most effective means of improving the antioxidant content of the diet is by apple cultivar selection. In addition to genetic factors, environmental factors before and after harvest can influence antioxidant content. Cultivar selection and the manipulation of cultivation could lead to specific conditions for optimal antioxidant enhancement. This would offer the consumer a wide selection of health-promoting cultivars and would result in premium prices for high antioxidant produce. Continued research will require integrated studies such as metabolomic and ionomic approaches to factor analyses that will identify specific conditions for enhancing the antioxidant content of apple products.

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Conflict of interest

No conflict of interest.

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Section 2

Molecular Mechanism of Flavonoids

Chapter 3

Molecular Mechanism of Flavonoids Using Fluorescence Spectroscopy and Computational Tools

Ana Paula Ribeiro Povinelli, Gabriel Zazeri and Marinônio Lopes Cornélio

Abstract

With more than 4000 compounds identified up to now, flavonoids are present in human diet since they can be found in fruits, vegetables, seeds, grains, and beverages, such as wine and tea. Over the years, medicinal properties of these polyphenolic compounds have been noticed. Consequently, the search for the biological targets and for the description of flavonoids action mechanism has been growing. Fluorescence spectroscopy and molecular docking are techniques based on physical theories which have been helping researchers to describe the interaction between flavonoids and biological targets. In this way, this chapter comes not only as an attempt to gather some works dedicated to explain flavonoid molecular mechanisms of action but also to introduce a brief theory of steady-state fluorescence spectroscopy and molecular docking.

Keywords: flavonoids, fluorescence spectroscopy, molecular docking, molecular mechanism, physical pharmacy

1. Introduction

More common than you might think, flavonoids are present in human diet since they can be found in fruits, vegetables, seeds, grains, and beverages, such as wine and tea [1]. They are most famous to beautify fruits and vegetables with vivid colors, but flavonoids most powerful actions are still unknown for most of population, which is unaware the antioxidant [2, 3], anticarcinogenic [4, 5], anti-inflammatory [6, 7], antiviral [8, 9], and antimicrobial [10, 11] effects provoked by these potent compounds. But, what are those compounds, after all?

Flavonoids, with over 4000 compounds identified until now [12], comprehend a wide group of molecules synthesized by plants as secondary metabolites responsible for ensuring vascular plant colonization and surviving on earth's environment [13]. These molecules play crucial roles to plants' life such as protection against insect attack and microbes invasion [14, 15] and by absorption of harmful ultraviolet radiation, attraction of insect pollinators by colorful anthocyanins synthesis [16], antioxidant action by inhibiting the generation of reactive oxygen species (ROS)

[17], involvement in pollen germination [13], involvement in biological communication in the rhizosphere [18] and action as regulators, involved in auxin transport and catabolism [19].

In general, flavonoids are polyphenolic compounds with the flavan nucleus as the structure skeleton, which consists of 15 carbon atoms arranged in 3 rings (C_6-C_3-C_6), labeled by A, B, and C (**Figure 1**) [3].

Flavonoids are classified into classes such as flavonol, flavone, flavanone, flavanol, isoflavone, and anthocyanidin, just to mention a few [20]. The basic structure of each class is shown in **Figure 2**. The differences between flavonoid class structures are in the oxidation level and C-ring substitution pattern, while the difference among flavonoids belonging to the same class is in the pattern of A- and B-ring substitutions [3].

As reviewed by Aidyn Mouradov and German Spangenberg [13], flavonoid synthesis in plants occurs commonly from the aromatic amino acids phenylalanine and tyrosine. At the beginning of synthesis, the amino acid is converted into coumaroyl-CoA by a number of enzymatic reactions involving *phenylalanine ammonia-lyase* (PAL), *cinnamate 4-hydroxylase* (C4H) and *4-coumarate-CoA ligase* (4CL). The *naringenin-chalcone synthase* (CHS) and *chalcone isomerase* (CHI)

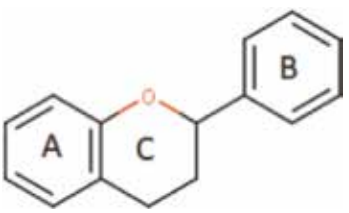

Figure 1.
Flavan nucleus, the structure skeleton of flavonoids.

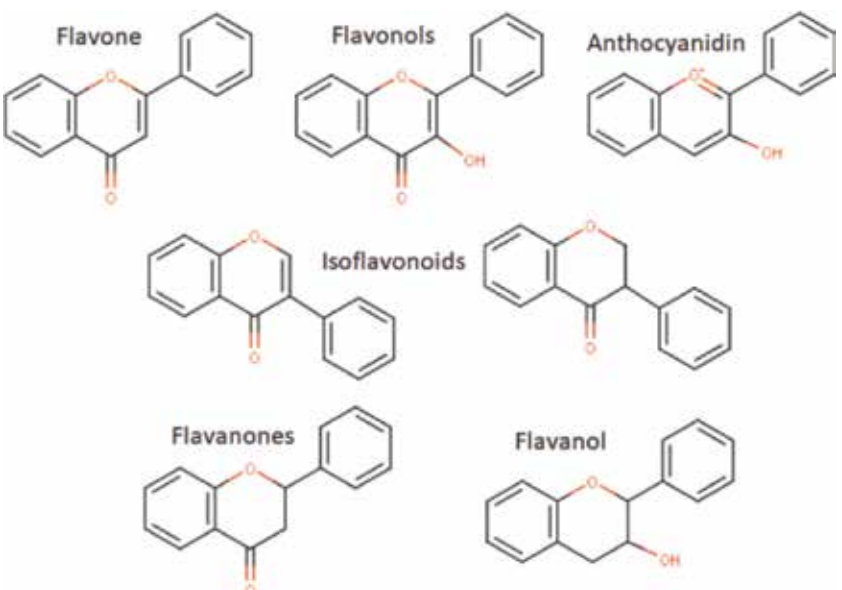

Figure 2.
Basic structures of different classes of flavonoids.

convert coumaroyl-CoA into naringenin; the pathway opens in different ways generating flavanones, dihydroflavonols, leucoanthocyanins, anthocyanidins, and flavan-3-ols by series of enzymatic reactions. These molecules can generate a couple of other compounds such as flavones and isoflavones and flavonols and anthocyanins. These flavonoids are generally found in plant epidermal cells, chloroplasts, vacuole, and nucleus [13].

Epidemiological studies reviewed by Romano and co-workers [21] have demonstrated an inverse relationship between dietary flavonoid intake and prevalence and risk of cardiovascular diseases and some types of cancer such as breast, colon, lung, prostate, and pancreas. Besides that, the authors emphasized the advances of the application of flavonoids in diseases related to the central nervous system, obesity, diabetes, inflammation, digestive system, and respiratory tract, as well as the effects of flavonoids in reproduction and the antimicrobial effects of these compounds.

Many efforts have been done to elucidate the mechanism of action of these compounds by using experimental approaches based on physical observable. Steady-state fluorescence spectroscopy is a technique widely used to follow the interaction of small molecules and biological targets. However, there are many others experimental techniques that have been used to describe biological systems, such as time-resolved fluorescence spectroscopy, nuclear magnetic resonance (NMR), isothermal titration calorimetry (ITC), circular dichroism (CD), and surface plasmon resonance (SPR).

Besides that, it has also used computational analysis in order to describe the interactions between flavonoids and molecular targets. Molecular docking and molecular dynamics are the most common computational techniques used to model biological systems.

In this way, this chapter comes not only as an attempt to gather some works dedicated to explain the molecular mechanisms of action of some flavonoids but also to introduce a brief theory of steady-state fluorescence spectroscopy and molecular docking.

2. Theory of steady-state fluorescence spectroscopy

The use of fluorescence spectroscopy experiments to understand the protein-ligand complex formation has become increasingly frequent in scientific community because many proteins have endogenous fluorescence probe such as tryptophan, tyrosine, and phenylalanine amino acids. Following the fluorescence signal of these probes, it is possible to characterize the molecular mechanism of complex formation [22].

As the name already suggests, this technique makes use of fluorescence as the physical observable. Fluorescence, by definition, is a photon emission mechanism which occurs by the decaying of molecule electrons from a higher energy singlet state to a lower energy singlet state with emission rate of the order of 10^8 s^{-1}, resulting in a typically fluorescence lifetime close to 10 ns [22].

The fluorescence spectroscopy, when applied in protein-ligand system, consists in analyzing the quenching of fluorescence signal in the presence of different concentrations of ligand. Quenching may be static or collisional (dynamic) depending on the nature of interactions. In collisional quenching, the fluorophore, which is in the excited state, is deactivated returning to its ground state because of diffusive encounters with some other molecule of the solution, called quencher. On the other hand, the static quenching occurs when a fundamental and non-fluorescent complex occurs [22].

In a typical experiment of fluorescence spectroscopy, the way to distinguish the quenching mechanism is analyzing the Stern-Volmer plot (Eq. (1)) in different temperatures, where F_o is the fluorescence intensity in the absence of the quencher and F is the intensity in different concentrations of the quencher, $[L_t]$ [22].

$$\frac{F_0}{F} = 1 + K.[L_t] \tag{1}$$

The increase in the constant K as a consequence of the increase in temperature is a strong indication that the quenching mechanism is collisional; in that case the constant is usually denoted by K_d. On the other hand, if the constant K decreases with the increasing in temperature, the characteristic mechanism is the static one, and the constant is usually denoted by K_{sv}. **Figure 3** shows the different behaviors of the static and collisional quenching [22].

If the mechanism determined was the static one, there are couples of models that can be applied in the system in order to obtain the association constant K_a or also called binding constant. The most common and simplest model reported in literature to study protein-ligand is the binding equilibria for a first-order reaction, where the ligands (L) are entering one by one at the binding site of the protein (P) [23].

$$\mathrm{P} + \mathrm{L} = \mathrm{PL} \rightarrow \mathrm{LP} + \mathrm{L} = L_2\mathrm{P} \rightarrow L_2\mathrm{P} + \mathrm{L} = L_3\mathrm{P} \ldots \rightarrow L_{n-1}P + L = L_nP$$

Assuming that the sites are equivalents and independents, the association constant is

$$K_a^n = \frac{[L_n.P]}{[L]^n.[P]} \tag{2}$$

where [P] is the free protein concentration, [L] is the free quencher concentration, and $[L_nP]$ is the concentration of protein-quencher complex. In this model, the protein is either free in solution or bound to quencher, then

$$[P_t] = [L_nP] + [P] \tag{3}$$

where $[P_t]$ is the concentration of total protein in the system [22]. Considering that the fluorescence intensity is only emitted by free proteins

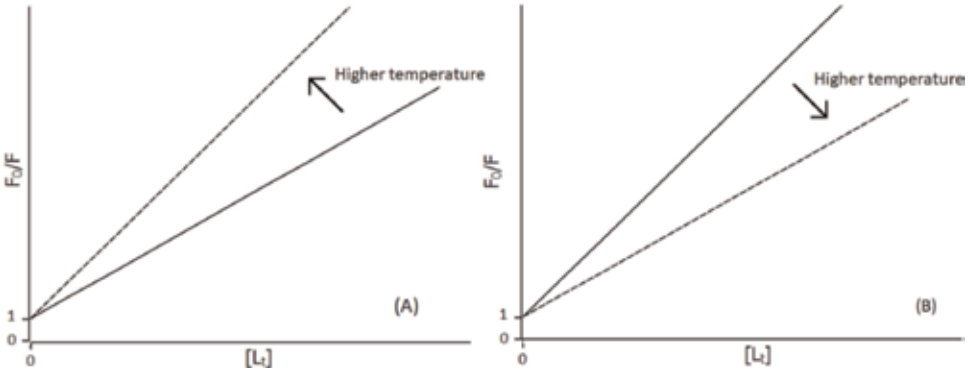

Figure 3.
Examples of different quenching mechanisms. (A) Collisional quenching and (B) static quenching.

$$\frac{[P_t]}{[P]} = \frac{F_0}{F} \tag{4}$$

Eq. (2) can be rearranged to

$$K_a^n = \left(\frac{F_0 - F}{F}\right).\left(\frac{1}{[L_t] - \left(\frac{F_0 - F}{F}\right).[P_t]}\right)^n \tag{5}$$

Applying the logarithm function in Eq. (5) and rearranging it

$$\log\left(\frac{F_0 - F}{F}\right) = n.\log K_a - n.\log\left(\frac{1}{[L_t] - \left(\frac{F_0 - F}{F}\right).[P_t]}\right) \tag{6}$$

Eq. (6) is known as double-logarithm equation, where one can calculate the binding constant K_a. The binding constant is related to thermodynamic parameter through the van't Hoff Equation [24]:

$$\ln K_a = -\frac{\Delta H}{R.T} + \frac{\Delta S}{R} \tag{7}$$

where R is the universal gas constant (≈ 8.31 J/mol.K) and T is the temperature. In a graphic of ln K_a versus T, the enthalpy change (ΔH) and the entropy change (ΔS) are obtained by the slope of the linear function and the linear coefficient, respectively.

Ross and Subramanian [25] associated the enthalpy variation and entropy variation with the most predominant interactions that stabilize the complex, as showed in **Table 1**.

Besides that, Gibbs free energy variation says about the spontaneity of the complex formation process, where $\Delta G > 0$ indicates a non-spontaneity process and $\Delta G < 0$ indicates a spontaneity process. The enthalpy variation, on the other hand, indicates if the system is either an exothermic process ($\Delta H < 0$) or an endothermic process ($\Delta H > 0$) [24, 25].

Unlike the binding equilibrium method that is based upon a first-order reaction predicting a single binding site, there is another method that does not make use of a previous model, and in addition to determining the number of binding sites, it can still determine the cooperativity, if any. This method was developed by Scatchard [26], and it is not as popular as binding equilibrium method to characterize protein-ligand interaction in the scientific community yet.

This method makes use of another technique to obtain preliminary data known as binding density function (BDF) [27], where a physical observable is chosen to follow the interactions, such as absorbance and fluorescence intensity, among others. Supposing that the intensity of fluorescence emitted by the protein was chosen as physical observable, in which in an aqueous system containing protein is titrated by the ligand. Considering the system in equilibrium, the average number of quencher bound by protein $\Sigma\nu_i$ is determined from a given free quencher

ΔH	ΔS	Predominant interaction
< 0	< 0	van der Waals and hydrogen bonds
> 0	> 0	Hydrophobic interactions
≈ 0	> 0	Ionic process

Table 1.
Expected signs of contributions to ΔH and ΔS.

concentration (L); in this context, if the free quencher concentration is the same for two or more solutions of different concentrations of total protein P_t, then the average distribution of the binding density of the quencher will also be the same, reaching in the expression for the mass conservation [27]:

$$[L_t] = [L] + (\sum \nu_i).[P_t] \quad (8)$$

where $[L_t]$ is the total quencher concentration, [L] is the free quencher concentration, and $[P_t]$ is the total protein concentration. In order to obtain the average number of quenchers bound ($\Sigma\nu_i$), a graphic of total quencher concentration $[L_t]$ versus the total protein $[P_t]$ can be plotted, in which by the angular coefficient, the $\Sigma\nu_i$ is obtained and by the linear coefficient of the linear function [L] is obtained. The values for $[P_t]$ and $[L_t]$ are obtained from the graphic of ΔF versus log $[L_t]$ shown in **Figure 4**, where ΔF is the suppression percentage [27].

Once the values of $\Sigma\nu_i$ and [L] were obtained from the graphic in the BDF method, the method developed by Scatchard [26] can be used to follow protein-ligand interaction. To determine all those information from the method, a graphic of $\Sigma\nu_i/[L]$ versus $\Sigma\nu_i$ is plotted, and from the function obtained in the graphic, it can be said that the sites have cooperativity or they are equivalents and independents.

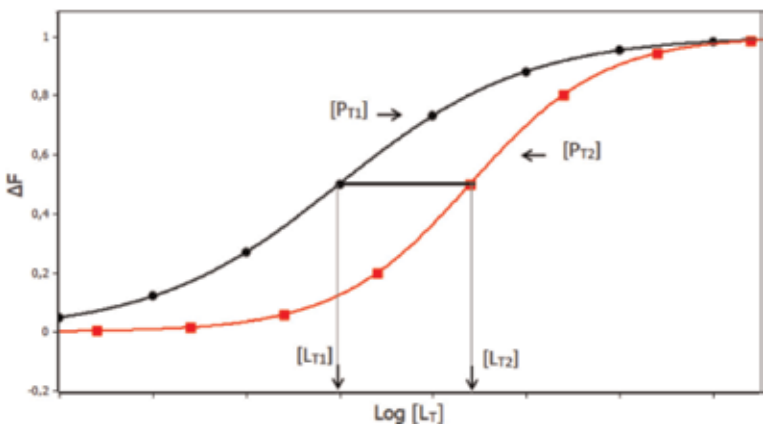

Figure 4.
Example of graph used in BDF theory. In this case, the percentage of quenching was measured in two different concentrations of protein, P_{T1} and P_{T2}.

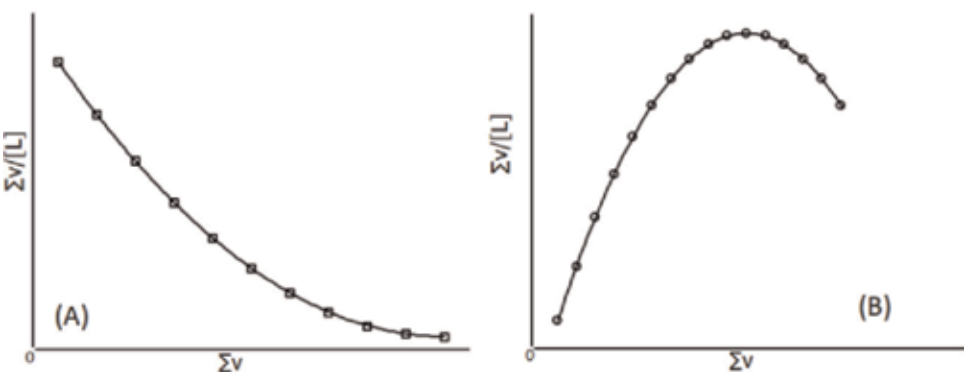

Figure 5.
Illustration of two Scatchard graphics. (A) Negative cooperativity and (B) positive cooperativity.

Bordbar and co-workers [28] reported the mathematical functions that describe the behavior of the system in the Scatchard plots with the type of cooperativity. They determined that if the function is polynomial with the positively concavity (**Figure 5(A)**), the protein has negative cooperativity between the sites. If instead there is a polynomial function with negative concavity, the protein has a positive cooperativity among the sites (**Figure 5(B)**) [28].

For these cases where the Scatchard graph shows a cooperativity function shape, one can apply the equation developed by Hill [24] to determine the number of sites n of each set of equal sites and the K_a association constant of each set of interaction sites:

$$\sum \nu_i = \frac{n_1.(K_{a1}.[L])^{H1}}{1 + (K_{a1}.[L])} + \frac{n_2.(K_{a2}.[L])^{H2}}{1 + (K_{a2}.[L])} \tag{9}$$

where [L] is the concentration of free quenchers and H is Hill's index. If H = 1 the system is noncooperative, H > 1 the system has positive cooperativity, and H < 1 the system has negative cooperativity [24, 28].

In the case that the function, which describes the system, is linear, the protein has no cooperativity among the sites, and all sites are identical and independent. For this behavior, Scatchard [26] developed his own mathematical model to find the number of sites n and the association constant K_a:

$$\sum \nu_i = \frac{n.K_a.[L]}{1 + K_a.[L]} \tag{10}$$

To rearrange in the form of linear equation to model the Scatchard graphic, it is given as follows:

$$\frac{\sum \nu_i}{[L]} = n.K_a - K_a.\sum \nu_i \tag{11}$$

3. Theory of molecular docking

Molecular docking is a powerful technique that has been used in association with experimental data to determine ligand binding sites in targets with pharmacological interest. It can also predict the ligand conformation in the binding site and consequently the interactions that stabilize the complex. In addition, molecular docking has been also used as an efficient and a cheap technique for virtual screening large molecule databases and selects compounds which bind with specificity in pharmaceutical targets before carrying out in vitro and in vivo experiments [29, 30].

Molecular docking consists in determining the most probable conformations of the complex composed by receptor ligand based on an energy ranking of each conformation. To obtain this energy ranking, the ligand is put to interact in an environment under a protein force field including non-covalent potentials such as van der Waals force, hydrogen bonds, and electrostatic nature forces [29]. There are several softwares to perform this kind of prediction such as AutoDock, GOLD, DOCK, and FlexX, just to mention a few.

Each of those mentioned software has its own potential mathematical function to elucidate the forces involved in the complex and calculate the energy score to be ranked. The following is an example of potential utilized by AutoDock04 [31] software:

$$V = I.\sum_{i,j}\left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}}\right) + J.\sum_{i,j}E(t).\left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}}\right) + K.\sum_{i,j}\frac{q_i.q_j}{\varepsilon.r_{ij}^{2}} + \Delta W_S \qquad (12)$$

The weighting constants I, J, E(t), K, and W are those optimized to calibrate the empirical free energy based on a set of experimentally characterized complexes. The first term is the Lenard-Jones potential, in which parameters A and B are taken from the Amber force field [31]. The second term refers to the hydrogen bond in which the parameters C and D are obtained to ensure a minimum energy of 5 kcal/mol in 1.9 Å for O-H and N-H and 1 kcal/mol in 2.5 Å for S-H. The function E(t) provides directionality based on the angle t of the geometry of an ideal hydrogen bond [31]. The third term is a shielded Coulomb potential for electrostatic interaction. The last term is the desolvation potential based on the volume of the atoms surrounding a given atom and shelter it of the solvent [31].

To reduce the computational costs, the programs usually make use of a precalculation type. The software creates grid maps for each type of atom present in the ligand to be used in the docking. Grid maps consist of a three-dimensional array of regularly spaced points centered on the active site of the protein or macromolecule under study. Each point inside the grid maps records the energy interaction of a test atom with the protein [31]. The complexity of finding the best conformation requires computational methods with the potential to effectively investigate a large number of possible solutions, aiming to find the best result. The search algorithms that are usually utilized by molecular docking softwares can be classified into three categories such as systematic, deterministic, and stochastic search methods [32].

In systematic search algorithms, each degree of freedom has a set of values, so that all degrees of freedom of the molecule are explored combinatorically during the search. Examples of systematic search algorithms are anchor-and-grow or incremental construction algorithm [32]. The deterministic search methods are characterized by the fact that, given the same initial input state, they always produce the same output. It happens because the initial state determines the possible movement to generate the next state, which has to have the same or lower energy than the previous state. Examples of deterministic search are energy minimization methods and molecular dynamics (MD) simulations [32]. Stochastic methods vary randomly in all degrees of freedom of the ligand (translational, rotational, and conformational) at each step, generating a great diversity of solutions. The solutions are evaluated according to a probabilistic criterion to decide if they will be accepted or not. In this way this method requires a large number of conformations to obtain a desired result. Monte Carlo, simulated annealing, and evolutionary methods are examples of stochastic search method, and they are the most common in molecular docking softwares [32, 33].

4. Molecular targets for flavonoids

Many efforts have been done to describe flavonoids' main biological targets involved in cellular processes such as inflammation and cancer. Flavonoid mechanism of action in cellular responses includes the inhibition of proteins in cytoplasm medium such as IκB kinase (IKK) complex and mitogen-activated protein kinases (MAPKs), as well as in extracellular medium such as interleukins and other cytokines.

In the pathway that triggers the activation of nuclear factor κB (NF-κB) in tumor necrosis factor (TNF) stimulated cells (**Figure 6**), IKK complex is a target for many flavonoids such as apinegin [34], luteolin [34], acacetin [35], ochnaflavone [35], delphinidin [35], and morin [36].

The inhibition of IKK complex results in failure in phosphorylation of Iκβ. As a consequence, NF-κB is not released to translocate to the nucleus. In the case of the flavonoids apinegin and luteolin, they can also inhibit NF-κβ activation by inhibition of MAPKs [34].

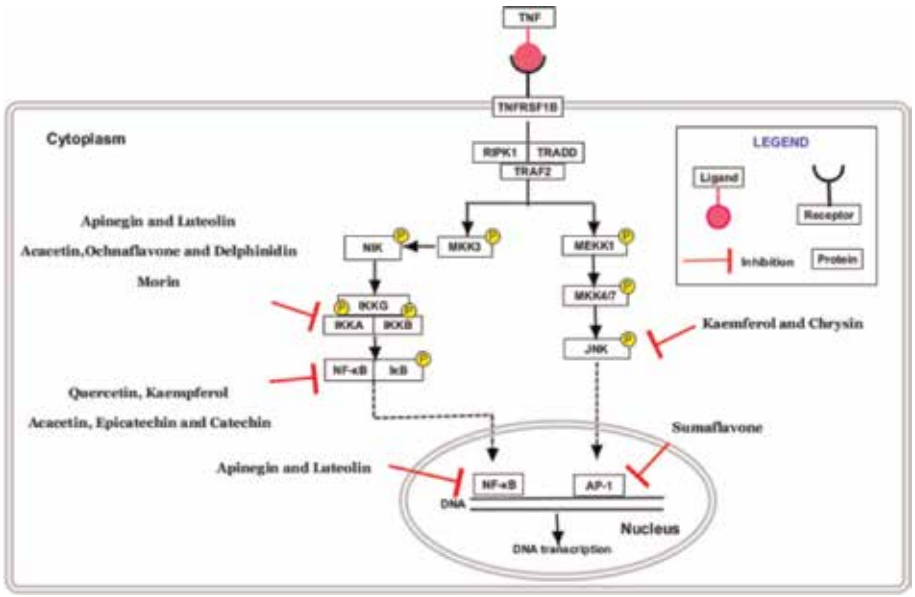

Figure 6.
NF-kB and AP-1 activation pathways induced by TNF.

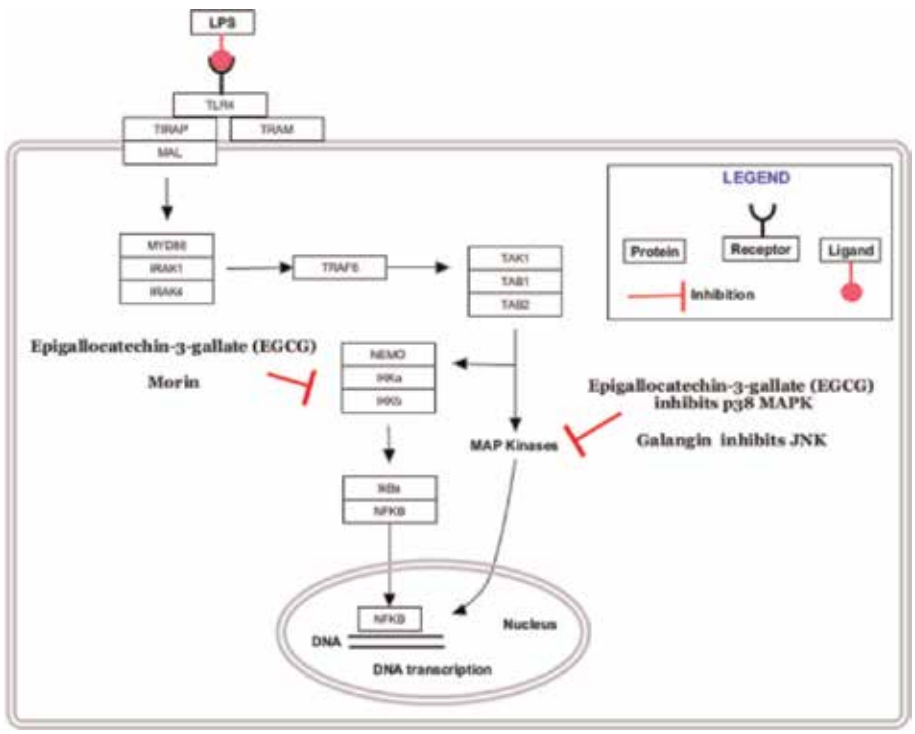

Figure 7.
NF-kB activation pathway induced by LPS.

In the pathway that triggers the activation of activator protein 1 (AP-1), c-Jun N-terminal kinase (JNK) is a target for flavonoids kaempferol and chrysin [34]. Such flavonoids bind to the protein and inhibit the activation of AP-1. Sumaflavone also suppresses the AP-1 activation, but the main target for this flavonoid has not been identified yet [35].

In LPS-stimulated cells (**Figure 7**), epigallocatechin-3-gallate (EGCG) [37] and morin [36] inhibited the activation of NF-κB activity by IKK complex inhibition. Besides that, EGCG also inhibited p38 MAP kinase activity [37]. Another molecular target in LPS-stimulated cells is JNK, which is inhibited for galangin [38].

The molecules genistein, kaempferol, quercetin, and daidzein inhibited the activation of STAT-1 and NF-kB in LPS-induced cells, while flavone, isorhamnetin, naringenin, and pelargonidin inhibited only the NF-kB activation. However the molecular targets for such flavonoids have not been identified yet [39, 40].

Cytokines can also be targets for flavonoids. As verified by Li and co-workers [41], baicalin is able to complex with a variety of chemokines such as SDF-1a, IL-8, MIP1-b, and MCP-2 and reduce the capacity of the cytokines to bind and activate their receptors.

Despite the works described above show which proteins are inhibited by flavonoids, the methodology used does not give information related to how strong is the affinity of the flavonoid for the protein, which amino acids of the protein participate in the interaction with flavonoids, which are the molecular driving forces involved in the interaction, the thermodynamic parameters of complex formation, how many binding sites there are in the protein for the flavonoids, and whether the protein presents cooperativity among their sites. In this way, the methodology of fluorescence spectroscopy and molecular docking described in Sections 2 and 3 come to help give this set of information that are of the utmost importance to drug discovery.

Among the target proteins mentioned above, NF-κβ (p50 and p65 subunits) has one tryptophan residue, while JNK have four tryptophan residues. Besides that, all the cytokines (SDF-1a, IL-8, and MCP-2) mentioned above have 1 tryptophan residue, except for MIP1-b which has 12. The presence of tryptophan makes the use of fluorescence spectroscopy possible, but the presence of more than one makes the interpretation of the spectral data more complicated. Different of the proteins mentioned above, AP-1 does not have tryptophan residue. On the other hand, there are tyrosine residues, which can also be followed in the experiments.

Besides the endogenous fluorescent probe, solubility is another important factor because fluorescence spectroscopy experiments are conducted with protein in solution. As the proteins mentioned above are found in cytoplasm or in extracellular medium, they are expected to be soluble in buffer.

5. Physical pharmacy in the description of molecular mechanism of flavonoids

As already discussed in the sections before, the stabilization of the complex is made by non-covalent interactions, such as hydrogen bonds, hydrophobic interactions, van der Waals and electrostatic forces, salt bridge, π stacking, and cation-π interactions. Consequently, the affinity and the thermodynamic parameters of each system are results of these interactions.

The complete characterization of a complex involves the description of these interactions, the affinity, and the thermodynamics. That is the reason why the use of techniques based on physical approaches in the pharmacy area has been growing.

Several authors characterized the complex formed by flavonoids and biological targets based on fluorescence spectroscopy data, as showed in Section 5.1. Some others used molecular docking to describe the complexes, as shown in Section 5.2.

Nevertheless, the most accurate description of the complex is reached by the association of data from fluorescence and docking, as one can see in Section 5.3.

5.1 The use of steady-state fluorescence spectroscopy

Based on the theory of fluorescence quenching described in Section 2, several authors investigated the molecular mechanism of interaction between flavonoids and proteins. The most basic method used to estimate the affinity of the ligand toward the protein is the Stern-Volmer plot, where one can calculate the Stern-Volmer constant (K_{sv}) and associate the magnitude of this constant with the binding affinity.

Papadopoulou and co-workers [42] used K_{sv} to evaluate the interaction of two flavonoids, quercetin and rutin, and the bovine serum albumin (BSA). The authors verified that quercetin demonstrated stronger affinity toward BSA compared to rutin. Such difference was explained based on the structure of both flavonoids, once rutin is a glucoside of quercetin. The authors concluded that hydrophobicity was an important factor in the change of affinity, once the incorporated disaccharide rutinose made the flavonoid less hydrophobic. Besides that, another important factor was the steric hindrance caused by the incorporated disaccharide.

With a similar methodology, Cao and co-workers [43] investigated the influence of glycosylation of quercetin and baicalein in the affinity toward BSA. The authors used the Stern-Volmer plot to classify the quenching mechanism as static or dynamic. Then they used the double-logarithm plot to calculate the binding affinity of the ligands studied. They verified that the glycosylation made the interaction weaker and suggested that the decrease in binding constant was an effect of steric hindrance caused by glycoside groups.

The influence of glycosylation in the binding affinity observed by Papadopoulou and co-workers [42] and Cao and co-workers [43] was attributed to steric hindrance. The steric hindrance suggested by the authors could be verified if they had used molecular docking to model the system.

The influence of ions Cu^{2+}, Al^{3+}, Mg^{2+}, and Zn^{2+} in the interaction of flavonoids and human serum albumin (HSA) was investigated by Bi and co-workers [23]. The authors calculated the binding constant by double-logarithm plot (Eq. (6)), and they concluded that binding constants were 14.2–99.6% of the ones without these metal ions. Therefore, the ion concentration would shorten the storage time of the compounds in blood plasma and enhance the effectiveness of the flavonoids. The influence of ions in the binding constant was also observed by Hu and co-workers [44] during experiments with morin and BSA. In this case, the authors observed that the constant decreased in the presence of Ba^{2+} and Hg^{2+}, but with the addition of the ions K^{+}, Li^{+}, Mn^{2+}, Fe^{2+} and Sn^{2+}, the constant increased.

Another experimental methodology has been used to characterize the interaction between protein and small ligands. As described in Section 2, thermodynamic parameters are calculated from fluorescence quenching data by using the van't Hoff equation.

Li and co-workers [45] used fluorescence experiments to compare the inhibition ability of three flavonoids (quercetin, isoquercetin, and rutin) toward *α-glucosidase* activity. They concluded that the complexes formed by the protein and the three flavonoids were spontaneous processes, driven mainly by hydrophobic forces, once ΔS and ΔH are both positive numbers. Based on the results obtained, the authors suggested that the flavonoids studied would be useful as an inhibitor of the enzyme and would help in the treatment of hyperglycemia and obesity.

Xi and co-workers [46] studied the interaction between hemoglobin and the flavonoids quercetin and rutin by using double-logarithm and Van't Hoff plots. The authors observed that for both flavonoids, the values of ΔH and ΔS were

negative and concluded that the acting forces are hydrogen bonding and van der Waals. As one can see, the results obtained by [45, 46] reveal that molecular forces that drive quercetin and rutin into *α-glucosidase* and hemoglobin are different. This difference is noticed because the same molecules interact in cavities with different characteristics.

Another molecular target for flavonoids in biological systems is nucleic acid. The study of the interaction between flavonoids and DNA using fluorescence spectroscopy is made by following fluorescence signal from flavonoids once DNA exhibits a very weak intrinsic emission.

The article published by Jana and co-workers [47] described the affinity of 3-hydroxyflavone toward DNA, by following the flavonoid fluorescence signal. The same methodology was used by Sengupta and co-workers [48] to describe the affinity of fisetin toward DNA. The interaction of polyphenolic compounds with DNA has a protective role, once flavonoids exhibit antioxidant properties.

DNA is also a target for luteolin, as shown in the article published by Chowdhury and co-workers [49]; the interaction between luteolin and nucleic acid is one of the causes of the decrease in affinity between DNA and *topoisomerase* I.

5.2 The use of molecular docking

The influence of flavonoid structure, the addition of organic group as well as the main interaction between the small molecules and biological targets can be described through the molecular docking technique.

The potential of some flavonoids as inhibitors of *α-amylase* verified by experimental assays was explained in the work published by Lo Piparo and co-workers [50, 51] using molecular docking. The authors observed that flavonols and flavones have the same scaffolds (**Figure 2**), and both subclasses possess a carbonyl group in the position C4 of the pyrone ring. The C2-C3 double bond is conjugated to the 4-keto group and is responsible for electron delocalization between the ring C and ring A. As a consequence, flavones and flavonol form a highly conjugated π-system that confers better stability of the protein-ligand complex. The authors also described a specific pattern of OH group, which interacts with the catalytic residues and promotes the inhibition of the enzyme.

The inhibitory effect of some flavonoids was also observed for *β-secretase*; the work published by Shimmyo and co-workers [52] showed that OH groups in myricetin, quercetin, kaempherol, morin, and apigenin stabilized the binding poses of flavonoids against the *β-secretase* active center by hydrogen bonds. In many cases, the OH directly interacted with the Asp catalytic residue and enhanced the inhibitory activity of polyphenolic compounds.

Si and co-workers [53] showed that some flavonoids inhibiting CYP2C9 activity may increase the risk of toxicity from coadministered drugs that are CYP2C9 substrates. The authors used molecular docking to give details about the interaction of CYP2C9–6-hydroxyflavone complex, showing that the 6-hydroxyflavone is bound by a π-π stacking interaction with the phenyl group of Phe 100 and by two hydrogen bonds with Leu102 and Phe100.

The examples above used molecular docking to describe the interaction between flavonoids and molecular targets in order to explain the results obtained from experimental assays. However, one can also use molecular docking in order to select the best candidates and then submit the chosen molecules to the experimental assays.

In the work published by Salam and co-workers [54], the authors built a library of 200 natural compounds including some flavonoids. With virtual screening, they selected the 29 best candidates to bind to the peroxisome proliferator-activated

receptors (PPARs) in the cell culture experiments. Besides that, docking studies showed that flavonoids are predicted to occupy the hydrophobic environment formed by residues Phe282, Phe360, and Phe360. They also showed that there is one specific OH group in all the flavonoids studied that is responsible to make a hydrogen bond with the receptor, except in the case of apigenin.

5.3 The use of fluorescence spectroscopy associated with molecular docking

Data from fluorescence spectroscopy offers a description of the complex in terms of binding constant, number of binding sites, cooperativity, and the thermodynamics of the complex formation. On the other hand, molecular docking offers details about the binding site environment, the ligand conformation, and the interactions that stabilize the complex. Some authors associated the fluorescence quenching data with the results from molecular docking in order to give a complete description of the biological system.

He and co-workers [55] verified the tryptophan fluorescence quenching of HSA caused by alpinetin. The authors used the van't Hoff model to calculate the thermodynamic parameters and concluded that the hydrophobic interactions were predominant to stabilize the complex. With molecular docking technique, the authors selected the best binding site of alpinetin in the HSA and showed that the binding site was close to tryptophan, which explains the static quenching. The authors also showed that the binding site was a hydrophobic cavity, which explains the data obtained by van't Hoff equation.

With a similar methodology, Kim and co-workers [56] showed the relationship between the number of OH groups in flavonoids and the affinity for mushroom *tyrosinase* by fluorescence quenching. Molecular docking studies showed that the dicopper catalytic site of *tyrosinase* is a preferential binding site for flavonoids, which explains the inhibitory activity of the polyphenolic compounds.

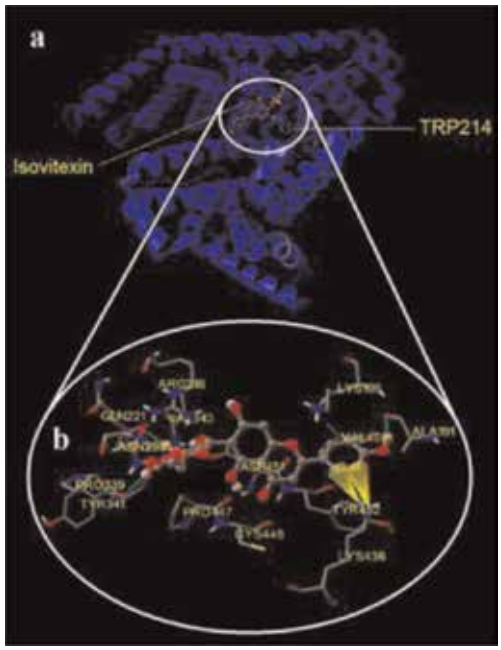

Figure 8.
(a) Binding site of isovitexin in subdomain IIA of HSA (b) Binding site microenvironment [57].

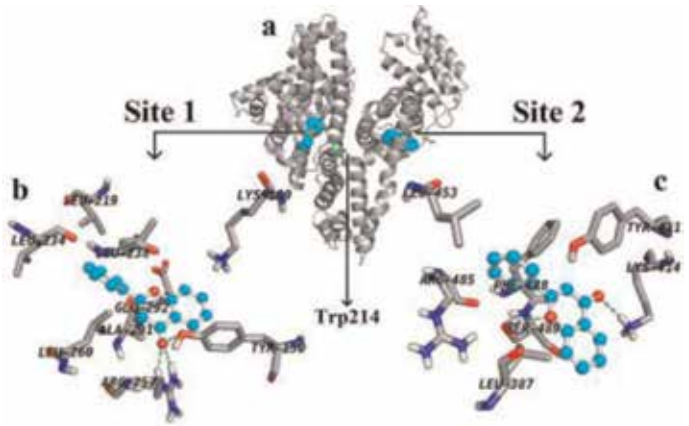

Figure 9.
(a) Location of the 2-phenylchromone molecules in HSA (b) Microenvironment of site 1, subdomain IIA of HSA (c) Microenvironment of site 2, subdomain IIIA of HSA.

Caruso and co-workers [57] used fluorescence spectroscopy to describe the interaction between isovitexin and HSA. The authors calculated the contribution of enthalpy and entropy by van't Hoff equation and concluded that the process was enthalpically driven. The complete description of the system was reached with molecular docking, as one can see in **Figure 8**. The oxygen atoms of Ala191 and Pro339 form hydrogen bonds with isovitexin, while neutral polar side chain residues like Gln221, Asn295, Tyr341, and Tyr452 are mainly taking part in electrostatic interactions (charge neutralization).

In this last article, the authors used the Scatchard method, which has been recently used to calculate the number of binding sites and to verify allosterism. With the Scatchard method, the authors concluded that there was one binding site for isovitexin in HSA, which was in agreement with the results obtained by the authors when they used the binding equilibrium model.

In another article, Caruso and co-workers [58] used a similar methodology in order to describe the interaction between 2-phenylchromone and HSA. In this case, the contributions of enthalpy and entropy calculated by van't Hoff equation showed that the process was entropically driven. With the Scatchard method, the authors concluded that there were two cooperative binding sites for 2-phenylchromone in HSA with two different binding constants. Note that this type of information could not be obtained from Stern-Volmer or double-logarithm models; only the Scatchard method can offer information about the system without the use of any binding model a priori.

Also in this study, molecular docking showed that 2-phenylchromone binds at subdomain IIA (Site 1) and subdomain IIIA (Site 2). As shown in **Figure 9**, at Site 1 amino acids Lys199, Leu (219, 234, 238, and 260), Ala291, and Glu292 are residues involved in the interactions with the molecule. At Site 2 amino acids Leu387, Tyr411, Leu453, Arg485, and Phe488 compose the microenvironment of interaction.

6. Conclusion

This chapter presents the real possibilities of combinations, between physical experimental techniques with computational tools which can contribute significantly to the advancement of the proposal of new drugs within the point of view of molecular action. Moreover, it has been demonstrated how to carry out such work,

combining the knowledge between fluorescence spectroscopy and molecular docking. These techniques may be added to pharmacology, pharmacokinetics, and pharmacognosy research fields, which represent the cradle of the search for new medicinal products and design.

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Conflict of interest

The authors declare that no conflict of interests exists.

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Chapter 4

Flavonoids as Modulators of Synaptic Plasticity: Implications for the Development of Novel Therapeutic Strategies for Healthy Lifestyle

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Abstract

Flavonoids are potential group of phytochemicals found in normal diets capable of mediating improvements in cognition and may reverse age-related declines in memory. Aging is associated with alteration of hippocampal synaptic plasticity and contribute to decline in cognitive functions. The current studies are directed at a greater understanding of how and why the brain modifies synaptic strength with dietary-derived phytochemicals (flavonoids) and age-related declines in cognitive functions (such as learning and memory). Flavonoids modulate neuronal function and thereby influence cognition. In addition, it has been suggested that flavonoids may delay the development of Alzheimer's disease-like pathology, anxiety, and depression disorders, suggesting a novel therapeutic strategy. Emerging evidence suggest that flavonoids are modulators of signaling pathways critical for controlling synaptic plasticity in the brain. For example, phosphatidylinositol-3 kinase (PI3K)/Akt, mitogen-activated protein kinase, protein kinase C, pathways could be involved Ca^{2+} signaling. Significants questions such as: (i) How does flavonoids affect plasticity? (ii) What receptors are modulating by flavonoids and how are they regulated? (iii) Do flavonoids have a neuroprotective effect in aging? are asked.

Keywords: flavonoids, synaptic plasticity, health lifestyle, brain

1. Introduction

Advances in medicine over the last century have resulted in a considerable increase in human life expectancy. Despite this positive outcome, with increase in age, comes a decline of metabolic and immune functions with impact on the cognitive functions. Although, some decline in cognitive function does occur with normal aging, there is also an increased age-associated risk of neurodegenerative disorders such as Alzheimer's disease (AD) [1]. At the same time, it highlights the need for a more comprehensive understanding of how different aspects of lifestyle such as physical exercise, meditation, musical experience, and diet may influence brain disorders in a preventative manner, affecting long-term neural function that affects cognitive performance [2]. In relation to the diet, flavonoids have been described as promising plant-based bioactives capable of modulating different aspects of neuroplasticity, resulting in improvements in memory in both rodents [2] and humans [3, 4]. For example, flavonoids have been correlated with their ability to modulate the phosphorylation state of intracellular proteins by the activation or inhibition of phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), mitogen-activated protein kinase (MAP), CAMP responsive element binding protein 1 (CREB-1), growth associated protein 43 (GAP-43), brain-derived neurotrophic factor (BDNF), or to alter expression of N-methyl D-aspartic acid (NMDA) receptors (NMDARs), $GABA_A$ receptors ($GABA_A$Rs), and 5-HT receptors (5-HTRs) [5, 6]. In addition, flavonoids can modulate epigenetic modifications [7]. These mechanisms are critical for the neuroplasticity and they are also related with inflammatory processes in the brain.

The aim of this chapter is to highlight the potential of flavonoid-rich food, flavonoid-rich extract, and medicinal plants that act as modulators of neuroplasticity in the central nervous system of mammals. We provide an outline of the neuroplasticity and how flavonoids affect this mechanism, and we will also describe their interaction in the neuroinflammation mechanism that affects the cognition. It will highlight the probable mechanisms that flavonoids promote neuroprotective effect in aging.

2. Flavonoids structure and brain bioavailability

Flavonoids are a large group of naturally occurring plant-based compounds that are commonly consumed through a diet rich in fruit, vegetables, tea, wine, and soy-based foods, being of considerable scientific and therapeutic interest. Flavonoids are responsible for numerous functions in plants. Among them, we can mention protection against ultraviolet rays, against insects, fungi, viruses, and bacteria, and the ability to provide the attraction of pollinating animals. In addition to these characteristics, many of these compounds also possess important pharmacological properties, such as antiviral, antitumor, anti-inflammatory, antioxidant, anti-inflammatory activity, and neuroprotective actions.

Flavonoids consist of two aromatic carbon rings, benzopyran (A and C rings) and benzene (B ring) (**Figure 1**). Flavonoids can be subdivided into different subclasses depending on the carbon of the C ring on which the B ring is attached and the degree of unsaturation and oxidation of the C ring on which the B ring is attached and the degree of unsaturation and oxidation of C ring. Thus, they may be divided in seven subclasses as: (1) flavones (e.g. apigenin, luteolin); (2) flavonols (e.g. kaempferol, quercetin); (3) isoflavones (e.g. daidzein, genistein); (4) chalcones (e.g. phloretin, chalconaringenin); (5) flavanones (e.g. naringenin, hesperetin); (6) anthocyanidins (e.g. delphinidin, cyanidin) and, (7) flavanols [e.g. catechin, epicatechin,

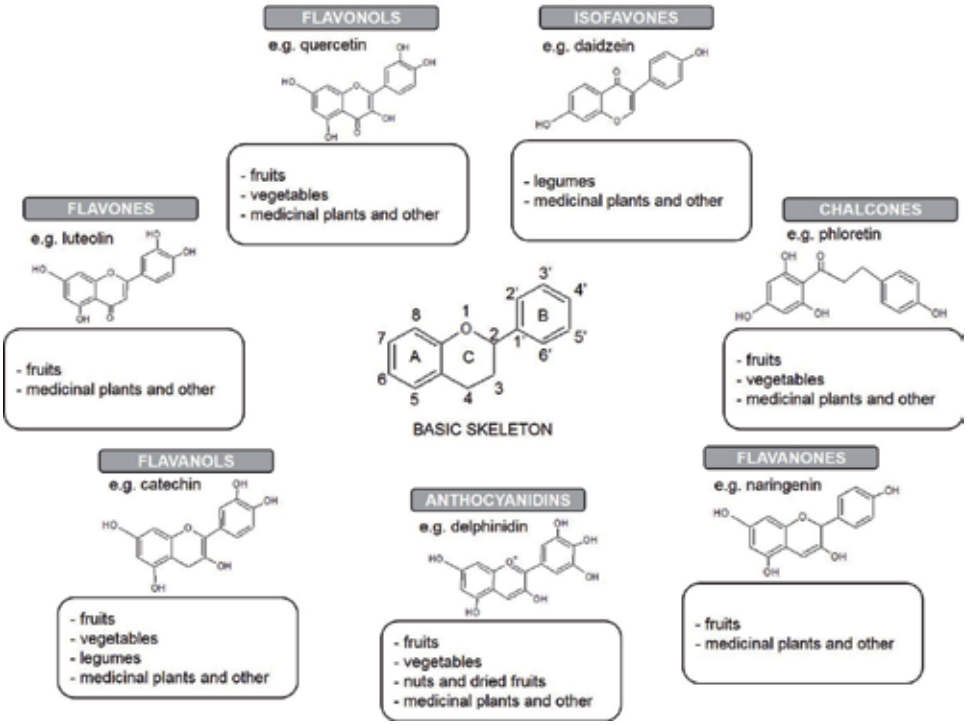

Figure 1.
Basic skeleton structure of flavonoids, subclasses, and their natural sources.

epigallocatechin, epigallocatechin gallate (EGCG)] [8]. For further information regarding the structure and classes of flavonoids, you can see references Andersen and Markhan [8].

Although flavonoids display directly modulate brain function, during absorption; they are extensive metabolized, resulting in a wide variety of metabolic derivatives. Do flavonoids access the brain?

In order to understand whether flavonoids are capable of modulating brain function, it is important to understand the bioavailability. Bioavailability is a crucial factor determining their biological activity *in vivo*. Therefore, information on the absorption and metabolism of dietary flavonoids in the digestive tract is important for determining their physiological functions, and what if flavonoids and their metabolic derivatives cross the Blood-Brain Barrier (BBB) [9–11]. This point is still a matter of debate, despite a number of studies shows the presence of flavonoids and their metabolites in brain tissue following oral administration of flavonol, e.g. (−) epicatechin [10], flavanones, e.g. hesperetin [11] and flavone, e. g. baicalein [12, 13]. The capacity of flavonoids and their metabolites to cross BBB is dependent on the degree of lipophilicity of each compound, i.e., less polar O-methylated metabolites may be capable of greater brain uptake than the more polar flavonoid glucuronides [14].

Bioavailability studies using flavonoids labeled with radioactive were found in various brain tissues such as hypothalamus, superior colliculus, cerebellum, striatum, and in limbic system structures as cortex and hippocampus [9, 11, 15], both important for memory formation, and are also adversely affected by aging and neurodegenerative diseases. It is a necessary work to discuss bioavailability of flavonoids in brain; however, some studies showed the direct and protective effects of flavonoids in modulating brain function, which will be discussed below.

3. Impact of flavonoids on cognition

The common medicine has focused on symptoms and treatment but the majority of chronical diseases are the result of unhealthy habits. The lifestyle medicine has focused on prevention, which means an increase in life quality, well-being, and avoids morbidity conditions [16]. Habitual consumption of dietary flavonoids has been consistently linked with improving cognitive functions [17–19]. For this reason, the flavonoids have been described as a class promised to maintain cognition functions and/or to delay in the progression of age-related cognitive. Despite a growing body of animal studies demonstrating positive effects in learning and memory after flavonoid intake (discussed in the next session), the human clinical trials are somewhat scarcer.

The neurobehavioral effects of phytoestrogens have been the limited data that exist regarding the influence of soy-derived dietary isoflavones on brain structure and function [20]. Clinical trial studies showed the efficacy of isoflavones on cognitive function in postmenopausal women. For example, Long term soy-isoflavone-based supplement (110 mg/d) for 6 months showed better verbal memory than the placebo control group [21]. Similarly, in women aged 50–65 found that intake of 60 mg/d for 3 months resulted in cognitive improvement in several categories related to frontal cortical functions [22]. Another study, involving younger postmenopausal women receiving 160 mg/d isoflavones for 6 months, and results showed an improvement cognitive flexibility [23].

With respect to anthocyanins, blueberry flavonoids supplement (579 mg/d) for 7 days induce cognitive improvements in young and aged adults [24]. Similar results were found after 3 months (long-term supplementation) with blueberry juice in older adults with cognitive impairment in working memory [25]. Some studies address the cognitive impact of a single dose of a blueberry juice in children (8–10 years old) [26]. This study showed for the first time a cognitive benefit for acute flavonoid intervention in children. Another study with 30 g of lyophilized anthocyanins, equivalent to 240 g or 1½ cups of fresh blueberries, demonstrated beneficial cognitive effects on memory and attention, not extending reading ability, in healthy children of 7–10 years of age. These findings increase the growing body of evidence that flavonoids are beneficial to healthy brain function [27].

Finally, precise estimation of nutrient intake is essential for establishing a relationship between diet and cognitive function. However, estimations of dietary flavonoid intake need to take into account their complexity and variability. More recently, a review reported wide range for mean total flavonoid intakes between 209 and 1017 mg/d (mean 435 mg/d) in European, US, and Australian adult populations [28]. In Brazil, the estimate is between 60 and 106 mg/d [29]. There are substantial variations in population estimates of dietary flavonoid intake, which may be associated to true differences in dietary patterns, such as differences in the food supply and cultural eating patterns between countries. Further studies are required to address and to detect effects of dietary interventions on human cognitive functions.

4. Mechanisms underpinning the actions of flavonoids as synaptic plasticity modulators

Flavonoid subclass has been extensively studied. For this reason, flavonoids have been recognized as promising agents capable of influencing different aspects of synaptic plasticity resulting in improvements in learning and memory. It is not completely clear how flavonoids affect synaptic plasticity. A growing body of evidence suggests that they can (1) modulate receptor function, and (2) promote

the expression of synaptic plasticity-related genes and proteins (**Figure 2**). The next sections outline the effects of flavonoids in synaptic plasticity and how these may underpin improvements in memory.

4.1 Flavonoid-receptor interactions

There are a number of studies that support the flavonoid-receptor interactions. For example, blueberry intake by young rodents increases the levels of GluN2b subunit of N-methy-D-aspartate receptor (NMDAR) in hippocampus [30]. Similarly, in young rodents, acute effect of oral flavonoid-rich fraction (Ff) intake up-regulated mRNA expression of the GluN2b subunit in dorsal hippocampus [5]. The flavonoid-rich fraction (Ff) containing flavones (Vitexin, Isovitexin, and 6-C-glycoside-Diosmetin) and improves learning and memory in young rodents. It is known that NMDARs are centrally involved in synaptic transmission, synaptic plasticity (long-term potentiation – LTP and long-term depression – LTD), and learning and memory [31]. According to this, quercetin resulted in improvements of hippocampal CA1 long-term potentiation in acute hippocampal slice from young rats [32].

The serotoninergic and GABAergic neurotransmissions are involved in learning and memory; the major targets are 5-$HT1_A$Rs and $GABA_A$Rs, and the modulation of these receptors in the hippocampus is essential for the acquisition and consolidation of memory [33–35]. Supporting, acute effect of oral Ff up-regulated mRNA expression of the 5-$HT1_A$R and GABA(A) alpha 5 receptor in dorsal hippocampus from young rats [5]. Molecular docking study showed that flavones such as Isovitexin and 6-C-glycoside-Diosmetin exhibited a strong interaction with the $GABA_A$ BZ binding pocket. Those flavones showed a lack of interaction with α1His101, which may explain the memory-enhancing effect identified in the behavioral test in young rats [36].

Flavonoids can modulate other receptors such as TrKB [37], δ-opioid [38, 39], nicotinic [40, 41], estrogen [42, 43], and adenosine [44–46]. For example, 7,8-dihydroxyflavone, a flavone, was shown high-affinity agonist of the TrKB receptor [47]. In addition, the flavones may improve cognition by modulating the acetylcholine and neurotrophic factors synthesis in hippocampus and frontal cortex [27, 48]. Those flavonoid-binding sites show many possibilities of flavonoids action mechanisms to modulate cognition and brain physiology.

4.2 Flavonoid-signaling pathways interactions

Receptor binding by flavonoids is responsible by changes in the up-regulated and down-regulated pathways such as, PI3K, PKC, MAP kinases, and nuclear factor-κB pathway. Their influence on such pathways suggests that they may modulate synaptic plasticity involved in neurodegenerative diseases, and memory acquisition, consolidation, and storage.

Short-term and long-term treatment of oral *Ginkgo biloba* (EGb) extract up-regulated mRNA and protein of the CREB-1 and GAP-43 in dorsal hippocampus

Figure 2.
Mechanisms underpinning the actions of flavonoids.

from young rats [49, 50]. These molecules are associated with molecular mechanism LTP, and it has been shown that the transcription factor, cAMP response element-binding protein (CREB), may regulate the synthesis of new proteins necessary for the formation of memory. Another potential protein associated with LTP is the protein 43-kDa growth-associated protein (GAP-43), and they dramatically enhances during LTP [51–53]. Quercetin-increased expression of activity-regulated cytoskeletal-associated protein (Arc/Arg3.1) [54] pathway is important for memory.

Flavanols and flavanones activate the ERK pathway [55–58] and ERK modulate cAMP response element binding protein (CREB) activation, which is involved in long-term changes in synapses and memory formation [37–39]. Similarly, acute effect of oral Ff up-regulated mRNA expression of the ERK in dorsal hippocampus from young rats [5].

In addition, the flavonoids may modulate the protein kinases, as MAP-kinase and PI3-kinase [59–62], the alteration on activation of kinase may influence directly on modulation of activity-dependent plasticity and morphological changes in synapses involved in memory acquisition, consolidation, and storage [63].

In summary, the ability of flavonoids to influence receptor activity and synaptic plasticity suggest that these might underpin enhancements in cognitive functions in both health conditions and neurological/psychiatric disorders. Additional approaches are required to understand the molecular mechanisms involved in these processes, for example, electrophysiological studies in rodents and human.

5. Prevention of cognitive decline through lifestyle interventions

There is increasing evidence that diet rich in flavonoid or supplements might delay the initiation of and/or slow the progression of cognitive decline related to aging and Alzheimer's diseases (AD). Among existing dietary patterns, adherence to a Mediterranean diet is associated with less cognitive decline, dementia, or AD [64, 65]. For example, a meta-analysis showed that greater adherence to a Mediterranean-style dietary pattern during older adulthood was associated with a lower risk of developing several different health outcomes such as CVD, neurodegenerative disorders, cancer, and overall mortality [66].

With regard to AD, the consumption of food rich in flavonoids such as red wine, fruit juice, and vegetables has been shown to delay the onset of AD [17, 67]. This is in accordance with previous studies linking high consumption of flavonoids to decline related to aging and dementia [19, 68]. A number of studies using animal models of AD have begun to investigate the possible mechanisms involved in these effects. For example, oral administration of the green tea flavonoid EGCG for 6 months to mice overexpressing the Swedish mutation of APP (Tg2576) reduced amyloid β (Aβ) pathology as well as improving cognition [69], and similarly long-term green tea catechin administration improved spatial learning and memory in senescence-prone mice [70]. Furthermore, feeding APP+PS1 double-transgenic mice blueberry from 4 months of age prevented deficits in Y-maze performance at 12 months, without altering the Aβ burden [71]. The myricetin and morin are successful to inhibit the β-sheet of Aβ oligomers. Apigenin, a flavone, and quercetin, a flavonol, have shown promising results with animal model of AD, and quercetin has shown to be benefit to early-stage AD patients [72].

The mechanism underlying these changes is not clear but might be linked to increase α-secretase activity [73] reported in vitro and in vivo after i.p. injection of EGCG [42, 74] or due to disruption of cAbl/Fe65 interactions [75]. Gallic acid and catechin-rich grape seed polyphenolic extract (GSPE) administered for 5 months to Tg2576 mice inhibited cognitive deterioration coincident with reduced levels of

soluble high-molecular-weight oligomers of Aβ [76]. Moreover, GSPE also inhibits tau fibrillization, promotes the loss of preformed tau aggregates, and disrupts paired helical filaments [77–80]. EGCG seems to have broadly similar effects. (−)-Epicatechin and hesperetin hold the potential to inhibit the development of tau pathology through an alternative mechanism relating to their ability to enhance Akt phosphorylation, thereby inhibiting GSK3β-induced tau hyperphosphorylation [55, 56]. Overall, this supports the claim that orally active flavonoids could have the utility in AD beyond anti-Aβ actions. The challenge ahead is to determine if flavonoids have efficacy in individuals affected by dementia.

6. Discussion: future directions

The consumption of flavonoid-rich foods and supplements throughout life may have the potential to limit or even reverse the progression of cognitive decline related of aging and potentially delay the onset and progression of dementia. The mechanisms by which flavonoids modulate cognitive functions are yet to be fully established.

In relation to synaptic plasticity, it will be particularly important to investigate the potential effect of flavonoids that mediate the induction of long-term depression (LTD), short-term potentiation (STP), and long-term potentiation (LTP) in hippocampus area. To dementia, it will be important to investigate the potential utility of flavonoids in the modulation of amyloid β pathology in more detail. In addition, it will be important to clearer dietary recommendations on flavonoid intake (including potentially a "recommended daily intake" level). Indeed, there are a number of important questions still to be resolved.

Conflict of interest

None.

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Section 3

New Trends in Therapeutic and Industrial Applications of Anthocyanins and Flavonoids

Chapter 5

Anthocyanins: Novel Antioxidants in Diseases Prevention and Human Health

Shang Yazhen, Wang Wenju, Zhu Panpan, Ye Yuanyuan, Dai Panpan, Zhao Wusen and Wang Yanling

Abstract

Anthocyanins are a category of water-soluble natural pigments that exist widely in all kinds of vegetables, fruits, and seeds. In fact, the chemical nature of anthocyanins is a group of compounds, and possesses antioxidant capacity like flavonoids. Anthocyanins show antioxidant activity by scavenging free radicals, activating antioxidant enzyme, and chelating metal ions. Anthocyanins, therefore, are recognized as one of the most effective natural antioxidant in the human body. Anthocyanins for a variety of disease prevention and health care are closely related to their strong antioxidant activity and scavenging free radical ability. The present chapter reviewed anthocyanins eliminating free radicals for preventing neoplasm, modulating antioxidant enzyme for preventing Alzheimer's disease, losing weight for preventing diabetes, regulating lipid metabolism for preventing cardiovascular disease, and inhibiting photoreceptor apoptosis for treating xerophthalmia and for other diseases treated. In addition, some healthy food added of anthocyanins was used as precaution for some diseases, else, there are some cosmetics added with anthocyanins, including sunscreen, creams, mouthwash, and shampoo. Specific creams for characteristics of Chinese old people skin in Chinese Company were developed and achieved anti-wrinkle and moisturizing efficacy. Simultaneously, anthocyanins can also be as a food additive to lactic acid milk, cakes, and other food.

Keywords: anthocyanins, antioxidants, disease prevention, human health

1. Introduction

Anthocyanins, also known as anthocyanidins, contain acidic and alkaline groups. It belongs to flavonoid compounds with phenolic substances. They exist in various plants in the form of water-soluble natural pigments, and they are the main colorant of plants. Anthocyanins are widely found in flowering plants (angiosperms) of 27 families of 72 species and major 6 anthocyanins, such as *Pelargonium hortorum* pigment, *Centaurea cyanus* L. pigment, *Consolida ajacis* (L.) schur pigment, and other anthocyanins pigment to be found in these plants [1]. In the present, many studies have indicated that anthocyanins are the most effective antioxidants, and their antioxidant property is 50 folds higher than vitamin E and 20 times higher than vitamin C [2]. Anthocyanins, as a natural antioxidant, have

been widely used in the medical treatment, health care, cosmetology, and food supplement and especially in human health and disease prevention.

2. Type and structure of anthocyanins

Anthocyanins are kinds of water-soluble natural pigment of flavonoids, and its molecular glycosylation type, position, and hydroxyl number are the main basis for distinguishing different anthocyanins. Right now, there are more than 20 kinds of anthocyanins known in plants [3], whose basic carbon skeleton is C6-C3-C6 with cationic structure of 3,5,7-trihydroxy-2-phenyl benzopyrane (**Figure 1**). Six anthocyanins of them are the most common, including *Centaurea cyanus* L. *anthocyanin*, *Consolida ajacis* (L.) Schur *anthocyanin, Petunia hybrida* (J.D. Hooker) Vilmorin *anthocyanin, Pelargonium hortorum Anthocyanin*, *Paenonia lactiflora Pall anthocyanin*, *and Malva sinensis* Cavan *anthocyanin* (**Figure 2**). The hydroxyl group in anthocyanins exists in the form of cationic ions in the cell solution in a lower pH value and shows a strong antioxidant capacity [4, 5]. Anthocyanins are highly contained in a lot of plants and natural medicinal materials, including grapes, apples, hawthorn, tea, peanuts, purple potatoes, and ginkgo. In addition, proanthocyanidins are also found in grape juice, red wine, chocolate, and beer [6]. Proanthocyanidins can convert into the anthocyanins by heating in an acidic medium. Under natural conditions, free anthocyanins are extremely rare and often exist in combination with a single or multiple glucose, rhamnose, or galactose to form glucoside, namely, anthocyanins [7]. It is in fact that anthocyanins are the primary pigment group of plant color, which produces a wider range of colors, ranging from light yellow to violet [8]. The double bonds and polyhydroxyl structure of anthocyanins are the foundation of their antioxidant activity.

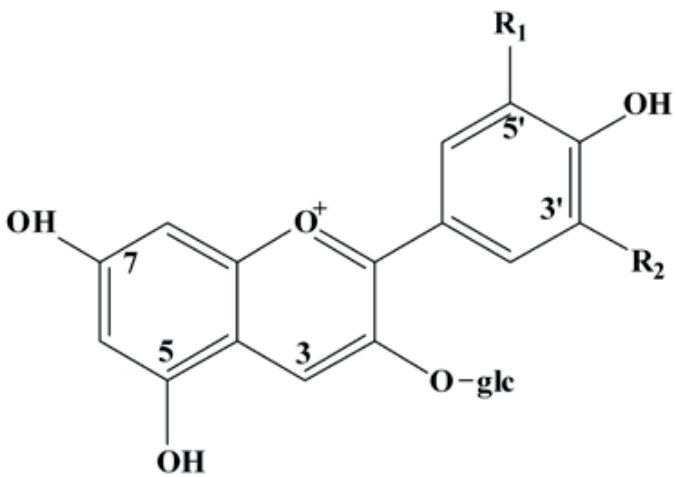

Figure 1.
The basic structure of anthocyanin.

3. Antioxidant activity of anthocyanins

Free radical is an uncoupled electron group or atoms that can independently exist, including superoxide anion radicals, hydroxyl radicals, lipoxyl radicals, nitric

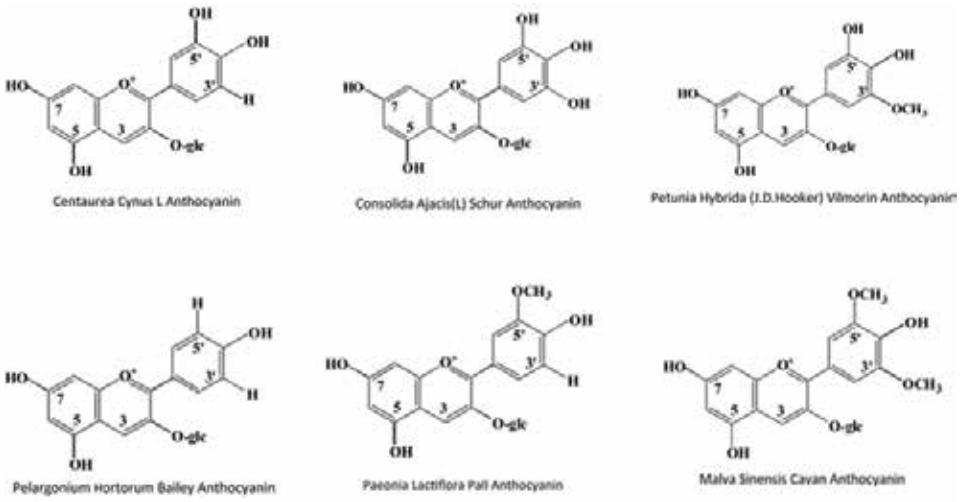

Figure 2.
The structure of anthocyanin.

oxide radicals, and other radicals. Free radicals are the normal metabolic products in an organism and also undertake the important function such as being responsible for transferring energy in the redox process of organism substance metabolism. However, the production of free radicals may be increased due to light, heat, radiation, and other factors. The excessive free radicals in the body are unstable and can capture the electron and show a strong oxidative ability and destroy the cell membrane, proteins, DNA, RNA, and other molecules, finally, which may result in aging and various diseases.

Oxygen free radicals, produced by oxidation respiratory chain, have a strong oxidation property [9]. The first way to remove oxygen free radicals is dependent on the endogenous free radical scavenging system in the body, which includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and other antioxidants. The related reactions are as follows [10]:

- SOD catalyzes superoxide anion radicals to produce hydrogen peroxide and oxygen (**Figure 3**).
- CAT catalyzes hydrogen peroxide to produce oxygen and water (**Figure 4**).
- GSH-Px catalyzes the reduced GSH to form oxidized GSH (GSSG) and two molecular waters (**Figure 5**).

In addition, there are other antioxidants such as reduced GSH, vitamin C, and vitamin E, which can also scavenge free radicals. These antioxidant enzymes and antioxidants can effectively eliminate free radicals to form lines of defense to prevent free radicals attacked to the body and maintain the balance of free radicals and finally to ensure the health of the body.

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Figure 3.
Catalytic reaction of SOD.

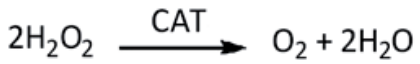

Figure 4.
Catalytic reaction of CAT.

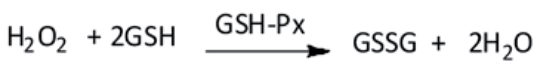

Figure 5.
Catalytic reaction of GSK-Px.

When there is an abnormal increase of free radicals in the body for some reasons, the body is unable to maintain its balance. The exogenous free radicals scavenger is needed to supplement and to help maintain the balance of free radicals to the body. The additional exogenous reduced antioxidants are oxidized by the increased free radicals and prevent the abnormal increased free radicals to attack the biomembrane and other bio-molecules. A series of studies have shown that anthocyanins exhibit its antioxidant activity from three aspects: the first is to directly scavenge free radicals, another is to regulate the activity of antioxidant enzymes, and the third is to chelate with metal ions. These three properties of anthocyanins are derived from the strong reducibility of the polyhydroxyl structure of anthocyanins. Many studies indicated that the ability of *Morus alba* L. anthocyanin, *Glycine max* (L) *Merr* anthocyanin, and *Bletilla striata (Thunb) Reichb.f.* anthocyanin for removing oxygen free radicals and hydroxyl free radicals was positively correlated with the content of anthocyanins, and their effects were higher than Vc [11–13]. Proanthocyanidins isolated from *Hippophae rhamnoides* L. and *Nelumbo nucifera* Gaertn can reduce malondialdehyde (MDA) level in serum and skin tissue of healthy rats, raise SOD and GSH-Px activities in serum and skin tissue, and significantly alleviate the liver lipid peroxidation injury by CCl4 [14]. In addition, metal ions, especially Fe^{2+}, can catalyze the transformation of oxygen radicals into hydroxyl radicals. When a substance can chelate with metal ions, the transformation from oxygen radicals to hydroxyl radicals can be blocked. Moreover, some anthocyanins have the ability to chelate with metal ions and play an antioxidant role. Procyanidins possess the "catechol" structure [10] and can strongly chelate with metal ions. The result of chelation is procyanidins can decrease free radical production from Fenton and Haber-Weiss reaction which is dependent on the necessary iron ions. Procyanidins block the free radical chain reaction and exert a strong antioxidant property. Oxygen free radical reacts with unsaturated fatty acids of the cell membrane to produce lipid peroxidation and the product to be MDA. MDA has a high toxicity and strong destructiveness to the cell membrane, which can change the cellular membrane fluidity and permeability and abnormal inside and outside ion distribution of cells and then destroy the function of various tissues and organs, which eventually leads to the cell irreversible damage and serious diseases. The polyhydroxyl structure of anthocyanins makes them have strong reducibility, which can play an important role in human health care and disease prevention by scavenging free radicals, regulating antioxidant enzyme activity and chelating metal ions in the body.

4. Effects of anthocyanins in the prevention and treatment of diseases

4.1 Antitumor effects of anthocyanins

Environmental pollution, excessive intake of junk food, irregular life, and other bad habits can lead the human body to induce certain carcinogens. The cancerogens

are metabolized and activated to produce free radicals to attack DNA to cause cancer; whereas, the carcinogenic ability of cancerogens is positively correlated to their ability of free radicals produced. At present, it has been confirmed that the increase of oxygen free radical and the change of antioxidant enzyme activity can result in the occurrence of tumors. Moreover, tumor patients usually show the imbalanced oxidoreduction state in the body and the interaction between tumors and antioxidant systems. Studies have shown that anthocyanins can exert antitumor activity by an antioxidant. The in vitro and in vivo experiments have exhibited that anthocyanins inhibit proliferation of tumor cell and development of tumor. The antitumor mechanism of anthocyanins may be related to their effective antioxidant capacity and cyclooxygenase inhibited. It is reported that *Nelumbo nucifera* Gaertn proanthocyanidins inhibited the colony formation and growth of melanoma B16 cells by a dose-dependent manner [15]. The in vitro studies by He et al. found that proanthocyanidins inhibited the proliferation of human colon cancer cell line SW620 in a concentration-dependent manner and activated the mitochondrial apoptosis pathway to promote the apoptosis of SW620 cells [16]. Many studies reported that proanthocyanidins can inhibit the proliferation and promote the apoptosis of SKOV3 cells by decreasing the expression of survival protein survivin and fight against the *Croton tiglium*-induced mouse skin papilloma formation and lessen tumor number and occurrence. Its mechanism of action is that proanthocyanidins can lower the content of NO of the skin in mice, and NO is regarded to be involved in the formation of dermal papilloma in mice [17, 18]. Studies by Zhang found that proanthocyanidins have a strong radiation sensitization action in SPC-A-1 cells of lung cancer [19]. In addition, proanthocyanidins showed good anticancer activity for liver cancer, prostate cancer, skin cancer, and other cancers. With the deepening of the research, proanthocyanidins will play a greater role in the prevention and treatment of cancer [20].

4.2 Anti-dementia effects of anthocyanins

A large number of studies have shown that the oxidative stress response is involved in the pathophysiologic process of Alzheimer's disease (AD) and a large amount of free radicals produced in AD patients' brain. If the large amount free radicals cannot be removed immediately, they will cause the lipid peroxidation of protein, nucleic acid, and other biomolecules and result in the neuronal apoptosis and aggravate the disease development of AD. *Lycium barbarum* L. anthocyanins can improve the mimic AD model rat memory impairment, increase the activity of antioxidant enzymes (SOD and CAT) and GSH content, and reduce MDA and protein carbonyl levels of serum and brain tissues [21]. Other experimental studies have confirmed that grape seed proanthocyanidins can prevent excessive production of β-amyloid protein (Aβ) in the brain and reduce cognitive decline with AD model rats and *Solanum tuberosum* anthocyanins ameliorate domoic acid-induced cognitive dysfunction, which may be used to the treatment of cognitive impairment caused by excitotoxicity and other brain diseases. *Solanum tuberosum* anthocyanins can also inhibit the nerve inflammation by blocking ERK, JNK, and NF-KB signals and show therapeutic effect on the acute encephalitis induced by lipopolysaccharide (LPS) in rats [22, 23].

4.3 Effects of anthocyanins in treatment of diabetes

Diabetes is a lifelong disease, and its incidence rate increases with the age, which seriously disturbs the quality of life to people. Studies have shown that the occurrence and development of diabetes are closely related to the abnormal metabolism

of free radicals in the body. Oxidative stress, deposition of glycosylated end products, and changes of vascular structure and function are all contributed to the increase of free radicals. Anthocyanins have antioxidant activity and can prevent and treat diabetes. Many studies showed that grape seed proanthocyanidin extract (GSPE) could significantly lower the blood glucose and glucose tolerance, increase the body quality, decrease the serum MDA level and increase the SOD activity to the mimic diabetic mice, inhibit fat deposition, and lower blood lipid to the fatty mice. Proanthocyanidins regulate fatty metabolism in mice by commonly influencing the expression of lipid metabolism-related genes, glucose, and insulin tolerance [24–26]. A series of studies by Bao et al. found that GSPE could improve renal function injury caused by diabetes and could improve the symptoms of diabetic nephropathy by antioxidative stress and inhibiting inflammation [27]. These studies suggest that GSPE have a strong hypoglycemic effect, and its hypoglycemic mechanism may be associated to its antioxidant capacity.

4.4 Effects of anthocyanins in preventing cardiovascular diseases

In recent years, cardiovascular diseases have become the common diseases that endanger human health. With the deepening of the research on oxygen free radicals in diseases, a large number of data have confirmed that the oxidative reactions mediated by free radicals and their products play an important role in the occurrence and development of cardiovascular diseases. As people age, the elastic fibers of arteries harden as they are oxidized, and the change is a major cause of cardiovascular disease in aging people. Proanthocyanidins mainly play an important role in preventing cardiovascular diseases by inhibiting the formation of artery atherosclerotic plaque and reducing the damage of free radicals induced by myocardial ischemia for protecting myocardial cell activity. Proanthocyanidins can protect blood vessels and reduce capillary permeability, and its function of preventing cardiovascular diseases is closely related to its antioxidative stress. Some results indicated that grape seed proanthocyanidins can effectively reduce the levels of LDL and cholesterol and the generation of MDA [28]. In addition, GSPE can protect vascular substances by capturing ROS and regulating enzyme activity [29]. Anthocyanins extracted from red wine can effectively remove superoxide free radicals and hydroxyl free radicals. The in vitro experiments indicated that anthocyanins can significantly inhibit the oxidation of LDL and the aggregation of platelets [30]. The animal and clinical studies have also found that proanthocyanidins can reduce blood pressure by lowering cholesterol level, reducing cholesterol deposition on blood vessel walls and improving vascular elasticity [31]. Some studies showed that *Nelumbo nucifera* Gaertn proanthocyanidins can increase the SOD activity of myocardial cells during ischemia reperfusion and reduce the production of MDA, thereby inhibiting apoptosis and protecting myocardial ischemia reperfusion [32]. Studies by Suda I et al. showed that anthocyanins from *Solanum tuberosum* can be absorbed by rats, enhance the antioxidant capacity of plasma, and play an important role in protecting cardiovascular system [33].

4.5 Protective effects of anthocyanins on liver and kidney

Studies have shown that gluttony can harm gastrointestinal tract, liver, and kidney as large amounts of free radicals are produced. Removing these deteriorated free radicals timely can protect liver cells and improve liver function. When fatty liver occurs, free fatty acids, oxidative stress, and free radicals are increased, which lead to the degeneration of proteins, DNA, and lipid in liver cells and lower the immunity. *Solanum tuberosum* anthocyanins were proven to alleviate liver injury caused by dimethylnitrosamine (DMN), protect the liver function, and resist

liver fibrosis [34]. In addition, *Solanum tuberosum* anthocyanins can also activate adenosine monophosphate to sensitize the protein kinase and inhibit liver fatty accumulation [35]. The results by Zhang et al. were also reported that anthocyanins from *Solanum tuberosum* can alleviate insulin resistance in liver caused by high-fat diet, and the effective mechanism is that anthocyanins can block oxidative and endoplasmic reticulum stress [36]. Studies showed that anthocyanins of *Solanum tuberosum* can inhibit oxidative stress in the kidney, and the molecular mechanism was to inhibit the activation of NLRP3 signaling pathway of inflammasome [37]. Studies by Sun et al. reported that *Solanum tuberosum* anthocyanins have a preventive effect on acute and subacute alcoholic liver injury and indicated that *Solanum tuberosum* anthocyanins have a certain antialcoholic effect [38].

4.6 Anti-inflammatory effects of anthocyanins

Inflammation is the body's defense against stimuli, which is usually to be regarded as beneficial but sometimes to be as harmful. The acute inflammation is a short-term self-limiting process, and the chronic systemic proinflammatory state can result in insulin resistance, atherosclerosis, type II diabetes, metabolic syndrome, cardiovascular disease, cancer, neurodegenerative disease, and other diseases [39]. When the body is in the acute inflammation, macrophages can effectively eliminate foreign materials by producing a large number of oxygen free radicals in a short time. However, when the acute inflammation changes into the chronic inflammation, the generation of reactive oxygen species (ROS) is out of control and dramatically increases, which will promote the inflammatory factors activated, aggravate the inflammatory response and gene mutations, and finally result in the occurrence of cancer [40]. Therefore, it is the important link to maintain the balance of free radicals and regulate the activity of SOD in the body against the inflammation [41]. Many studies indicated that anthocyanins showed strong anti-inflammatory activity in both in vivo and in vitro, and their effective mechanism may be the ability to remove ROS and regulate SOD activity [42]. Anthocyanins from *Ligustrum x vicaryi* Hort can inhibit the mice's auricle swelling induced by xylene, raise the serum SOD activity of mice, and then exert its anti-inflammatory and analgesic properties. Studies by Kim et al. found that anthocyanins from *Glycine max* (L) *Merr* can inhibit ROS level in human gastric epithelial cells infected by *Helicobacter pylori* and in a dose-dependent manner, and the inhibition was significant [43]. In conclusion, anthocyanins have been proven to express high antioxidant properties, and the anti-inflammatory effect is primary from its inhibiting ROS production and enhancing SOD expression [39].

4.7 Effects of anthocyanins in the male reproductive system

Smoking, drinking, staying up late, or genital tract infections, exposing to the phenols chemicals, heavy metals, and the external environmental high ionizing radiation, may significantly increase free radicals production in the male reproductive system. The high free radicals can cause many diseases to the reproductive system in the male. GSPE, as a highly effective antioxidant, plays an important role in the prevention and treatment of related male reproductive diseases. GSPE can antagonize the reproductive toxicity of male mice caused by heavy metals, fluorine, and semicarbazide, enhance the sperm survival rate, and reduce the sperm malformation rate. It is also confirmed that GSPE can increase the activity of lactate dehydrogenase X and glutamyl transpeptidase in the mice's testis of semicarbazide infected and reduce the activity of acidic phosphatase, in which it is proven that GSPE possesses a good repaired function for the testis injuries. In addition, GSPE

can elevate the SOD activity of testicular tissue induced by testicular torsion reduction, decrease the MDA level, and appear to be an obvious protection to mice reproductive function injury. Meanwhile, GSPE can also inhibit the spermatogenic cells apoptosis in experimental cryptorchidism male rats, and the effective mechanism is also derived from the antioxidant capacity of GSPE [44].

4.8 Applications of anthocyanins in ophthalmology

With the popular of various electronic devices such as mobile phones, computers, and LED lights, the blue light harm is the more and more to people. The long-term blue light irradiation to retina can cause a lot of free radicals produced. These free radicals can result in retinal pigment epithelial cell apoptosis, intraocular metabolic abnormalities, toxin trash accumulation, and hindering of the blood circulation, which cause myopia, cataracts, macular degeneration, ocular fundus diseases, vitreous opacity, floaters, retinopathy, and other eye diseases. Proanthocyanidins can effectively eliminate oxygen free radicals, which is beneficial to the treatment of ophthalmological diseases. Studies showed that anthocyanins can significantly improve visual fatigue and the early myopia and the distant vision of mild myopia [45]. This result indicated that proanthocyanidin eye drops have good effect for the treatment of xerophthalmia [46]. Muthenna et al. also provided that *Cinnamomum cassia* Presl proanthocyanidin extract B2 can improve the cataract of diabetic rats, ameliorate the optic nerve blood perfusion, and block the optic nerve cell apoptosis induced by ischemia. The possible effective mechanism of anthocyanins is that anthocyanins have a strong scavenging ability for free radical and inhibiting intracellular calcium overload and then protect the optic nerve structure and function [47]. In addition, anthocyanins also have therapeutic effects to glaucoma, in which the effective mechanism of anthocyanins is both removing free radicals and reducing intracellular calcium overload and also is related to the enhancement of SOD activity. Furthermore, anthocyanins can alleviate further injury of the optic nerve in the eye surgery, which is also associated to raising SOD activity [48, 49].

5. Functions of anthocyanins in health care and cosmetology

5.1 Enhancements of anthocyanins in the immune system

Free radicals attack the immune system or lymphocytes to damage them, which can result in the decline of cell-mediated immunity and humoral immunity. Free radicals also lead to the decline of immune recognition and the emergence of autoimmune diseases. Studies by Gabriela et al. showed that GSPE can improve the immune suppression induced by ultraviolet irradiation in mice, which may be one of the mechanisms by which GSPE inhibits the light of carcinogenesis [50]. By regulating the differentiation of inflammatory T cells, GSPE can reduce the secretion of interleukin IL-17, IL-21, IL-22, IL-26, and other cytokines and reduce the incidence of inflammation and diseases [51]. Studies showed that proanthocyanidins from *Hippophae rhamnoides* L seed could significantly increase the mice immunity, enhance the mice carbon clearance ability, raise the mice T lymphocyte activity, and promote the hemolysin formation [52]. Hao et al. added proanthocyanidins extracted from *Sorghum* to the feed of ablactation piglets. The IgG, IgM, C3, and IL-2 concentrations of piglets serum significantly increased, which was conducive to improving piglets immunity [53]. In addition, The Institute of Shanghai Nonghao Biological Technology, which studied proanthocyanidins from *Pinus tabulaeformis* Caar as a feed additive, found that proanthocyanidins have antigenic properties,

produce specific immune responses, improve the immune function, and reduce the incidence of livestock and poultry [54].

5.2 Antiaging effect of anthocyanins

Senescence is a complex physiological process. As early as 1956, Harmon proposed the free radical theory of senescence [55], which believed that the senescence was primarily caused by the attack of free radicals to cell components, and then maintaining the balance of antioxidants and free radical scavengers in the body could delay senescence. There are many studies indicated that proanthocyanidins can obviously reduce the generation of spontaneous MDA in liver and brain tissue of rats and have a significant antagonistic effect in liver lipid peroxidation induced by free radical initiators CCl_4, H_2O_2, and iron ions, lower the depletion of GSH in liver tissue, and reveal obviously anti-lipid peroxidation and free radicals' scavenging ability [56]. The result by Sato et al. reported that GSPE can promote the recovery of cardiac systolic function and decrease the area of myocardial infarction after ischemia reperfusion and the possible mechanism of this proanthocyanidins are related to directly removing peroxide free radicals, enhancing SOD activity, decreasing MDA level, and inhibiting the damage from free radical lipid peroxidation to myocardial membrane [57]. Proanthocyanidins, as natural antioxidants, can alleviate the aging of the body by adjusting the free radical oxidation system [58].

5.3 Effects of anthocyanins on cosmetology and skin care

Young people's skin is nutritious and white than aged peoples. With the increase of age, the skin will get rougher, wrinkled, and darker and gradually senile plaques form. This is the reason why the skin has some substances, like SOD, CAT, and GSH-Px, that can prevent the skin from aging. There are other antioxidants such as vitamin E and vitamin C also to assist regulating the balance of oxygen free radical for preventing skin aging and damage. With the increase of age, the enzyme activity that removes oxygen free radical drops and the antioxidant content also drops in the skin. Then, the excessive harmful oxygen free radical is able to bring about the cell damage. If the complement of a few exogenous free radical scavengers is not immediately provided, the balance of free radical of the skin is hardly maintained, and good skin is hardly possessed [10]. The present studies indicate that proanthocyanidins are good antioxidant and have free radical scavenging abilities and also can promote the covalent cross-linking of collagen molecules, inhibit the elastase production and prevent the degradation of elastin, prevent skin aging and laxity, and reduce the excessive secretion of sebaceous glands to skin. In addition, proanthocyanidins can combine with proteins by the form of hydrophobic bonds and hydrogen bonds, which can shrink skin pores and tighten skin. Proanthocyanidins can reduce the o-benzoquinone structure of the melanin to phenolic structure to fade the pigment and inhibit the key enzyme tyrosinase phthalidomide activity of melanin synthesis to achieve white skin and decrease spot efficacy. Proanthocyanidins can absorb ultraviolet light and inhibit the process of lipid peroxidation. Supplement of external grape seed proanthocyanidins can decrease Fas protein expression and increase the skin bcl-2 protein expression after the irradiation of skin and tend the skin to normal skin status, which indicates that proanthocyanidins from grape seed can reduce the sun damage to a certain extent [59–61]. In addition, proanthocyanidins also have the effects in treating skin inflammation, moisturizing and antiaging skin. In the present, proanthocyanidins, as raw materials, are added to successfully produce the night cream, skin whitener, sunscreen, mouthwash, and shampoo in France, Italy, and Japan market [62, 63]. Proanthocyanidins isolated from grape

seeds, a face cream based on the characteristics of Chinese elderly people's skin in the Chinese market was developed, which has been proven stabile to achieve anti-wrinkle and moisturizing efficacy.

5.4 Anthocyanins for food additives

Anthocyanins, as a kind of natural food pigment, have advantages of high security and abundant resources, as well as certain nutritional and dietary functions, as compared with synthetic pigments. In recent years, people pay more and more attention to food safety and health, and then anthocyanins have shown to have more and more concern for consumers and researchers. Currently, anthocyanins have been allowed to be put into production and used natural edible pigments including grape skin pigment, berry pigment, purple sweet potato pigment, perilla pigment, cabbage pigment, purple corn pigment, and other edible pigment [2]. Anthocyanins from blueberry fruits, as natural food additives, have been widely used in the lactic acid milk, cakes, and other food.

6. Conclusions

In recent years, a large number of studies have confirmed that anthocyanins have strong antioxidant properties and anthocyanins, as safe natural pigment and effective antioxidants, have been a widely used. This chapter systematically summarizes the application of anthocyanins in human disease prevention and health care based on anthocyanins' structure and species. However, at present, there is no report upon the anthocyanins as a clinical drug used. Then, the intensive research and innovation should be performed in order that anthocyanins specifically apply to the prevention of clinical diseases and health care in the future.

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Conflict of interest

The authors report no declarations of interest.

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Chapter 6

Flavonoids: A Promising Therapy for Obesity Due to the High-Fat Diet

Monica Michicotl Meneses
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Abstract

Currently, metabolic diseases are the main public health problem. Obesity is a metabolic imbalance that leads to insulin resistance, hyperinsulinemia, dyslipidemia, hypertension, and proinflammatory and prothrombotic states that can trigger type 2 diabetes as well as cardiovascular diseases. Obesity is the excess of adipose tissue and is considered a risk factor to develop metabolic syndrome. The obesogenic environment that is lived promotes the search for specific solutions that help to control, eradicate, or minimize the negative results in health, making use of the properties of flavonoids, as important sources of antioxidants, with anti-inflammatory, antithrombotic, and antihypertensive effects. Given the above, the objective of this chapter is to highlight the effects of flavonoids on the modulation of lipolysis and lipogenesis altered by a hyperlipidic diet.

Keywords: flavonoids, lipolysis, lipogenesis, obesity, inflammation

1. Introduction

Obesity is a major health problem worldwide. It is the result of the combination of genetic factors, inadequate nutrition, and lack of regular physical activity. The ingestion of a diet of high energy density is the main cause of visceral or central obesity, since the excess energy is stored in adipocytes, which increase in size and number, or both, especially the visceral ones, producing an increase in the rate of lipolysis, which, in turn, stimulates the secretion of cytokines by the infiltration of leukocytes, macrophages generating inflammation in the adipocytes, and leads to proinflammatory state, insulin resistance, and endothelial dysfunction. Thus, adipose tissue dysfunction represents the etiopathogenic mechanism (**Figure 1**) in the development of cardiovascular disease, type 2 diabetes, and renal disease initiated by visceral obesity [1].

The WHO defines overweight and obesity as "abnormal or excessive accumulation of fat that can affect health." A person is defined as normal weight if their BMI is 18.5–24.9 kg/m^2, overweight if the BMI is 25–29.9, or obese if the BMI is 30 or more [2].

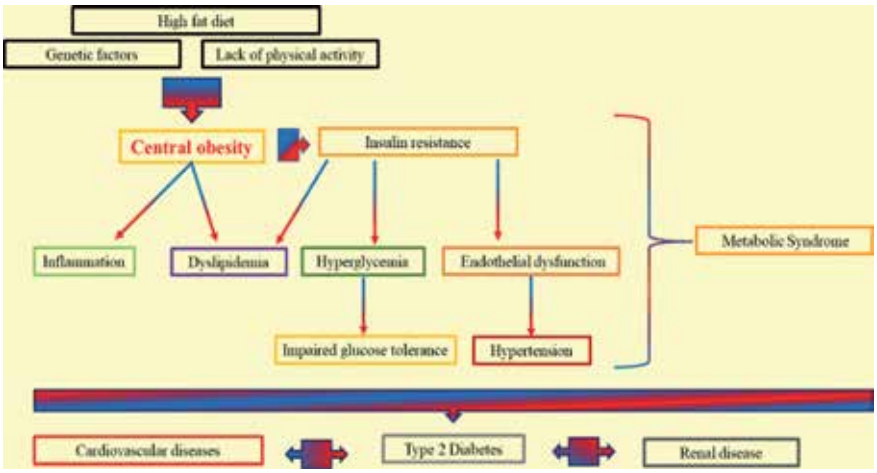

Figure 1.
Etiopathogenic and pathophysiological mechanisms of obesity.

2. Mechanisms present in obesity

The number of adipocytes increases in the early age but reaches a maximum in youth and remains constant regardless of weight changes. In overweight or moderate obesity, the cells grow by the accumulation of lipids, without increasing their number. However, when obesity increases to more severe levels, the number of adipocytes will always increase [3].

Adipose tissue can be made up of large adipocytes (hypertrophy) or many small ones (hyperplasia). These two mechanisms contribute to the expansion of adipose tissue. In adults, hypertrophy is the mechanism that predominates and has been detected, which is strongly related to diet, while hyperplasia depends on genetics [4].

When there is an imbalance between the amount of energy consumed and that used by the body, it begins to store the excess energy in the form of triglycerides inside the adipocytes. These adipocytes begin to become hypertrophic, which causes free fatty acids to be released into the circulation (lipotoxicity), as well as the adipocytes changing their immunological balance which promotes the production of proinflammatory cytokines [5].

Obesity is a chronic state of low-grade inflammation. During the development of obesity, macrophages infiltrate the visceral white adipose tissue, causing chronic inflammation of low intensity that is characterized by the upregulation of proinflammatory adipokines such as TNFα, and decrease the concentration of anti-inflammatory adipokines such as adiponectin. In addition, saturated fatty acids and TNFα, derived from adipocytes and macrophages, result in a cycle that leads to chronic inflammation of fat cells [6], as shown in **Figure 2**.

Also, during obesity, adipose tissue produces a greater amount of reactive oxygen species (ROS) which causes oxidative stress. This stress in turn leads to the abnormal production of adipokines (chronic low-grade inflammation), where it has been shown, for example, that the concentration of adiponectin is inversely related to the concentration of ROS [7].

2.1 Abdominal obesity

The white adipose tissue is metabolically active itself that participates in the metabolic regulation and physiological processes such as the inflammatory response, vascular function, and the secretion of hormones and adipocytokines [8].

Different epidemiological studies have shown that the increase in the consumption of diets high in saturated fats and simple carbohydrates leads to the progressive accumulation of intra-abdominal fat mass, accompanied by alterations in their pattern of adipocytokine secretion and in the homeostasis of the metabolism of lipids [9, 10].

There are two main types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT) (**Figure 3**). White adipocytes are the most common fat cells, are present in visceral and subcutaneous adipose tissues, and are responsible for the expansion of fat mass in obesity. On the other hand, brown adipocytes are smaller cells that play a central role in the process of thermogenesis; their deposits are mainly focused on the interscapular and perirenal and around the great vessels, although their deposits are limited according to age advances [10].

In the particular case of obesity and the capacity shown by white adipocytes to adapt their metabolism to the energy demands of the organism, it will depend on the BAT to adequately perform its function as an energy reservoir (uptake of circulating fatty acids, esterification, and deposition as triglycerides (TG)) and prevent ectopic deposits of lipids and, therefore, lipotoxicity in tissues such as the liver and

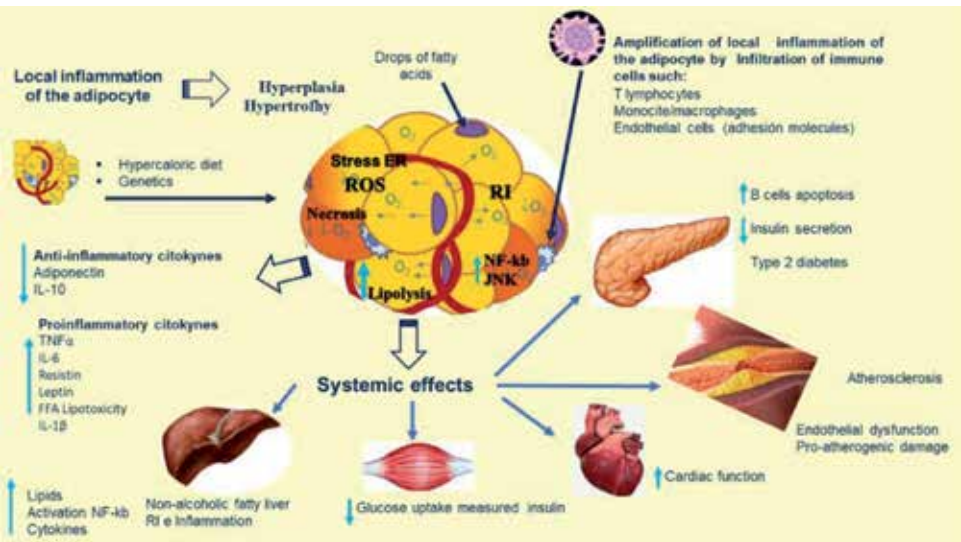

Figure 2.
Mechanisms of inflammation in adipose tissue. ↓Decrease, ↑increase. Interleukin 10 (IL-10), interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα), Jun kinase (JNK), necrosis factor kappa B (NF-κb), free fatty acids (FFA).

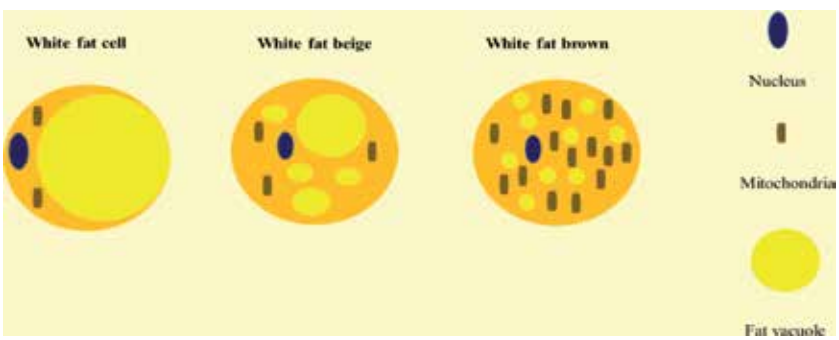

Figure 3.
Types of adipose tissue.

skeletal muscle. It is important to note that in lipogenesis and TG synthesis in the liver due to a hypercaloric diet, the synthesized fatty acids are incorporated into the triacylglycerides, leading to an increase in the synthesis and secretion of very-low-density lipoproteins (VLDL), as shown in **Figure 4**.

When the capacity of the WAT to store TG is exceeded, the activity of lipoprotein lipase of the adipocyte (LPLa) begins to decrease and with it the hydrolysis of TG that is transported by chylomicrons from the small intestine. In the long term, this generates an elevation in plasma levels of triglycerides and c-VLDL. Similarly, the progressive hypertrophy of adipocytes promotes tissue hypoxia, inflammation, and infiltration of macrophages that induce an increase in the secretion of various proinflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL −6), plasminogen inhibitor 1 (PAI-1), C-reactive protein (CRP), and monocyte chemoattractant protein 1 (MCP-1), among others [11, 12].

The peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor that plays a central role in the regulation of adipogenesis (differentiation and proliferation of adipocytes), allowing the expansion of the TAB in response to a positive energy balance through the increase in the number of small adipocytes with high sensitivity to insulin. In addition to this, PPARγ induces the expression of genes involved in uptake (lipoprotein adipocyte lipase (LPLa)), transport (CD6 fatty acid transporter (FAT/CD36), fatty acid-binding protein in adipocytes (aFABP)), esterification, and deposition of TG (acyl-CoA synthetase (ACS), perilipin 1 (Plin1)) and inhibits the expression of proteins involved in lipolysis (hormone-sensitive lipase (HSL), monoglyceride lipase (MGL)) [13, 14]. The above functions prevent the secretion of free fatty acids (FFA) into portal and systemic circulation and, consequently, lipotoxicity and insulin resistance in peripheral tissues. On the other hand, PPARγ contributes to maintain an adequate sensitivity to insulin through induction in the secretion of adiponectin (ApN) and leptin and the increase in the expression of the substrate of the insulin receptor (IRS1/2) and the transporter GLUT4 [15].

The oxidative function of WAT is generally underestimated. However, this is relevant during the process of differentiation of adipocytes in which the gene expression of PGC-1α is increased, a master regulator of mitochondrial biogenesis and oxidative metabolism [16].

The lipid droplets that are concentrated in the cytoplasm of adipocytes are surrounded by structural proteins and enzymes that respond to hormonal stimuli for lipolysis. Cyclic AMP (cAMP) is a second messenger that activates lipolysis by stimulating protein kinase A (PKA) which, in turn, is responsible for phosphorylating membrane protein perilipin 1 (Plin1). The latter activates the hydrolysis of the

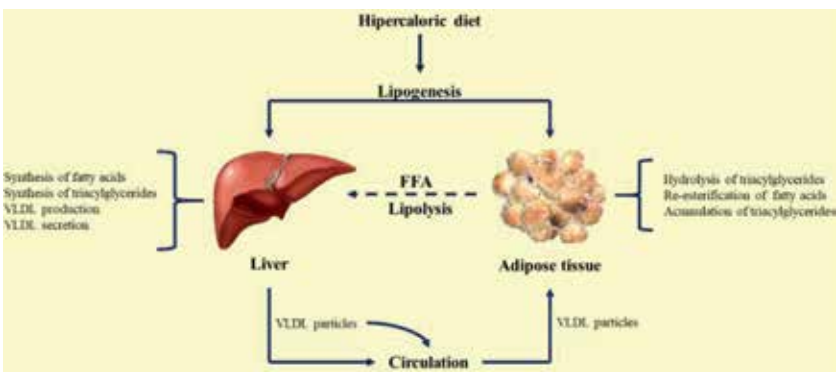

Figure 4.
Lipogenesis stimulation by hypercaloric diets.

TG stored in the lipid vacuoles through the induction of CGI-58, the coactivator of acyl triglyceride lipase (ATGL). Subsequently, PKA activates the hormone-sensitive lipase (HSL) that is responsible for hydrolyzing diacylglycerol (DAG) to monoacylglycerol, which will finally be hydrolyzed to obtain a non-esterified fatty acid (NEFA) and a glycerol molecule [10] (**Figure 5**) [55].

Insulin acts as a physiological inhibitor of catecholamine-induced lipolysis, since after stimulation of the insulin receptor (IRS-1/2) and phosphatidylinositol-3 kinase (PI3K), PKB is activated that phosphorylates phosphodiesterase-3B (PDE-3B) producing the hydrolysis of cAMP. The reduction of cAMP levels and PKB activity that accompany the activation of PDE-3B results in net dephosphorylation and decreased activity of HSL, leading to decreased hydrolysis of stored TAGs [56].

The adequate regulation of the process of lipolysis will lead to the fatty acids released into the circulation being captured by peripheral tissues, activated (addition of an acyl-CoA group), and transported, by carnitine palmitoyl transferase-1 (CPT-1/2), inside the mitochondrial matrix for its oxidation. However, the imbalance that is generated in the metabolism of adipocytes in obesity causes a lipogenic state and insulin resistance that decreases the rate of oxidation and favors lipolysis. The latter favors the ectopic deposits of lipids in the liver, a chronic state of inflammation and systemic resistance to insulin [10].

Abdominal obesity and IR in the WAT promote lipolysis and secretion of FFA into the portal circulation. In addition to this, an increase in plasma concentrations of remnant chylomicrons (rich in TG) is observed due to the absence of LPL induction in the liver and WAT. The hypertriglyceridemia is further accentuated by an increase in the synthesis and secretion of hepatic VLDLs, secondary to an increase in the portal flow of FFA and an increase in the production of apolipoprotein B-100 [17]. The hypertriglyceridemia of MS is directly related to a reduction in the plasma concentration of HDL and an increase in small and dense LDL particles (sdLDL) [18].

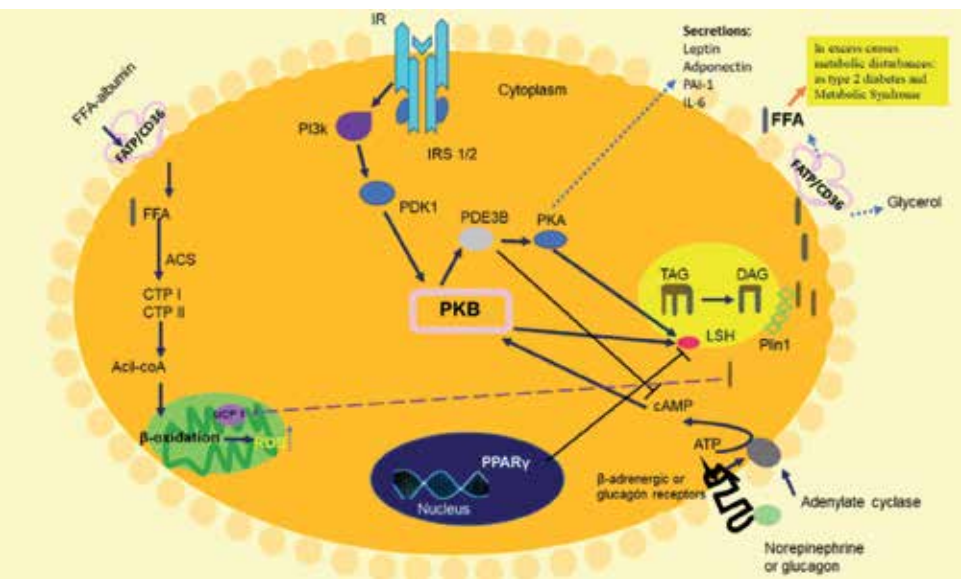

Figure 5.
Control of lipolysis in the human adipocyte. Receptors β y α_{2A}-adrenergic (RA), Kinase A protein (PKA), hormone-sensitive lipase (LSH), insulin receptor (IRS-1/2), Kinase B protein (PKB), kinase (PI3 [PI3-k]), phosphodiesterase type 3B (PDE 3B), kinase protein (PDK1), perilipin (Plin 1), diglyceride (DG), monoglyceride (MG), fatty acid transporter protein (FATP), free fatty acids (FFA), acyl co-A synthetase (ACS), Carnitine palmitoyltransferase (CTP), reactive oxygen species (ROS). The solid arrows indicate the effects that appear beyond the activation of kinases. The sign indicates → stimulation and —| indicates inhibition.

The decrease in plasma levels of HDL is the result of a decrease in the concentration of HDL3 particles (in maturation) and in a greater degree of HDL2 (mature). In hypertriglyceridemia, VLDL becomes prone to the reciprocal transfer of cholesterol esters and triglycerides with HDL2, by the action of cholesterol ester transferase protein (CPTE). This causes the conversion of HDL2 into smaller HDL3 particles rich in triglycerides that become suitable substrates for hepatic lipase (LH).

In IR, the greater induction of LH activity increases the hydrolysis of TG and phospholipids of HDL3 and promotes the formation of even smaller and poor HDL particles in cholesterol esters. They are more susceptible to renal dissociation and excretion of their apolipoprotein A-1 (apoA-1) [19, 20].

The decrease in plasma concentration of HDL reduces the reverse cholesterol transport (RCT) from the peripheral tissues to the liver for biliary excretion. As a result, the atherogenic processes and the elevation in total cholesterol concentrations are favored [19].

At the level of the pancreatic β cell, the accumulation of intracellular cholesterol (due to alterations in the ABCA1 cholesterol transporter) has been shown to influence the reduction of insulin secretion and cellular degeneration that culminates in apoptosis [21].

IR and decreased levels of HDL are related to an increase in sdLDL levels, which have a great atherogenic potential that increases the risk of developing CVD. Its formation is the product of an increase in CPTE activity that favors the exchange of cholesterol esters of LDL by TG of VLDL and consequently the formation of TG-rich LDL particles.

Skeletal muscle is another tissue that undergoes serious metabolic alterations due to the elevation of plasma FFA and TG levels, since it favors the uptake and excessive accumulation of TGIM that diminish mitochondrial oxidation capacity and contribute to the development of IR in this tissue [19, 20].

It has been suggested that the increase in intramyocellular accumulation of lipids is mediated by an increase in the translocation of the FAT/CD36 protein responsible for transporting long-chain fatty acids from the extracellular space to the outer mitochondrial membrane, where it favors its transport by the CPT-1 for its subsequent oxidation. However, when the uptake of fatty acids increases, there is a decrease in the rate of oxidation (by increased levels of malonyl-CoA that inhibit CPT-1) that promotes their accumulation in the form of TG drops. This is directly related to the development of IR and hyperglycemic states, since the accumulation of fatty acid metabolites (long-chain acyl-CoA, DAG, ceramides) induces kinase involved in the phosphorylation of amino acid residues of the insulin receptor or of its substrates resulting in the interruption of the insulin signaling cascade [20, 22].

2.2 Cytokines secreted by fatty tissue

Some of the cytokines secreted by fatty tissue are listed below:

Adiponectin: It is a protein that is synthesized mainly in adipocytes [23] and is the cytokine most secreted by adipose tissue. Its concentration is linked to the ADIPOQ gene [24] and is associated with inflammation processes.

Alpha tumor necrosis factor (TNFα): It is a cytokine that is associated primarily with the inflammatory response related to obesity. It also has an effect on lipid and glucose metabolism [25]. Studies have been conducted in humans with obesity showing high expression of TNFα in adipose tissue and a decreased expression of this factor after losing weight. It has also been shown that TNFα suppresses the transcription of adiponectin [26].

IL-6: It is a cytokine whose synthesis is induced by inflammatory stress and is involved in atherogenesis. It is believed that high levels of IL-6 are responsible for

the increase of proteins in obese patients, particularly CRP. This protein decreases the activity of lipases, which increases lipid consumption by macrophages. It has also been directly associated with IL-6 with the index of body mass, waist circumference, and visceral fat in obese patients [27].

MCP-1: It is a chemokine that is secreted in response to the proinflammatory cytokines that has the function of recruiting monocytes and macrophages in case of inflammation or tissue damage [28]. It is involved with obesity and is secreted by adipose tissue. Normally the adipose tissue of slender people contains 5–10% of macrophages, while in obese the content of macrophages in the adipose tissue can reach as high as 50% of the total cells [6].

PAI-1: It is an inhibitor of serum proteases whose main function is antifibrinolytic (the ability to promote the formation of clots). It has been discovered that this molecule is associated with the differentiation of pre-adipocytes to adipocytes, with the fat content in the vesicles of adipocytes and the level of leptin circulating in plasma [29].

Leptin: It is a hormone produced mainly by adipose tissue that is released into the bloodstream. Leptin levels decrease during fasting; after food intake, leptin is produced which sends a signal to the hypothalamus that inhibits the appetite [30]. However, it has been observed that in obese patients there is a phenomenon of resistance to leptin since in their blood high levels of this hormone have been found.

2.2.1 PPARɣ

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to a superfamily of nuclear receptors that regulate the metabolism of glucose and lipids.

PPARɣ is a receptor that is abundantly expressed in adipose tissue and one of the main regulators of glucose and insulin metabolisms. It also plays an important role in the transcriptional activation of adipokines, including adiponectin [31]. PPARɣ directly controls the expression of many genes related to the key functions of adipocytes, such as lipid transport and metabolism, as well as the production of adipokines. It also affects the expression of genes involved with lipid metabolism such as lipid transport (FABP4), fatty acid absorption (LPL, FATP/SLC2/A1, OLR1), recycling of intracellular fatty acids (PEP-CK/PCK1, GK, AQP7), and lipolysis (GPR81) [32].

2.2.2 Oxidized low-density lipoproteins (LDLox)

The increase in vascular production of EROS not only causes a decrease in the synthesis and bioavailability of endothelial NO but also can react and oxidize small, dense low-density lipoproteins (sdLDL) that infiltrate and easily adhere to proteoglycans in the basal vascular lamina [33].

The presence of LDLox constitutes a crucial factor in the development of proinflammatory processes in the arterial vascular wall. Once these molecules are captured by membrane receptors of endothelial cells, they promote a series of proapoptotic and remodeling processes that favor the development of atherosclerosis and endothelial dysfunction. The increase in LDLox concentrations has also been associated with an increase in the proteasomal degradation of eNOS, changes in the ratio of eNOS: iNOS expression and with protein oxidation [34].

Similarly, LDLox are recognized and phagocytosed by macrophages that during the process undergo changes in their conformation and become foam cells. These cells adhere to the smooth muscle cells of the endothelium and continue to accumulate lipids, which favors the formation of lipid striae that progress to form atheromas [35].

It was shown that the incubation of cell cultures with (−)-epicatechin had a protective effect against the oxidative damage generated by the presence of LDLox. This reduces the activation of endothelial cells that promote inflammatory responses (release of cytokines, chemokines, and angiogenic factors) and the production of cell adhesion molecules that facilitate the migration of macrophages to the vascular intima to phagocytose LDLox [36].

2.2.3 Effects of flavanols on hyperglycemia and insulin resistance

In addition to the anti-inflammatory effects that cocoa flavanols have shown, there are recent publications indicating that these also have beneficial effects on hyperglycemia and insulin resistance. These alterations are closely related to dyslipidemia and the presence of abdominal obesity and, consequently, to the pathogenesis of the metabolic syndrome [37, 38].

In a study with hypertensive patients, a vasodilator effect was observed, as well as a decrease in blood pressure and an improvement in blood glucose and fasting and postprandial insulin response, after the daily consumption of dark chocolate rich in flavanols [40, 41].

In another study in mice with type 2 diabetes (DT2) and obesity, it was observed that the administration of cocoa liquor rich in procyanidins (CLPr) decreased the hyperglycemia in a dose-dependent manner. The proposed mechanisms involve an increase in the translocation of GLUT-4 toward the cell membrane, an increase in phosphorylation of AMPK, and the induction of gene expression of UCP-2 in skeletal muscle [39, 40]. Another phenolic compound, ellagic acid, increases the expression of the type 4 glucose transporter (GLUT4) and the peroxisome proliferator-activated gamma receptor (PPAR-γ). Activation of the latter by pioglitazone upregulates adiponectin, but when combined with pure ellagic acid, this positive regulation is achieved at lower drug concentrations, i.e., ellagic acid is responsible for antidiabetic activity [41].

These results are consistent with those obtained in two studies in which it shows that the administration of a flavanol-rich cocoa extract in an animal model with DT2 has hypoglycemic and lipid-lowering effects [39, 42]. In a similar study, it was evaluated whether supplementation of a high-fat diet with CLPr could attenuate the development of obesity, insulin resistance, and hyperglycemia induced by a high-fat diet and that glucose levels were obtained at different doses of CLPr. Plasma fasting decreases, compared to the group fed with a high-fat diet without supplementation. Also, when performing the oral glucose tolerance test, it was observed that supplementation with 2% of CLPr manages to reduce hyperglycemia and postprandial hyperinsulinemia [43].

Phosphatidylinositol-3-kinase (PI3K) and AMPK are the two main molecules involved in the regulation of GLUT4 translocation. Thus, the increase in the activation of AMPK by the administration of CLPr was related to an increase in the expression and translocation of GLUT4 and, therefore, with a higher glucose uptake.

Finally, the effect of the administration of CLPr on the protein expression of UCP1 (brown adipose tissue) and UCP2 (white adipose tissue and liver), involved in the regulation of thermogenesis and energy metabolism, was studied. The results showed that both concentrations of CLPr increase energy expenditure, through an increase in protein expression of UCP1 and UCP2 [44].

2.2.4 Effects of flavanols on alterations in lipid metabolism

Atherogenic dyslipidemia (increased levels of TG, c-LDL, and c-VLDL, accompanied by decreased HDL) not only constitutes one of the central criteria of the

metabolic syndrome but has also been shown to be directly related to the development of CVD. In addition to its beneficial effects on oxidation, inflammation, and endothelial function, cocoa flavanols have also been shown to have lipid-lowering effects that attenuate the development of NCDs associated with alterations in lipid metabolism. There are meta-analyses of clinical trials that have shown that the consumption of products derived from cocoa (cocoa and dark chocolate) has beneficial effects on the lipid profile of patients with some type of CVD or with metabolic risk factors. Most studies are consistent in showing a decrease in plasma levels of CT and c-LDL; however, in relation to the increase in HDL-c levels, the results are heterogeneous [45–47].

Other studies in animals and humans (healthy or with CV risk) have also reported a significant decrease in plasma levels of TG, CT, and c-LDL and an increase in c-HDL levels, after a period of chocolate consumption dark or cocoa [48, 49].

In an animal study, the hypocholesterolemic effects of a mixture of epicatechin and catechin and another mixture of oligomeric procyanidins of cocoa were evaluated, after the ingestion of a high cholesterol diet for 4 weeks. The results showed that only the procyanidin mixture significantly reduced the plasma concentrations of TG and increased the fecal excretion of bile salts and cholesterol, compared to the control group. Through an in vitro study with procyanidin B2, B5, C1, and A2, it was determined that a possible mechanism to explain the previous results is a decrease in the solubility of cholesterol in the micelles that allow intestinal absorption [50, 51].

In a recent in vitro study by Gu et al., the inhibitory effects of cocoa extracts and monomeric, oligomeric, and polymeric flavanols on the activity of pancreatic lipase and phospholipase A2 were evaluated. The results showed that the different extracts had inhibitory effects on the activity of both enzymes and that said effects are proportional to the total polyphenol content and the degree of polymerization of the flavanols [52].

In the liver, alterations in lipid metabolism promote an increase in its fatty infiltration and lipotoxicity that culminate in the development of nonalcoholic steatohepatitis (NASH), one of the most severe comorbidities of the metabolic syndrome [40, 54]. In an experimental study with rats, the preventive or palliative effects that cocoa supplementation could have on the development of NASH induced by a diet high in fat and deficient in choline were evaluated. The results showed that the supplementation with cocoa reduces the degree of steatosis, liver fibrosis, and portal inflammation. In this same study, it was observed that the supplementation with cocoa reduced the accumulation of fat in the liver, due to an increase in the levels of gene and protein expression of the fatty acid-binding protein (LFABP) in the hepatocytes of rats with NASH.

Another flavonoid that has positive effects in obesity is morin, suppressing lipogenesis, gluconeogenesis, inflammation, and oxidative stress, tending to modify the concentration of triacylglycerides in the liver.

A preclinical study showed that morin acts as an inhibitor of fatty acid synthase (FAS) by regulating the SREBP1-c protein binding element, in addition to regulating the liver increase of carnitine palmitoyl transferase 1a (CPT1a) [51, 52].

It is important to mention that morin could interact with various receptors involved in metabolic diseases as well as ligand of altered genes in obesity and therefore in the present inflammation. However, the mechanism of action of the flavonoid is still unknown since there are no major reports of research related to the mechanism and effect of it.

2.2.4.1 Effect of flavonoids of marine algae on obesity

The content of flavonoids in *Undaria pinnatifida* is equivalent to 42% of the total phenols, and several studies have shown that the main phenolic compounds

and flavonoids contained in this alga are rutin, caffeic acid, catechol, quercetin, and morin with approximately 3.1 mg/g of sample. Although there are not many reports regarding the content of flavonoids in Undaria, in other brown algae, the content varies from 0.9 to 6.3 mg/g of sample [53].

2.2.4.2 Effects of flavonoids on body fat

The content of epididymal adipose tissue (ATe), retroperitoneal (ATr), mesenteric (ATm), and total (%) is modified by the intake of phenols from marine algae, such that in the standard group with 5.10% adipose tissue, and the group that was given a high-fat diet, 11.33% was reached, in contrast to the group that consumed free phenols, the content was reduced to 8.9%.

The effect of the phenols was the reduction of the concentrations of triacylglycerols in 57% with respect to the group fed with a high-fat diet; only 10% above the group was fed a normal diet. In relation to changes in total cholesterol levels, phenols decreased it by 75% with respect to the group with a high-fat diet, remaining only above the group with a normal diet by 20% [58].

2.2.4.3 Clinical studies on the effect of flavonoids

Recent studies have shown the importance of the intake of flavonoids and their relation to the risk of chronic diseases, where the intake of flavonoids and obesity were inversely associated in both men and women using multivariate models in a study in the USA. Adults in the highest quartile of flavonoid intake had a significantly lower body mass index and waist circumference than those in the lowest quartile of flavonoid intake ($P < 0.03$ and $P < 0.04$, respectively); and the ingestion of flavonoids was inversely related to the levels of C-reactive protein in women (trend p, 0.01). These findings support a growing evidence that the consumption of flavonoids may be beneficial for the prevention of diseases [57].

Scientific evidence has been found in clinical studies of the effect of flavonoids present in fruits that can be consumed regularly and be part of the diet, suggesting a beneficial effect for health, as is the case of anthocyanins, punicalagin, and ellagic acid, present in the pomegranate fruit.

The effect of flavonoids present in pomegranate juice on the function of adipocytes has been studied. Using increasing doses of juice and by radiometric methods, the activity of the amino oxidase was determined, and with colorimetric methods the influence of the juice on the lipogenic and lipolytic activities of the human adipose tissue was evaluated. The results showed a dose-dependent response of juice to inhibit the monoamine oxidase and the activity of the amino oxidases present in the human adipose tissue sensitive to semicarbazide. The juice also inhibits lipogenesis and lipolysis in human and mouse adipose cells [58].

Oral supplementation with pomegranate extract on biomarkers of inflammation and oxidative stress in plasma, as well as serum metabolic profiles in overweight and obese people, for 30 days, resulted in a significant decrease in serum glucose, insulin, and blood levels, total cholesterol, concentration of low density lipoproteins LDL-c, MDA and IL-6. It is concluded that the consumption of pomegranate extract can reduce complications related to obesity [59].

The functionality of flavonoids in various diseases has been demonstrated, and these have been part of our diet all the time, since they are found abundantly in fruits, vegetables, and grains that we consume, such as apples, grapes, blueberries, pomegranates, oranges, broccoli, spinach, thyme, cocoa, nuts, and soybeans, to name a few.

However, the concern that the benefits of these compounds have aroused in the food industry is wide, since, from these natural sources, products containing

flavonoids are manufactured, enriching, fortifying, or increasing the concentration of flavonoids present in various products to have a positive effect on health. In the market there are various products rich in flavonoids such as fruit and vegetable juices, wines, cereals, milk formulas, soy milk, almond milk, confectionery, rice drinks, relaxing drinks, food supplements, and capsules containing extracts of flavonoids. The development of new functional products is increased due to the need to contribute to the health welfare.

3. Conclusions

Although there is sufficient evidence on the beneficial effect of flavonoids in relation to the improvement in health status in metabolic diseases such as obesity, where flavonoids contribute to recover the lost balance due to the deregulation of lipogenesis and lipolysis, much remains to be done to clarify aspects such as the adequate concentrations suitable for use in drug design, the interactions between the different compounds present, as well as the modifications in the absorption of them depending on the changes in the health states. It is also necessary to deepen into the reaction mechanisms involved for a better management of these compounds according to each pathology.

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Conflict of interest

There is no conflict of interest to declare.

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Chapter 7

Natural vs Synthetic Colors

Luis Alberto Cira Chávez, Raquel García-Barrientos, Laura Elisa Gassos Ortega, Octavio Dublan Garcia and Maria Isabel Estrada Alvarado

Abstract

Anthocyanins are the most important group of water-soluble compounds responsible for the red, purple, and blue colors seen in flowers, fruits, and other parts of the plant. For centuries, these compounds have been consumed by man without obvious detrimental effects due to their bright colors and anti-inflammatory and antioxidant properties. Anthocyanins are an important alternative for synthetic food colorings that have been banned in foods, because they have been associated with certain diseases. Anthocyanins can be extracted from different plant tissues; the usual method of obtaining is solid-liquid extraction. However, it is worth mentioning the existence of other methods. Thus, Japanese scientists developed an alternative methodology that consists of extracting anthocyanins by fermenting the matrices that contain them. The stability of anthocyanins in processed products has been studied, and it has been shown that certain acid anthocyanins are stable after extraction. Anthocyanins are antioxidants that play an important role in reducing the risks of several human degenerative diseases.

Keywords: anthocyanins, antioxidants, natural colors

1. Natural versus synthetic colors

Anthocyanins are the most important group of water-soluble compounds responsible for the colors red, purple, and blue that appear in flowers, fruits, and other plant tissues. For centuries, these compounds have been ingested by humans due to their bright colors, anti-inflammatory, and antioxidant properties, without any evident harmful effects. Anthocyanins are a good potential replacement for synthetic food colorings, particularly for those that have been banned because of their association with disease.

Anthocyanins are extracted from vegetable tissues, and the most common method is liquid-liquid extraction. However, other methods are also available, such as an alternative methodology developed by Japanese scientists who extracted these compounds by fermenting vegetable matrices.

The stability of anthocyanins in processed products has also been a topic of research, and it has been shown that certain acid anthocyanins are highly stable after the extraction. Since these compounds are antioxidants, they play an important role in reducing the risk of several human degenerative diseases.

2. What are anthocyanins?

Anthocyanins belong to a class of substances known as flavonoids, one of the largest categories of phenolic compounds. The basic structure of anthocyanins is made up of a flavylium cation (C6-C3-C6), which may be attached to different sugars, as well as to hydroxyl and methoxy groups, resulting in over 635 different anthocyanins identified to date. The most common sugar associated to anthocyanins is glucose, although rhamnose, xylose, galactose, arabinose, and rutinose have also been found as part of these molecules [1]. Anthocyanins may be mono-, di-, or tri-glycosides, depending on the number of sugar molecules they contain. Durst and Wrolstad [2] reported that anthocyanins are glycoside groups that belong to the family of flavonoids; their structure contains two aromatic rings A and B, joined by a three-carbon link (**Figure 1**). The structural variations that occur in ring B result in six different anthocyanins as shown in **Table 1**.

Anthocyanins are the most important natural pigments, soluble in water, that give colors red, purple, and blue to flowers, fruits, and other parts of the plant. Besides coloring, these pigments play other roles in plants, such as attracting pollinizers in order to disperse pollen and seeds, as well as protecting the tissue against UV radiation and harmful virus and bacteria. Given the above, the scientific interest on anthocyanin pigments has increased in the past few years, particularly on their role in the reduction of heart disease, cancer, diabetes, anti-inflammatory effects, and improvement of visual acuity [3]. For centuries, these compounds have been a part of the human diet due to their attractive bright colors, anti-inflammatory, and antioxidant properties, without any evident harmful side effects. Anthocyanins are regarded as a potential alternative in the replacement of artificial food colorings, some of which have been associated to certain diseases. Several sources of anthocyanins have been studied in order to find acidified anthocyanins with greater stability at different pH conditions, at an affordable cost. Stability is a relevant factor since the color of these compounds is easily affected by several conditions, mainly pH [4]. Predominant structures of anthocyanins at different pH values are shown in **Figure 2**.

The color of anthocyanins depends on the number and orientation of hydroxyl and methoxy groups. Increases in hydroxylation produce color changes toward the blue side of the color spectrum, while increases in methoxylation produce red colorations [3].

The color changes in anthocyanins given by variations of pH are due to the glycoside substitutions (mono-, di-, or tri-saccharides) in positions 3 and/or 5 of the B ring (**Figure 1**), and this also helps to increase solubility. Some examples of glycosylated saccharides are glucose, galactose, xylose, arabinose, rutinose, sambubiose, and

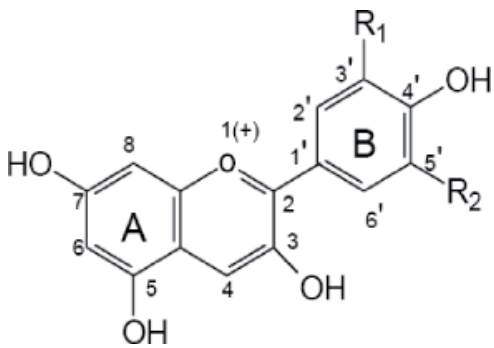

Figure 1.
Structure of anthocyanins [2].

Aglycone	Substitution R1	Substitution R2	Absorbance (nm) visible spectrum
Pelargonidin	H	H	494 (orange)
Cyanidin	OH	H	506 (orange-red)
Delphinidin	OH	OH	508 (blue-red)
Peonidin	OCH_3	H	506 (orange-red)
Petunidin	OCH_3	OH	508 (blue-red)
Malvidin	OCH_3	OCH_3	510 (blue-red)

Table 1.
Substituents of the six types of anthocyanins.

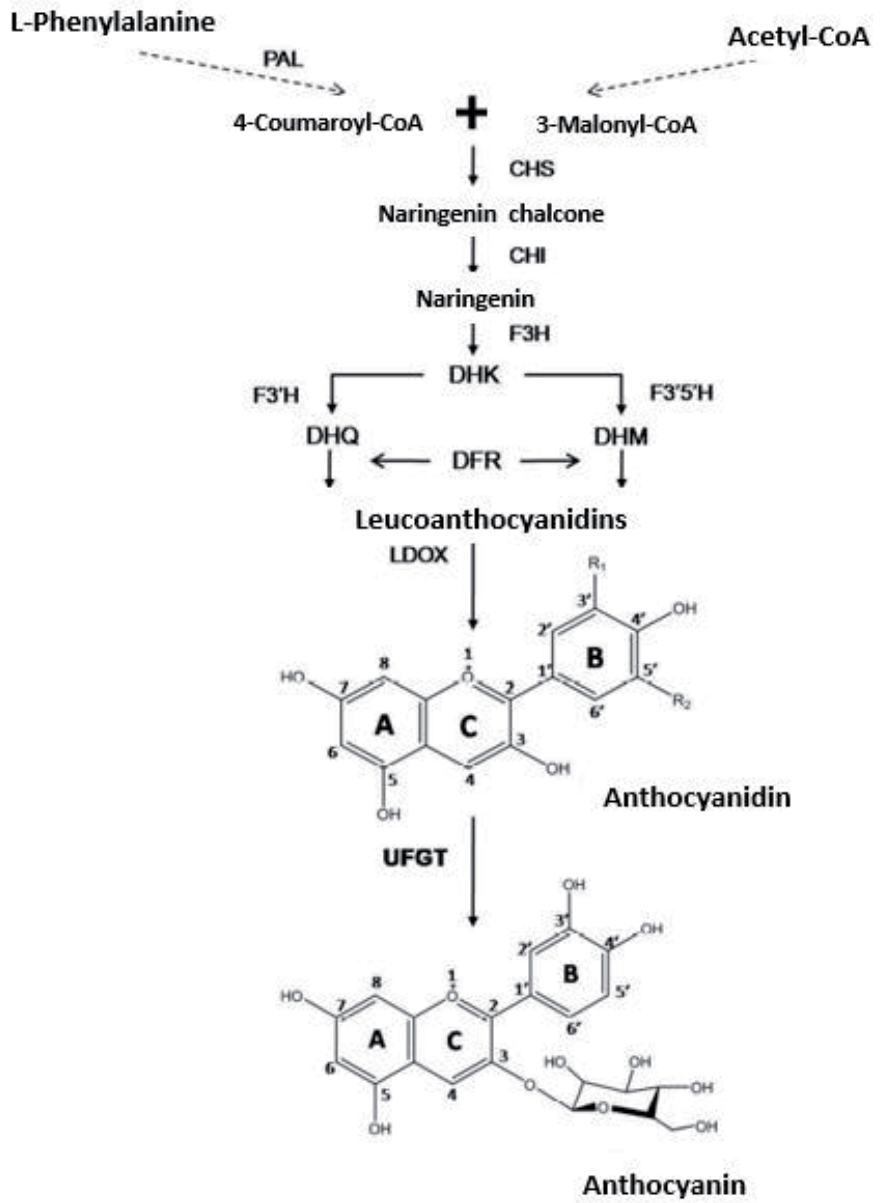

Figure 2.
Anthocyanins biosynthesis pathway [5].

gentiobiose. Another cause of the color displacement toward purple in the molecule is the aromatic acylations in the position 5 of carbon B in the structure [6].

Figure 2 shows the biosynthesis of anthocyanins as established experimentally, where ring A is synthesized via the malonic acid pathway, by condensing three molecules of the malonyl-CoA. On the other hand, ring B is synthesized via the shikimic acid pathway. The enzyme phenylalanine ammonia lyase (PAL) reacts with phenylalanine, which converts into *p*-coumaric acid by the loss of NH_3. Afterward, a condensation reaction of three molecules of malonyl-CoA results in an intermediate 15-carbon compound, which is transformed into a flavanone. Then, the flavanone is converted into an anthocyanin by the hydroxylation of carbon 3 and the subsequent dehydration. Finally, the molecule is stabilized by glycosylations of the heterocycle, and the reaction is catalyzed by the glycosyl transferase enzyme and then by methylation reactions followed by acylations [7].

The use of natural anthocyanin pigments as food colorings in processed products is getting increasing attention, since they are very attractive to consumers, while having beneficial health effects. Anthocyanin pigments are permitted as natural food colors in the United States under the fruit (21 CFR 73.250) and vegetable categories (21 CFR 73.260) [8].

3. Anthocyanin sources

Hibiscus flower (*Hibiscus sabdariffa* L.) is a source of vitamins C and E, polyphenolic acids, anthocyanins, and flavonoids, all of which are known to have antioxidant activity, as they are capable of reducing free radicals [9].

Different varieties of *Hibiscus sabdariffa* L. are available in Mexico, and each one is characterized by its anatomy, color, and physicochemical properties. The content of active compounds varies according to the chalice, as well as to the extraction method. These pigments may be an alternative to the industry of colorings, cosmetology, and processed food, while providing extra health benefits [10].

Nowadays, the food and cosmetic industries demand an ample variety of additives and colorings, in order to improve the appearance of a product and make it attractive to consumers. Industrial food colorings are found in many products that we use or buy on a daily basis, such as juice, jellies, pastries, soft drinks, paints, cosmetics, and more. Most of these additives are synthetically produced and may cause adverse health consequences: allergic reactions, digestive problems, cancer, and asthma, among others [11].

Natural food colors are found in fruits such as acai, cherries, cranberries, elderberries, raspberries, blueberries, black and blue grapes, plums, strawberries, figs, pomegranate, and red apple [12]. Other important sources are found in vegetables: beets, purple lettuce, green onion, radish, purple cabbage, red bell peppers, eggplant, as well as cereals such as blue corn (*Zea mays* L.) [13], blue wheat [14], and rice [15]. Recently, other plants and flowers have been studied as potential sources of antioxidants [16, 17], such as elder (*Sambucus nigra*) [18], perilla fruit from Japan (*Perilla frutescens*) [19], and flower petals: iris (*Iris dichotoma*, *Iris domestica*) from China [20], Damascus rose (*Rosa damascena*) [21], cyani flower (*Centaurea cyanus*) [22], dahlia (*Dahlia mignon*) [23], and viola (*Viola tricolor*) [16].

4. Anthocyanins as natural colorings

The growing concern about the use of synthetic colorings in processed foods, cosmetics, and pharmaceuticals is caused by their potential harmful effects. Countries like Australia, Japan, Norway, and Switzerland have banned the use of

some synthetic colorings, such as Red No. 20 and 40, since they have been related to hyperactivity in children of school age. This effect may be considered as an acute neuronal illness; however, these food additives are still being used in the United States [24].

Regulatory policies dealing with the use of colorings derived from anthocyanins vary from country to country. The United States is the most restrictive country on the use of anthocyanins as natural colorings, where four out of the 26 colorings that are approved for their use in foods are derived from grape peel, vegetable, and fruit juice [25]. In Mexico, there is no regulator policy for natural colorings at this point.

In the European Union, Chile, Colombia, Iran, Israel, South Korea, Malta, Peru, Saudi Arabia, and the Emirates, all colorings derived from anthocyanins are regarded as natural [26].

5. Functional properties of anthocyanins

The stability of anthocyanins in processed products has been studied, demonstrating that some acid anthocyanins are stable after extraction. These compounds have antioxidant properties and play an important role in reducing the risk of developing several human degenerative diseases [27].

The interest in anthocyanin pigments is not only due to a potential replacement of artificial food colorings, but is also due to their pharmacological and therapeutic properties. **Table 2** shows different investigations on the biological properties of anthocyanins from several substrates.

Biological property	Studies	Authors
Therapeutic	Reduction of coronary heart disease, anticarcinogenic effects, antitumor, anti-inflammatory, and antidiabetic	Miyazawa et al. [28]
Antioxidant activity	Stabilization of oxygen reactive species, inhibition of lipoprotein oxidation, and platelet aggregation (wine anthocyanins)	Ghiselli et al. [29]
Antioxidant activity	Anthocyanin-rich foods show a high antioxidant activity against hydrogen peroxide (H_2O_2), peroxide radicals (ROO), superoxide (O_2), hydroxyl (OH), and singlet oxygen (O_2)	Wang and Jiao [30]
Antitumor and anticarcinogenic activities	Sweet purple potatoes and blue cabbage were fed to lab rats causing tumor suppression	Hagiwara et al. [31]
Antitumor effects	Soy red bean extract, containing cyanidin conjugated with glucose and rhamnose, was fed to rats	Koide et al. [32]
Anticarcinogenic activity	Fractions of red wine anthocyanins suppressed cancer HCT-15 cells from human colon and carcinogenic gastric AGS cells.	Kamei et al. [33]
Anticarcinogenic activity	Essays demonstrate that cranberries inhibit the initiation, promotion, and progression stages of carcinogenesis	Chang et al. [34]
Anti-inflammatory activity	Concentrated anthocyanin extracts showed inhibitory effect in the production of nitrous oxide in activated macrophages	Wang and Mazza [35]
Anti-inflammatory activity	Anthocyanin extracts from raspberry inhibited EG2 prostaglandin, a synonym of anti-inflammatory activity	Vuorela et al. [36]

Table 2.
Functional properties attributed to anthocyanins.

6. Anthocyanin extraction methods

Anthocyanins may be extracted from different vegetable tissues, the most common method being the solid-liquid extraction. However, novel methods have been developed, such as the methodology developed by Japanese researchers where anthocyanins are extracted after fermenting the vegetable matrices that contain them [37].

Nowadays, anthocyanin extracts are usually applied without separating the individual components, since all the compounds have shown antioxidant activity, not only those with color [38].

On the other hand, the polar nature of the anthocyanin molecule permits solubility in a variety of solvents, such as alcohols, acetone, and water. However, their stability is easily affected by structural modifications by hydroxyl and methoxy groups, glycosides, and particularly acyl groups, as well as by environmental factors such as temperature and light [25].

Amongst those technologies, those currently available for anthocyanin extraction are the use of polar organic solvents, such as ethanol and methanol, and sometimes acidified media. In many cases, the solvents or chemical synthesis involved in the extraction are derived from petroleum, which leaves a strong carbon print on the environment [39].

Extraction of natural colorings by organic solvents has been the method of choice for decades. The toxicity of these solvents complicates the marketing of the final product due to their toxicity and environmental concerns. Many technologies for exploitation of agro-industrial residues have been developed out of the need of solving the problem of accumulation of solid organic residues. Green and clean technologies focus on lessening the environmental impact, while helping the processing and marketing of the final products [40].

Novel methods for the extraction of anthocyanins are ultrasound-assisted extraction [41] and extraction using supercritical fluid CO_2 [42].

A viable method for anthocyanin extraction is the use of hydrolytic enzymes, which accelerate the reaction at which a substance is broken down into simpler components when reacting with water. This is the case of cellulase and pectinase that hydrolyze cellulose and pectin, respectively; both are found in the cell wall of fruits and vegetables [43].

A response surface methodology based on the Box-Behnken design may also be used to optimize an extraction method [44]. Identification and quantification of anthocyanins are based on the use of chromatographic methods, mainly the HPLC and UHPLC liquid analyses; mass spectrophotometry is also very helpful for the identification of individual compounds [44].

7. Antioxidant capacity highlights

Antioxidants are molecules that inhibit or delay the oxidation in two ways: by trapping free radicals, in which case they are known as primary antioxidants (phenolic compounds) and are destroyed in the induction process, or by mechanisms such as chelation with heavy metals, capture of oxygen, conversion of hyperoxides into nonradical species, absorption of UV radiation, or inactivation of singlet oxygen; substances exhibiting these properties are known as secondary antioxidants [45].

Antioxidant activity is defined as the capacity of one or several compounds within a substance to inhibit oxidative degradation of another compound, acting mainly on free radicals [46].

8. Free radicals

Most of the chemical compounds of biological relevance are made by atoms joined together by covalent bonds, where two different atoms share a pair of electrons in the same orbital, and each electron rotates in the opposite direction to its pair. In the cells, chemical reactions that break these bonds heterolytically take place continuously, making one of the parts take two electros and generating unstable nucleophilic or electrophilic compounds, known as anions and cations. However, some chemical reactions, electromagnetic radiation, and other factors may break bonds homolytically, resulting in two parts that have one electron each; these are known as free radicals [47].

Generally speaking, a free radical is an atom or molecule that has one or more unpaired electrons in the external orbitals and is capable of existing independently. It is very reactive and tends to reduce in order to stabilize, which means that subtracts an electron from stable atoms or molecules that are in turn oxidized. Once the free radical has obtained its missing electron, the stable molecule is oxidized and is left with an unpaired electron, which makes it a new free radical that initiates a chain reaction [6].

9. Mechanisms of antioxidant defense

Figure 3 is a summary of the oxidation mechanisms of a cell, as well as the action that the antioxidant exerts to prevent oxidation. Cell respiration is shown, where molecular oxygen is converted into a superoxide anion, followed by hydrogen peroxide, then a hydroxyl radical and finally water, while the central illustration explains how cell metabolism may form free radicals (superoxide anion and hydroxyl) [48].

According to Londoño [46], when the superoxide anion suffers a mutation catalyzed by the superoxide dismutase enzyme, it becomes less reactive but is still

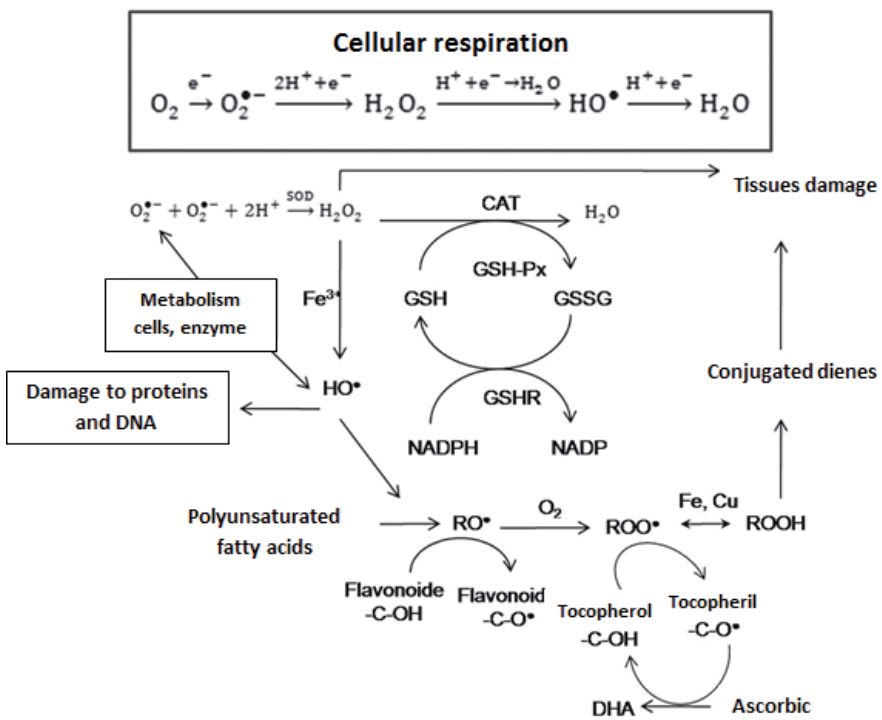

Figure 3.
Pathways of free radical production and action of antioxidants [46].

toxic for tissues; therefore, it is converted into water by the action of the catalase enzymes, which reduce hydrogen peroxide by oxidizing glutathione; this in turn is generated by the action of glutathione reductase, which uses NADPH as a cofactor. Hydrogen peroxide may also be converted into a hydroxyl radical via a Fenton-type reaction, which is catalyzed by iron. Once produced, the hydroxyl radical attacks proteins, nucleic acids, and mainly polyunsaturated fatty acids, thus generating lipid radicals that quickly react with oxygen to produce peroxyl radicals.

Stabilization of free radicals derived from lipids may be accomplished by phenolic antioxidants such as flavonoids and tocopherols, which stabilize the free radicals (phenoxyl and tocopheryl, respectively). Stabilization may happen inside the molecule by displacement or by reaction with ascorbic acid to generate a reduced compound [49].

10. Safety and toxicology of anthocyanins

Consumption of anthocyanins is generally recognized as safe for humans, since they have been a part of our diets for generations, and so far, no harmful effects have been reported. This may be associated with their low absorption and bioavailability. Nevertheless, the use of nutritional supplements based on anthocyanins is a growing trend among consumers, and this has raised some concern because the doses recommended by manufacturers are generally much higher than that given by natural foods. Furthermore, no regulation is available for such dietetic supplements in the United States, among other countries, which may result in fraudulent/adulterated products. It is also likely that people looking to benefit from anthocyanins are also using other supplements or pharmaceuticals. Anthocyanins are treated as xenobiotics [50] and, therefore, are able to modulate biochemical activities or compete for several enzymes that metabolize or transport medications [51]. This increases the risk for potential adverse effects and toxicity due to interactions with pharmaceuticals. However, so far, no reports have demonstrated adverse effects on anthocyanins in levels associated to a healthy diet.

11. Perspectives of inclusion of anthocyanins in processed foods

Several studies show the possibility of replacing artificial food colorings for anthocyanins, such as those derived from flowers. When mixed into dairy matrices such as yogurt, there are some improvements in the production and final product [17]. Furthermore, the addition of anthocyanins is not only recommended for their color and bioactivity, since recent studies propose their use during processing and/or storage of the final product by their inclusion into intelligent films based on biodegradable polymers, which work as biosensors due to their high sensitivity to pH changes. In this manner, freshness of meat and fish may be monitored [52, 53]. Both studies used *Hibiscus* flower extract due to its low cost. Other researchers have studied anthocyanins from purple cabbage (*Brassica oleracea*) as temperature indicator when incorporated into chitosan films; this study also suggests its application in the production of smart food packages [54].

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Flavonoids with over 6000 natural colorful compounds are a unique class of phytonutrients found in almost all vegetables, fruits, and herbs. This book discusses the nature and role of these compounds by studying the molecular mechanism of flavonoids using spectroscopy and computational tools. The book also addresses the characteristics of natural vs. synthetic colors from both chemical and biological points of view. More importantly, a lengthy chapter explains in full detail the usefulness of these natural coloring properties to provide a safe, efficient, and economic therapy and/or prophylaxis of many health problems, e.g. obesity and cardiovascular disorders. This book poses a balance between developments in scientific research and the idea that researchers must be able to absorb and link scientific advances with clinical practice so that the management of diseases can be based on sound physiological concepts.

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