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Phenolic Compounds

Biological Activity

Edited by Marcos Soto-Hernandez,
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PHENOLIC COMPOUNDS - BIOLOGICAL ACTIVITY

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Contributors

Alice Grigore, Tatsuro Egawa, Satoshi Tsuda, Katsumasa Goto, Tatsuya Hayashi, Rieko Oshima, Xiao Ma, Ayumi Goto, Ruben San Miguel, Castillo Juárez, García Contreras Rodolfo, Pérez López Macrina, Muñoz Cazares Naybi, Israel Castillo Juárez, Giuseppina Lima, Cristine Vanz Borges, Igor Otavio Minatel, Maria Izabela Ferreira, Hector Gomez-Gomez, Chung-Yen Oliver Chen, Jonathan Delgado-Adámez, Sara Martillanes, Javier Rocha-Pimienta, Manuel Cabrera-Bañegil, Daniel Martín, Anabela Romano, Sandra Gonçalves, Basli Abdelkader, Belkacem Nacim, Amrani Iman, Anthony Ananga, James Obuya, Joel W. Ochieng, Violetka Tsolova, Manuela Ciocoiu, Laurentiu Badescu, Magda Badescu, Andreea Arsene, Daniela Elena Popa, CRISTINA MANUELA DRĂGOI, Ion Bogdan Dumitrescu, Alina Crenguta Nicolae, George Traian Alexandru Burcea Dragomiroiu, Bruno Ștefan Velescu

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Meet the editors

Dr. Marcos Soto-Hernández is a pharmacist from the National University of México and completed his PhD degree at the University of Wales, Cardiff, UK, and now is a full professor at the Colegio de Postgraduados where he conducts research in phytochemistry and bioactivity natural products. He has established collaboration with research groups in the UK, the Netherlands, and Spain and other groups in México; he has received several awards locally and abroad. At present, his main line of research is related with bioguided isolation of secondary metabolites with its importance in medicine and agriculture and the potential of the local aromatic plants is part of his recent research.

Dr. Mariana Palma-Tenango is an engineer agronomist from the Universidad Autónoma Chapingo; holds a PhD degree in Plant Physiology from the Colegio de Postgraduados, México; and has teaching duties in the National Autonomous University of Mexico. She is an assistant professor of Phytochemistry at the Colegio de Postgraduados, and she is a reviewer of several journals and an editor. She has participated in the organization of meeting and symposiums in México and is a supervisor of master and PhD degree students. Her research line is phytochemistry, medicinal, and aromatic plants.

Dr. María del Rosario García-Mateos is a chemist of the University of Puebla, México, and holds a PhD degree from the Colegio de Postgraduados. Her research line is related with the nutraceutical properties and biological activity of horticultural crops. She has supervised master and PhD thesis, has published more than 71 papers, and has been a reviewer of several scientific journals. She has established collaboration with many groups national and international.

Contents

Preface

Phenolic compounds comprise a broad class of natural products formed mainly by plants, but also microorganisms and marine organisms that have the capacity to form them. They are one of the main classes of compounds formed in plants as part of their response to biotic or abiotic factors, and their structure varies from compounds of low molecular weight as phenolic acids, phenylpropanoids, or stilbenes to those of higher molecular weight, for example, the polyphenols known as tannins.

Nowadays the interest in these compounds has increased mainly due to their diverse chemical structure and wide biological activity valuable in the prevention of some chronic or degenerative diseases. The functional foods are a rich source of these phytochemicals, and this is the starting point for this book which shows the state of the art of the phenolic compounds and their biological activity.

This book integrates eleven chapters that show diverse biological activity of the phenolic compounds present in some crops or fruits from the review of the recent developments and future opportunities of grape seed nutraceutical for the prevention of human diseases or their novel antivirulence properties until other chapters that show the recent experimental evidences linking phenolic acids to glucose metabolism and the upstream signaling events in the skeletal muscle. Another chapter of the book shows a review of the state of the art of phenolic antioxidant capacity, important to consider the potential of the phenolic compounds as antioxidant agents. Also their immunomodulatory or anticancer properties are described in detail in this book.

We hope that the information will be useful and allow the interaction with diverse areas of knowledge related with these compounds.

Dr. Marcos Soto-Hernández
Department of Botany
Postgraduate College
Texcoco, México

Dr. Mariana Palma-Tenango
Science Faculty
National Autonomous University of Mexico
Mexico City, México

Dr. María del Rosario García-Mateos
Department of Plant Science
Institute of Horticulture
Chapingo Autonomous University
Texcoco, México

Chapter 1

Phenolic Compounds: Functional Properties, Impact of Processing and Bioavailability

Igor Otavio Minatel, Cristine Vanz Borges,
Maria Izabela Ferreira,
Hector Alonzo Gomez Gomez,
Chung-Yen Oliver Chen and
Giuseppina Pace Pereira Lima

Additional information is available at the end of the chapter

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Abstract

In this chapter, we discuss the influence of the processing methods on the content of phenolic compounds in fruits and vegetables. The intake of fruits and vegetables based-foods are associated with delayed aging and a decreased risk of chronic disease development. Fruits and vegetables can be consumed *in natura*, but the highest amounts are ingested after some processing methods, such as cooking procedures or sanitizing methods. These methods are directly methods are directly related to alteration on the phenolic content. In addition, the postharvest conditions may modify several phytochemical substances. Phenolic compounds are referred to as phytochemicals found in a large number of foods and beverages. The relative high diversity of these molecules produced by plants must be taken into account when methods of preparation are employed to obtain industrial or homemade products. Phenolic compounds comprise one (phenolic acids) or more (polyphenols) aromatic rings with attached hydroxyl groups in their structures. Their antioxidant capacities are related to these hydroxyl groups and phenolic rings. Despite the antioxidant activity, they have many other beneficial effects on human health. However, before attributing health benefits to these compounds, absorption, distribution, and metabolism of each phenolic compound in the body are important points that should be considered.

Keywords: cooking procedures, sanitizing methods, postharvest, cultivating conditions, health benefits

1. Introduction

Most of the vegetables and some fruits are preferably consumed after some kind of processing, which can cause favorable or disadvantageous changes in the flavor and texture, increasing the food's palatability and affecting the quantity and quality of bioactive compounds, such as phenolics. The biological, physical, and chemical modifications that occur during some processing methods, as the cooking, are predominantly related with sensorial, nutritional, and textural alterations, which may be beneficial or harmful to the human health. A high temperature can inactivate microorganisms, decreases anti-nutritional factors, increases the digestibility of foods, and modifies the bioavailability of the phenolics. In contrast, the thermal processing may have negative effects on these bioactive compounds. Furthermore, other processing methods have been adopted for fruits and vegetables, whether for domestic consumption or in the food industry, for example, fresh-cut, drying, blanching, pasteurization, use of electric fields and membranes, among others. In this chapter, we will address the influence of some processing methods in plant-based food based on the phenolic content, as well as on their bioavailability.

2. Functional properties of phenolic compounds

Phenolic compounds are a main class of secondary metabolites in plants and are divided into phenolic acids and polyphenols. These compounds are found combined with mono- and polysaccharides, linked to one or more phenolic group, or can occur as derivatives, such as ester or methyl esters [1]. Among the several classes of phenolic compounds, the phenolic acids, flavonoids, and tannins are regarded as the main dietary phenolic compounds [2]. Many studies have shown a strong and positive correlation ($p \leq 0.05$) between the phenolic compound contents and the antioxidant potential of fruits and vegetables [3–5]. This antioxidant mechanism, present in the plants, has an important role in the reduction of lipid oxidation in (plant and animal) tissues, because when incorporated in the human diet, not only it conserves the quality of the food, but it also reduces the risk of developing some diseases. Studies have shown that a diet rich in fruits and vegetables contributes to the delay of the aging process and to the decrease of the inflammation and oxidative stress risk, related with chronic diseases (e.g., cardiovascular diseases, arteriosclerosis, cancer, diabetes, cataract, disorders of the cognitive function, and neurological diseases) [6–8].

The antioxidant activity of phenolic compounds is attributed to the capacity of scavenging free radicals, donating hydrogen atoms, electrons, or chelate metal cations [9]. Molecular structures, particularly the number and positions of the hydroxyl groups, and the nature of substitutions on the aromatic rings, confers to phenolic compounds the capacity of inactivating free radicals, which is referred to as structure-activity relationship (SAR). The hydrogen atoms of the adjacent hydroxyl groups (*o*-diphenol), located in various positions of the rings A, B and C, the double bonds of the benzene ring, and the double bond of the oxo functional group (-C=O) of some flavonoids, provide these compounds their high antioxidant activity (**Figure 1**). This characteristic can be observed in quercetin and catechin. Both compounds

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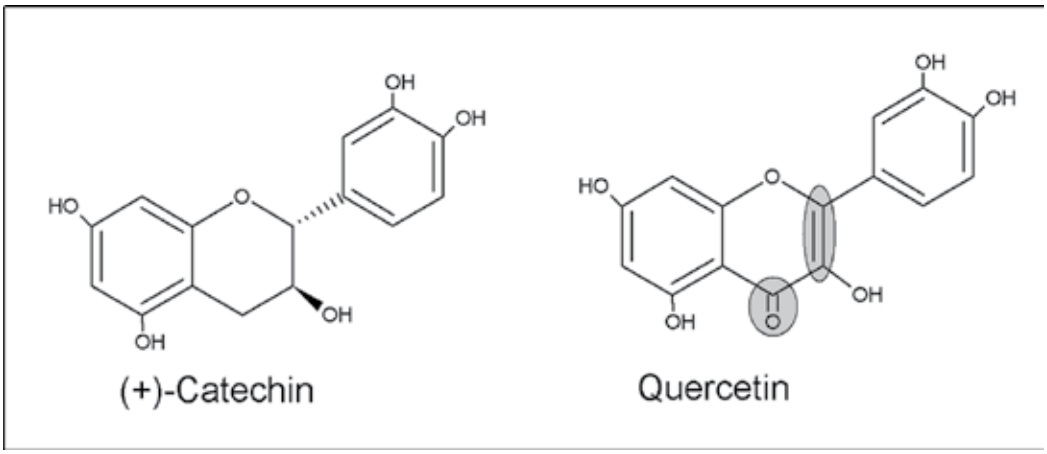

Figure 1. Double bonds of the benzene rings and the oxo function (gray background).

share a similar number of hydroxyl groups, at the same positions, however, quercetin also contains a 2,3-double bond in the C ring and the 4-oxo function [10]. The advantage of this structure is an enhancement of the TEAC (Trolox equivalent antioxidant capacity) value, when compared to the saturated heterocyclic ring of catechin with approximately half the antioxidant activity.

Even though there are innumerous studies comparing the biological actions and *in vitro* antioxidant activity of phenolics, and the function of its content in vegetables and consequently in human, there is no consensus about the best way of preparing/consuming fruits and vegetables intending preservation or to increase their antioxidant activity.

3. Processing methods and their impact on phenolic compounds

The functionality and stability of the phytochemical compounds in the human body depends, not only on the quantity, but also on the bond and/or interaction of these compounds with other molecules, on the location in the food matrix, and in the presence of other bioactive compounds. In plants, the phenolics can be found linked to the cell membranes/walls or can be free, and the food processing methods such as the use of high temperatures or freezing, can cause the release of these compounds, which is implied by an increase of its bioavailability in the human body. Some reports show that heating affects the content of some polyphenols, including flavonoids, due to the extractability alteration by the rupture of the cell wall. In this way, polyphenols linked to the wall could be released more easily on cooking than from the raw material [11]. Other authors confirm the release of these compounds by heat treatment, as described in sweet corn [12] and in citrus peel [13], due to breakdown of the matrix.

Domestic or industrial food preparation include a variety of processes, such as preparation (peeling, washing, and chopping), boiling, frying, and baking (traditional oven or microwave), among others [14]. Several research studies have shown increase in the phenolic compound

levels, as well as in the antioxidant activity after cooking [15–17] or after other processes, while other studies related the thermal processing with the decrease of phenolic contents [18, 19]. Changes in the phenolic composition are of great complexity, because they vary according to their structure, to the analyzed food and, mainly, according to cooking method used in the preparation. There are indications that the retention of phytochemicals and the antioxidant properties after the cooking vary considerably between the different vegetables and methods used in their preparation [20–22]. Initially, it was believed that the thermal processing applied to several foods was prejudicial regarding the retention of the nutrients (e.g., antioxidants). However, the nutritional and bioavailability increased, and a higher antioxidant activity was observed in vegetables and/or fruits that went through thermal processing [22]. The heating may disrupt the cell membrane, leading to the release of membrane-bound phytochemicals, what may result in an increase in bioavailability Nevertheless, we will discuss forward that these compounds are not always increased. Many times, they are decreased and other times, the thermal processing does not affect the phenolic content.

Most of the studies show that the boiling may result in a decrease of the total phenolic compound content, while steam techniques and stir frying may promote an increase of these compounds. This may be explained by the fact that phenolics are highly soluble in water [23, 24] and, in the boiling process, they may be lost by leaching. However, heat treatment may soften the vegetal tissues and facilitate the extraction from the cellular matrix [25]. The rupture of the cell wall and of the other subcellular compartments, during the boiling, facilitates the migration of cellular components with the consequent release of these molecules into the boiling water.

Variations in the phenolic compounds may occur influenced by the food matrix used, as the verified reduction in the phenolic compounds content in broccoli (*Brassica oleraceae* L. cv. *gemmifera*) when submitted to thermal processing in water [26]. In tomatoes, the cooking by boiling, baking, and frying induced significant reductions ($p < 0.01$) in the total phenolic content [27]. Phenolic losses upon boiling or blanching were observed in several cruciferous vegetables, as kale (*Brassica oleracea* var. Acephala, cv. Winterbor), broccoli (*Brassica oleracea* var. botrytis italica, cv. Sebastian), brussels sprouts (*Brassica oleracea* L. var. gemmifera, cv. Maczuga), cauliflower (*Brassica oleracea* var. botrytis), white cauliflower (cv. Rober), green cauliflower (cv. Amphora) [28, 29], as well as spinach (*Amaranthus sp.*), cabbage (*Brassica oleraceae*), swamp cabbage (*Ipomoea reptans*), and shallots (*Allium cepa*) [30]. Broccoli (*Brassica oleraceae* L. cv. Lord) subjected to boiling, showed losses of flavonoids and phenolic acids by 72 and 52%, respectively, as compared with fresh broccoli [31].

In contrast to the described losses, some researchers relate an increase of the phenolic content in broccoli, green beans, and pepper when subjected to the boiling process [32]. The increase of phenolic content described in vegetables after cooking, has been attributed to the higher extractability of the cellular matrix compounds, as well as to the tissue dehydration [33, 34]. However, low correlation coefficients ($R = 0.5$) were observed between the increases in the content of total phenolic compounds in cooked peppers and the weight loss during cooking [35], suggesting that besides dehydration of peppers, others factors were involved in such increases.

In broccoli (*Brassica oleraceae* L. cv. Lord)) subjected to steaming, there was an increase of the total polyphenol content (1.6 times), flavonoids (1.5 times) and phenolic acids (1.3 times) in comparison to *in natura* broccoli [31]. This effect may be explained due to the rupture of complexes between the polyphenolic compounds and other compounds (e.g., proteins), resulting in a better availability by steaming extraction.

The steam-cooking process has proved to be effective in the maintenance or increase of the phenolic content in some vegetables. In study using kale (*Brassica oleraceae* L. var. acephala D.C) and red cabbage (*Brassica oleraceae* L. var. capitata), it was verified that steam cooking resulted in higher contents of bioactive compounds and higher antioxidant activity, which can be attributed to the absence of loss by leaching [21]. Corroborating with these results, studies realized on tubercles showed an increase in the total phenolic compounds contents (e.g., anthocyanin) after thermal treatments that do not use water in the process [36, 37]. The thermal treatment effect preserves or causes a little increase in the anthocyanin content in different potato "cultivar" (*Solanum tuberosum* L.) when cooked in microwave (9 min, 900 W), steamed (pressure cooker, 15 min), boiled (in water in a pressure cooker, 15 min), and baked (in a hot air oven, 40 min, 180°C) [38]. This increase may be attributed to the inactivation of the polyphenol oxidase (POD) due to the thermal treatment. Some cooking methods like microwave can destroy the potato cellular microstructure and induces a better extraction of the compounds from the cell matrix [39, 40].

Many studies on cooked potatoes showed that the total phenolic content may be maintained or even improved, based on the cooking method used [25, 40, 41]. In the study with different cultivations of potatoes cooked in microwave (2 min 30 s, 1100 w), baking (375°C, 30 min), and boiling (18 min), an increase in the quantity of chlorogenic acid, rutin, and kaempferol-rutinose was verified [25]. In opposition, another research on cooked potatoes by boiling (60 min), microwave (20 min) and baking (204°C, 60 min) showed reduction of 44, 55, and 53% for the total phenolic content, respectively [18]. In commercial processing, the pretreatment commonly used, such as blanching and dices processing, can also induce a significant loss of total phenolics [42]. Thus, cooking methods with lower temperatures should be preferred in order to maintain the phenolic compound contents in potatoes [43]. Steaming and microwaving are the cooking methods that retain the highest quantities of total phenolic compounds and antioxidant activity in cooked potatoes [44]. The stir-frying method induced the highest loss of these compounds (72.44%), followed by baking (40.51%), air frying (32.52%), and frying (14.08%).

Among the factors that affect the leaching of matrix compounds, we can include the polarity of medium used. Polar mediums, such as water, allow changes in the phenolic compound levels. In contrast, if the medium is nonpolar (use of oil for frying, in both deep frying and pan frying), the loss of compounds is lower due to the lack of diffusion or migration to the medium [19]. The use of extra virgin olive oil (EVOO) did not induce loss of the total phenolic compound content in fresh potato (*Solanum tuberosum*), pumpkin (*Cucurbita moschata*), tomato (*Licopersicum esculentum*), and eggplant (*Solanum melongena*). However, the retention of phenolic compounds was directly affected by the cooking technique used, deep frying > sautéing > boiling > boiling water/EVOO mixture [45]. Addition of oil during the cooking process may

result in transference of some compounds present in the oil to the food. Comparing different oils (olive oil and sunflower oil) used to fry with grilling method, an increase in phenolic compounds can be observed [46]. This may be due to the thermal destruction of cell wall and other subcellular components, during the cooking process, stimulating the release of these compounds. At the same time, some mechanisms have been proposed to explain the variations found in the phenolic compounds in foods after thermal processing, such as the rupture of the phenol-sugar glycosidic bonds, leading to the formation of phenolic aglycons [47].

The phytochemical quantity retained in fruits and vegetables, after the processing, depends on the stability of these compounds during the different food preparations. Molecular modifications induced by processing and the transformations that occur before the consumption are mainly related to the sensibility of the compounds to oxidation and/or isomerization [17]. Reduction of phenolic amounts may occur due to isomerization of certain compounds caused by the high temperature. In catechin, the isomerization are clearly demonstrated [48], where epimerization changes the epistructured catechin to nonepistructured catechin and vice versa. Therefore, others methods of food processing that influence phenolic content, and not used in conventional domestic processing, should be considered.

3.1. Blanching

One of the first steps of vegetable and fruits processing, as well as before the extraction of juices, is the blanching process used mainly in order to inactivate enzymes and to remove undesirable microorganisms. Blanching is the treatment that can be used to inhibit the phenolic oxidation (browning). The browning reaction involves different compounds and follows chemical pathways that include reactions promoted by those enzymes not involved in catalytic processes, as the Maillard reaction that results in melanoidin formation [49]. Blanching may induce alterations in the content of some bioactive compounds in fruits and vegetables and is used to inactivate some enzymes, such as laccase, lipoxygenase, polyphenol oxidase, and peroxidase that affect the quality during storage. These enzymes can promote deterioration reactions and consequent undesirable changes in nutritional value, flavor, and color (including dark pigments).

Polyphenol oxidase (PPO, EC 1.14.18.1) and peroxidase (POD, EC 1.11.1.7) are involved in the phenolic compound oxidation and are very important in preserving the food quality. The phenolic compound oxidation induces the production of dark compounds (browning), that induces rejection by the consumers, and decreases the antioxidant capacity of foods [50]. The browning reactions have generally been assumed to be a direct consequence of phenolic compound oxidation by PPO action [51]. However, at least a partial role may be attributed to the action of peroxidase [52–54]. The mixing of phenolic compounds with polyphenol oxidase and peroxidase enzymes in the presence of oxygen produces colored pigments [55]. When there is disruption of the cell, some compounds such as phenolic acids suffer the action of the polyphenol oxidases that induces oxidation of phenolics and results in dark compound formation [56]. In addition, polyphenol oxidases catalyze two different reactions. The first is the *o*-hydroxylation of monophenols and diphenols (monophenol oxidase, cresolase activity) and the second reaction promotes the o-diphenol oxidation to *o*-quinone (diphenolase/

catecholase activity) [55]. This quinone formation suffers polymerization and causes yellow and brown coloring.

The peroxidase enzyme is one the most important enzymes responsible for polyphenol degradation. This enzyme is generally considered as the reference enzyme for blanching treatments, due to its high thermal resistance and high concentration in most vegetables. Residual POD activity could still be detected after a high-temperature blanching, and its inactivation in food product can indirectly indicate that other enzymes are likely to be inactivated [57]. In order to inhibit the polyphenol oxidases and peroxidases some treatments have been used, including the addition of ascorbic acid or chemical agents (sulfites), exclusion of oxygen, refrigeration, and nonthermal treatments. PPO is relatively thermolabile, temperatures above 50°C and proper time of treatment decreases its activity. At higher temperatures (above 80°C), these enzymes may be completely inactivated [58].

In blueberry fruits processed into juice, blanching induced an increase in the retention of anthocyanin levels (23% instead of 12%) and the total anthocyanin content of juice from blanched blueberry is twice the nonblanched one [59]. The blanching treatment thus demonstrated to be extremely effective in reducing the PPO activity and maximizing anthocyanin recovery. This effect should be a result of the complete inactivation of the PPO enzyme, or of the greater extraction yield linked to the increase of fruit skin permeability caused by the heat treatment [60]. In some eggplant genotypes, cooking processes as boiling and grilling induced a drastic decrease in PPO activity, with a little residual PPO activity [61]. Whereas, in wheat flour, the PPO showed the maximum decrease in its activity when processed in microwave (81.4%), compared to hydrothermal treatment (48.3%). The strong decrease of the PPO activity, after microwave, can be attributed to the higher heating uniformity and higher penetration power of the microwave.

The activity of the enzymes PPO and POD are closely related, acting in a combined form in the darkening of fruits and vegetables. The phenol oxidation by PPO produces hydrogen peroxide (H_2O_2), independently on the substrate used. The POD catalyzes the phenolic compound oxidation, since there is a high affinity between the H_2O_2, produced by the PPO, that acts as an electron acceptor and the vegetal phenolic compounds that work as electron donators (substrates: catechin, quercetin and its glycosides). This process promotes the oxidation of phenolic compounds and produces quinones that affect color, flavor, texture, and loss of the nutritional and functional quality [53, 62]. The PPO is more thermolabile when compared to the POD, however, variations may occur depending on the food matrix. In some cases, the inactivation can be obtained at 80°C, which would explain, partially, the reason why the amount of phenolic compounds increases when the product is taken to high temperatures, as used during pasteurization or other procedures [62–65].

Generally, blanching is a thermal process used in combination with other methods and carried out by treating the fruits and vegetables with steam or hot water for 1–10 min at 75–95°C. Time/temperature combination may vary according to vegetable or fruit used [66]. Blanching performed on *Lagenaria siceraria* fruit before preparing the juice, using water at 90°C for 5 min, resulted in a total phenol content of 644 mg/100g, values similar to that found in unprocessed fruit (640 mg/100 g) [63]. However, when the combination of blanching and sonication was

applied, there was an increase in the total phenolic compounds of 63% and there was also an alteration in the structure of the molecules after the process, indicating that the use of both methods increases the quantity of these compounds.

3.2. Processing methods applied to beverages

3.2.1. Clarification

This process is regularly used in beverage industries to remove phenolic constituents that give color, astringency, and bitterness to the juices. In addition, these compounds, high-molecular weight proteins, and pectins contained in juices may cause turbidity (haze formation) and, consequently, decrease the product acceptance by the consumer [50]. To remove all undesirable molecules, the clarification process consists of the addition of gelatin, bentonite, polyvinylpolypyrrolidone (PVPP), and kieselsol, among others. After that, the juice samples are subjected to ultrafiltration several times, a process used in beverages to remove high-molecular weight proteins and other compounds added during clarification process (gelatin, bentonite, PVPP, kieselsol). Employing this method helps to avoid the formation of cloudy appearance during processing and storage. However, besides the direct reduction of phenolic compounds induced by clarification, the ultrafiltration process can remove the phenolic compounds that are complexed with proteins [67].

3.2.2. Pasteurization process and emerging technologies

Beyond the clarification, many juices are submitted to the pasteurization process (heat process used to inactivate pathogenic microorganisms) that, not only eliminates microorganisms, but also affects the PPO activity, which indirectly degrades monomeric anthocyanin by forming *o*-quinones from polyphenols during enzymatic browning [68]. On the other hand, pasteurization is one of the methods used in industrial scale that causes the highest losses of bioactive compounds. According to Azofeifa, after the pasteurization of the blackberry (*Rubus adenotrichus* Schltdl) juice, many phenolics may be lost [69]. This study described a decrease from 191 to 181 mg of cyanidin-3 glucoside per gram of extract, when the temperature was increased from 75 to 92°C, even using the least time in the highest temperature. Another study showed that the increase of pasteurization temperatures promotes loss of phenolic compounds in orange juice [70].

New emerging technologies have been used in the beverage industry, such as high intensity pulsed electric field (HIPEF), high pressure, and ultrasound. All of these technologies are nonthermal processes that help stabilizing the beverages from microorganism-induced damages or enzymatic degradation, and have few or any negative effect on the phenolic compounds [71–74].

Another processing that affects the amount of phenolic compounds is the fermentative process. In wine, for example, the phenolic compounds not only have functional properties, but also have important functions for the product's sensorial quality, impacting the color, flavor, smell, and aging [75–77]. The different steps involved in the process, such as the wine-making technique used, the maceration characteristics (temperature, enzymes, and chemical reagents

used), fermentation, presence of alcohol, and aging cause alterations in the phenol concentration, mainly in the anthocyanins, which are pigments responsible for the color and have great biological importance [75, 76].

3.3. Sanitizing methods

Currently, in the food processing, there is a great concern about the residues formed by the action of some compounds used during the sanitation. During harvest, transportation, or processing, the tissues may be mechanically injured that facilitates the food contamination [78]. In many countries, the use of chlorinated compounds, particularly the hypochlorite salts, is very common to minimize the pathogen infestation rate. Sodium hypochlorite is a potent sanitizer that has oxidant action and is used in domestic or industrial food processing. However, the use of chlorine or chlorine-based products, has some disadvantages, such as the formation of organochlorinated compounds, chloroform, trihalomethanes, and haloacetic acids, that have known or suspected carcinogenic or mutagenic effects, with proven toxicity to liver and kidneys [79]. Due to these effects, its use has been forbidden in organic foods. The relation between products containing chlorine and the phenolic compound content has been investigated and there is still no consensus about the results. In mushrooms, the use of sodium hypochlorite at room temperature induced the disappearance of phenolics and the formation of their oxidation products [80]. The losses of flavonols (23%) and anthocyanins (13%) due to leaching were detected after sanitation using sodium hypochlorite at 50°C in red onion slices [81]. In contrast, in carrots, washing in chlorinated water (100 mg/L) did not induce alterations in the phenolic compound content [82].

One of the alternatives for hypochlorite is ozone (O_3), used as an antimicrobial agent since the end of the nineteenth century to purify potable water. The use of O_3 has many advantages over other chemical oxidants, its precursors are numerous and economically profitable and can be used in gaseous or aqueous state, depending on the product [83, 84]. Beyond its antimicrobial activity against a wide range of microorganisms, O_3 can destroy chemical residues and convert nonbiodegradable organic materials into biodegradable materials [85]. At the same time, due to its fast decomposition into oxygen and to the fact that it does not form residues in the treated products, its use in the food processing is authorized by the organic certification [86]. Various research groups have studied the relation of the ozone action with the phenolic compounds, but contradictory results about its action have been described. Certainly, some fruits and vegetables may be more susceptible to the action of this gas and may show different responses, mainly regarding the stress caused by the oxidant action.

Both the time and ozone concentration may cause different responses. In pineapple, banana, and guava, the application of gaseous O_3 induced a significant increase in total phenolics, whereas in bananas and pineapples, the flavonoid content increased in response to up to 20 min of ozone treatment. For guava fruits, the flavonoid content increased and total phenolic decreased inversely when these fruits were exposed up to 10 min [87]. The authors attribute the increase of these compounds to the activation of the enzyme phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), which may have increased due to the stress. The increase of the phenolic compounds and flavonoid contents in these fruits may be caused by other factors, such

as the modification of the cell wall that occurs when a plant cell is exposed to ozone, increasing the extractability and release of the phenolics bonded to the cell wall. In kiwifruit stored in an enriched environment with ozone and under refrigeration for 30 days or for 3 months followed by 12 days of shelf life, there was an increase in the content of phenolic compounds [88]. In this study, the authors affirm that ozone acts as a potent elicitor to anti-free-radicals found in oxygen and nitrogen-reactive species.

The decrease in the content of some polyphenols caused by the treatment with ozone in grape juice has been described [89]. After the use of ozone at low concentration (1.6% w/v) for 10 min, there was a significant decrease of 78.0, 95.0, and 99.0% for cyanidin-3-O-glucoside (Cy3Gl), delphinidin-3-O-glucoside (Dy3Gl), and malvidin-3-O-glucoside (My3Gl), respectively. However, at higher ozone concentrations (7.8% w/w) only Cy3Gl and Dy3Gl were observed to be stable. The anthocyanin degradation influenced in the color of grape juice due to ozone processing can be attributed to the strong oxidizing potential of ozone [90].

Thus, ozone can induce increase or decrease of some phenolic content. In organic and conventional Palmer mangos, sanitized with ozonized water, no effect in phenolic content was observed [91]. The variations in the contents of these compounds were in function of the cultivation mode (organic or conventional) of the fruit. A similar result was described in organic or conventional cabbage treated with ozonized water. There were no variations in the total phenols or flavonoid contents due to sanitizing treatment [92].

Other chemical treatment used to inactivate pathogenic or spoilage microorganisms is the ultraviolet-C light (UV-C), a non-ionizing germicide radiation with wavelength range from 200 to 280 nm. Treatment with UV-C radiation has been widely studied as a fruits and vegetables disinfectant, and offers an alternative for chemical sterilization and preserves food quality [93]. UV-C radiation delays the maturation, decreases the senescence, maintains the quality of the products for a longer time, and reduces the postharvest deterioration of fruits and vegetables [94, 95]. The UV-C has been suggested as a suitable stress-promoting technology. This treatment may also accelerate the ethylene production and, consequently, activate the expression of ethylene response factor (ERF) genes. This response is consistent with the fact that UV-C is a stress agent in plants and generally increases the ethylene production under stress, probably by acting on system 2 autocatalytic ethylene [95, 96]. Even though the UV-C increases the ACC (1-aminocyclopropane-1-carboxylate) oxidase transcription and stimulates the ethylene production, the maturation evolution is still delayed [95].

The application of hermetic doses of UV-C not only is capable of improving the storage time, but can also increase the nutritional and functional properties of fruits and vegetables. The alteration of the ERF expression, through hormonal induction or abiotic stress, may induce secondary metabolic pathways, e.g., the phenolic compounds production [95, 97, 98]. Low UV-C doses may efficiently reduce the microbial population in fresh-cut products, which is one important sector of the food market. The UV-C treatment (0, 1, 3, 5, and 10 min) increased the total phenol and flavonoid contents in fresh-cut mangoes during storage for 15 days at 5°C, and this effect can be attributed to the increase of the phenylalanine ammonia-lyase activity. In addition, the irradiation improved the antioxidant capacity, which is probably related to the increase of the phenolic compound content [99]. The UV-C radiation (1.5 and 3.0 kJ/m^2),

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in minimally processed Satsuma mandarin, promoted an increase in the flavonoid content (22.20 and 21.34% for narirutin, 11.75 and 33.25% for hesperidin) and total phenolic compounds (5.73 and 8.13%), after 3 days of storage [100]. According to the authors, this flavonoid increase may be related to the citrus defense mechanism in reaction to the stress induced by the UV-C application.

Another type of radiation that has been widely used in the maintenance of the quality of fresh and dry foods is the ionizing radiation, which can be constituted by electrons of high energy, X-rays, or gamma rays (^{60}Co or ^{137}Ce) [101]. Among the gamma radiation effects, we can highlight the delay in the maturation, the reduction of the microorganisms in grains, cereals, fruits and spices, reduced storage losses, and extended shelf life. However, the irradiation may induce stress signals and stress responses in fruits and vegetables that increases the antioxidant compounds [102]. In juices of carrot (*Daucus carota* var. sativa) and kale (*Brassica oleraceae* var. acephala), treated with gamma-irradiation (10 kGy), there were an increase in the total phenol contents and antioxidant capacity, when compared to nonirradiated juice [103].

The alterations induced by the gamma-irradiation in dry herbs and spices are related with the radiation dose applied, and generally, result in an increase of the total phenolic compound contents [104], whereas by using gamma-irradiation at 10 kGy, in *Thymus vulgaris* L., no modification in the phenolic profile and bioactive properties, were observed [105]. On the other hand, the gamma-irradiation can either decrease or improve the bioactivity of irradiated samples, depending on the changes in the structure of different antioxidant molecules and/or breaking some chemical bonds [106]. The influence of different radiation doses (1, 5, and 8 kGy) were also verified on the color, organic acids, total phenolics, total flavonoids, and antioxidant activity of dwarf mallow (*Malva neglecta* Wallr.) [107]. Irradiation at 5 kGy increased the amounts of citric and succinic acids, and decreased the fumaric acid levels. In contrast, in the decoction prepared, the antioxidant properties and levels of total phenolics and flavonoids were decreased with the 8 kGy dose.

3.4. Drying methods

The traditional/conventional drying process is accomplished by heat, and in this process, two transport phenomena occur simultaneously. The first is a moisture movement and the second is a heat transfer [108]. In this method the increase in temperature may induce reduction of phenolic compounds, however, depending on the air velocity and on the heat exposure time, the antioxidant compounds content, as well as the antioxidant activity, can be affected [109, 110]. The effect of temperature (40, 60, and 80°C) and air velocity (0.5, 1.0, and 1.5 m/s) on the drying kinetics and quality attributes of apple (var. Granny Smith) slices during drying were studied [109]. The authors found that the total phenolics decreased with temperature, but a reduced thermal degradation was observed at high air velocity. On the other hand, there was the least destruction of phenolic content at 80°C and 1.5 m/s, probably due to short drying time and, therefore, less exposure of the phenolics to thermal effect. Even though the quantity of phenolic compounds decreased with the increasing temperature, the same effect was not observed for the antioxidant activity (DPPH). At 80°C, the antioxidant activity values did not differ from that measured at 40°C. This effect can be explained by the development of

Maillard browning reaction occurring concomitantly with other events, contributing to generation and accumulation of different antioxidants. Similar results were observed in quinoa, in which a moderate correlation between the phenolic compound content and the antioxidant activity DPPH, were observed at 40 and 80°C [109].

The scientific community has turned its attention to advances in the dehydration methods that preserve and retain the nutrients, stimulated by the increasing demand of dehydrated food products with higher quality, and resulting in more efficient methods and operations. New dehydration methods can retain the amount of nutrients in dry fruits and vegetables in a similar content to the fresh vegetables [111]. Total phenolic compounds were either unaffected or actually increased in concentration and/or extractability after high-pressure preservation treatments and microwave preservation. The results were similar, total phenolic contents either declined (4–91%) or increased (104–125%), depending on the particular food species. Nevertheless, at microwave vacuum preservation, total phenolics were retained at higher levels than in those fruits and vegetables that were air-dried; since the microwave power is less than 500 W. Interestingly, when total phenolic levels at microwave vacuum preservation is compared to freeze-drying, the results showed that freezing-drying is a better dehydration method than microwave vacuum drying.

4. Bioavailability of phenolic compounds

The major sources of phenolics are fruits, vegetables, and beverages, such as coffee, tea, wine, and fresh-fruit juices. Besides its potential effects in protecting against several chronic diseases, it is essential to understand the modifications occurred to these compounds after food processing, and its bioavailability. Better knowledge of the modifications induced by different processing methods in phenolic compounds are essential to evaluate appropriately the bioavailability of these compounds.

The bioavailability of bioactive compounds is the absorptive process of these molecules across the intestine into the circulatory system, after food ingestion. In a review study, reported values of several polyphenols ingested pure (isolated compound) or in foods, ranged from 0.072 to 5 μM, when reached the plasma. The total intake of polyphenols, in the studies grouped for this review, ranged from 6.4 to 1000 mg/day [112]. In elderly Japanese population, the consumption of polyphenols ranged from 183 to 4854 mg/day, with 665 to 1492 mg/day on an average. Beverages such as coffee and green tea were the largest source of these compounds [113].

Usually, from the total phenolics ingested, phenolic acids account for approximately one-third and flavonoids account for the two-thirds remaining. Phenolic and polyphenolic compounds, in isolate or associated to vitamins, such as carotenoids, vitamin E, and vitamin C, are reducing agents that protect human body's specific tissues against oxidative stress. However, polyphenols are the most abundant antioxidants in diets based on fruits and vegetables. The most abundant benzoic acids ingested in human diet are gallic, ellagic, protocatechuic, and 4-hydroxybenzoic acids. Therefore, cinnamic acids are mainly represented by caffeic, ferulic, sinapic, and p-coumaric acids. Diets based on plant foods are a

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rich source of polyphenols that have health benefits and avoid the development of chronic diseases. However, food processing, such as blanching and thermal treatments, may influence its levels and induce its conversion to secondary compounds. In addition to molecular modifications occurring in phenolics during food processing, the absorption and metabolism of these compounds are triggered by enzymatic and nonenzymatic reactions (**Figure 2**). These molecules can also suffer conjugation reactions that may increase or decrease their bioavailability.

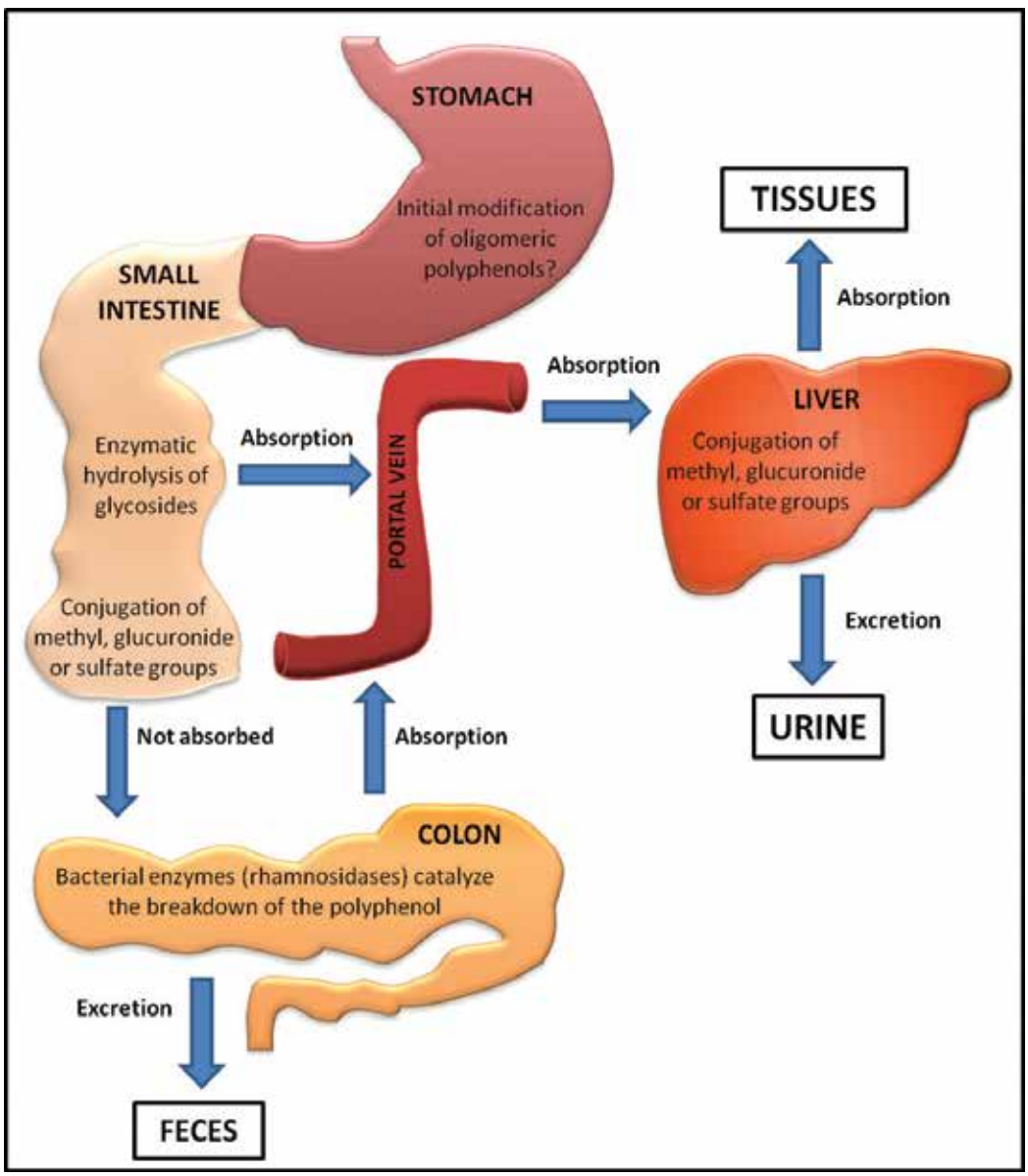

Figure 2. Predicted routes for absorption of dietary phenolics.

Oligomeric polyphenols may suffer initial modifications induced by gastric acid from stomach during the absorption process. In the small intestine, the glycosidic polyphenols are cleaved to release the glycoside radical before absorption. This process is mediated by enzymes, that have affinity for glucose, xylose, and galactose, such as lactase phlorizin hydrolase (LPH) and cytosolic β-glucosidase (β-CBG) [114]. However, the polyphenols resistant to the action of these enzymes are not absorbed in the small intestine and may be cleaved by intestinal bacteria to produce small molecules as phenolic acids. The structures of polyphenols can still pass by conjugation reactions with addition of methyl, glucuronide, or sulfate groups. Remaining polyphenols, mainly that attached to rhamnose, are modified for α-rhamnosidases produced by colonic microflora.

After these absorptive processes the phenolics follow to four possible pathways: 1—excreted through feces; 2—absorbed by intestine/colon mucosa, pass through portal vein and reach the liver; 3—are further conjugated in the liver with methyl, glucuronide or sulfate groups and released in blood stream for tissues absorption; 4—excreted in urine.

5. Conclusion

Processing methods has been associated with changes in the quantity and quality of (poly) phenols. The high diversity of these molecules produced by plants must be taken into account when processing methods of preparation are employed to obtain industrial or homemade products. There are innumerous studies comparing the biological actions and *in vitro* antioxidant activity of phenolic compounds in function of its content in plant-based foods and consequently, in humans. The phytochemical amount retained in fruits and vegetables after the processing, depends on the stability of these compounds during different food preparations. Molecular modifications induced by processing, and transformations that occur before the consumption, are mainly related to the sensibility of the compounds to oxidation and/or isomerization.

The physical and chemical transformations that occur during the thermal processing in each species and between different species can vary, depending on the processing method used, as well as on the temperature and time employed. In general, the thermal processing methods as the beverage pasteurization result in loss of the phenolic compound content, due to the high temperatures employed, as well as the cooking of vegetables in water, because it promotes the leaching of the phenolic compounds. Even though it is not possible to affirm that these effects are observed in all foods, the thermal processing methods such as microwave cooking, steam cooking, air frying, oil frying, and grilling induce alterations in the food matrix, promoting the extraction of these compounds and increasing its bioaccessibility.

Methods such as blanching can minimize the phenolic compounds oxidation by inactivating the enzymes (i.e., PPO and POD) responsible for the darkening of the vegetables and can be used as a preprocessing in order to avoid the loss of these compounds during the process of hot-air drying. The drying at microwave vacuum induces total phenolics retention at higher levels than in those fruits and vegetables that were air-dried. Regarding dehydration, the best method seems to be freeze-drying.

Beyond the thermal processing, the sanitizing methods such as the use of sodium hypochlorite and ozonization can also affect the amount of phenolic compounds, in a dependent manner of the food matrix, compound/method employed, concentration, and sanitation time. The use of ionizing and nonionizing radiation in the food sanitation, cause modifications in the profile of the phenolic compounds and the results vary according to the dose. However, the application of this technology usually induces the increase of the phenolic content, which may be related to the vegetable defense mechanism on reaction to the stress induced by the radiation application.

Better knowledge of the modifications induced by different processing methods in phenolic compounds is essential to properly evaluate the bioavailability of these compounds. The molecular modifications occurring in phenolics during food processing, the absorption and metabolism of these compounds are triggered by enzymatic and non-enzymatic reactions, and these molecules can suffer conjugation reactions that may increase or decrease their bioavailability and consequently, affect the beneficial effects to human health.

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Author details

Igor Otavio Minatel[1], Cristine Vanz Borges[1], Maria Izabela Ferreira[1], Hector Alonzo Gomez Gomez[1], Chung-Yen Oliver Chen[2] and Giuseppina Pace Pereira Lima[1]*

*Address all correspondence to: gpplima@ibb.unesp.br

1 Sao Paulo State University, UNESP, Botucatu, Brazil

2 Jean Mayer USDA, Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA

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Chapter 2

The Relationship Between Phenolic Compounds from Diet and Microbiota

Daniela Elena Popa, Cristina Manuela Drăgoi,

Andreea Letiția Arsene, Ion Bogdan Dumitrescu,

Alina Crenguța Nicolae, Bruno Stefan Velescu and

George T.A. Burcea-Dragomiroiu

Additional information is available at the end of the chapter

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Abstract

All multicellular organisms live in a strong bond with the microorganisms from around the world, and the humans are not the exceptions. Human microbiota (a complex bacterial community) contains about 10^{14} microbial cells, 10 times more than the content of the cells from our body and the microbial genome named microbiome, 1000 more that the human genome. It colonises any surface of the human body, above our skin, in the genitourinary tract, gut and airways. From all this, the gut is the most colonised organ, with an amount of almost 70% of the human microbes. Considering the large size of the gut, compared with a tennis terrain, filled with substances that plays a key, nutritive role for the microbes, polyphenols are micronutrients from our diet, with an emerging role in the modulation of the colonic microbial population composition and activity. Therefore, many studies underline that long-term consumption of diets rich in plants polyphenols offers protection against cancer, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases. This chapter reviews the biological effects of plant polyphenols in the context of relevance to human health, especially considering the food functionality area, together with the complexity of the human microbiota and the bioavailability highly dependent on their intestinal absorption.

Keywords: dietary polyphenols, microbiota, microbial metabolism, human health, interactions

1. Introduction

Phytochemicals are the richest resources for human consumption because of their various applications. By using phytochemicals, new and novel products for the treatment and prevention of serious diseases could be improved.

Bioactive plant secondary metabolites play a crucial role in plant growth, development and physiology. The effective components of medicinal plants are usually the secondary metabolites, and the synthesis of them is affected by a variety of factors, such as biotic and abiotic effectors. Also, secondary metabolites help plants to survive, defence and compete with others. Thus, the production of the majority of plant phenolic compounds could be induced by the applications of abiotic and biotic elicitors. Due to this knowledge, the usage of elicitors is usually preferred to the optimisation of rare phytochemical sources.

Polyphenols are secondary metabolites from plants, characterised by two aromatic rings, tailored by hydroxylated phenyl moieties, in large amounts in fruits, vegetables, cereals and beverages. The compounds are showing a large diversity, including chlorogenic acids, tannins and flavonoids (flavonols, flavanones, flavan-3-ols, anthocyanins, isoflavones and flavones). Dietary polyphenols are substrates for gut and colonic microbiota. They and their metabolite allow the maintenance of gastrointestinal health, interacting with epithelial cells and modulating the microbial complexity of the gut. Polyphenolic compounds play the role of promoting factors of growth, proliferation or survival for beneficial gut microbiota, exerting beneficial prebiotic activity and inhibiting the proliferation of some pathogenic bacteria (*Salmonella*, *Helicobacter*). Although in the raw material, phenolic compounds occur as glycosylated derivatives, in order to exert their pharmacological effect, these polyphenols undergo various intestinal transformations, allowing the digestive enzymes and also the microbiota metabolism to eliminate the sugar component or other hydroxyl moieties and to release the aglycons that are further absorbed into the blood circulation, and from there, they became relevant for the organs, exhibiting strong biological effect.

2. Polyphenols, gut microbiota and health

The intestinal tract is colonised with a whole bacterial community (microbiota) that has more than 800 different bacterial species [1]. They exert a metabolic effect, due especially to the dietary compounds that provide energy, a supply of nutrients for the organism and transformations of xenobiotics. A normal composition for the gut microbiota implies a good life for the host, while imbalances are tightly associated with metabolic disorders. The factors that influence the gut microbiota are environmental (diet, the intake of antibiotic and xenobiotic) and endogenous (the adult-type microbiota is different from the child-type microbiota).

It develops a large number of functions—protective, immune and metabolic—correlated with the health status of the host. The intestinal microbiota is essential for the postnatal development that is for the content of T cells, intraepithelial T cells and immunoglobulins [2]. It is well known that prebiotics and prebiotic-like phytonutrients may exert a beneficial growth activity of bacteria and inhibit pathogenic bacteria, as a target for the biological compounds orally administered. It is also to mention the genes involved in the utilisation of the carbo-

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hydrates and lipids (the microbiome), allowing the enzymes to utilise carbohydrates, host-derived glucuronides (like mucin), deconjugate and dehydroxylate the bile acids, reduce the cholesterol, synthesise some vitamins (K and B groups) and also participate to the amino acids and xenobiotic metabolism.

The gut colonisation starts immediately after birth, being dominated by *Bifidobacterium* population, due to the breast feeding, and stabilises for the first 12 months. Analyses show that adults and weaned children have *Bacteroides* population in their microbiota, followed by *Firmicutes*, like *Eubacterium*, *Ruminococcus*, *Clostridium* and *Bifidobacterium*. In infants, *Escherichia coli*, *Raoultella* and *Klebsiella* form the bacterial complex, dynamic and susceptible to changes, made by dietary factors and other diverse disorders [3].

It is to underline that two categories of enzymes are exerting an important activity of degrading the polysaccharides and xenobiotics: β-glycosidases and β-glucuronidases, which may act a beneficial or harmful role. β-Glycosidases are involved in the nutrient utilisation, which lead to body energy, by fermentation of dietary polysaccharides, thus resulting mainly short-chain fatty acids, with different functions (acetate and propionate access the portal circulation and impact the lipid metabolism, butyrate is used by enterocytes and positively influence the cell growth and differentiation). Unlike the β-glycosidases, β-glucuronidases release the toxins and another endogenous particle that have been glucuronide in the liver and excreted with the bile into the gut. Studies performed on about 40 bacterial strains, dominant in human faeces, showed a low β-glucosidase activity for Gram-positive *Firmicutes*, while the β-glucuronidase activity is noted for some *Firmicutes*, with clostridial cluster [4].

Intestinal enzymes are also related to the cholesterol and bile acid metabolism. Following this pathway, cholesterol is degraded to coprostanol, and bile acids are converted into secondary bile acids through deconjugation and dehydroxylation (deoxycholic acid and lithocholic acid). For the obtaining of this metabolites, incriminated for their carcinogenetic role, are primary mentioned *Bacteroides intestinalis* and secondarily *Bacteroides fragilis* and *Escherichia coli*.

Also, colonic strains are involved actively in the protein synthesis, highlighting the source of amino acids for the human organism. At least 1–20% of circulating lysine and threonine in adults are derived from intestinal microbiota [5].

The colonisation of germ-free mice reduces the levels of circulating fasting-induced adipose factor (Fiaf) and the skeletal muscle and liver levels of phosphorylated AMP-activated protein kinase, contributing to fat storage [6].

There can be described a straight correlation between the polyunsaturated fatty acids and gut microbiota. In vitro studies performed with *Lactobacillus* strains (*L. rhamnosus*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus*) have shown that different concentrations of these acids (10–40 μg/mL) inhibited growth and adhesion to mucus of all tested strains, while low concentration of gamma-linoleic and arachidonic acid (5 μg/mL) exerted a positive effect on the growth and mucus adhesion of *L. casei* [7]. Fatty acids affect the sites for the gastrointestinal microbiota, modifying their composition in the intestinal wall. In clinical trials, atopic eczema children diet supplemented with formulation containing *Bifidobacterium Bb-12* or *Lactobacillus GG* exerted different effects on plasma lipids; the first one increased the level of alpha-linoleic acid, suggesting that interactions between prebiotics and dietary polyunsaturated fatty acids could be established, although further studies in vivo are needed to confirm this hypothesis [8].

Briefly, plant polyphenols have a bioavailability and exert their beneficial effects depending on their transformation by gut microbiota, taking into account the polyphenol type and identifying the responsible microorganism.

It is well known that dietary polyphenols and their metabolites contribute to the health by modulating the gut microbiota, stimulating the beneficial bacteria and inhibiting the pathogens ones, exerting prebiotic-like effects [9].

3. Microbial metabolism of polyphenols

We can distinguish among polyphenols flavonoids and nonflavonoids (**Figure 1**).

Flavonoids are classified into further classes according to their chemical structure: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols, anthocyanidins, isoflavones and proanthocyanidins [11].

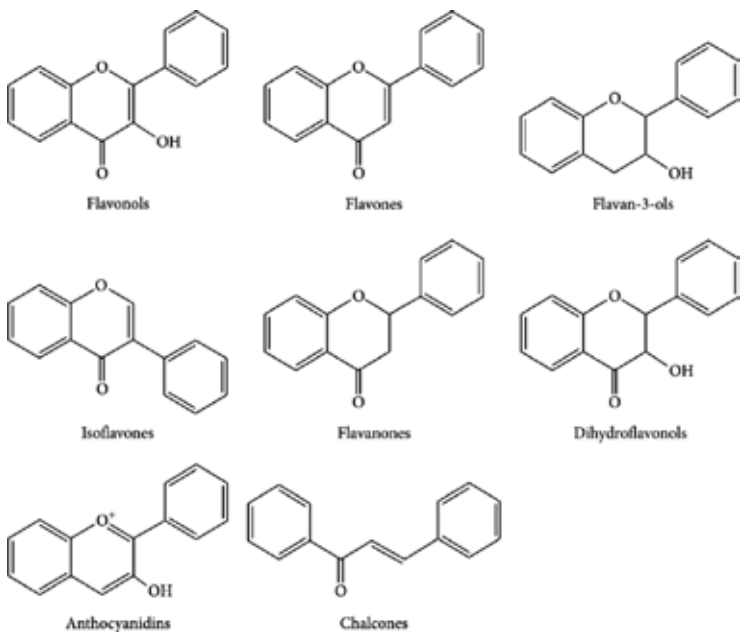

Figure 1. Phenolic compounds encountered in food [10].

Non-flavonoids can be divided into simple phenols, phenolic acids, benzoic aldehydes, hydrolysable tannins, acetophenones and phenyl acetic acids, hydroxycinnamic acids, coumarins, benzophenones, xanthones, stilbenes, lignans and secoiridoids. They can be found in foods and are important to human health. Among these, resveratrol is unique in the grapes and red wine; ellagic acid is found in berry fruits (strawberries and raspberries) and in the skins of different tree nuts. Lignans can be found in flax, sesame and many grains. Curcumin is a strong antioxidant from turmeric. Rosmarinic acid is a dimer of caffeic acid and ellagic acid is a dimer of gallic acid. While gallic acid and ellagic acid are found in the free form, their glucose esters, known as hydrolysable tannins, also exist in plants. The C6-C3 hydroxycinnamate derivatives occur mainly as conjugates with tartaric acid or quinic acid and can be found as

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chlorogenic acids. These last compounds, principally 3-O-, 4-O- and 5-O-caffeoylquinic acids, form 10% of green coffee beans (*Coffea canephora*). Regular consumers of coffee may provide a daily intake in excess of 1 g of chlorogenic acids, and these may conclude the major diet.

Usually, they are metabolic products of chloroplasts, as defence against oxidative damage during photosynthesis [12], but can also be produced by sexual organs as defence against solar UV, at the root level or as defence against virus, bacteria and fungi [13].

After ingestion, depending on their chemical composition, phenolic compounds are absorbed in the small intestine or, in some cases, can be found in the unchanged form in the colon. Only a small percentage (5–10%) of the total polyphenol intake is absorbed in the small intestine. The remaining polyphenols (90–95%) accumulate in the intestinal lumen and, following the bile pathway, represent the target for the gut complex, subject for the enzymatic activity.

Bacteria is well represented in the colon, where around 300–500 different species live. The most common bacteria are *Bacteroides*, in an amount of 30% of all gut, followed by *Clostridium*, *Prevotella*, *Eubacterium*, *Ruminococcus*, *Fusobacterium*, *Peptococcus* and *Bifidobacterium*. The lowest concentrations are described for *E. coli* and *Lactobacillus*. There is a direct established correlation between the levels of *Prevotella* and a children high-fibre diet. Also, there are data that confirm the same higher levels of *Prevotella* in diets' full field with polysaccharide foods, in contrast with a long-term diet rich in saturated fat, in which the microbiota is represented by *Bacteroides* strains [14]. The colonic pH (especially the decrease from 6.5–5.5) plays an important role, with the tendency to suppress *Bacteroides* spp. and promotes butyrate-producing Gram-positive bacteria [15]. After absorption into the small intestine, the phenolic compounds are involved into Phase I reaction (oxidation, reduction and hydrolysis) or, particularly, Phase II biotransformation (conjugation) in the enterocytes and hepatocytes, thus resulting water-soluble metabolites (methyl, glucuronide and sulphate derivatives), released into the blood circulation in order to achieve the organs where the effect is developed or excreted into the urine. But, since all human hosts have their own unique signature of the intestinal complex of bacteria, like the fingerprint, human intestinal microbiota composition can modulate the polyphenol impact on health (**Figure 2**).

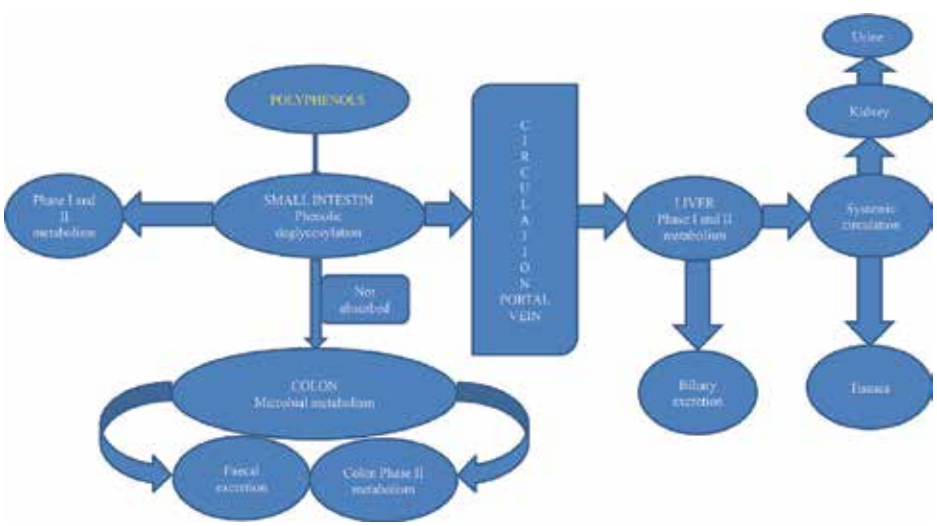

Figure 2. Routes for dietary polyphenols and their metabolites in humans.

4. Influence of phenolic compounds in gut microbiota composition

The level of biotransformation complies with two factors: the chemical structure of the polyphenol, related to the metabolite that can be absorbed and the properties that can generate a beneficial effect, and the composition of gut microbiota, because specific biotransformation requires particular species or strains with special genes for specific enzymes (**Figure 3**).

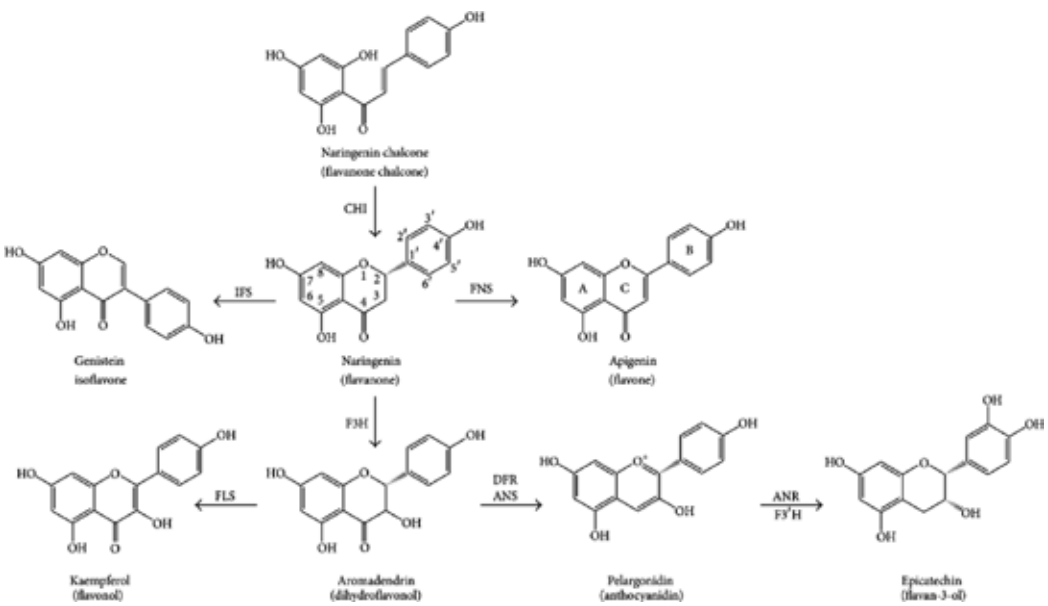

Figure 3. Biosynthesis of the flavonoid families [12].

4.1. Flavonoids

4.1.1. Flavanones and flavonols

The most common flavanones are: hesperitin, naringenin, naringin, hesperidin, abundant in citrus fruits and tomatoes. Well-known flavonols are quercetin and rutin, found as glycosylate derivatives in onions, capers, apples, broccoli, grapefruit and plums. The position of the hydroxyl group may influence the degradation of the compound, related with the glycosidic bond (C- or O-glycoside) and the degradation rates, which seems to be much slower for the C-glycosidic metabolism than for the hydrolysis of the O-glycosidic bond (**Figure 4**). From this point of view, the slow-degrading compound will be more bioavailable, because they can be greater absorbed than the ones that are quicker degraded in the colon.

In different studies involving six bacterial strains (*Bacteroides galacturonicus*, *Lactobacillus* sp., *Enterococcus caccae*, *Bifidobacterium catenulatum*, *Ruminococcus gnavus* and *E. coli*), different concentrations exerted an inhibitory effect on the growth of all analysed bacterial species. For hesperetin, the effect was weaker. This fact was explained by the dependency of the potential of these compounds on the sugar presence/absence in the moiety [16].

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In the presence of different flavonols (galangin, kaempferol, fisetin, quercetin), *Bifidobacterium adolescentis* is conducted to an anti-inflammatory effect, in the presence of nitric oxide, to which fisetin was the first responsive, by decreasing the nitric oxide with 76% [17].

Another study that underlines the viability of four bacterial strains (*E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium* and *L. rhamnosus*) was tested among pure polyphenols, in a range of concentrations between 62.5 and 1000 μg/mL. All tested flavanols decreased the bacterial growth but especially quercetin and naringenin, with the lowest minimum inhibitory concentration (MIC) values. *S. aureus* was the most sensitive, while *L. rhamnosus* required a MIC of 125 μg/mL [18].

Following their bioavailability, flavonols and flavanones are antiviral, in vitro assays performed with quercetin showed an increased bacterial cell membrane, diminishing cell motility, an important factor of the bacterial virulence [12]. From literature data results, quercetol and its heteroside (rutoside) have the greatest therapeutic potential. From this point of view, flavones, especially quercetol, are evaluated for their antioxidant capacity correlated to their use in treatment of different diseases induced by reactive oxygen species (ROS) liberation. These substances act at cellular membrane level (reduce lipid per oxidation and increase membrane resistance) and endoplasmic reticule.

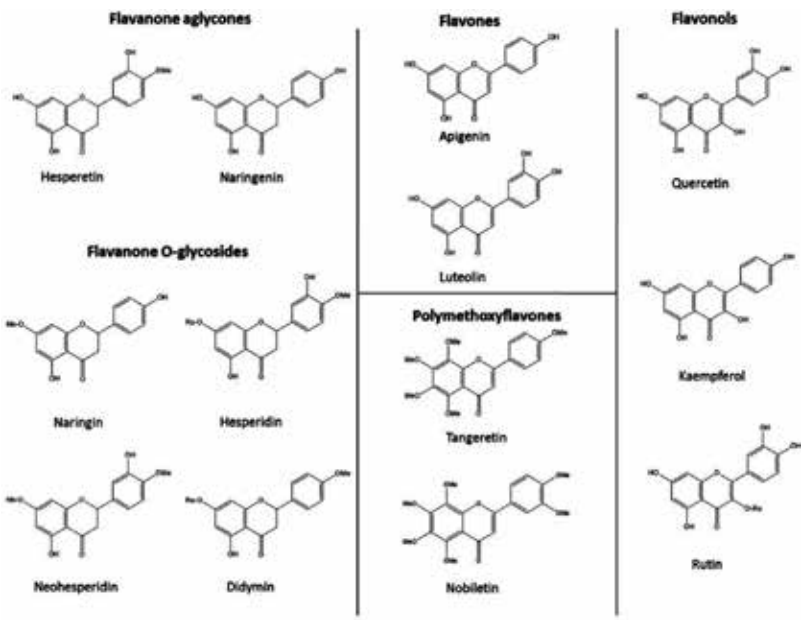

Figure 4. Molecular structure of *Citrus* flavonoids [12].

Antioxidative capacity of flavones from selective dried extracts can be determined in aqueous as well as in different concentrations of alcoholic solutions.

The methods used for antioxidant capacity assessment are based upon antioxidants' possibility to annihilate free radicals action by hydrogen atom transfer (HAT) method or an electron

transfer single electron transfer (SET). Using these methods they can reduce different oxidant substances. HAT methods are solvent type and pH range independent and most rapid than SET methods. The most cited methods for antioxidant capacity determination are ferric reducing antioxidant power (FRAP) (from SET methods) and trolox equivalent antioxidant capacity (TEAC) (from SET and HAT methods).

Previously conducted researches on mice poisoned with lead acetate and treated with quercetol revealed quercetol role in lowering enzymatic activity of superoxide dismutase (SOD), catalase, demonstrating indirect action as possible antidote (ratio of lead acetate bioinactivation/ quercetol = 2.18).

4.1.2. Flavanols

(−) Epicatechin and (+) catechin, flavanol monomers, in a range of concentrations of 150–1000 mg/mL promoted the growth of *Eubacterium rectale*, *Clostridium coccoides*, the lowest concentration being registered for *Lactobacillus* spp. and *Bifidobacterium* spp. [19].

The characteristic carbonyl group lacks from the structure of flavan-3-ols aglycones, at C4 (as found in flavonols and flavanones). This may be the reason to understand its transformation by colonic microbiota which modifies other types of flavonoids, as *Eubacterium ramulus*.

Once the initial gallate esters have been metabolised, the aglycones give rise to diphenylpropan-2-diol through a C-ring opening, which is further converted into 5-(3′, 4′-dihydroxyphenyl)-γ-valerolactone. This lactone ring opens and gives rise to 5-(3, 4-dihydroxyphenyl)valeric acid. Further transformations generate OH-phenylpropionic and hydroxy-benzoic acids (**Figure 5**) [2].

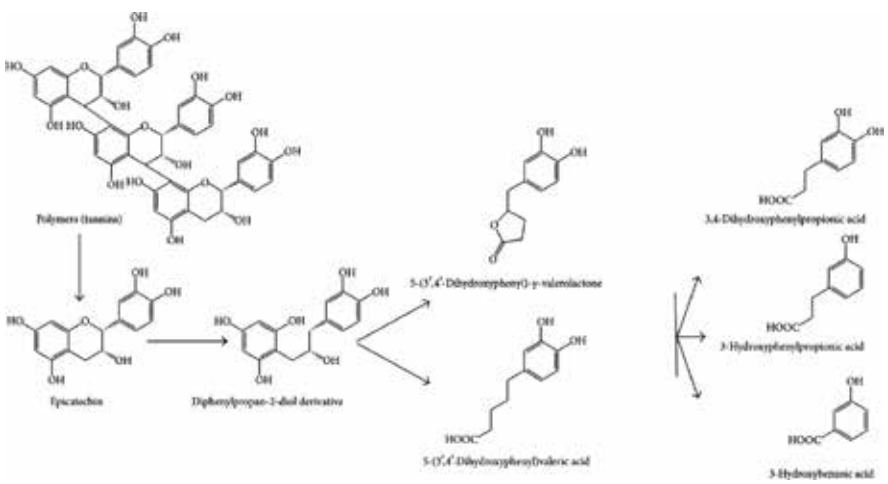

Figure 5. Colonic degradation of epicatechin tannins.

Several studies are focused on the main catechin of green tea leaves, epigallocatechin-3-gallate, that have been reported for the anti-infective properties. The inhibition effects refer to hepatitis

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C virus, HIV-1, influenza virus, adenovirus, Epstein-Barr virus and herpes simplex virus. The mechanism involved is related to the attachment of virions to cells downregulating CD4 cell surface receptor expression; the inhibition of the proviral genome at the time of integrating into the host cell, by binding between the integrase and the viral DNA; also the inhibition of endosomes and lysosomes required for the fusion of viral and cellular membranes; and the inhibition of muraminidase activity responsible for preventing self-aggregation of virus particles [12].

It is to underline that epigallocatechin-3-gallate inhibits epimastigotes' growth of *Trypanosoma cruzi* and the binding of *Plasmodium falciparum* to the ICAM-1 cellular receptor, related to malaria. The lethal mitochondrial damage above *Leishmania donovani* and *Leishmania amazonensis* has been explained by the inhibition of the parasite arginases.

In vitro studies, the main tea phenolic aglycones (3-O-methylgallic acid, gallic acid and caffeic acid), on pathogenic intestinal bacteria inhibited the growth of *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides* subspp. Caffeic acid exerted a strong inhibiting effect above *E. coli*, *Salmonella*, *Pseudomonas*, *Clostridium* and *Bacteroides*.

4.1.3. Isoflavones

Most of the studies performed on isoflavones showed an important effect in the postmenopausal period. Greater concentrations of isoflavones are found in plants from the Fabaceae family (soy, lentils, beans, chickpeas).

All isoflavones (daidzein, genistein, formononetin) are glycosylated and therefore are not absorbed across enterocytes, because they have great polarity and molecular weight. Their bioavailability implies the conversion of glycosides into bioactive aglycones, following the pathway of β-glucosidases from the small intestine (*Lactobacillus*, *Bifidobacterium*). The most important isoflavone, daidzein, is metabolised depending on the gut microbiota. Some individuals produce (s)-equol through dihydrodaidzein and tetrahydrodaidzein, and other produce O-desmethylangolensin, generated by *Clostridium* sp. We can distinguish two types of individuals: (s)-equol producers and non-producers (**Figure 6**). Because of the antioxidant potential performed by the non-polar molecule, due to the penetration more reliable in the cell membrane, (s)-equol binds to the estrogenic receptors, downregulating their activity, with a potential application in breast and prostate cancer therapy.

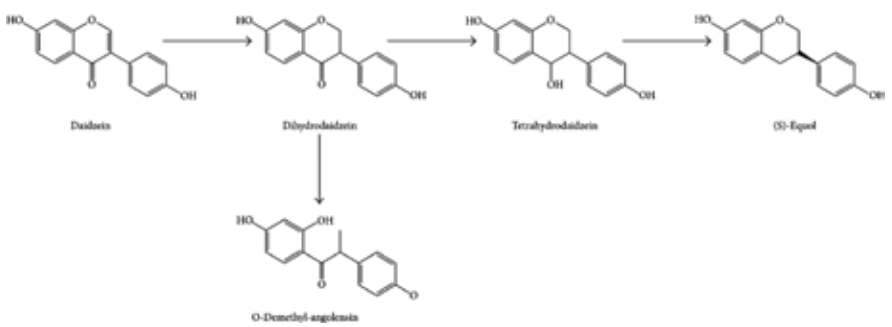

Figure 6. Colonic formation of (s)-equol and O-demethylangolensin from the isoflavone daidzein [12].

4.1.4. Condensed tannins (proanthocyanidins)

The studies performed using tannins introduced in rat diet concluded that animals with gut microbiota enriched with *Enterobacteriaceae*, *Prevotella* and *B. fragilis* had a condensed-tannins diet [20].

Mostly, proanthocyanidins, in their monomeric, oligomeric or polymeric forms, are responsible for the red, blue and purple colour of fruits, of flowers and in a lower manner of leaves, showing an important antioxidant role. Although a lot of these compounds enter the colon and they are depredated by β-glycosidase by the gut microbiota (especially by *Lactobacillus casei*, *Lactobacillus acidophilus* and *Bifidobacterium lactis*), the mechanism that can explain the antimicrobial mechanism is correlated with the disintegration of bacterial membrane, with the presence of amounts of cytoplasmic material and membrane debris outside the cells. Extracts from bilberries and blueberries showed inhibitory effects on the growth of Gram-positive and Gram-negative bacteria but without a biological effect on yeasts.

4.2. Non-flavonoids

4.2.1. Stilbenes

Resveratrol (3, 5, 4′-trihydroxy-trans-stilbene) has antimicrobial effect against pathogenic agents, shown after the administration of this compound to a DSS-induced colitis rat model. An important increase in lactobacilli and bifidobacteria, associated with a decrease of enterobacteria, was observed after a trial of 20 days [21].

4.2.2. Hydrolysable tannins

This class includes gallotannins and ellagitannins, present in raspberries, cranberries, strawberries, grapes and pomegranates. The difference between the two compounds arises at the gut microbial hydrolysis level, glucose and gallic acid for the first one and ellagic acid for the second one (**Figure 7**). This is metabolised in the colon to urolithin A and this monohydrox-

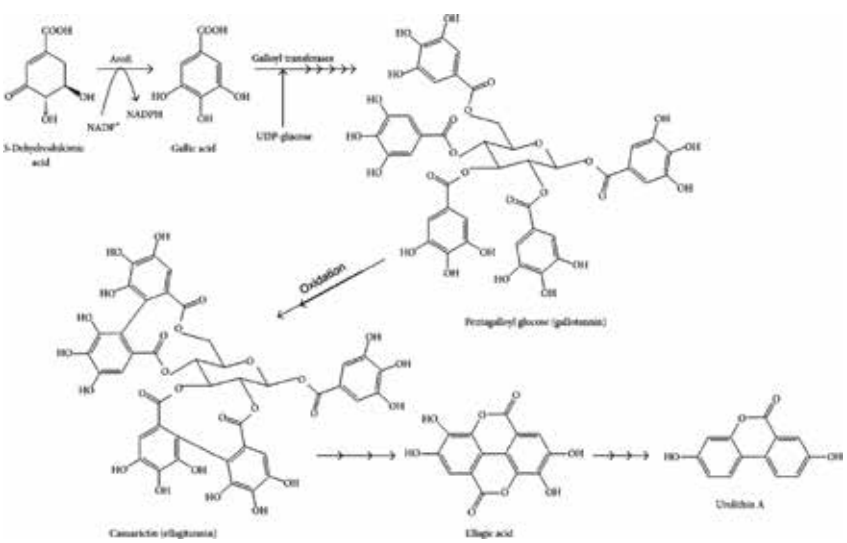

Figure 7. Biosynthetic steps for generation of two hydroxycinnamic acid polymers: ellagitannins and gallotannins [12].

ylated derivate to urolithin B. Of course, the complexity of the microbiota from the colon is responsible for the ellagitannins' metabolisation.

Pomegranate (*Punica granatum*) polyphenols have a suppressive activity against influenza virus A, due to punicalagin, who blocks the replication process of the virus and inhibits the agglutination of red blood cells by the virus. Also, the ellagitannins from this fruit are effective against *S. aureus*, *Salmonella*, *Listeria monocytogenes* and *E. coli* [22], and the ellagic acid inhibits the biofilm of methicillin-resistant *S. aureus* [23].

For ellagic acid, it was described an in vitro antimalarial activity, especially *P. falciparum* strains, regardless the levels of chloroquine and mefloquine resistance, influencing the mature trophozoite and young schizont stages, respectively, the proteins and nucleic acid synthesis. Also, ellagic acid potentiates the activity of current antimalarial drug [24].

4.2.3. Chlorogenic acids

Caffeic acid, ferulic acid and p-coumaric acid are abundant in peaches, plums and coffee seeds.

The main microbial metabolites of caffeic acid are 3-hydroxyphenylpropionic and benzoic acids, under the action of *E. coli*, *B. lactis* and *Lactobacillus gasseri*. The metabolites formed by ferulic acid in the colon are 3-(4-hydroxy-phenyl)-propionic acid and vanillin.

The role played by the gallic acid is underlined against the synthesis of the biofilms by different bacteria (*E. coli*, *Pseudomonas aeruginosa*, *S. aureus* or *Listeria monocytogenes*), the human rhinoviruses, enteroviruses and herpes simplex virus type 2. Gallic acid from *Terminalia nigrovenulosa* bark has shown strong antifungal activity against *Fusarium solani* and *Meloidogyne incognita* [25, 26].

5. Polyphenols-microbiota relationship, at a glance

The interaction between the phenolic compounds as dietary components and gut microbiota has gained a lot of attention in the last years, due to their bioavailability and the interest in human health. Although we acknowledge about this relationship from data literature, studies are lacking, making difficult understanding the exact mechanism of each compound. In vivo studies, focused on the ethical and economic issues, are also difficult to translate into in vitro conditions. It is clear that dietary polyphenols contribute to the maintenance of the human health, especially the gut, by stimulating the growth of beneficial bacteria and the inhibition of the pathogen ones, exerting prebiotic-like effects. A better understanding of the interaction between dietary polyphenols and gut microbiota through the emerging advances would be essential in order to identify genes and micro-organisms involved in polyphenol inactivation and conversion and, thus, to elucidate the implications of diet on the modulation of microbiota for achieving health benefits.

Acknowledgements

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Author details

Daniela Elena Popa[1]*, Cristina Manuela Drăgoi[2], Andreea Letiția Arsene[3], Ion Bogdan Dumitrescu[4], Alina Crenguţa Nicolae[2], Bruno Stefan Velescu[5] and George T.A. Burcea-Dragomiroiu[1]

*Address all correspondence to: danielafarmacie@yahoo.com

1 Department of Drug Control, Faculty of Pharmacy, University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania

2 Department of Biochemistry, Faculty of Pharmacy, University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania

3 Department of General and Pharmaceutical Microbiology, Faculty of Pharmacy, University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania

4 Department of Pharmaceutical Physics and Informatics, Faculty of Pharmacy, University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania

5 Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania

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Chapter 3

Application of Phenolic Compounds for Food Preservation: Food Additive and Active Packaging

Sara Martillanes, Javier Rocha-Pimienta,
Manuel Cabrera-Bañegil,
Daniel Martín-Vertedor and
Jonathan Delgado-Adámez

Additional information is available at the end of the chapter

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Abstract

Phenolic compounds are well known for their health benefits related to antioxidant activity. In addition, this kind of compounds can be extracted from natural sources, such as olives, grapes, fruits, vegetables, rice, spices, herbs, tea and algae, among others. In this way, these compounds have increased their popularity and, little by little, the consumers are more interested in these compounds due to the fact that they come from natural sources and because they have health biological activity. In fact, other important characteristics associated to phenolic compounds are the antimicrobial activity, because phenolics have the capacity of retarding the microbial invasion in some products and avoiding the putrefaction of others, mainly fruits and vegetables. These properties allow phenolic compounds to be suitable for numerous food preservation applications. Therefore, different kinds of products can be fortificated with phenolic compounds to extend the shelf life of some foods, to turn them in functional food or to incorporate them in food packaging. Active packing is an innovative strategy where phenolic compounds can play an important role for improving the global assessment and extend the shelf life of commercial products.

Keywords: biopreservatives, sampling, antioxidant, antimicrobial, food packaging

1. Introduction

For the food industry, phenolic compounds have potential use as biopreservatives. In fact, phenolic compounds have been extensively studied for their application in the food industry for improving the shelf life of perishable products. The use of phenolic compounds from natural sources in food is an interesting opportunity for the application of their biological activities

and allows the production of food without synthetic additives for consumers, because the current concern about the impact of food on health has been influencing the consumer choice of food based on its formulation.

The organoleptic properties and safety of perishable food could be altered by several processes, which include microbial spoilage or oxidation processes. The value of the food is decreased by the destruction of substances, such as proteins, vitamins or fatty acids that have beneficial properties. Production of off-flavors and odors is also decrease the value of the products and, in the worst case, foodborne illnesses transmission related to unsafe food intake. Moreover, changes in food consumption patterns and markets internationalization have caused changes in the retail and distribution practices, with an increased distribution distance and longer storage time. Accordingly, the improvement in the shelf life of perishable foods has become a challenge for the agri-food industry worldwide. The biological activity of phenolic compounds provides a protective effect against deterioration of foods.

In recent years, consumers demand natural food ingredients because of its safety and availability. In this sense, phenolic compounds are beginning to replace chemical additives in food and are perceived to be safer and claimed to alleviate safety concerns. There are many methods of application, but the main is direct addition of phenolic compounds to products. Despite this, a large amount of initiatives have been made to find alternative solutions to the aim of avoiding undesirable inactivation. Spraying, coating and dipping treatment of food are currently applied to product prior to packaging as valid options. The numerous experimental applications of phenolic compounds to various fresh perishable foods demonstrate that they are well-suited to be utilized as preservatives in foods and could be often valid alternatives to synthetic food additives.

Thus, the potential value of phenolic compounds as biopreservatives is considered for the safe extension of perishable products shelf life and these substances can be used to delay or inhibit the oxidation and growth of microorganisms. However, in food applications, the phenolic compounds could be influenced by food components, processing and storage. This chapter will be review the recent research on the application and mode of action of phenolic compounds in perishable food.

2. Phenolic compounds and antioxidant activity

Phenolic compounds, commonly known as polyphenols, are secondary metabolites of plants generally involved in defense against ultraviolet radiation or aggression by pathogens. This wide family of compounds can be found in an extensive range of food and by-products food, as well as beverages, medicinal herbs or spices. In the last part of twentieth century, the interest in food phenolics has increased due to their antioxidant and free radical-scavenging abilities [1] and a large amount of assays has been developed to measure these properties, including hydrogen atom transfer (HAT), single electron transfer (ET), reducing power, and metal chelation, among others. Total phenolic content is a parameter that provides an indirect measure of antioxidant activity. Last trend is the determination of antioxidant activities in food models [2].

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There are many sources of antioxidant, and their values are going to be listed below:

Olives (*Olea europaea* L.) and olive oil are well known for their health benefits related to its large amount of phenolic compounds; verbascoside, ligstroside and oleuropein are some examples. The antioxidant ability of phenolic extracts of olive fruits in different maturation stages and different cultivars have been studied by many researches, like Ziogas et al. [3]. By-products obtained in the olive oil extraction are an excellent source of phytochemicals because of their low toxicity, limited costs, broad availability. In this sense, Ramos et al. [4] and Martín-Vertedor et al. [5] have studied olive mill and leaves extract like natural antioxidant, respectively.

Grapes (*Vitis vinifera* L.) and their products are particularly rich on phenolic antioxidant, Carlsen et al. [6] found an antioxidant content between 0.69 and 1.74 mmol/100 g in different grape juices. For its part, grape seeds contain polyphenols, mainly the monomerics catechin, epicatechin and gallic acid, and the polymeric and oligomeric procyanidins. Many authors have focused their investigations in the antioxidant potential of this by-product. Delgado-Adámez et al. [7] aimed to investigate the antioxidant activity of natural extracts of grape seeds extracted from juice and grape seeds extracted after wine manufacture. The antioxidant activity of olive and grapes is showed in **Table 1**.

Matrix	Antioxidant activity	Assay	Reference
Olive fruits	19.0–49.6 μmol AA/g FW	FRAP	[3]
Dry olive mil residue	EC50 24.7–29.56 μg/mL	DPPH	[4]
Olive leaves	15.60 mmol Trolox/kg extract	ABTS	[5]
Grape seed extracts	57.48 ± 3.6% inhibition	DPPH	[7]

Table 1. In vitro antioxidant activity of olives, grapes and their by-products.

The number of studies focused on other fruits and vegetables like natural antioxidant sources have been increased last years. Fruits such as pomegranate, cherry, berries or citrus and a wide range of by-products obtained from these peel, seeds or stones have been studied for this purpose. According to Brat et al. [8], vegetables with the highest polyphenol concentration were artichokes, parsley and Brussels sprouts. Other vegetables found in the bibliography because of their antioxidant activity were spinach, broccoli, cauliflower and eggplants. In **Table 2**, antioxidant activity[22] of many fruits and vegetables can be observed.

The healthy benefits associated with the regular consumption of whole grain products have been reported during the last decades. These healthy properties are due to the presence of secondary metabolites with high antioxidant activity, including phenolic compounds. In this sense, rice (*Oryza sativa* L.) and rice bran, a by-product, have been used to obtain phenolic extracts with antioxidant activity. Its antioxidant activity is mainly due at phenolic acids such as ferulic or *p*-coumaric acid [23]. Butsat and Siriamornpun found between 86.7 and 85.9% of inhibition in 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) [9].

Matrix	Antioxidant activity	Assay	Reference
Pomegranate peel	5.50 μg/mL	DPPH	[16]
Cherry	EC50 6065.68 g.f.w./g DPPH	FRAP	
Blackberry	EC50 2142.42 g.f.w./g DPPH	FRAP	[17]
Strawberry	EC50 3778.94 g.f.w./g DPPH	FRAP	
Blueberry	EC50 7775.4 g.f.w./g DPPH	FRAP	
Orange	36.57 mg Trolox/L extract	ABTS	[18]
Artichokes wastewaters	43 mM de Trolox	DPPH	[19]
Parsley	EC50 2.62 mg/cm3	DPPH	[20]
Cauliflower	1.5 mmol TEAC/100 g f.w.	DPPH	[21]

Table 2. In vitro antioxidant activity of some fruits and vegetables.

The healthy benefits of tea are related to its high phytochemicals content with antioxidant properties. Among these, catechins are the most abundant and powerful compounds in tea [10]. Last years, many researchers have been aimed to obtain tea extracts with high polyphenolic content for different uses. In this way, new techniques as pulsed electric field treatment have been used in recent years [11] for the polyphenol extraction from fresh tea leaves with good yields. Moreover, Sousa et al. [12] suggest the use of ultrasound-assisted ultrafiltration process to purify catechins from green tea extracts.

Spices and herbs are traditionally used because of their antioxidant properties for food conservation. These properties are due, among others, to phenolic compounds. For example, rosemary extracts have been exhaustively investigated and applied due to its high concentration of two phenolic compounds, rosmarinic acid and carnosic acid. On the other hand, Carlsen et al. [6] found high antioxidant activities in clove, mint and cinnamon, among others. In the same way, Lu et al. [13] proved that antioxidant activity of black garlic come from phenolic compounds. In **Table 3**, we can observe the antioxidant activity of many spices.

Matrix	Antioxidant activity	Assay	Reference
Rosemary leaves	EC50 17 ± 9 μg/mL	DPPH	[22]
Clove	277.3 mmol/100 g	FRAP	
Mint	116.4 mmol/100 g	FRAP	[6]
Cinnamon	77.0 mmol/100 g	FRAP	
Black garlic	30% inhibition	DPPH	[13]

Table 3. In vitro antioxidant activity of spices and herbs.

Finally, we cannot forget algae as source of phenolic compounds. Many authors have studied the potential antioxidant effects of edible marine algae, as well as their phenolic compounds,

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some of them like phlorotannins, a type of tannins found in brown algae and bromophenols, exclusive to seaweed [14, 15].

3. Phenolic compounds and antimicrobial activity

Nowadays, regarding food, there is the tendency to consume more and more fresh and healthy products, and as similar to its original condition. Therefore, it has emerged the need to seek conservation alternatives, because it has been associated the consumption of chemical preservatives with poisoning. Many foods contain natural compounds with antimicrobial activity. In nature, these compounds can play the role of prolonging the life of food, even many of them have been studied for their potential as direct food antimicrobials. The antimicrobial agents of natural origin interest (vegetable derived) is increasing, so now it seeks a combination of two or more factors which interact additively or synergistically controlling the microbial population. In general, more and more plants or parts of these that contain natural antimicrobial are discovered, for example, which include phenolic compounds from bark, stems, leaves, flowers,... so not only we have more security, but better quality, because this type of antimicrobials are considered as potentially safe sources [23]. Herbs and plants antimicrobial activity is generally attributed to the phenolic compounds present in extracts or essential oils, and it has been observed that fat, protein, salt concentration, pH and temperature affect the antimicrobial activity of these compounds [24]. It is estimated that 1–10% of about 500,000 species of plants in the world have use as food. Most of food antimicrobials are only bacteriostatic (prevent the development of germs) or fungistatic instead of bactericides (destroy germs) or fungicides, so its effectiveness is limited. On the other hand, due to that some microorganisms cannot be inhibited or destroyed by conventional doses of antimicrobials used individually, it is preferable to use a combination of them, expanding its spectrum of action [23]. The problem of microbial food spoilage has obvious economic implications for manufacturers, distributors and consumers [25]. The food quality is affected by physical, chemical, biochemical and microbiological factors, and the control of these factors, in particular microbiological factor, is essential for food preservation. The use of food additives of natural origin involves the isolation, purification, stabilization and incorporation of these compounds to food with antimicrobial purposes, without this affecting adversely the sensory characteristics, nutritional and its health guarantee.

3.1. Phenolic compounds

Phenolic compounds such as caffeic acid, chlorogenic, *p*-coumaric and ferulic are present in parts of plants that are used as spices. The antimicrobial activity of these and other acids as hydroxycinnamic and cinnamic may retard microbial invasion as well as fruit and vegetable putrefaction. Gram-positive and Gram-negative bacteria molds and yeasts commonly found as deteriorative organisms are sensitive to hydroxycinnamic acid derivatives. Caffeic, ferulic and *p*-coumaric acids, for example, inhibit *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. Other phenolic compounds, which have demonstrated antimicrobial activity,

are tannins and tannic acid, the latter for example is inhibitory to *Listeria monocytogenes*, *E. coli*, *Salmonella enteritidis*, *S. aureus*, *E. faecalis* [25] and *Aeromonas hydrophila* [26].

Phenolic compounds such as flavonols, typically present in fruit and green tea, have antibacterial activity. Thereby, Puupponen et al. [27] showed that myricetin, which is used as pure chemical compound, inhibited the growth of lactic acid bacteria derived from the flora of the gastrointestinal tract of humans, but not affect the growth of *Salmonella*, whereas extracts prepared directly from strawberries, raspberries and others were strong inhibitors of *Salmonella* and *E. coli* [28].

3.2. Spices and herbs

Many spices and herbs exhibit antimicrobial activity, such as celery, coriander, laurel, basil, angelica, leeks, horseradish, mint, thyme, among others. The compounds in spices and herbs that have antimicrobial activity are simple and complex phenol derivatives. Generally, spices are more effective against gram-positive, that against gram-negative organisms:

- Cinnamon, cloves and mustard: great preservative power.
- Black/red pepper, ginger: weak inhibitors against a wide variety of microorganisms.
- Pepper, bay leaf, coriander, cumin, oregano, rosemary, sage and thyme: intermediate activity.
- Other: anise, mint, fennel, celery, dill, turmeric.

The conservative function is due to the essential oils of these spices and herbs, whose composition have phenols as thymol or carvacrol, as well as other compounds as eugenol or cinnamic aldehyde with antimicrobial power.

But there is a major drawback in its use and the problem is that it is necessary a high concentration to obtain a preservation effect, and therefore, there are alterations in flavor. Consequently, the use of herbs as antimicrobial agents is limited to food in which the change in taste is considered desired [29].

3.2.1. Oregano

There are a lot of studies about the antimicrobial activity of extracts of different types of oregano. Specifically, it has been evaluated the antimicrobial activity of the individual components as well as the essential oil. The carvacrol and thymol phenols have the highest levels of activity against Gram-negative microorganisms, such as *Salmonella typhimurium, E. coli, Klebsiella pneumoniae, Enterobacter cloacae* and *Yersinia enterocolitica*; thymol being the most active. Also against Gram-positive bacteria such as *Staphylococcus aureus, Staphylococcus epidermidis, L. monocytogenes* and *Bacillus subtilis* [30]. And against fungal species such *as Candida albicans, C. tropicalis, Torulopsis glabrata, Aspergillus niger, Rhodotorula* and *Geotrichum*.

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3.2.2. Cinnamon extract

Cinnamic aldehyde is a phenolic compound of some species, including cinnamon. It is generally recognized as safe for use in foods, and it is used in many foods as a flavoring. This not only exhibits antibacterial activity but also inhibits the growth of molds, production of mycotoxins and it may reduce aflatoxin production by 99%.

3.3. Other extracts

Plant extracts from grape seeds, grapefruit seeds and green tea are important sources of polyphenolic compounds and phenolic acids with significant antibacterial and antioxidant activity [31]. These compounds impart an inhibitory effect against gram-positives.

3.4. Phenolic compounds and antimicrobial mode of action

Phenolic compounds mode of action has not yet been determined. However, it has been observed that these can inactivate essential enzymes, react with cell membrane or alter the function of the genetic material.

The active components of essential oils can vary in its composition because it can be affected by certain variables such as plant genotype, different extraction methods, geographical location as well as environmental and agronomic conditions. Hydroxycinnamic acids and esters, due to their propenoid side chain, are less polar than the corresponding hydroxybenzoic acids which, facilitates their transport across the cell membrane [32].

Antimicrobials attack mechanism within a cell is carried out in parts and/or important functions for cell survival. It can be carried out in the cell wall, cell membrane, protein synthesis and genetic elements; this can cause irreparable damage to a cell. Several natural antimicrobials are not yet known its mode of action, but as these act differently, combinations of these can lead to better results [33].

Part of polyphenols antimicrobial properties has been attributed to their chelating properties complexing metal ions that are essential for the bacterial growth. The cell walls of Gram-negative bacteria represent a great barrier for polyphenols to get into cell cytoplasm; only in some studies grape seed extracts have been reported to inhibit Gram-negative bacteria [34].

There are few studies focused on understanding the mechanism involved in inhibition of microbial by spices and essential oils. However, phenolic structure of many of the compounds with antimicrobial activity present in the spices and their essential oils, suggest that the mode of action should be similar to other phenolic compounds.

In many cases antimicrobials may have no effect until a critical concentration is exceeded. Thyme extracts were used at different concentrations to inhibit *Salmonella typhimurium* by Raibaudi [28]. These researchers found that there was a critical concentration in which the extract had antimicrobial effect, and at lower concentrations the phenolic compounds did not have antimicrobial activity. The authors indicated that the phenolic compounds can sensitize

the cell membrane, and saturate the sites of action, therefore, the cell suffers a serious injury, causing the membrane collapse.

4. Phenol fortification of food products

Referring to meat industry, the meat products are vulnerable to lipid oxidation. One way of measuring the degree of lipid oxidation is the thiobarbituric acid reactive substances (TBARS) method. Although, initially, synthetic antioxidants have been used with the purpose of avoiding the lipid oxidation, the new tendencies are finding them in natural sources and applying into the meat. In order to prevent undesirable lipid oxidation changes, a 0.2% of bee pollen was effective in retard lipid oxidation in pork sausage during 30 days of storage at 4°C. It showed lower values of TBARS along the experiment than control [35] being the highest percentages of decrease in TBARS after 10 days of storage. Cocoa and grape seed extracts allowed to enrich in phenol Spanish dry fermented sausages without affecting the sensory profile [36] and the incorporation kordoi (*Averrhoa carambola*) fruit juice extract in pork nuggets allowed increasing the storage life of them from 21 to 35 days compared to the pork nugget without extract [37] and the TBARS values of enrichment of 4% juice and 6% of extract were lower during the 35 days compared to control. In addition to lead lo attenuation effect against lipid oxidation, although the only addition of green tea extract directly in hamburger allowed reduction of TBARS during 8 days of storage, the incorporation of green tea extracts in combination with chitosan in hamburgers achieved more resistant to lipid oxidation at the end of storage and to microbiological deterioration [38]. A common strategy of enrichment of phenol content in food is the olive leaves extracts. The treatment consisted of incorporation of olive leave extract in pork patties, whose TBARS values were significantly decreased by increasing amount of grape seed flour ranged from 0.5 to 5.0%. Consequently, it also delayed protein and lipid oxidation [39]. The addition of spice extracts in chicken meat not only was effective against microbial growth and lipid oxidation but CIE a* values and sensory color and odor were also improved referred to the use of butylated hydroxyl toluene during storage for 15 days at 4°C [40]. Over storage period, TBARS values were lower in the samples control with of 1.0 and 0.5% of different spices extracts in chicken meat. This kind of addition was more effective and provided TBARS values considerably lower during 20 days of storage than the chicken meat without spice extract fortification in storage temperature the range from 4 to 20°C [41]. A typical non meat product susceptible of oxidation is the margarines but it could reduce in relation with vitamin E-enriched margarine with using *Opuntia ficus-indica* peel extract [42].

Although pomegranate peel extract was also effective on retarding lipid oxidation and protein oxidation beef meatballs [43], another kind of application of pomegranate of peel extract is its capacity as melanosis inhibitor during the refrigerated storage of Pacific white shrimp in addition to retard the mesophilic, psychrophilic, lactic acid bacteria and enterobacteriacea counts [44]. Noticeable improvements of color of foodstuff such as strawberry and red radish can be achieved by intermolecular co-pigmentation phenolic mango peel extracts [45].

In relation with vegetables oils, phenolic extract can be used for improving their frying characteristical by means of enrichment in oregano whose phenols such as rosmarinic acid protected the endogenous antioxidants from degradation [46]. In this same line, a concentration of at least

400 mg/kg of polyphenols extracted from olive vegetation is enough to reduce oxidation of the tocopherols of refined oils in more efficient way than synthetic antioxidant [47] and the incorporation of 100, 200, 400 and 1200 mg/kg of determined groups of phenolic compounds allowed induction period determined by Rancimat test to increased from 4.15 (refined oil control) to 5.96, 6.90, 8.50 and 12.3 h, respectively. The incorporation of olive leaf extract in concentration of 120 and 240 mg per kg of oil improved the oxidative stability of frying olive oils as results of high retention of microconstituents [48]. In this study, the oxidative stability of fresh sunflower, olive and palm oil with addition of 240 mg per kg increased from 5.1, 13.3 and 34 from 7.7, 20.8 and 43.1 h, respectively, while the same oils submitted to fry ranges from 3.4, 9.8 and 31.0 to 5.9, 17.6 and 36.7, respectively. In an essay where natural bioactive compounds of olive leaves were profited, Delgado-Adámez et al. [49] found the oxidative stability of Arbequina olive oils increased by incorporation 100 mL of olive leaf extract referred to control, being the maxima oxidative stability (80 h) where these extracts where used in combination with lecithin as emulsionant. Furthermore, the incorporation of rosemary methanol extract in oil used for frying showed an enhanced stability retarding the oil oxidation throughout its use in frying due to phenolic compounds mainly carnosol and carnosic acid [50]. The olive oil enriched by phenolic compounds from thyme increased in more than 10 h of oxidative stability than control oil [51].

During Maillard reaction, which is necessary for the appearance and taste of foods, advanced glycation end-products can be generated and increase the risk for development of health disorders such as diabetes [52]. In this context, polyphenols fractions from decaffeinated tea and grape seed extract have been added in the elaboration of breads preventing the formation of the harmful advanced glycation products [53, 54]. In connection with other enzymatic reaction, rice bran extract phenols have the ability of inhibiting polyphenol oxidase and enzymatic browning activity effects on potato and apple [55]. In **Table 4** are summarized the principal quality improvement of phenolic compounds in food matrix.

Type of phenol extract added	Type of food matrix	Quality improvement
Bee pollen	Pork sausage	Retarding lipid oxidation in pork sausage during 30 days of storage at 4°C
Cocoa and grape seed extracts	Spanish dry fermented sausages	Enrichment in phenol without affecting the sensory profile
Kordoi fruit juice extract	Pork nuggets	Increasing the storage life of them from 21 days to 35 days
Green tea extracts in combination with chitosan	Hamburgers	Achievement more resistant to lipid oxidation at the end of storage and to microbiological deterioration
Olive leave extract	Pork patties	Delaying protein and lipid oxidation
Spice extracts	Chicken meat	Effectiveness against microbial growth and lipid oxidation. Improvement of sensory color and odor
Opuntia ficus-indica peel extracts	Margarines	Reduction of oxidation in relation with margarine with vitamin E
Pomegranate peel extract	Beef meatballs	Retarding lipid oxidation and protein oxidation

Type of phenol extract added	Type of food matrix	Quality improvement
	Pacific white shrimp	Capacity as melanosis inhibitor during the refrigerated storage. Retarding the mesophilic, psychrophilic, lactic acid bacteria and enterobacteriacea count
Mango peel extracts	Strawberry red radish	Improvements of color by intermolecular co-pigmentation
Oregano	Vegetable oils	Improvement frying characteristical. Protection the endogenous antioxidant from degradation
Olive vegetation	Refined oil	Reduction of oxidation of the tocopherols in more efficient way than synthetic antioxidant. Rancimat test to increased from 4.15 (refined oil control) to 12.3 h
Olive leaf extract	Sunflower oil Olive oil Palm oil	Improvement the oxidative stability both fresh and frying oil
	Arbequina olive oils	The oxidative stability increased
Rosemary methanol extract	Oil for frying	Enhancement stability retarding the oil oxidation
Decaffeinated tea and grape seed extract	Bread	Prevention the formation of the harmful advanced glycation products
Rice bran extract	Apple potato	Inhibition polyphenol oxidase and enzymatic browning activity effects

Table 4. Main quality improvement of phenolic compounds in food matrix.

5. Use of phenolic compounds in active food packaging and edible films

Bio-based and synthetic polymers are being functionalized with well-known antioxidants and antimicrobials such as phenolic compounds, showing excellent opportunities to prolong shelf life when used directly in contact with the food matrix or as part of combined strategies. However, the evaluation of the efficiency consists of a multidisciplinary approach and its effect in a food has to be studied case by case. This section provides an overview on the most recent scientific advances and a critical view of the benefits, limitations and future trends of the use of phenolic compounds in the active food packaging and edible films.

During distribution, the quality of the food product can be deteriorated biologically and chemically as well as physically. Traditional food packaging concepts are reaching their limits in prolonging shelf life. Therefore, novel packaging concepts, as active packaging, enable a further prolongation of shelf life. Active packaging can be defined as a t'ype of packaging that interacts with the packaging condition, extending shelf life and improving safety or sensory properties while maintaining food product quality [56]. Active food packaging has focused

their attention on bio-based functional packaging materials incorporating natural active compounds and ingredients. [57] Different incorporation mechanisms are currently being used:

- Addition of emitting sachets: This technique has been applied for volatile compounds such as timol and carvacrol. No direct surface contact occurs, and volatile compounds are released into the headspace of the package.
- Incorporation of absorbent pads: pads are one of the most successful applications of active food packaging. The food product is placed on the absorbent pad, and pad soaks up fluid or juice exuded by the food product that would otherwise collect on the bottom of food packaging or tray. The unsanitary juices immobilized in pads might, however, originate undesirable odors, spoil the food quality or promote the propagation of foodborne pathogens. Phenolic compounds may be incorporated into the absorbent pad, adding functionality to packaging.
- Dispersion of phenolic compounds in the packaging polymer: Phenols can be incorporated by extrusion, heat-press or casting. The main disadvantage of extrusion is the use of high temperatures and shearing forces that can reduce biological activities (antioxidant and antimicrobial).
- Coating or dipping: Coatings and edible films serve as carriers of phenolic compounds and are in direct contact with the food surface. The advantages of this method are that the compounds are not exposed to excessive heat and can be applied at any stage of the food supply chain.

Independently of the technique used for incorporation, the phenols packaging systems are divided in two main categories: (1) those in which the phenolic compounds migrates from the package into the food, and (2) those in which the phenolic compounds remains immobilized in the package.

5.1. Scope of applications

Phenolic compounds can be incorporated into polymers or into carriers that may be extruded or coated into packaging materials. Applications of active packaging are numerous and growing. **Table 5** and **Table 6** provide a synopsis of current and future applications of the use of phenolic compounds in the active food packaging and edible films.

5.2. Recent trends and regulatory aspects

While in the United States, Japan and Australia, active packaging systems are already being successfully applied to extend shelf life or to monitor food quality and safety, in Europe, the development and application of active packaging systems are limited. This can be explained by the legislative restrictions, as there are no specific regulations on the use of active packaging in Europe [76]. In addition, fear of consumer resistance, lack of knowledge about effectiveness, economic and environmental impact of active packaging have to be taken into account.

Sources of phenolic compounds	Main results	References
Several phenol compounds: acids, essential oils components and dopamine.	Some pathogens were significantly reduced (*Staphylococcus aureus and Escherichia coli*)	[58]
Carvacrol	Carvacrol could therefore be used in active packaging formulations as its release from the polymer matrix can be controlled.	[59]
Aqueous green tea extract	Enhanced polyphenolic content and antioxidant activity of the films.	[60]
Tea polyphenols	Increased the antioxidant activity of the films.	[61]
Zataria multiflora Boiss and Mentha pulegium essential oils	Exhibit antimicrobial activity	[62]
Essential oil of oregano, rosemary, and garlic.	The results of this study suggested that the antimicrobial activity of some spice extracts were expressed in a WPI based edible film	[63]
Rosemary essential oil	Exhibit antimicrobial activity	[64]

Table 5. Examples of phenolic compounds for potential use in food packaging materials.

Sources of phenolic compounds	Type of food matrix	Main results	References
Barley husks	Salmon	Increasing the oxidative stability	[65]
Barley husks	Blue shark muscle	Exhibit antioxidant capacity	[66]
Brewery residual stream extract and commercial rosemary extract	Beef	Active packaging films enhanced oxidative stability of beef during refrigeration.	[67]
Catechin and quercetin	Different food simulants	Can improve food stability	[68]
Green tea extract	Pork sausages	Shelf life extension	[60]
Green tea extract	Several foods	Exhibit antioxidant capacity	[69]
Essential oil of cinnamon, oregano, and clove.	Different food simulants	Exhibit antimicrobial activity	[70]
Rosemary and oregano	Fresh lamb steaks	Extended fresh odor and color from 8 to 13 days compared to the control.	[71]
Grape seed extract	Pork loins	The active packaging in a significant shelf life extension.	[72]
Zataria multiflora Boiss essential oil and grape seed extract.	Mortadella sausage	The active packaging in a significant shelf life extension.	[73]
Grapefruit seed extract	Ground beef	Preserving beef quality	[74]
Grapefruit seed extract	Table grapes	Exhibits antifungal and antioxidative activity	[75]

Table 6. Relevant examples of phenolic compounds applied to active food packaging and edible films.

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The legislation that applies to traditional packaging also use for active packaging, which requires that compounds are registered on positive lists and that the overall and specific migration limits are respected. This is more or less contradictory to the concept of some active packaging systems that require a sensor of some kind being in direct contact with food products and some substance may migrate from the sensor into foods [76]. These migrations could be intentional or unintentional, and the type, amount and possible health effects of the substance must be determined in order to regulate the use of them.

Meanwhile, the U.S. Food and Drug Admisnitration (USFDA) (1997) classifies essential oils and the majority of natural extracts as generally recognized as safe (GRAS). However, there are regulatory limitations on the accepted daily intake of essential oils or essential oil components, so before they can be used in food products, a daily intake survey should be available for USFDA. In Europe, essential oils fall under Regulation 1334/2008 on natural flavorings; and natural plant extracts have been considered under Directive 2002/46/EC on food supplements.

The food industry main concern about introducing active components to packaging seems to be that consumers consider the components dangerous. Before the food industry can decide on the best active packaging technique, studies are needed in markets to evaluate consumer attitudes towards these techniques. It is also important that the food producer, retailer, and the consumer be in tuned with the compounds used in active packaging. Attitudes must be willing to accept new technologies and those involved in each step of the food chain must be sure that the new system is safe and true for the user. Phenolic compound are often perceived favorably by consumers as healthy or natural foods.

Recent technological advances in active packaging are discussed, and food related applications are presented.

Furthermore, the benefits of active packaging need to be considered in a global approach to environmental impact assessment. The environmental effect of plastics-based active packaging will vary with the nature of the product/package combination. The additional ingredients need to be evaluated for their environmental impact. Active packaging is an emerging area of technology which can confer many preservation benefits on a wide range of foods. As more companies become aware of the economic advantages of using absorbent technology, and consumers accept this approach, the technology will likely emerge as the preservation technology of the twenty first century.

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Author details

Sara Martillanes, Javier Rocha-Pimienta, Manuel Cabrera-Bañegil, Daniel Martín-Vertedor and Jonathan Delgado-Adámez*

*Address all correspondence to: jonathan.delgado@gobex.es

Agrifood Technology Institute of Extremadura (CICYTEX-INTAEX), Badajoz, Spain

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Chapter 4

Phenolic Antioxidant Capacity: A Review of the State of the Art

Rubén San Miguel-Chávez

Additional information is available at the end of the chapter

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Abstract

There are many evidences pointing to oxidative stress as the promoter of the development of many degenerative diseases such as cancer, cardiovascular diseases, and neurodegeneration. It has been suggested that a diet rich in antioxidants would be beneficial to human health. To determine the antioxidant capacity of the different sources of antioxidants, they have different chemical methods used, in vitro cells, laboratory animals, and recently nanoparticles. This chapter provides an account of the main antioxidant evaluation methods applied to phenolic compounds, recounting their advantages and disadvantages, as well as a reflection on the parameters that should always care to obtain reproducible results.

Keywords: antioxidant capacity, phenolics, free radicals, standardized methods

1. Introduction

Phenolic compounds, or polyphenols, are a wide group of metabolites that originate from the secondary metabolism of plants. They contain one or more hydroxyl groups attached to a benzene ring and have an important role in the defense against plant pathogens and abiotic stressors [1].

This is one of the largest groups given its high chemical diversity. The basis of their structure is precisely a phenol group, that is, a hydroxyl attached to an aromatic ring [2] (**Figure 1**). Phenolic compounds are a chemically heterogeneous group, with the following chemical properties: some compounds are water soluble, some are soluble in organic solvents, some are found as glycosides, and some others are large insoluble polymers. Another characteristic is that this is a chemical group with high antioxidant activity (AOA) [3].

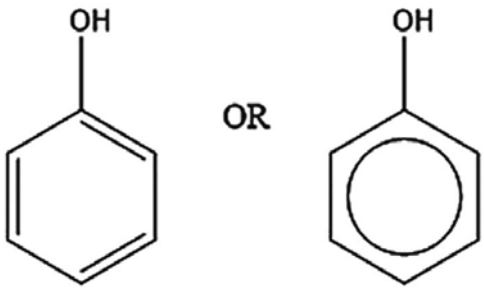

Figure 1. Phenol group, the basic structure of phenolic compounds.

2. Antioxidants

The term "antioxidant" is more important every day in modern society, since it is being associated with a whole series of benefits to human health. Despite that, antioxidants have applications not only in health but also in fields such as the chemical industry, where they are used as additives in the manufacture of rubbers and plastics to delay damage by the action of oxygen [4]. Also, in the food industry adding these antioxidant molecules results in prolonged shelf life, as in the case of fats, which become rancid due to the action of reactive oxygen and nitrogen species [5]. In the widest sense, an antioxidant molecule can be defined as a substance capable of preventing or delaying the oxidation of other molecules, such as lipids, proteins, or nucleic acids [6]. In the preceding definition, it is understood that the antioxidant molecule per se can perform such activity, as is the case of molecules as big as proteins and enzymes, or smaller molecules including vitamins, carotenoids, and phenolic compounds, of which flavonoids have an important role. However, authors like [6] mention that a more updated definition of what an antioxidant is would have to include not only the molecules that scavenge or reduce an oxidizing chemical compound but also those that act as chemical signals that induce the synthesis of enzymes related to the antioxidant mechanism of the cell.

Such oxidation can be carried out by two types of chemical reactive species: free radicals and other molecules that, without being radicals, due to their reactive nature can induce oxidation in molecules as the ones already mentioned.

3. Free radicals and reactive oxygen species (ROS) and their reaction mechanisms

It is widely known that in the atom, the electrons are ordered in their energy orbitals, with an even number of them in the last, most external level. This distribution gives the atom stability and a low possibility of reaction with a nearby atom. However, under certain conditions, the last level of energy can lose its stability by losing or gaining an electron. When this happens, the last orbital shows an unpaired electron, making the atom a free radical. This characteristic results in

a drastic increment of its ability to react with other atoms and/or molecules present nearby; in the cell environment, these molecules include lipids, proteins, and nucleic acids. When chemical interaction between free radicals and the aforementioned molecules occurs, changes in the structural properties of macromolecules can result, which eventually affect their function [7].

It is important to mention also the reactive oxygen species (ROS). This term includes all those reactive molecules, free radicals or not, that center their reactivity on an atom of oxygen. In spite of the delimitation that the presence of oxygen [8] gives, this title also includes chemical species with chemical reactivity centered on or derived from atoms that are different from oxygen. Such is the case of species that contain nitrogen or chlorine, atoms that are responsible for their chemical reactivity [9] (**Table 1**).

Free radicals	Nonradical reactive species
Superoxide O_2^-	Hydrogen peroxide H_2O_2
Hydroxyl HO^-	Hydroperoxide ROOH
Alkoxy RO^-	Hypochlorite ClO^-
Peroxy-OOH	Singlet oxygen $^1O_2$
Nitric oxide NO	Ozone O_3
Nitric dioxide NO_2	Peroxynitrite NO O_2^-
Modified from Ref. [5].	

Table 1. Examples of free radicals and reactive oxygen, nitrogen, and chlorine species.

4. The presence of free radicals and reactive oxygen species in living organisms

In mammals, the reactive chemical species generated are nitric oxide (NO^-). This free radical is a product of the enzymatic action mediated by the nitric oxide synthase located in the cytosol of the cell; it is continuously produced by vascular endothelial cells [10].

Regarding the endogenous generation of ROS, it is part of the normal working of the aerobic organism. Under normal physiological conditions, animal tissues produce significant amounts of ROS. Among the most produced ROS, the free radical superoxide (O_2^-) prevails [11]. This radical is produced through the electron transport chain in the mitochondrion (during the interaction between oxygen molecules and complexes I and III) [12]. It is necessary to remember that the electron transport chain is a series of reactions oriented to producing, between the matrix and the intermembrane space, a proton gradient that is used by the cell to synthetize ATP from ADP. During the functioning of such chain, from 1 to 3% of oxygen that enters the mitochondria is transformed to superoxide (O_2^-), that is, it gains an electron. Despite that, and thanks to the presence in the mitochondrion of the superoxide dismutase, the levels of O_2^- diminish,

becoming oxygen and hydrogen peroxide. Hydrogen peroxide too is quickly reduced to water inside the mitochondrion by the action of the enzyme glutathione peroxidase, and the hydrogen peroxide that is not reduced exits the mitochondrion to be eventually reduced by another class of peroxidases present in the cytoplasm and by catalase in the peroxisomes [12].

5. Methods for the evaluation of antioxidant activity

In the modern world, many scientists around the globe attribute the origin of many diseases to oxidative stress; there is much evidence to support this theory [13, 14]. For this reason many nutritionists recommend the consumption of at least a minimum of foods such as fruits, vegetables, some drinks like grape wine, and spices and also food supplements from natural and synthetic origin containing antioxidants to help keep an individual healthy [15].

The antioxidant molecules present in these foods, drinks, and supplements, among which phenols are included, have been characterized as antioxidants by means of several methods and under different experimental conditions. Despite that, sometimes the results from the same molecule may vary when different methods are used [5]. This can be understood in two ways: on the one hand, inside living systems there are multiple radicals and reactive chemical species, as well as mechanisms involved in oxidative stress; on the other hand, when an in vitro method is used, it is important to take into account the chemical nature of other molecules being tested to employ the most adequate assay in order to get results that are closest to reality. For these reasons, there is no simple and universal method to characterize the antioxidant chemical abilities of all molecules [7]. Each proposed method will always have advantages and disadvantages, which need to be taken into account in terms of complexity, required facilities and equipment, the chemical mechanism that it tests, the quantification method, and its relevance in biological systems.

Several methods have been proposed to evaluate the antioxidant activity (AOA) of a molecule, which can be classified in several ways. In this chapter, they will be divided according to their reaction mechanism.

Antioxidants can deactivate radicals basically in two ways: (a) by a single-electron transfer (SET) and (b) by a hydrogen atom transfer (HAT). In the first case, the method will evaluate the capacity of the possible antioxidant to transfer an electron and reduce certain compound, including carbonyls, metals, and radicals [4, 7]. In the second case (HAT), the capacity of an antioxidant to scavenge free radicals by proton donation is measured (**Figure 2**).

In the case of HAT, several inconveniences can arise during the evaluation, since the presence of reducing agents, including metals, can generate errors by an apparent reactivity [5]. Also, the result can be affected in SET by the presence of contaminating metals, and given that the SET reaction is normally very slow and requires quite a long time to finish, secondary reactions can occur, which may contribute to a high variability and poor repeatability of the results [5].

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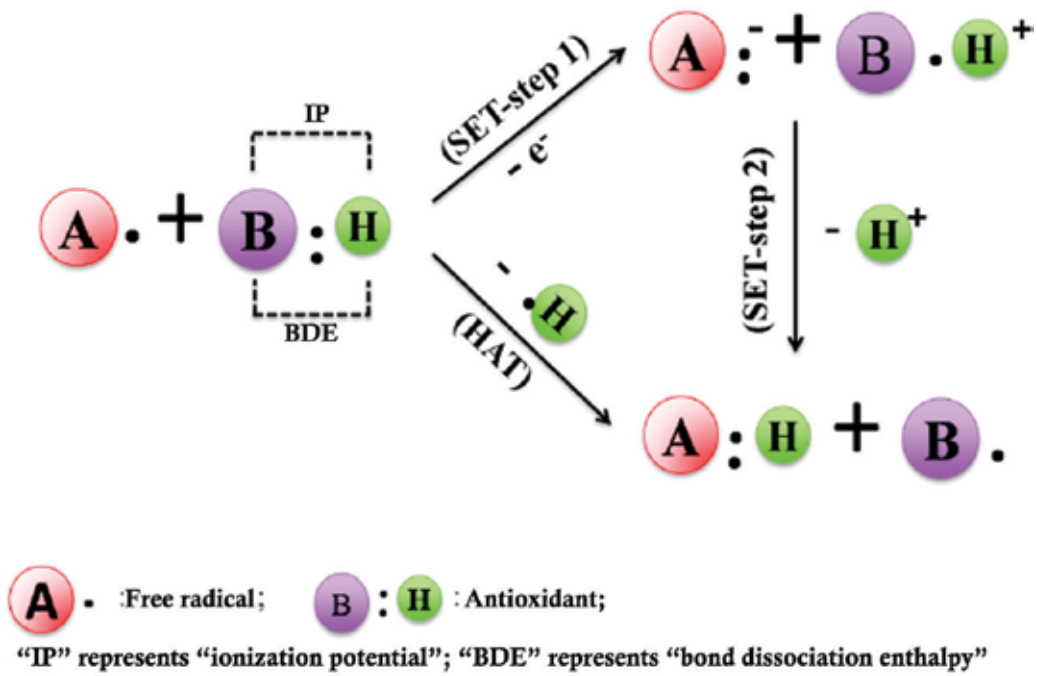

Figure 2. Reaction mechanisms of single-electron transfer (SET) and hydrogen atom transfer (HAT) [16]. Both mechanisms almost always occur together in all samples, with the balance determined by antioxidant structure and pH.

There are several methods reported in the literature to determine the antioxidant activity of polyphenols; however, in this chapter only the most common methods related to antioxidant activity of phenols will be discussed.

5.1. Methods based on the HAT reaction mechanism

5.1.1. Method with phycoerythrin

The substrates that are employed in this assay are two proteins found in several species of red algae: β-phycoerythrin and R- phycoerythrin [17, 18]. Their most important trait, relevant to the assay, is that they are fluorescent; this fluorescence diminishes on contact with peroxyl radicals, product of heat decomposition of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and the decrease is proportional to the amount of those radicals. But, when molecules of an antioxidant are added, the loss of fluorescence is decreased. However, phycoerythrin presented serious disadvantages identified in [19], such as the variability in the quality of this fluorescent protein varied from one extraction to another; it is also photoblanched under plate-reader conditions, and finally this protein interacts with polyphenols due to the nonspecific protein binding and loses fluorescence even without added radical generator [4]. For this reason, another fluorescent molecule that did not have such disadvantages being the fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3H],9′[9H]

thioxanthene]-3-one), a nonprotein synthetic molecule, was the most appropriate [19]. Using this method, the automated oxygen radical absorbance capacity (ORAC) of several products, including fruit juices and nectars, has been reported using Trolox as standard; these results have been published as equivalents of such standard [20]. The results involve both the time that the inhibition of the oxidation lasts and the concentration of the substrate that can be inhibited.

5.1.2. Comments about this method

When this method is used to evaluate phenolic acids, they show a low activity against peroxyl radicals compared with some flavonoids, which have several hydroxyl groups. Despite this appreciation, even flavonoids in form of glycogens can show ORAC activity. This shows that several factors are involved in the antioxidant activity of the molecules, such as their propensity to donate hydrogens or oxygens, which is directly related to the reduction of their potential [20].

5.1.3. Total radical-trapping antioxidant parameter (TRAP)

This method evaluates the capacity of antioxidant compounds to block the potential reaction between peroxyl radicals originated from the 2,2′-azobis(2-amidinopropane) (ABAP) and the R-phycoerythrin by measuring its fluorescence or from the 2,2′-azobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by measuring its absorbance [21, 22]. Using this method, the antioxidant capacity is determined as the time needed for all the antioxidant to be consumed, by the increment of the time needed for the oxidized products to appear when the antioxidants are added or also as the percentage of reduction of the oxidation reaction. The values of this test are generally expressed as the increment of the time needed for the oxidized products to appear or the reaction time of the antioxidant, compared with the times of Trolox [23].

5.1.4. Comments about this method

When one wishes to compare the results obtained in different laboratories, there is the problem that the time allowed for the reaction to occur is different. This problem can be overcome by reducing the time of observation when testing a specific antioxidant and also by using more adequate equipment. It is necessary to mention that this assay has also been criticized for using nonphysiological oxidative stress (water-soluble peroxyl radicals) [24].

5.2. Methods based on the SET reaction mechanism

5.2.1. FRAP method

The initials FRAP stand for "ferric reducing antioxidant power method." This method is based on the reductive capacity of the iron that is part of the compound Fe $(TPTZ)^{3+}$ [25]. When this compound is reduced to Fe $(TPTZ)^{2+}$, a blue color appears, and its absorbance can be measured at 593 nm. The medium of the reaction is acid, and the results can be expressed

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in the form of Fe^{2+} equivalents, or as in other methods, by using a standard compound. This method was initially developed to be used in plasma, but nowadays it is employed in other liquids such as fruit juices and pulps and other foods.

5.2.2. Comments about this method

The most criticized part of this method is that it assumes that the maximum reaction time is between 4 and 6 min [5], but in the case of phenolic compounds including acids such as caffeic, tannic, and ferulic, or quercetin, the reaction can last for more than an hour.

Also, according to [20] since this method is used in complex liquid mixtures such as fruit nectars, all the molecules that are part of that fluid take part in the reduction of Fe^{3+}; therefore, it is not possible to determine the individual participation of the different components from the mix of antioxidants in the total antioxidant activity.

5.2.3. Copper reduction assay

This assay is based on the reduction of Cu(II) to Cu(I). In this reaction, all the antioxidant molecules from a sample are involved. There are basically two methods that use copper. The first of them is the assay Bioxytech AOP-490, where the molecule bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) forms a complex 2:1 with copper (Cu(I)), and a chromophore is formed, with a maximum of absorbance at 490 nm [21]. The amount of bathocuproine that contains Cu(I), product of the reaction of Cu(II), causes a variation in the absorbance of the color complex at 450 nm. Similarly, the assay with CUPRAC uses neocuproine (2,9-dimethyl-1,10-phenanthroline), and the complex with Cu(I) will be the one that presents coloration at 450 nm. Uric acid is used in the standard curve, and, therefore, the results will be expressed as equivalents of that acid [5].

5.2.4. Comments about this method

The advantage of copper over iron is that every class of antioxidant, including thiols, will be detected with very little interference from free reactive radicals, and the kinetic of the reaction when using copper is faster compared with iron (FRAP) [5]. An inconvenience that can arise is that the phenanthroline is not water miscible, and, therefore, it must be mixed with organic solvents such as 95% methanol [26].

5.2.5. Trolox equivalent antioxidant capacity/ABTS radical cation decolorization assay

By using a spectrophotometer in this assay, the loss of color can be measured when an antioxidant is added to the chromophore ABTS. + (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). When the antioxidant molecule reduces ABTS. to ABTS, the solution diminishes its blue-green coloration, tending to be colorless. [27]. The usual way to prepare and use ABTS is by adding 80 mg of manganese dioxide to a stock solution of 5 mM ABTS prepared in a buffer of 17 mM Na/K at pH 7. As an antioxidant standard, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is used. The standard curve is made with at least six concentrations, ranging from 0 to 350 μM. The samples to be tested are diluted in the buffer of Na/K pH

7 and then mixed with 200 μL of the ABTS solution in a 96-well plate. The absorbance at 750 nm is recorded. Trolox equivalent antioxidant capacity (TEAC) values are calculated from the standard curve with Trolox, and the results are expressed in Trolox equivalents (mM) [27].

5.2.6. Comments about this method

Currently, this method is widely used in many laboratories around the world due to its simplicity of use [28–30]. Thus, the antioxidant activity of a vast range of compounds has been reported using this method. The assays can be carried out in media with both organic and inorganic solvents without affecting ABTS activity. It can be carried out in plates with wells, greatly reducing the use of reagents and making this method environmentally friendly. However, this radical is not naturally found in living organisms, and, thus, the results may not be considered representative of those that take place in living organisms. Finally, regarding the thermodynamic properties of phenols, one compound can reduce ABTS only if its redox potential is lower than that of ABTS; the potential of phenols is lower, so they can reduce ABTS, and it can be used as an antioxidant test for these molecules [5].

5.2.7. 2, 2′-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay

DPPH is a nitrogenous organic radical with a delocalized electron, and this characteristic gives it a purple coloration, with a maximum absorbance at 515 nm [31]. The assay is based on the measurement of the capacity of antioxidants to reduce DPPH [32]. Such reduction can be measured by the decolorization of the purple color in its absorbance. This assay was first reported by [33], and according to [34], to carry it out, it is necessary to dilute 200 μL of the sample in methanol and mix it with 2 mL of 0.5 mM DPPH. After 30 min, the absorbance is measured at 515 nm in a spectrophotometer. The percentage of DPPH radical scavenging is calculated with the expression:

$$\% \text{ inhibition of DPPH radical} = ((Abr - Aar)/Abr) \times 100 \quad (1)$$

where Abr is the absorbance before the reaction and Aar is the absorbance after the reaction occurred.

5.2.8. Comments about this method

As ABTS, DPPH is also widely used in many laboratories in the world due to its simplicity and the ease to carry it out. However, the fact that the absorbance is read at 515 nm can cause interferences with compounds that absorb at the same wavelength, which may complicate the interpretation of the results [5]. On the other hand, other chemical characteristics of DPPH make understanding the results more difficult. This assay is not competitive because DPPH is a radical and an oxidant at the same time, so decolorization of the reactive can be attributed both to the reaction of the radical and to a reduction of the steric accessibility, with the latter being determinant for the reaction. Thus, small molecules, with better accessibility to the site of the radical, would seem to have better antioxidant capacity. In contrast, larger molecules with much faster reaction times with DPPH than some smaller molecules could apparently

show lower antioxidant capacity. For these reasons, the results with DPPH must be interpreted with caution.

5.2.9. Folin-Ciocalteu AOC method or total phenolic assay

This is a classic method for the detection of total phenols in the laboratory [35]. However, during color development an oxidation-reduction reaction takes place; due to that, this method has been proposed as a method for the detection of antioxidant activity, particularly in phenols [36]. It was originally proposed in 1927, using molybdotungstate as a reagent for phenol reduction, with which a colorized compound was obtained; this compound was read at wavelengths between 745 and 750 nm [36].

This simple, sensitive and precise method, when the reaction occurred in an acid medium, it was much too slow. For this reason [37] improved the method by substituting the molybdotungstate for molybdotungstophosphoric heteropolyanion, which reduces phenols more specifically, and the colorized product is read at 765 nm. The experimental conditions proposed in [37] consist in mixing 1 mL of the sample diluted in at least 60 mL of water and 5 mL of the Folin-Ciocalteu reagent; afterward, the mix is agitated, and 15 mL of Na_2CO_3 is added, mixed, and diluted to 100 mL with water; finally, it is incubated for 2h at 24°C, and the absorbance is measured at the indicated wavelength. Despite the simplicity of the instructions, several recent articles report variations in the incubation period, the concentrations of the reagents, and especially the interchange of gallic acid for some other standards, among which can be cited acids like tannic, chlorogenic, caffeic, vanillic, and ferulic, among others. This can lead to variations in the results, sometimes of several orders of magnitude [38].

5.2.10. Comments about this method

The advantage of the method is that it can be used with practically any plant sample, but only when the aforementioned conditions are controlled [38]. There is also a list of molecules, both inorganic (hydrazine, iron ammonium sulfate, manganese sulfate, sodium cyanide, sodium sulfite, and xanthine, among others) and organic that include amino acids such as adenine, sugars such as fructose, proteins, and fatty acids. If said interferences are controlled, then it is possible to consider the results of this method to report the antioxidant activity of the samples.

5.3. Assessment of the antioxidant capacity in cell culture

The methods used to assess antioxidant capacity that have been mentioned so far are carried out mainly in vitro, using only one oxidizing chemical species and a single reagent to show oxidant and antioxidant activity by means of color development, fluorescence, etc. However, it is necessary to know the real role of antioxidant molecules in living systems [39]. Cell culture is being used to address that issue; in them, chemical molecules that cause oxidation per se can interact with each other, as well as with molecules that do not cause oxidation by themselves, but that generate a disturbance in the redox balance of the cell, provoking in the end oxidative stress [40]. One of the advantages of this system is its ease of handling, compared with laboratory animals, which are harder to obtain and handle given the strict regulations for their ethical management. In this type of assays, it is important to verify the

final concentration accumulated in the cell that is a product of their addition in the culture medium and/or its direct addition to cells, since not all antioxidants are incorporated into the cell in the same way, as it is cited by [40]. An example could be tocopherol and tocotrienol, because in several studies it has been reported that apparently, tocotrienols have a higher antioxidant activity compared with tocopherols; however, if the concentration of these antioxidants is adjusted, both compounds have the same antioxidant capacity. The apparent disparity in antioxidant activity is due to the fact that tocotrienols can enter the cell more easily given their short chain compared with tocopherols.

5.4. Nanotechnology-aided assays

With the advent of the nanotechnology in the decades of 1980–1990 [41], the technology that is being developed has also proposed the use of a method that uses nanoparticles to evaluate antioxidant activity as well. Scampicchio proposed, in one of the first works in the field [42], the assessment of some phenolic compounds by the generation and growth of gold nanoparticles (AuNPs) from a gold solution (AuIII) in solution ($HAuCl_4$) by the generation of a sharp plasmon absorption band at 555 nm. The optical properties of the AuNPs correlate well with the reduction potential of phenolic acids, something that can be determined by voltimetric measurements, and this method is proposed to evaluate antioxidant capacity of pure compounds or their mixtures [6].

When [43] conducted an experiment where they characterized the kinetic of AuNP generation at an absorbance of 540 nm and described a sigmoid curve as a function of the concentration of polyphenols, they proposed the following equation:

$$A_{540} = A_{max}/1 + e - KAuNPs(X - XC_{50}) \quad (2)$$

XC_{50} = the concentration of polyphenols that give half of the maximum plasmon resonance absorption; KAuNPs = the number of AuNPs produced by concentration unit of polyphenols.

The authors proposed that KAuNPs be used as a parameter to estimate antioxidant activity. This method has already been employed in the determination of antioxidant activity of several products including honey [44], wine [45], tea [46], apples [47], etc. For their part, [48] also used silver nanoparticles for the same goal. The method, whose name is silver nanoparticle antioxidant capacity (SNPAC), uses Trolox as its standard. The rationale of this method is that polyphenols are able to reduce Ag^+ ions in the presence of citrate-stabilized silver seeds; the intensity of the plasmon, visible at 423 nm, is evaluated, and thus antioxidant capacity [49] is quantitatively assessed. With respect to polyphenols, this method is more robust and repeatable than assays that use a direct reduction of metallic ions by antioxidants. As the gold method, this method has already been employed in several fruit juices and teas [50]. Some of its main advantages are good linearity with the concentration of the sample (polyphenols) and the lack of interference by molecules present in the samples such as reducing sugars, fruit acids, or amino acids.

6. Conclusions

Throughout the development of different methods to determine antioxidant activity of several molecules, polyphenols among them, one can observe an evolution, which goes from the involvement of the antioxidant with its substrate, its exposition to several antioxidants, and a medium that shows the changes that take place during the reaction, to more sophisticated methods where the kinetic of the formation of nanoparticles show the presence or absence of antioxidant activity.

In the light of so many methods, a question arises: Why is not there a universal method to evaluate the antioxidant activity of any molecule? The answer may not be simple, but it would have to do with the goals that are sought; they may be the simple detection of antioxidant activity in a sample, or they may include the comparison of antioxidants with each other and the understanding of the process inside a living system such as cells in culture or more complex systems as laboratory animals and of course the human body.

Many authors recommend that certain steps be considered to select and report the results of antioxidant activity (**Table 2**). These include carrying out the assay in a systematic way, always respecting the conditions established for its development: controlling the source of the sample that is subject to experimentation regarding its origin, management during its collection and transport to the laboratory, and always controlling the standard used to compare the results of the sample. By adhering to these steps, variations in the results obtained by different laboratories can be reduced when the antioxidant activities from samples of the same origin are compared.

Method	Required equipment	Biological relevance	Mechanism	End point	References
Fluorescein	Sophisticated	Medium	?	Fixed time	[19]
TRAP	Sophisticated	High	HAT	Lag phase	[21, 22]
FRAP	Medium	Low	SET	Time varies	[25]
Copper reduction	Medium	Low	SET	Time	[26]
TEAC/ABTS	Simple	Low	SET	Time	[27]
DPPH	Simple	Low	SET	IC_{50}	[31, 32]
Folin-Ciocalteu AOC	Simple	Medium	SET	IC_{50}	[36]
Cell culture	Medium	High	?	?	[39]
Nanotechnology	Sophisticated	High	?	?	[42, 43]

Modified from Ref. [5].

Table 2. Relevant characteristics of the methods to evaluate the antioxidant capacity of phenolic compounds.

In the same way, the selection of one of the many available methods must be based on the following criteria, to optimize the results: (a) select a relevant source for the sample that is

abundant enough to repeat the results; (b) select a method that is as simple as possible, taking into account the facilities, the equipment, and the reagents that are available; (c) choose a method where reaction times are clear, as well as the type of mechanisms that will be carried out; (d) select an assay with good repeatability; (e) consider if the antioxidants are of hydrophilic or lipophilic nature; and (f) take into account the nature of the oxidants.

Many of the proposed assays that have been traditionally used to give a value to a molecule or group of molecules, in the case of crude extracts, are complex, since several factors can participate. For example, particle size has an effect over the determination of its activity, the pH, and the medium of dissolution. This can lead us to declare that simple tests that need little equipment are not always the most adequate. This is the case of DPPH; since it is a test that requires only a methanol, DPPH, glassware, and spectrophotometer, it could give the best results when assigning an antioxidant value to a molecule.

Another important aspect that could be a source of confusion in the literature is the interchangeable use of the terms activity and antioxidant capacity. During the compilation of articles for the preparation of this chapter, it was noted that several authors titled his research as antioxidant activity and reported figures rather denote the ability of extracts or molecules for antioxidation, reporting them in units of a standard as can be equivalent of Trolox or per unit of time. So that being strict about definitions, if the antioxidant activity of a sample is reported, it should simply describe whether or not presented such a phenomenon, something similar to what is done when colorful preliminary tests are done to detect the presence or no major group of secondary metabolites. As suggested be careful in handling of such terms and keep in mind that in the case of the antioxidant activity refers to whether or not an oxidation retardant of a substrate regardless of the magnitude. On the other hand, it indicates how much capacity has antioxidation an extract or molecule to a substrate, in this case being reported in% inhibition, IC_{50}, XC_{50}, Trolox equivalents, etc., units that can be compared to know which sample is more suitable to prevent oxidation of said substrate.

The rise all over the world of the number of people with chronic-degenerative diseases caused by oxidative stress and a lack of antioxidants in the diet is more and more alarming, especially if the high cost that this implies for the health sector of any country is considered, due to the need to treat everyday more cases of cancer, diabetes, arteriosclerosis, hypertension, etc. Finding new sources of antioxidants, especially from natural sources, is paramount, and comparing them with the traditional molecules is also important; therefore, it is desirable to employ assays that show if an extract or molecule has or not antioxidant activity and that are able to quantify their antioxidant capacity.

Author details

Rubén San Miguel-Chávez

Address all correspondence to: sanmi@colpos.mx

Colegio de Postgraduados, Campus Montecillo, Texcoco, Estado de México, Mexico

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Chapter 5

Plant Phenolic Compounds as Immunomodulatory Agents

Alice Grigore

Additional information is available at the end of the chapter

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Abstract

Immunology is a source of continuous discoveries; Immunology was and still is a source of continuous discoveries. Immunomodulation encompasses all therapeutic interventions aimed at modifying the immune response. Immunostimulation is desirable to prevent infection in states of immunodeficiency and to fight infections and cancer. On the other hand, immunosuppressive agents inhibit the activity of the immune system, and they are used to prevent the rejection of transplanted organs and tissues and to treat autoimmune diseases or diseases that are most likely of autoimmune origin (e.g., rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, ulcerative colitis, etc.), or other nonautoimmune inflammatory diseases (e.g., allergic asthma). The discovery of immunomodulatory agents from medicinal plants devoid of toxic side effects, with enhanced bioavailability and that can be used for a long duration, is of great actuality. Research on natural immunomodulators provides a therapeutic solution that addresses a multitude of disorders. Plant phenolic compounds already proved beneficial effects in cardiovascular diseases, diabetes, and cancer, exerting mainly antioxidant and anti-inflammatory effects. The concepts of "immunomodulatory," "anti-inflammatory," and "antioxidant" are often strongly related, and a review of phenolic compound action on immune system should be analyzed in a context, revealing their mechanism of action on effector cells and also on the system as a whole.

Keywords: immunomodulation, immunostimulation, immunosuppression, phenolic compounds, bioavailability

1. Introduction

Immune response is one of the most complex mechanisms of the living body, involving the strong cooperation of a large variety of cell types for defending against any potential dangerous agent. Perturbation of this well-adapted process results in a cascade of disorders

and even the occurrence of chronic diseases, making the regulation of the immune system a key factor in maintaining a healthy equilibrium of the body. The discovery of immunomodulatory agents from medicinal plants devoid of toxic side effects, with enhanced bioavailability and that can be used for a long duration, is of great actuality.

In terms of molecular weight, phytocompounds are classified in high-molecular compounds such are peptides, polysaccharides, and low-molecular compounds—terpenes, alkaloids, and also phenolics. Plant phenolic compounds already proved beneficial effects in cardiovascular diseases, diabetes, and cancer, exerting mainly antioxidant and anti-inflammatory effects. Most of the plant-derived phenolics influence the nonspecific immune response mainly by enhancing phagocytosis and proliferation of macrophages and neutrophils. The concepts of "immunomodulatory," "anti-inflammatory," and "antioxidant" are often strongly related, and a review of phenolic compounds action on immune system should be analyzed in a context, revealing their mechanism of action on effector cells and also on the system as a whole.

2. Overview on the immune system

Immune response is controlled both by direct interaction of different types of cells (lymphoid cells: B and T lymphocytes, T helper (Th) cells, natural killer (NK) cells; myeloid cells: neutrophils, basophils, monocytes, macrophages) and by-products of synthesis they secrete (immunoglobulins, cytokines: interleukines, colony-stimulating factors, growth factors, interferons, etc).

Although innate and adaptive immunities work complementarily to provide an overall protection to the human body, they appeared at different times in evolution. Basic mechanisms of the innate immunity are found both in vertebrates and invertebrates and even in plants, while adaptive immune response is specific to vertebrates [1].

Innate immunity has no capacity for immunological memory and employs an antigen-independent defense mechanism that provides host defense immediately or within hours after exposure to pathogens. Cells involved in this response comprise phagocytic cells (neutrophils, monocytes, and macrophages), cells secreting inflammatory mediators (basophils, mast cells, and eosinophils), and natural killer (NK) cells. Pathogen-associated molecules (called pathogen-associated immunostimulants) stimulate two types of innate immune responses—inflammatory responses and phagocytosis by cells such as neutrophils and macrophages [1], processes regulated by soluble mediators known as cytokines. The mechanism is complex, and a precise delimitation of immunity, inflammation, and oxidation cannot be set. Innate immunity can also stimulate adaptive immune response with the help of a group of specialized cells known as antigen-presenting cells (APCs) such are dendritic cells (DCs). APCs display the processed antigen to lymphocytes and collaborate with them to elicit the immune response. Unlike innate immunity, the adaptive immune response involves antigen-specific antibodies, and a certain time interval is required for the maximal response to be achieved after exposure to the antigen.

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Adaptive responses are mainly conducted by T cells, facilitated by APCs in cell-mediated immunity and B cells in antibody-mediated immunity. The T lymphocyte group represents 60–80% of total lymphocytes and has a very high lifetime and is mainly involved in eradication of intracellular pathogens by activating macrophages and by killing virally infected cells. These lymphocytes recognize the primary structure of an antigen, a mechanism different from that of B lymphocytes and plasma cells, which recognize the antigen by the spatial structure. T helper (Th) lymphocytes represent 2/3 of total lymphocytes and are of special importance because they secrete interleukins, messenger molecules that facilitate the communications between immune system cells. Depending on the type of cytokines that they secrete, Th1 cells producing interleukin-2, IFN-γ, and TNF-α and triggering inflammatory reactions and Th2 cells producing interleukins 3, 4, and 5, the main stimulator of immunoglobulin A and E synthesis, are distinguished [2].

In antibody-mediated immunity, activation of B lymphocytes conducts to plasma cells synthesizing immunoglobulins or memory B cells leading to immunological memory.

3. Overview on phytophenols

Phytophenols are secondary metabolites based on a common carbon skeleton structure—the C6–C3 phenylpropanoid unit [3]. Among this group, several classes are described:

Flavonoids are natural phenolic substances of C6–C3–C6 type, derivatives of 2-phenylbenzopyran (flavan) or 3-phenylbenzopyran (isoflavan). Flavonoids are present in plant organs as glycoside or aglycone. Flavonoid aglycones are based on 2-phenylbenzo-γ-pyrone core (2-phenylchromane) grafted with hydroxyl, methoxyl, dimethylallyl, etc. groups.

Most of the compounds are hydroxylated on A ring, at C5 and C7 positions. On the ring B are grafted 1–3 phenolic groups at C4′, 3′, and 5′. Depending on the degree of oxidation and substituent type of segment, several classes are classified:

- Flavones—double bond between C2 and C3 (e.g., apigenin, luteolin)
- Flavonols—flavones 3-hydroxylated (e.g., kaempferol, galangin, quercetin, miricetin)
- Flavanones—flavones 2,3-dihydrogenated (e.g., naringenin, hesperetin)
- Flavanonols—flavonols 2,3-dihydrogenated (e.g., taxifolin, dihidrokaempferol) [4]

Also, other varieties of flavonoids are:

- Biflavonoids are dimer of flavonoids. The monomers are linked in positions 6 and 8, which are highly reactive. The links established can be C–C (amentoflavone, bilobetol, ginkgetol) or C–O–C (hinokiflavona). The hydroxyl groups may be free or, most often, methylated.
- Isoflavones are 3-phenylbenzo-γ-pyrone derivatives (3-phenylchromone), specific for Fabaceae family (species of this family contain a specialized enzyme responsible for converting 2-phe-

nylchromane to 3-phenylchromane). Isoflavones are usually found in free state (daidzein, genistein) and very rare as heterosides (mainly O-heterosides) (daidzein, puerarin) [4].

– Chalcones (1,3-diaryl-2-propen-1-ones) are α,β-unsaturated ketones, comprised of two aromatic rings, that function as precursors in the synthesis of flavonoids and isoflavonoids (phloretin, arbutin).

In regard to **phenolic acids,** two classes can be distinguished: derivatives of benzoic acid (C6–C1 structure) and derivatives of cinnamic acid (C6–C3 structure). Hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids having C6–C1 structure [5]. The hydroxycinnamic acids are more common than are the hydroxybenzoic acids and consist mainly of *p*-coumaric, caffeic, ferulic, and sinapic acids [6] and also of esters of caffeic acid with quinic acid (chlorogenic acid), tartaric acid (cichoric acid), and 2-hydroxydihydrocaffeic acid (rosmarinic acid).

Lignans are compounds resulted from the condensation of two to five molecules of phenylpropane derivatives (C6–C3). Dietary lignans are metabolized by the intestinal microflora to enterodiol and enterolactone, compounds associated to many positive effects for human health [6]. This class comprises valuable antineoplastic agents—podophyllotoxin, matairesinol, and immunostimulants—syringaresinol, arctigenin, etc.

Tannins, both procyanidins and hydrolysable tannins, are so named for their use in the tanning of leather or hides based on their ability to bind and precipitate proteins. There are a vast number of processes for which plants employ tannins, ranging from herbivore protection to hormone regulation. Procyanidins and hydrolysable tannins differ in their core polyphenol structure and have differing functions both in the plant and on mammalian cells [7]. Hydrolysable tannins contain a sugar core surrounded by phenolic groups such as gallic acid residues. These residues can be subsequently modified by further addition of phenolic groups, oxidation reactions, or other polyphenols [8, 9], thereby generating increasingly complex polyphenols. The procyanidins are produced by assemblage into oligomers; up to 28-mers of procyanidins have been recorded [10]. These oligomers are formed via combinations of the monomer subunits epicatechin or catechin and are often modified by the addition of gallic acid residues. In grapes, for example, about 20% of the residues are galloylated [10].

Stilbenoids contain two phenyl moieties connected by a two-carbon methylene bridge (C6–C2–C6) [11], the most known representative compound of this class being resveratrol.

4. Aspects related to the structure-activity relation of phytophenols

It was showed that there are differences in immunomodulation exerted by flavonoid glycosides and their corresponding aglycones. While quercetin is able to activate concomitantly lymphocytes and secretion of IFN-c, the similar flavonoid rutin (quercetin-3-rutinoside) significantly stimulates the secretion of IFN-c, but do not elevate the proliferation of human peripheral blood mononuclear cells (PBMC), indicating the sugar moiety as the key point for different responses [12].

The importance of the sugar at position 3 for the selective immunosuppression by astilbin (taxifolin 3-rhamnoside) was highlighted by Guo et al. Most of the flavonoid glycosides have glucose attached to aglycones and are usually hydrolyzed by glucosidase. In the case of astilbin, the sugar attached to aglycone is rhamnose, which is likely difficult to hydrolyze, and it is suggested that this phytocompound may show a different metabolic route from other flavonoids, owing to the type and position of sugar attached [13, 14].

Hydroxylations of flavonoids at positions 5 and 7, together with the double bond at C2–C3 and the position of the B ring at 2, appear to be associated to the highest inhibition of pro-inflammatory cytokine expression [15]. Luteolin and apigenin contain hydroxyl groups in their backbone, and it was suggested that these may be involved in immunomodulatory activities since luteolin, which contains hydroxyl groups both at the 3′ and 4′ positions in ring B, exhibits stronger immunomodulatory properties than apigenin that has only a 4′ hydroxyl group in ring B [16]. For chalcone class, trimethoxy chalcones at the A ring with fluoro, chloro, and bromo substitution in the B ring, like 2′-hydroxy-3-bromo-6′-methoxychalcone, 2′-methoxy-3,4-dichlorochalcone, flavokawain A, or flavokawain B, are considered better inhibitors of NF-κB [17]. The number and position of methoxy group seems to be correlated to immunomodulatory capacity as in the case of coumarins. For instance, two methoxy groups (isopimpinellin) are correlated to lymphocyte activation, while one methoxy group (xanthotoxin) conducts to IFN secretion; bergapten (5-methoxypsoralen) is a better IFN-γ activator than xanthotoxin (8-methoxypsoralen) [12].

Due to the fact that the immune response is very complex, some studies were focused on antioxidative immune-mediated mechanisms, and it was shown that the most important feature is the presence of a C–2,3 double bond in combination with a 4-oxo group as it is proved by the higher antioxidant activity of luteolin comparing to apigenin [16]. Souza et al. [18] showed that flavonoid aglycones have high-antioxidant inhibitory activities, while C-glycosylated flavonoids have no significant effect even at the highest concentration tested (50 μmol/L).

Another factor that appears to be important for the influence on the immune response, in particular stimulation of T-cell cytokine production by polyphenols, is the size of the polyphenol molecule [19, 20]. Schepetkin et al. [19] showed that molecular subunits of oenothein B with smaller molecular weights do not have the same leukocyte immunomodulatory capacity, and also procyanidin oligomers, but not monomers, are able to stimulate innate lymphocytes [7]. Also, the chain length of flavanol fractions has a significant effect on cytokine release from both unstimulated and LPS-stimulated PBMCs. Long-chain flavanol fraction and short-chain flavanol fraction, in the absence of LPS, stimulated the production of GM-CSF and increase expression of the B-cell markers CD69 and CD83. The oligomers are potent stimulators of both the innate immune system and early events in adaptive immunity [21].

5. Aspects related to phytophenol bioavailability

Research regarding bioavailability of phytophenols is essential for the establishment of dietary management of diseases [22]. Increased intake of flavonoids with higher in vitro activity is not a guaranty for a strong pharmacological effect in vivo because low absorption and rapid elimination

cause a limited bioavailability. In most of the published data regarding this issue, the concentration of polyphenols in blood and urine after ingestion of phenols rich food was measured as an indicator of their absorption [23], but there are complex reactions of metabolization hindering the biological activity of the parent compounds [24]. Many of these phytophenols with high activity in vitro are not object of industrial investment because of their oral bioavailability below 30% [25].

The absorption of some, but not all, dietary polyphenols occurs in the small intestine. Before the absorption, these compounds must be hydrolyzed by intestinal enzymes. It is believed that the phenolic compounds are absorbed by a passive diffusion mechanism (aglycones) or by carriers present in the intestine [26]. Polyphenols that are not absorbed in the small intestine reach the colon, where they undergo substantial structural modifications by colonic microflora that hydrolyzes glycosides into aglycones and degrades them to simple phenolic acids [27]. Once absorbed, and prior to the passage into the bloodstream, the polyphenol-derived aglycones undergo other structural modifications due to the conjugation process [23]. Glucuronidation and sulfation conjugation reactions are described to have a significant impact on the bioactivity of polyphenols. In particular, the low oral bioavailability of some phenolic substances could be explained by glucuronidation [28]. The low absorption profile of curcumin was demonstrated in human and rat models [29], and it was attributed not only to the poor solubility of this compound but also to the glucuronidation or sulfation processes.

These conjugation reactions significantly reduce the polyphenol antioxidant activity, since both sulfation and glucuronidation occur at the reducing hydroxyl groups in the phenolic structure. It was already shown that these groups are mainly responsible for the antioxidant and immunomodulatory properties of polyphenols [30]. Nevertheless, conjugation reactions might enhance certain specific bioactivities. For example, Koga [31] described that the plasma metabolites of catechin have an inhibitory effect on monocyte adhesion to interleukin-1 in beta-stimulated human aortic endothelial cells, while catechin had no effect [32]. Lignans, for example, need to be biotransformed by gut microflora to be biologically active [26].

Manach et al. [33] suggested that among the most well-absorbed phytophenols in humans are gallic acid and isoflavones, catechins, flavanones, and quercetin glucosides, while the least well-absorbed are proanthocyanidins, the galloylated tea catechins, and the anthocyanins.

The efficiency of absorption of phenolic acids is markedly reduced when they are present in the esterified form rather than in the free forms as it was observed in patients with colonic ablation where caffeic acid was better absorbed than chlorogenic acid [34]. Moreover, it was shown that the occurrence of ferulic acid and antioxidant activity in plasma is increased following intake of food matrix with ferulic acid bound to arabinoxylans compared with results after intake of free ferulic acid, proving that the action of gut microbiota may lead to improved bioavailability [35].

6. Interaction of phytophenols with the immune system

6.1. Dendritic cells

Dendritic cells (DCs), as essential component of the innate immune system, are the most potent antigen-presenting cells (APCs), allowing the critical decision between immune activation and

tolerance. Aberrant activation of DCs can cause detrimental immune responses; thus, agents effectively modulating their functions are of great clinical value. Several plant phenolic compounds proved their ability to influence DC function, especially in a suppressive way. Because Th1 cells are either functionally immunogenic or provide protection against invading pathogens, the inhibition of DC-mediated Th1 polarization may constitute an associated immunosuppressive mechanism [36].

Similar modes of action were established for daidzein (isoflavone) [37], silibinin (flavonolignan) [38], fisetin (flavonol) [39], apigenin [36], and baicalin (flavone glycoside) [40] in LPS-stimulated DCs, all compounds exhibiting immunosuppressive activity by inhibiting cell maturation and activation. They significantly and dose-dependently inhibit the expression levels of maturation-associated cell surface markers including CD40, costimulatory molecules (CD80, CD86), and major histocompatibility complex class II (I-A(b)) molecule. An impaired induction of the T helper type 1 immune response and a normal cell-mediated immune response induced by the abovementioned compounds were noticed as it was previously found in the case of curcumin [41]. This well-known phytophenol is also a potential therapeutic adjuvant for DC-related acute and chronic diseases being highly efficient at Ag capture, via mannose receptor-mediated endocytosis [41]. The suppressive effect on DCs was also showed for another phenolic compound belonging to ellagitannins class, oenothein B; it was associated with the induction of apoptosis without the activation of caspase-3/7, 8, and 9; and this was supported by the morphological features indicating significant nuclear condensation [42].

6.2. Lymphocytes

Stimulation of cell-mediated immune response is one of the most studied effects of plant phenolic compounds, the experiments being carried out both in vitro and in vivo on different species: humans, fish, bovine, etc. In this respect, it was showed that oenothein B, a polyphenol isolated from *Epilobium angustifolium* and other plant sources, is known to activate myeloid cells and stimulate innate lymphocytes, including bovine and human γδ T cells and NK cells, resulting in either increased CD25 or CD69 expression [43]. Moreover, it enhances IFNγ production by both bovine and human NK cells and T cells [44]. Low concentrations of dihydroquercetin (0.025 and 0.0125%) as food supplements are able to increase the immune status—high phagocytic and respiratory bust activities of gilthead sea bream [45].

Stimulation of both humoral and cell-mediated seroresponse was observed (increases of the antibody titers, lymphocyte, and macrophage cells) also in chicks, after administration of an 80% aqueous methanol extract from the leaves of *Jatropha curcas* L. (Euphorbiaceae) and a biflavone di-C-glucoside, 6,6″-di-C-beta-D-glucopyranoside-methylene-(8,8″)-biapigenin) (0.25 mg/kg body wt) [46].

In healthy well-nourished humans, it was showed that consumption within the usual daily intake range of orange juice and its major polyphenol hesperidin (daily 500 mL of orange juice or an isocaloric control beverage with hesperidin (292 mg in a capsule) for 3 weeks) do not induce immunomodulation of cell immune function [47].

Differences between cell-mediated immune response modulations of different compounds belonging to the same class were found in the case of isoflavones.

Daidzein potentiates proliferation of mixed splenocyte cultures activated with ConA or LPS and the secretion of interleukins 2 and 3, while genistein have no influence, although a significant cooperation between these compounds may occur [48]. Contradictory findings regarding genistein (25, 250, 1250 ppm) were presented by Guo et al. [49], which showed that exposure to genistein increases the number of splenic B cells (L), macrophages (L and M), T cells (H), T helper cells (L and H), and cytotoxic T cells (M and H). It was suggested that genistein may modulate the immune system by functioning as either an estrogen agonist or antagonist. The differential effects of genistein on thymocytes in F(1) male and female mice indicate that genistein immunomodulation might be related to its effect on thymus [50].

6.2.1. B cells

Several studies show that epigallocatechin gallate (EGCG) enhances the mitogenic activity of B lymphocytes but not T lymphocytes. Gallic acid and tannic acid induced some enhancement, but rutin, pyrogallol, and caffeine did not, indicating that the galloyl group on EGCG was responsible for enhancement [51].

Cumella et al. [52] found that quercetin, but not taxifolin (dihydroquercetin), inhibited mitogen-stimulated immunoglobulin secretion of IgG, IgM, and IgA isotypes in vitro with an IC50 of approximately 30 mM for each isotype.

6.2.2. T cells

These cells express TCR on their surface to recognize specific antigens processed by APCs, such as dendritic cells, macrophages, and fibroblasts. Activated T cells differentiate into either cytotoxic T cells (CD8+ cells) or Th cells (CD4+). Cytotoxic T cells participate in the destruction of infected cells by secreting perforin, granzyme, and granulysin. Th cells have no direct killing activity in the infected cells but direct other immune cells to act against pathogen-infected cells, mainly by secreting several cytokines. After infection with a certain pathogen, the immune system must select the best defense mechanism, which involves the differentiation of Th cells into Th1 (to promote the bactericidal activities of macrophages) and Th2 cells (to activate or recruit IgE-producing B cells, mast cells, and eosinophils).

Th balance

Intake of representative polyphenols (flavones, flavone-3-ols, catechins, antocyanidins, flavanones, procyanidins, and resveratrol) can improve a skewed Th1/Th2 balance and suppress antigen-specific IgE antibody formation [53]. This was suggested as one mechanism of action of quercetin contributing to its anti-inflammatory and immunomodulating properties having potential of being utilized in several types of allergic reactions. Quercetin is able to inhibit IL-6 and IL-8 better than cromolyn (antiallergic drug disodium cromoglycate) [54], and it ameliorates experimental autoimmune encephalomyelitis, which is associated with Th1-mediated immune responses [55].

A preventive effect on IgE synthesis mediated by Th2 cells was suggested for cocoa. On the other hand, cocoa intake modifies the functionality of gut-associated lymphoid tissue by means of modulating IgA secretion and intestinal microbiota [56].

In allergic diseases, besides the influence on Th2 activation, regulatory T cells represent another possible target for polyphenols activity [57].

Jaceosidin, a flavone isolated from *Artemisia vestita*, exerts an immunosuppressive effect both in vitro and in vivo through inhibiting T-cell proliferation and activation, which is closely associated with its potent downregulation of the IFN-γ/STAT1/T-bet signaling pathway [58]. Naringenin also alleviates symptoms of contact hypersensitivity by its inhibitory effects on the activation and proliferation of T cells. In vitro, naringenin reduces CD69 (the protein level) and cytokines such as IL-2, TNF-alpha, and IFNγ (the mRNA level) expressions, which highly expressed by activated T cells and induces T-cell apoptosis by upregulation of Bax, Bad, PARP, cleaved caspase-3 and downregulation of phosphorylated Akt, Bcl-2 [59].

Kawamoto et al. showed that 6-gingerol suppresses the expression of Th1 cytokines even in strong Th1-polarizing conditions in vitro and also the expression of Th2 cytokines due not to enhancement of Th1 cytokine production but to inhibition of the general pathway for cytokine expression. Another phenolic phytocompounds that contribute to Th1 polarization of the immune response are procyanidin C1 [60] and proanthocyanidin 1 [39].

An immune shift from Th1 to Th2 is suggested for tea polyphenols taking into consideration increased serum concentrations of anti-inflammatory cytokine, such as IL-4. A T lymphocyte transformation test (LTT) demonstrated that dietary tea polyphenols promote the proliferation and activation of T lymphocytes, reflected by elevation of CD4+/CD8+ ratio, inhibition of pro-inflammatory IL-1, and IFNγ expression caused by oxidative stress [61]. Also, umbelliprenin (UMB) and methyl galbanate (MG), terpenoid coumarins isolated from *Ferula szowitsiana*, reduced remarkably PHA-induced splenocyte proliferation and both preferentially induced T(H)2 IL-4 and suppressed T(H)1 IFNγ secretion [62]. Auraptene, a citrus fruit-derived coumarin, has been reported to exert valuable pharmacological properties, including suppression of cell cycle progression, which contributes to inhibiting T-cell proliferation and cell division. Administration of auraptene decreases the CD3/CD28-activated T lymphocyte secreting T helper (Th)1 cytokines at lower levels (10 and 20 μM), and it could decrease Th2 cytokine IL-4 at a higher level (40 μM) [63]. The dose administrated is essential also in the case of curcumin, which at 2.5 μg/ml inhibits ConA, PHA, and PMA-stimulated human spleen lymphocyte proliferation at 77, 23, and 48%, respectively, over controls, reaching 100% inhibition in higher dose (5 μg/ml) [64].

The mechanism of decreasing the activity of effector Th1 cells proposed for cirsilineol (a trimethoxyflavone isolated from *Artemisia vestita*) is the selective inhibition of IFNγ signaling, mediated through downregulating STAT1 activation and T-bet expression in colonic lamina propria CD4(+) T cells. Therefore, it is strongly suggested that cirsilineol might be potentially useful for treating T-cell-mediated human inflammatory bowel diseases [65].

Treg cells

Besides the cytotoxic T cells and Th cells mentioned above, there are the regulatory T (Treg) cells, which are critical in maintaining immune tolerance and suppressing autoimmunity.

Green tea and its active ingredient, epigallocatechin-3-gallate (EGCG), have been shown to improve symptoms and reduce the pathology in some animal models of autoimmune diseases. Mice treated with EGCG had significantly increased Treg frequencies and numbers in the spleen and lymph nodes and had inhibited T-cell response [66, 67] and dose-dependently attenuated the disease's severity [68].

6.3. Macrophages

Macrophages are the main cells responsible for the innate immunity, and their activation by lipopolysaccharide (LPS) from Gram-negative bacteria or IFNγ from host immune cells is important for controlling infections. Activation of mononuclear cells and increase of the phagocytic response are induced by several phytophenols, mainly via influencing of MAPK and nuclear factor κB (NF-κB) signaling pathways: daidzein at high doses (20 and 40 mg/kg) [69], coumarin (1,2-benzopyrone) [70], procyanidin A1 [39], procyanidin C1 (max dose 62.5 μg/ml) and procyanidin dimer B2 [60], kaempferitrin from *Justicia spicigera* extracts at 25 μM [71], biflavone isolated from 80% aqueous methanol extract of *Jatropha curcas* L-di-C-glucoside,6,6″-di-C-beta-D-glucopyranoside-methylene-(8,8″)-biapigenin (0.25 mg/kg body wt to 1-day-old specific-pathogen-free (SPF) chicks) [46], oenothein B [19], morin hydrate (5, 10, and 15 μM) [72], geraniin, and isocorilagin (up to 12.5 lg/ml) [73].

As macrophages are stimulated to secrete a battery of inflammatory mediators and cytokines, regulation of their activity ensures an appropriate immune response. Inappropriate or prolonged macrophage activation is largely responsible for various inflammatory states. There are also phytophenols that inhibit the secretion of various pro-inflammatory molecules from macrophages or their migration: grape polyphenols [74], cianidol [75], EGCG [76], fisetin [77], quercetin, kaempferol, daidzein, genistein [78], xanthohumol [79], etc.

Orange juice and hesperidin, a flavanone glycoside contained in the juice, showed different immune responses, suggesting that hesperidin displays a suppressive effect on inflammation generated by LPS, while the juice seems to enhance the functions of macrophages associated with antimicrobial activity [80].

6.4. Neutrophils

These cells provide rapid response and nonspecific protective effect against invading pathogens, and the exposure of antigen by APCs is not required to activate these cells [38]. Most of the effects exerted by phytophenols on neutrophils are based on inhibition of superoxide anion production: biflavonoids like procyanidin, fukugetin, amentoflavone, and podocarpusflavone isolated from *Garcinia brasiliensis* showed potent inhibitory effects on the oxidative burst of human neutrophils, inhibiting reactive oxygen species (ROS) production by 50% at 1 μmol L^{-1} [81], catechol (1–10 μM) [82], broussochalcone A—a prenylated chalcone [83], and viscolin [84].

The effects of flavonoids on human neutrophils are complex and suggest several sites of action depending upon the flavonoid's subcellular distribution and pathway of stimulation [85].

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6.5. Modulation of soluble factor secretion

6.5.1. Immunoglobulins

Humoral immunity is mostly quantified by serum levels of specific immunoglobulins. A stimulatory effect on IgM- and IgG-mediated humoral immune response was observed in the case of green tea, a well-known rich source of polyphenols [86]. Serum IgM and IgG levels are also significantly increased, whereas specific IgA and IgE are not changed after ellagic acid (a natural phenolic compound found in fruits and nuts) treatment [87].

IgG response is increased after treatment with pomegranate extract rich in polyphenols (16.9% gallic acid equivalent (GAE) per day in calves) [88] and red wine (Negroamaro) pre-treatment of lymphomonocytes [57]. Immunoglobulin synthesis is induced also by cianidol [75], its O-methyl-derivative [89] and daidzein [69].

Humoral immunity measured by anticomplement activity showed an increase in inhibition of the complement system after the addition of morin (natural flavonoid that is the primary bioactive constituent of the family Moraceae) hydrate (significant effect at 15 μM concentration) [72].

6.5.2. Interleukins

6.5.2.1. IL-2

Catechin, epigallocatechin gallate (EGCG), epicatechin (EC), luteolin, chrysin, quercetin, and galangin increase IL-2 secretion, while EGC, apigenin, and fisetin inhibit the secretion. There was no obvious structure-activity relationship with regard to the chemical composition of the flavonoids and their cell biological effects [90]. Contradictory results were obtained by Xiao et al. [91], which reported the inhibitory action of chrysin on splenic mononuclear cell secretion of interleukin-2, after oral administration of the phytocompound from day 1 to day 16 (50 mg/kg once daily), while, for therapeutic treatment, rats received chrysin from day 7 to day 16 at the same dose once daily).

Inhibitory effects on IL-2 production have also equol (4′,7-isoflavandiol) [14], quercetin by IL-2R alpha-dependent mechanism [55] and curcumin, which inhibits IL-2 synthesis in ConA, PHA, and PMA stimulated SP-L in a concentration-dependent manner with an ED50 measured at 3.5 μg/ml. Exogenous IL-2-stimulated SP-L proliferation is also inhibited by curcumin in a concentration-dependent manner with an ED50 of 2 μg/ml [64]. 8-Methoxypsoralen (140 μM) induces a dose-dependent decrease in IL-2 receptor expression on PHA-stimulated lymphocytes, explaining the mechanism by which this compound impairs lymphocyte function, since IL-2 receptors play a central role in lymphocyte proliferation and immune reactivity [92, 93].

6.5.2.2. IL-12

IL-12 is the most important factor driving Th 1 immune responses. An interesting dynamic was showed in the case of the orange juice and its main component, hesperidin. In non-LPS-

stimulated macrophages, IL-12 level was increased by orange juice by 143% and hesperidin by 72%. For LPS-stimulated macrophages, the orange juice treatment did not alter IL-12 level, while hesperidin treatment decreased IL-12 level by 29%, suggesting that hesperidin displays a suppressive effect on inflammation generated by LPS [80]. Curcumin exhibits impaired IL-12 expression in DCs [41]; quercetin blocks IL-12-dependent JAK-STAT signaling in Th cells [55]; ellagic acid reduces IL-12 production both ex vivo and in vivo treatment [87]; chrysin [91], licochalcone E [94], xanthohumol, shows the strongest inhibitory effect on IL-12 production in LPS-stimulated xanthohumol 4′-O-beta-D-glucopyranoside (XNG) being less effective, followed by isoxanthohumol and 8-prenylnaringenin while (2S)-5-methoxy-8-prenylnaringenin 7-O-beta-D-glucopyranoside have no effect [95]. macrophages, xanthohumol 4′-O-beta-D-glucopyranoside (XNG) being less effective, followed by isoxanthohumol and 8-prenylnaringenin, while (2S)-5-methoxy-8-prenylnaringenin 7-O-beta-D-glucopyranoside [94] and licochalcone E have no effect [95].

6.5.2.3. IL-1β

Inhibitory effect on IL-1b secretion has apigenin [96] and flavokawain A in the LPS-stimulated cells [97]; curcumin in DCs [41]; curculigoside in B16F10-induced metastatic tumor progression in experimental animals [98]; ellagic acid in ex vivo and in vivo experiments [87]; chrysin, in splenic mononuclear cells [91]; equol (4′,7-isoflavandiol) [14]; and kurarinone and kuraridin in RAW264.7 macrophages [99]. There are also phenolic phytocompounds, which promote pro-inflammatory IL-1b secretion: oenothein B [43], polyphenols contained in red wine (Negroamaro) [57], and 1% dietary EGCG [67].

6.5.2.4. IL-4

Quercetin [54], 6-gingerol [100], and ellagic acid [87] suppress interleukin IL-4 production, one of the key cytokines secreted by Th2 cells.

6.5.2.5. IL-6

Suppression of LPS-induced expression of pro-inflammatory cytokine IL-6 is induced by licorice flavonoids [101]; 1% dietary EGCG [67]; flavokawain A [97]; curcumin [41]; quercetin attenuates TLR7-induced expression, effect of mediated by HO-1 [102]; curculigoside [98]; syringic acid or vanillic acid [103]; licochalcone A [104]; chrysin [91]; apigenin, through modulating multiple intracellular signaling pathways in macrophages and prevents LPS-induced IL-6 production by reducing the mRNA stability via inhibiting ERK1/2 activation [96]; and luteolin at transcriptional level [13].

6.5.2.6. IL-17

It is well known that IL-17 is an essential factor involved in autoimmune diseases, and some synthetic inhibitors are already in clinical testing. As regards phytophenols, grape seed proanthocyanidin extract (GSPE) shows promising results, attenuating clinical symptoms in a model of collagen-induced arthritis in mice [105].

6.5.3. TNF-α

Based on the inhibitory effect on TNF-α secreted by LPS-stimulated cells, flavonoids were classified in four groups: strong (flavones, flavonols, chalcones), moderate (flavanones, naringenin, antocyanidin, pelargonidin), weak (genistein), and inactive (eriodictyol) [106]. Several phenolic phytocompounds successfully suppress the expression of pro-inflammatory cytokines such as TNF-α; flavokawain A [97]; curcumin [41]; quercetin [102]; curculigoside [98]; ellagic acid [87]; syringic and vanillic acids [103]; apigenin [96]; chrysin [91]; kurarinone and kuraridin [99]; luteolin [13]; equol (4′,7-isoflavandiol) [14]; and cardamonin, a chalcone derivative isolated from *Artemisia absinthium* L. [107]. An enhancement of TNF-α production is noticed for 1% dietary EGCG, while no effect was exhibited by lower concentrations of compound (0.15–0.3%) [67].

6.5.4. IFNγ

Chrysin inhibited the splenic mononuclear cell secretion of IFNγ [91]; quercetin is suggested to exert T-bet-dependent IFNγ suppression [55], ellagic acid [87], syringic and vanillic acid [103], equol (4′,7-isoflavandiol) [14]. On the other hand, other phytophenols enhance IFNγ level: curculigoside [98], oenothein B, by both bovine and human NK cells and T cells, alone and in combination with IL-18 [44], and a response not observed with other commonly studied polyphenols [43].

6.6. Influence on transcription factors

Nuclear factor κB (NF-κB) plays an important role in inflammatory processes, in autoimmune response, apoptosis, and cell proliferation, by regulating the genes involved in these processes. This factor is activated mainly under conditions of oxidative stress, under the action of various pathogenic stimuli (viruses and bacteria but also inflammatory cytokines). Because of its effects on vital biological processes, modulation of its activation pathway is of great therapeutic potential.

Curcumin inhibits PMA-stimulated NF-κB activation in lymphocytes by 24, 38, and 73%, respectively, at final concentrations of 2.5, 5, and 10 μg/ml, respectively [64], and fisetin also inhibits LPS-induced nuclear factor κB activation and JNK/Jun phosphorylation [77]. In LPS-stimulated macrophages, activation of NF-κB that is inhibited was reported for caffeic acid phenethyl ester [108]; licochalcone E, a constituent of licorice [94]; luteolin [13]; kurarinone and kuraridin [99]; astragalin (kaempferol-3-O-glucoside) [109]; naringin [110]; nodakenin, a coumarin isolated from the roots of *Angelica gigas* [111]; quercetin (100 ppm) [112]; carnosol (20 μM) [113]; and apigenin [36]. The main mechanism of inhibition consists in the degradation of inhibitor κB and nuclear translocation of NF-κB p65 subunit. These events are strongly linked with modulation of reactive oxygen species generation. A correlation between antioxidant and immune function was presented for equol (4′,7-isoflavandiol), an isoflavandiol metabolized from daidzein, which at an optimal concentration of 40 μmol/L exerts mainly antioxidant effects in chicken macrophages by increasing T-SOD, GSH levels but collateral immune enhancement by increasing expression of TLR4 and genes encoding cytokines [14].

It was found that both phenolic acids and other phenolic compounds found in free form in cereal grains are significant modulators of NF-κB activity, but only their combinatorial action

gives the desirable effect. Although ferulic and p-coumaric acids alone are effective modulators of NF-κB activity, a mixture of ferulic, caffeic, p-coumaric, and sinapic acids in low concentrations has significant synergistic, enhanced, and additive effects on NF-κB activity [35].

7. Conclusions

Considerable attention is currently focused on the development of natural medicines with less or no side effects, maximum efficacy, and low cost. Plant phenolic compounds proved to be competitive candidates for therapy in several disorders, and some of them undergone clinical trials (e.g., quercetin, curcumin). Modulation of immune system is a challenge, due to complex mechanisms involved and to route of administration, knowing that most of the plant-derived compounds are given orally in the form of medicines or even as functional foods.

Several aspects resulted from reviewing the literature: plant extracts are not always well characterized or standardized, making difficult to assign the immunomodulatory effect to a single compound; high concentrations of phenolic compounds are used for in vitro studies, and substances that have proven effective on laboratory scale are often ineffective in clinical trials, often due to bioavailability aspects (as these compounds are an important part of the human diet). Up to now, few human trials were carried out, most of them being focused on proving only the antioxidant or anti-inflammatory effect. This review reveals that phenolic compounds are a rich source of valuable potential therapeutic agents for immune system modulation, but further work needs to be carried out in order to establish therapeutical doses, precise mechanism of action, and optimal ways of administration.

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Author details

Alice Grigore

Address all correspondence to: alicearmatu@yahoo.com

National Institute of Chemical-Pharmaceutical Research and Development, Pharmaceutical Biotechnologies Department, Plant extracts laboratory, Bucharest, Romania

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Chapter 6

Inhibitory Properties of Phenolic Compounds Against Enzymes Linked with Human Diseases

Sandra Gonçalves and Anabela Romano

Additional information is available at the end of the chapter

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Abstract

Some drugs currently used are inhibitors of enzymes involved in mediating many disease processes. Concerns over the toxicity and side effects of synthetic enzyme inhibitors have led to a search for new safe and effective inhibitors particularly from natural sources. Owing to their wide range of biological effects, plant phenolic compounds are one of the most studied families of natural products. This chapter aims to provide an overview of the potential of phenolic compounds as enzyme inhibitors. Extensive research has been conducted to study the enzyme inhibitory capacity of many phenolic compounds against several enzymes linked with important human conditions. Investigations conducted are mainly focused on the inhibition of angiotensin I-converting enzyme, α-amylase and α-glucosidase, lipase, cholinesterases, proinflammatory enzymes (cyclooxygenases and 5-lipoxygenase) and tyrosinase, which are related with hypertension, type II diabetes, obesity, Alzheimer's diseases, inflammation and skin hyperpigmentation, respectively. Overall, among phenolics, flavonoids are probably those with great capacity to inhibit the activity of the enzymes revised. Several studies demonstrated the potent antioxidant and anti-inflammatory properties of flavonoids, which highlight the therapeutic potential of these compounds. Although our literature survey showed that a huge number of phenolic compounds have been studied and there are some promising compounds depending on the enzyme, more *in vivo* tests and subsequent steps to be a drug candidate are required before therapeutic application.

Keywords: Alzheimer's disease, diabetes, flavonoids, hyperpigmentation, hypertension, inflammation, obesity

1. Introduction

Due to their essential catalytic role in several physiological processes, enzymes are considered to be one of the most attractive targets for drug intervention in human diseases [1]. Indeed,

the therapy of some important human aliments, namely hypertension, metabolic disorders, inflammatory diseases and neurodegenerative diseases, includes the use of enzyme inhibitors. Nevertheless, some of the inhibitors currently in use (**Table 1**) are reported to have side effects, including hepatotoxicity, gastrointestinal disturbances and diarrhea [2–4]. Consequently, there is a great interest in finding new effective natural inhibitors without undesirable effects.

Diseases	Enzyme	Main standard inibitor(s)
Hypertension	Angiotensin-converting enzyme	Aptopril, benazepril, enlapril
Diabetes	α-Amylase and α-glucosidase	Acarbose
Obesity	Pancreatic lipase	Orlistat
Alzheimer's diseases	Cholinesterases	Tacrine, donepezil, rivastigmine, galantamine
Inflammation	Cyclooxygenases and 5-lipoxygenase	Indomethacin
Skin hyperpigmentation	Tyrosinase	Kojic acid

Table 1. Some standard enzyme inhibitors commonly used.

The discovery of enzyme inhibitors to be used in human therapeutics is an active and actual area of research. Several studies provided evidence about the beneficial effects of phenolic compounds in human health due to their wide range of biological properties, namely antioxidant, anticancer, and antimicrobial [5]. The biological actions of phenolic compounds involve different mechanisms including the interaction with enzymes [6]. In the last years, a great number of reports were published describing the inhibitory potential of phenolic compounds against human enzymes. The present chapter aims to systematize the information about the enzyme inhibitory properties of phenolic compounds against key enzymes associated to several human diseases, namely angiotensin I-converting enzyme, α-amylase, α-glucosidase, lipase, cholinesterases, cyclooxygenases (COXs), 5-lipoxygenase (5-LOX) and tyrosinase.

2. Human diseases and phenolic compounds

Chronic diseases or non-communicable diseases are adverse health conditions of long duration and, generally, also of slow progression, causing 63% of all deaths worldwide (36 million out 57 million global deaths) [7, 8]. These diseases can be classified into five main groups: cardiovascular diseases; cancer; chronic respiratory diseases, such as chronic obstructed pulmonary disease and asthma; diabetes; and neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases. Hypertension is one of the major risk factors for cardiovascular diseases and is estimated to affect one-third of the Western population [9]. Obesity is also considered a global epidemic problem and is associated with multiple chronic diseases [10]. Overproduction of oxidants and chronic inflammation is responsible for the pathogenesis of many chronic diseases, and experimental and epidemiological studies demonstrated that plant antioxidants play an important role in the prevention and treatment of these diseases.

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Plant-derived antioxidants, particularly phenolic compounds, can reduce the oxidative stress in the body maintaining, a balance between oxidants and antioxidants due to their reducing, free radical scavenging or metal chelating properties [8]. Phenolic compounds can easily donate hydrogen from hydroxyl groups positioned along the aromatic ring to terminate free radical oxidation of lipids or other biomolecules, and the aromatic phenolic ring can stabilize and delocalize the unpaired electron within its aromatic ring [11].

More than 8000 different structures of phenolics have been identified up to now [12]. Phenolics may be classified into different groups depending upon the number of phenol rings and the structural elements that bind these rings to one another [13]. The main groups of phenolic compounds are flavonoids, phenolic acids, tannins, stilbenes and lignans [13]. Flavonoids are probably the most important group of phenolic compounds. These are molecules of low molecular weight that have a common C6–C3–C6 structure consisting of two aromatic rings (A and B) linked through a three carbon chain, usually organized as an oxygenated heterocycle (ring C) (**Figure 1**) [6]. Flavonoids can be characterized as flavanones, flavones, flavonols, isoflavones, flavanols (essentially, flavan-3-ols) and anthocyanidins. This classification depends on the degree of unsaturation and oxidation of the oxygenated heterocycle [6]. Many authors demonstrated that the position and number of substituents in the flavonoid basic structure significantly affect the biological function [6, 14].

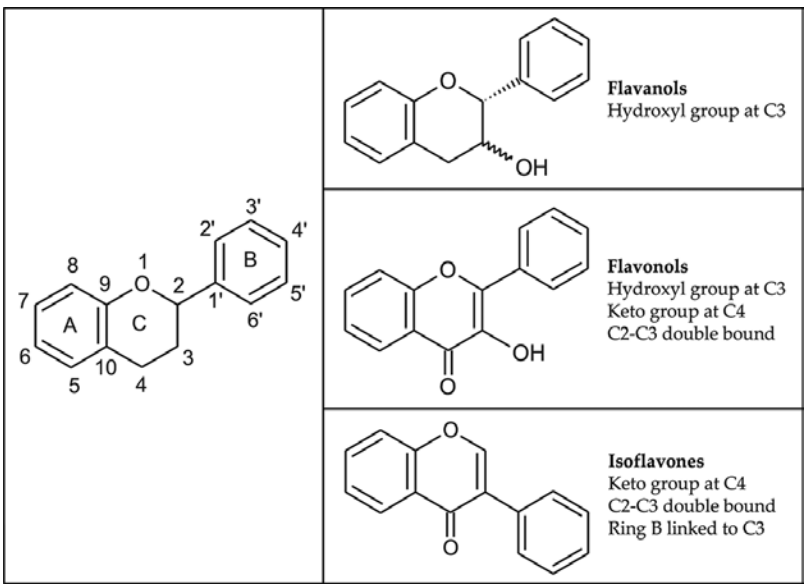

Figure 1. Chemical structure of selected flavonoids. Adapted from Fraga et al. [6].

The biological action of phenolic compounds involves different mechanisms including nonspecific and specific mechanisms [6]. Nonspecific mechanisms are for instance the free radical scavenging and metal sequestration capacity of phenolic compounds and their interactions with membranes. On the other hand, specific mechanisms include

the interaction of phenolics with enzymes, with transcription factors or with receptors. The complexity of some diseases leads to the recent search for alternative therapeutic strategies based on combined therapy protocols and multifunctional compounds. Thus, phenolic compounds, showing a wide range of biological effects through different mechanisms, have a great potential to be used in the prevention and treatment of several human diseases (**Figure 2**).

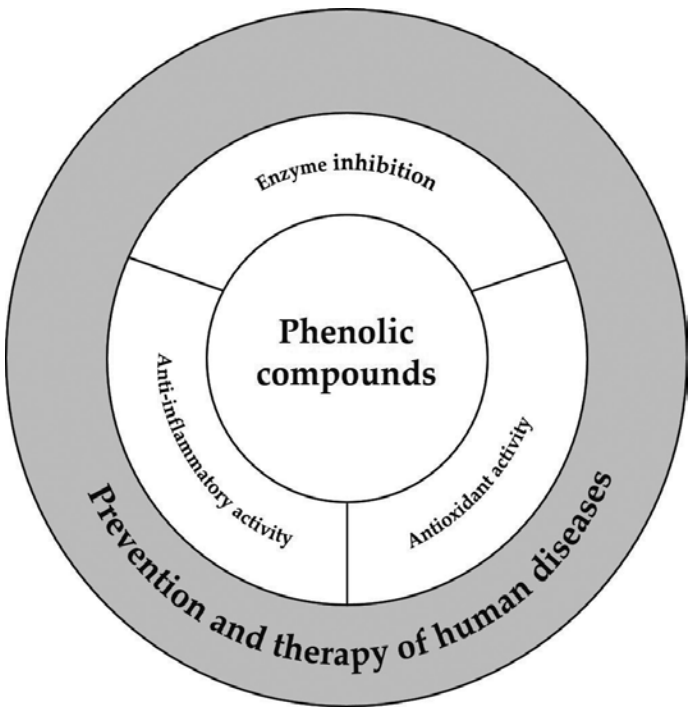

Figure 2. Snapshot of some of the health benefits of phenolic compounds.

3. Enzyme inhibitors

Enzyme inhibitors can have many applications in pharmaceutical, environment and biochemical industries, having a great impact on healthcare and medical sector. The control of some important human diseases includes the use of enzyme inhibitors which represent a great part of the drugs in clinical use. Specific enzyme inhibition will remain a major focus of pharmaceutical research for the foreseeable future [1]. Enzymes are protein molecules acting as catalyst in biological systems. Enzymes specificity assures high coordination and harmonious interplay among different metabolic activities essential to sustain life. The enzyme activity depends on numerous factors, for example, the most important the enzyme concentration,

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the amount of specific enzyme substrate, the electrochemical reaction of medium for enzyme activity (pH) and the presence of activators or inhibitors.

Enzyme inhibitors prevent enzymes from their catalytic function by interfering with any step in the catalytic cycle. They are low molecular weight compounds that in small quantity can reduce or completely inhibit the enzyme activity [15]. Some human enzyme inhibitors, such as antithrombin and antitrypsin, control the enzyme activity in the body, and under physiological conditions, they guarantee their action. Among natural enzyme inhibitors, there are intermediary products produced during some metabolic pathways. The inhibition of products is a restricted way of control or modulation of substrate flux through the pathway. If enzymes are sensitive to product inhibition, the output of end product of the pathway will be suppressed [16].

An inhibitor can modify one amino acid or several side chain(s) required in enzyme catalytic activity. To protect enzyme catalytic site from any change, ligand binds with critical side chain in enzyme. Enzyme inhibitors are conceptually classified as specific and nonspecific. Inhibitors can reduce or completely inhibit the enzyme catalytic activity reversibly or irreversibly. Irreversible inhibitors usually change the enzyme chemically. Reversible inhibitors bind non-covalently to produce different types of inhibition, depending on whether these inhibitors bind the enzyme, the enzyme-substrate complex, or both. Most drugs that function through enzyme inhibition interact with their target enzyme through simple, reversible binding mechanisms [1]. Reversible inhibitors can be classified into competitive, noncompetitive or uncompetitive (**Figure 3**). In competitive inhibition, the substrate and inhibitor cannot bind to the enzyme at the same time; therefore, the competitive inhibitor competes with the substrate for the active site. A noncompetitive inhibitor binds equally well to both free enzyme and the enzyme-substrate complex. An uncompetitive inhibitor binds exclusively to the enzyme-substrate complex yielding an inactive enzyme-substrate-inhibitor complex.

4. Phenolic compounds as inhibitors of enzymes linked with human diseases

The use of plant-based enzyme inhibitors is encouraged nowadays because there is concern about the critical side effects of synthetic pharmaceutical agents. In the following sections, the enzyme inhibitory properties of phenolic compounds are reviewed. Investigations have been mainly focused in angiotensin I-converting enzyme, α-amylase and α-glucosidase, lipase, cholinesterases, proinflammatory enzymes (COXs and 5-LOX) and tyrosinase, which are linked with hypertension, type II diabetes, obesity, Alzheimer's diseases, inflammation and skin hyperpigmentation, respectively (**Table 2**). These were selected to be included in this chapter although there are evidences of the inhibitory properties of phenolic compounds against other enzymes like monoamine oxidase and catechol-*O*-methyl transferase.

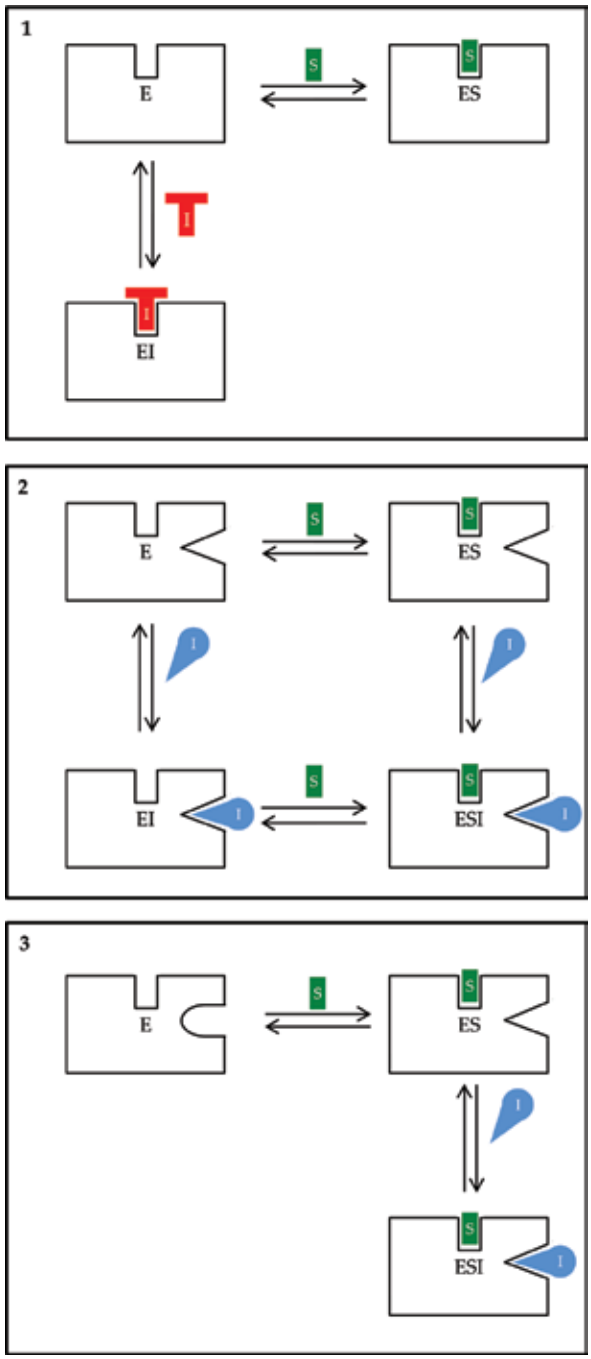

Figure 3. Scheme representative of the three major forms of reversible inhibitor interactions with enzymes: (1) competitive inhibition; (2) noncompetitive inhibition; (3) uncompetitive inhibition. S: Substrate; E: free enzyme (E); ES: enzyme-subtract complex; I: inhibitor; ESI: enzyme-subtract-inhibitor complex. Adapted from Copeland [17].

Diseases	Enzyme	Compound(s)	Important references
Hypertension	Angiotensin-converting enzyme	Flavonoids	[14]
		Tannic acid	[19]
		Anthocyanins	[27]
		Proanthocyanidins	[28]
Diabetes	α-Amylase and α-glucosidase	Flavonoids	[37, 39, 40]
Obesity	Pancreatic lipase	Flavonoids, phenolic acids	[47]
Alzheimer's diseases	Cholinesterases	Flavonoids	[53, 54]
		Coumarins	[59]
Inflammation	Cyclooxygenases and 5-lipoxygenase	Flavonoids	[62, 64–67]
		Stilbenes	[61, 62]
Skin hyperpigmentation	Tyrosinas	Flavonoids	[71, 73]
		Stilbenes	[72, 74, 75]
		Chalcones	[72]

Table 2. Main phenolic compounds described as inhibitors of enzymes linked with human ailments.

4.1. Hypertension: inhibition of angiotensin-converting enzyme (ACE)

Hypertension is a common and often progressive disorder that poses a major risk for cardiovascular diseases and related complications [18]. Hypertension is an important and increasing public health problem worldwide, and some data indicate that about one-quarter of the adult population suffers from this condition [19]. Inhibition of angiotensin-converting enzyme (ACE) is considered to be a target for discovery of lead antihypertensive agents and an important therapeutic approach in the treatment of high blood pressure [20]. ACE catalyzes the conversion of the precursor angiotensin I into angiotensin II, a peptide responsible for triggering vasoconstrictive effects, and it degrades bradykinin, a potent vasodilator [21, 22]. Some of the widely used ACE inhibitors such as aptopril, benazepril, enlapril and other [23] have revealed certain limitations like susceptibility to proteolytic degradation leading to side effects. Therefore, research has been conducted to find new ACE inhibitors from natural sources particularly from plant origin. Many investigations indicate that polyphenol-rich food is effective in the protection and treatment of hypertension namely via ACE inhibition [24]. In a recent review, Patten et al. [25] described 74 families of plants that exhibited significant ACE inhibitory activity. Also, Field and Newton [26] have shown that cocoa polyphenols are bioavailable molecules that induce an antihypertensive response including through ACE inhibition.

Guerrero et al. [14] evaluated the ability of 17 flavonoids belonging to five structural subtypes to inhibit ACE and showed that the highest activity was obtained for luteolin. Results from these authors allow concluding that the combination of substructures on the flavonoid skeleton that increase ACE activity is made up of the catechol group in the B-ring, the double

bond between C2 and C3 at the C-ring and the cetone group in C4 at the C-ring. Al Shukor et al. [19] investigated the ACE-inhibitory capacity of 22 phenolic compounds belonging to different classes and subclasses. Among analyzed compounds, tannic acid exhibited the highest ACE-inhibitory activity. Results indicated that the number of hydroxyl groups on the benzene ring plays an important role for activity of phenolic compounds and that substitution of hydroxyl groups by methoxy groups decreased activity. Furthermore, phenolic acids and flavonoids inhibit ACE via interaction with the zinc ion, and this interaction is stabilized by other interactions with amino acids in the active site. Resveratrol and pyrogallol may inhibit ACE via interactions with amino acids at the active site blocking the catalytic activity of ACE [19].

Several studies suggest anthocyanins as important ACE inhibitors. Ojeda et al. [27] demonstrated the ACE-inhibitory capacity of the anthocyanins delphinidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside isolated from *Hibiscus sabdariffa*, a plant used in folk medicine, as antihypertensive. Moreover, kinetic determinations suggested that those compounds inhibit the enzyme activity by competing with the substrate for the active site. Studies from Eriz et al. [28] with *Vitis vinifera* extracts demonstrated that the inhibitory activity of proanthocyanidins (condensed tannins) against ACE is associated to a higher availability of OH groups, larger mean degree of polymerization and presence of epicatechin gallate.

4.2. Type II diabetes mellitus (DM): inhibition of carbohydrates-hydrolyzing enzymes

Disorders of carbohydrate uptake may cause severe health problems such as diabetes, obesity and oral diseases [29]. Diabetes mellitus (DM) is one of the most serious and chronic diseases and can be attributed to hyperglycaemia, a condition characterized by an excessive concentration of glucose circulating in the blood. Two types of DM are known, type I that is characterized by insufficient insulin production and type II that results from ineffectiveness of insulin [30]. Type II DM accounts for approximately 90% of diabetes cases worldwide and is attributed to greater prevalence of sedentary lifestyle, unhealthy diet and rise of obesity within modern society as well as an increasing number of elderly populations [31, 32]. Type II DM has become a serious medical concern worldwide and is frequently correlated with many complications such as cardiovascular diseases, hypertension, kidney failure, blindness, and neurological complications [33].

One therapeutic approach for treating DM type II is to decrease the postprandial glucose levels, which could be done by retarding the absorption of glucose through the inhibition of the carbohydrates-hydrolyzing enzymes, α-amylase and α-glucosidase, in the digestive tract. These enzymes are responsible for the breakdown of oligosaccharides and disaccharides into monosaccharides suitable for absorption. Inhibitors of these enzymes delay the rate of glucose absorption by preventing carbohydrate digestion and consequently blunting the postprandial plasma glucose rise [34]. The use of synthetic agents as acarbose is an important clinical strategy for controlling postprandial glycemia [35]. Acarbose reduces blood glucose levels and is approved by Food and Drug Administration (FDA); however, it causes critical side effects, such as liver disorders [4], and its use is now restricted [36]. To avoid or decrease the adverse effects of currently used synthetic inhibitors, it is necessary to employ naturally

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occurring alternatives. In the last years, several reviews have been published focusing on the antidiabetic potential of natural products including on the inhibitory properties of phenolic compounds against carbohydrates-hydrolyzing enzymes [29, 30, 37, 38]. Phenolic compounds have been receiving much attention for controlling the digestibility of starch [30], and flavonoids are widely studied as α-amylase inhibitors [37]. Tadera et al. [39] tested several flavonoid compounds for their inhibitory activity against α-amylase and showed that, in general, the potency of inhibition is correlated with the number of hydroxyl groups on the B ring of the flavonoid scaffold. The structural requirements for the inhibition of human salivary α-amylase by 19 flavonoids were studied by Lo Piparo et al. [40]. Author's findings demonstrated that the flavonols and flavones enzyme inhibitory capacity depend on hydrogen bonds between the hydroxyl groups of the polyphenol ligands and the catalytic residues of the binding site and formation of a conjugated α-system that stabilizes the interaction with the active site. Recently, Xiao et al. [37] revised the structure-activity relationship of polyphenols inhibiting α-amylase and concluded that the hydroxylation galloylation of flavonoids, including catechins, improved the inhibitory effects against α-amylase. Moreover, these authors also observed that the glycosylation of the hydroxyl group, methylation, methoxylation and the hydrogenation of the C2-C3 double bond on flavonoids decreased the inhibition of the enzyme.

There are also some reports describing the inhibitory properties of other classes of phenolic compounds, like hydroxycinnamic acids and tannins, against α-amylase and α-glucosidase; however, overall they are less effective than flavonoids [29, 37].

Phenolic compounds, in particular flavonoids, may provide a protective effect against hyperglycemia-induced chronic diseases through a dual protection: inhibition of starch digestion and antioxidant effect. The co-application of phenolics with synthetic enzyme inhibitors for controlling prosprandial glycemia may reduce the effective dose of synthetic inhibitors required [30].

4.3. Obesity: inhibition of pancreatic lipase

Obesity is considered a global epidemic problem by the World Health Organization (WHO) and is recognized as the main life style disorder in developing countries, being termed as the "New World Syndrome." It is associated with multiple chronic diseases and disabilities such as dyslipidemia, fatty liver disease, osteoarthritis, hypertension, obstructive sleep apnea, gallstones, type II diabetes, reproductive and gastrointestinal cancers, coronary artery disease, heart failure and stroke [10, 41, 42].

The methods used to reduce body weight include diet, exercise, drug therapy, bariatric surgery or combinations of several of these methods. Currently, orlistat (Xenical) is the only drug approved by FDA for long-term treatment of obesity [43, 44]. Orlistat reduces intestinal triglyceride absorption through inhibition of pancreatic lipase. Its long-term administration is associated with a small but statistically significant weight loss of about 3% more than diet alone in overweight and obese people [45]. Moreover, it can also decrease blood pressure, prohibit the onset of DM type II and improve oral glucose tolerance [44]. However, some adverse gastrointestinal effects of orlistat have been reported as steathorrhea, bloating, oily spotting, fecal

urgency and fecal incontinence, as well as hepatic adverse effects [2]. Thus, there has been an increase interest in the search for new natural substances that show potent inhibitory activity against pancreatic lipase with fewer side effects. As a result, many plant extracts and compounds have been screened for their capacity to inhibit pancreatic lipase [44, 46, 47]. Among plant compounds, proteins, polysaccharides, saponins, triterpenes and phenolic compounds have shown capacity to inhibit this enzyme [46]. Phenolic compounds have some potential efficacy for preventing obesity by inhibiting the activity of enzymes related to fat metabolism as pancreatic lipase, lipoprotein lipase and glycerophosphate dehydrogenase [48]. Examples of compounds with pancreatic lipase inhibitory capacity are the flavonoid hesperidin from the peels of *Citrus unshiu*, proanthocyanidins from *Cassia mimosoides* and tea catechins [47, 48]. A compilation of recent and significant results of phenolic compounds as pancreatic lipase inhibitors can be found in the review recently published by Buchholz and Melzig [47]. Flavonoids and phenolic acids are probably the most studied chemical classes of phenolics showing this effect [47]. The lipase inhibitory capacity has been documented for more than 70 different flavonoids, and the inhibitory effect depends on the number and position of phenolic hydroxyl groups [47]. In the class of phenolic acids, several hydroxybenzoic and hydroxycinnamic have shown capacity to inhibit the enzyme. However, hydroxybenzoic acids are less effective than hydroxycinnamic acids, and the influence of methoxy groups (less efficient) in the molecule and hydroxyl groups (more efficient) can be seen. The carboxy group takes part in the activity of these compounds, and the size of the molecule influences the activity [49].

4.4. Alzheimer's diseases (AD): inhibition of cholinesterases

Alzheimer's disease (AD), the most common form of dementia, is a progressive age-related neurodegenerative disorder. AD rates are predicted to increase enormously, especially in developing regions, considering the accelerated aging of human society and the increase in life expectancy worldwide. Although the exact pathogenesis of AD still remains to be fully elucidated, it is currently considered to be a multifactorial disease. In the early 1970s, postmortem studies revealed that choline uptake and acetylcholine release are reduced in the brains of AD patients being these reductions associated with substantial presynaptic cholinergic deficits [50]. This led to the establishment of the "cholinergic-deficit hypothesis," which posits that the impairment in the cholinergic function is of critical importance in AD especially in the brain areas dealing with learning, memory, behavior and emotional responses that include the neocortex and the hippocampus. The levels of acetylcholine, a neurotransmitter responsible for the conduction of electrical impulses from one nerve cell to another nerve cell, are decreased due to its rapid hydrolysis by acetylcholinesterase (AChE) enzyme [3, 50]. Butyrylcholinesterase (BChE) is an enzyme closely related to AChE and serves as a co-regulator of cholinergic neurotransmission by hydrolyzing acetylcholine [51]. Some studies have shown an increased BChE activity in the most affected areas of the brain during the development of AD. The inhibition of both AChE and BChE has been documented as critical targets for the management of AD by an increase in the availability of acetylcholine and decrease in the Aβ deposition. However, the brain region contains a small quantity of BChE since it is mostly localized in the peripheral tissues. Therefore, the potential advantage of selective inhibition of AChE over BChE may include lesser degree of associated side effects

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due to peripheral inhibition of cholinesterase enzyme [52]. The first drug developed for AD based on the cholinergic-deficit hypothesis was tacrine that was approved for the treatment of cognitive loss in patients with AD by the FDA in 1993 [3]. Later, other cholinesterase inhibitors, like donepezil (1996), rivastigmine (2000) and galantamine (2001), have been approved and used for the management of AD [3, 52]. Due to the limited efficacy and gastrointestinal side effects of these drugs, such as nausea and diarrhea, there has been a continuous effort to find more effective cholinesterase inhibitors to be used to prolong and improve the life of the AD patients [3]. In this sense, plant extracts and compounds have been investigated for their role in prevention and treatment of AD including as cholinesterase inhibitors. Several investigations described the anticholinesterase effects of many naturally occurring flavonoids and newly synthesized flavonoids analogues. Uriarte-Pueyo and Calvo [53] reviewed the acetylcholinesterase inhibitory capacity of 128 flavonoids and conclude that flavones and isoflavones are the compounds with higher activity, proving that the carbonyl group at C4 seems to be important in this activity. Later on, Anand and Singh [54] discussed the data on the effects of flavonoids in various enzyme targets that play an important role in the pathogenesis of AD. Some conclusions arising are that isoflavone analogues demonstrate high AChE inhibitory activity, as compared to chalcone, flavones and flavanone analogues, suggesting that the nature of flavonoid moiety affects AChE inhibition in a great extent. Moreover, different moieties capable of interacting with catalytic site of AChE, including benzyl piperidine, piperidine, pyrrolidine, have been linked to paraposition of ring B of flavonoid scaffold using appropriate spacer to obtain dual-binding AChE inhibitors. Flavonoids may be attractive lead compounds for the development of effective and safe anti-AD drugs by modulating the enzyme targets in the disease [54].

Other compounds with potent AChE inhibitory activity are coumarins [55], and similar to flavonoids, several coumarins analogues have been synthesized, and their enzyme inhibitory capacity evaluated. Data on the inhibitory effects of coumarins against cholinesterases have been reviewed by several authors [56–58]. Various coumarins obtained from plants particularly from the *Angelica* genus revealed potent cholineterases inhibitory capacity (**Figure 4**) [56–59]. Taking into consideration structure-activity relationships, coumarins that small moieties such as hydroxyl or methoxy attached at C-7 of the coumarin skeleton seem to have a lower AChE inhibitory effect, in comparison with coumarins of greater substitutions, such as benzyloxy, $O–CH_2–C_6H_5$, positioned at the same carbon [60]. Additionally, a cyclized isoprenoid moiety at C-6 greatly contributes to the increase in AChE inhibition of some coumarin derivatives [59]. Moreover, research suggests that furanocoumarins are selective BChE inhibitors [56].

Numerous studies showed that phenolic compounds not only exhibit cholinesterase inhibitory capacity as potent antioxidant and anti-inflammatory properties, acting to scavenge radicals and regulate inflammatory responses; moreover, some of these compounds readily cross the blood-brain barrier to act on specific targets that have been implicated in the pathogenesis of AD.

4.5. Inflammation: inhibition of proinflammatory enzymes

Several diseases such as diabetes, obesity, cancer, osteoarthritis, atherosclerosis and Crohn's disease are associated with chronic inflammation. The mechanisms of inflammation involve

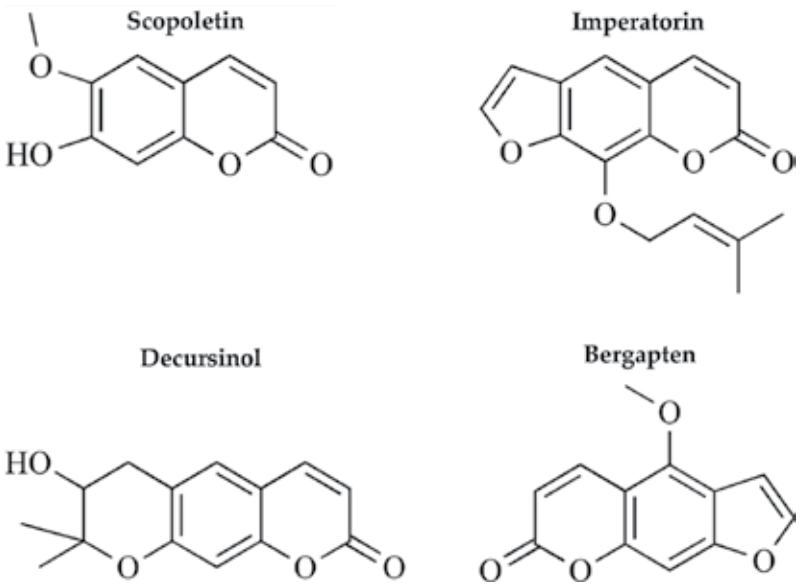

Figure 4. Chemical structure of some coumarins with cholinesterase inhibitory capacity.

a series of events in which the metabolism of arachidonic acid plays an important role [61] COX-1 and COX-2 enzymes catalyze the conversion of arachidonic acid to prostanoids. The second pharmacologically relevant metabolic pathway of arachidonic acid is mediated by 5-LOX. This enzyme is involved in the biosynthesis of inflammatory mediators named leukotrienes. COXs and LPXs are considered proinflammatory enzymes; the former affects platelet aggregation, vasoconstriction, vasodilatation and later, the development of atherosclerosis [62]. Both COXs and 5-LOX have received considerable attention because they are putative targets for cancer prevention. Nonsteroidal and steroidal anti-inflammatory drugs exert their action by inhibiting these proinflammatory enzymes through different mechanisms [63]. The nonsteroidal and steroidal anti-inflammatory drugs currently in use effectively manage the acute inflammatory reaction; however, in chronic inflammatory states, the long-term treatment with these drugs is followed by severe adverse effects. This justifies the search for new and safe anti-inflammatory agents being plant compounds good candidates. Recently, there has been interest in the antiinflammatory/immunomodulatory potential of flavonoids including their capacity to inhibit the activity of proinflammatory enzymes [64–66]. Li et al. [66] demonstrated the anti-inflammatory activity of the flavonoid baicalin. This compound inhibits COX-1, COX-2 and 5-LOX activities, decreases production of proinflammatory eicosanoids and attenuates edema in an *in vivo* model of inflammation. Butein, another flavonoid, decreased COX-2 expression in cancerous lung cells [67]. In addition, flavocoxid, a mixed extract containing the flavonoids baicalin and catechin, acts as a dual balanced inhibitor of COX-1 and COX-2 with a significant inhibition of 5-LOX [65]. It exerts beneficial effects in several experimental models of inflammation, and it has a significant efficacy in management of osteoarthritis and a good gastrointestinal tolerability [65].

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Stilbenes are another important group of plant compounds with anti-inflammatory properties. Kutil et al. [61] recently demonstrated that several natural stilbenes are potent inhibitors of proinflammatory enzymes and not only the most extensively studied compound of this group, resveratrol (3,5,4′-trihydroxy-trans-stilbene) found in red wine. The same group [62] evaluated the inhibitory potential of several wines and 33 phenolic compounds commonly occurring in wine against COX-1, COX-2, and 5-LOX. Authors observed that red wines were potent inhibitors of all three tested enzymes but the results obtained with isolated compounds could not fully explain the overall activities of the wine. Although trans-resveratrol considerably inhibits both COX-1 and COX-2, the activity of this compound alone could not be responsible for the overall inhibitory activity. In addition, results also showed that piceatannol, luteolin, quercetin, and myricetin were potent inhibitors of 5-LOX, but considering the ratio between their IC_{50} values and their concentration in wine only piceatannol could substantially contribute to the overall activity of red wines. Authors hypothesize that wine proanthocyanidins could also contribute to its overall potential since their inhibitory capacity against these enzymes was previously described.

4.6. Skin hyperpigmentation: inhibition of tyrosinase

The color of mammalian skin is mainly determined by the degree and distribution of melanin pigmentation. Melanin plays an important role in protecting skin from ultraviolet UV damage; however, overproduction of melanin poses not only an esthetic but also a dermatological problem. Indeed, some dermatological disorders, such as melasma and age spots, result in the accumulation of an excessive level of epidermal pigmentation [68].

Melanin is formed by several oxidative reactions which involve tyrosine and tyrosinase [69]. Tyrosinase catalyses three reactions in the biosynthetic pathway of melanin in melanocytes: the hydroxylation of tyrosine to L-DOPA and its oxidation to dopaquinone. Following a series of oxidoreduction reactions, the intermediate dihydroxyindole DHI and dihydroxyindole carboxylic acid are produced and polymerized to form melanins [70]. The inhibition of tyrosinase is one of the major strategies used to treat hyperpigmentation; however, concerns over the toxicity and side effects of synthetic inhibitors have led to a search for new safe and effective tyrosinase inhibitors. Moreover, tyrosinase is also responsible for browning in fruits and vegetables, and thus, inhibitors of this enzyme are frequently applied to plant-based foods.

Search of tyrosinase inhibitors is crucial for the development of skin whitening agents but also anti-browning and insect control substances. A number of researchers have been dedicated to identify inhibitors from natural sources including plants. The largest group of phytochemicals with potent tyrosinase inhibitors belongs to phenolics ranging from the simple ones to polyphenolics [71]. Furthermore, flavonoids occupy the largest portion in newly discovered natural tyrosinase inhibitors, and their structure is compatible with roles of both substrates and inhibitors of tyrosinase [71, 72]. Some flavonoids, such as kaempferol, quercetin, and morin, show inhibitory activity against tyrosinase, while others such as catechin and rhamnetin behave as substrates and suppress tyrosinase activity by being a cofactor (catechin) or acting as a free radical scavenger (rhamnetin) [72]. For instance, steppogenin, a flavanone derivative isolated from *Cudrania tricuspidata*, showed tyrosinase inhibitory activity much

higher than kojic acid, a known inhibitor of this enzyme [73]. The presence of two hydroxyl groups located on the aromatic ring at positions 2 and 4 in flavonoids was concluded to be necessary for tyrosinase inhibitory activity [71].

Some stilbenes, such as resveratrol, oxyresveratrol, chlorophorin and andalasin, have also been reported as having tyrosinase inhibitory properties [72, 74, 75]. The most promising inhibitor appears to be oxyresveratrol (32-fold higher inhibitory activity than kojic acid). Some of these compounds are sensitive to photo-oxidation, which limits their use in cosmetic formulations; however, the acetylated resveratrol derivative triacetyl resveratrol is noted to be comparably effective and much more stable than resveratrol [75].

There are also a number of chalcones (the precursors of flavonoids and isoflavonoids), such as licochalcone A, kuraridin, kuraridinol, 2,4,20,40-tetrahydroxy-3-(3-methyl-2-butenyl) and morachalcone A, with remarkable tyrosinase inhibitory activity [72]. Additionally, due to the versatile bioactivity and unique structural motif of chalcones, a number of derivatives have been developed as effective tyrosinase inhibitor candidates.

Although a great number of phenolic compounds revealed tyrosinase inhibitory capacity, the necessity to clarify the viability of these inhibitors in terms of their skin-whitening efficiency has become an urgent task.

5. Concluding remarks

The control of some important human diseases includes the use of enzyme inhibitors; however, there is some concern about the use of synthetic inhibitors due to their side effects. Thus, the use of natural enzyme inhibitors, particularly from plant origin, is encouraged nowadays. Indeed, up to date, extensive research has been conducted to study the enzyme inhibitory properties of phenolic compounds. Data revised showed that different phenolic compounds are efficient inhibitors of the activity of a broad number of enzymes linked with important human conditions, such as hypertension, type II diabetes, obesity, Alzheimer's diseases, inflammation, and skin disorders. It is unmistakable that phenolic compounds are multifunctional compounds that provide a wide spectrum of biological actions beneficial for human health. These compounds exert their action by different mechanisms and have a huge potential in the prevention and treatment of several human diseases. Our bibliographic survey also indicates that flavonoids are probably the group of phenolic compounds with great capacity to inhibit the activity of all the human enzymes analyzed. Several structure-activity relationships studies can be found for some flavonoid compounds. In addition to the enzyme inhibitory capacity, several studies demonstrated the strong antioxidant and anti-inflammatory properties of flavonoids which highlights the relevance of these compounds for the prevention and control of diseases involving oxidative stress or inflammation.

For the future, standardized protocols to search potential inhibitors should be designed in order to minimize the differences among obtained results. Despite the vast number of phenolic compounds studied *in vitro,* few compounds have continued to *in vivo* tests. In addition, further studies should be performed to predict drug-likeness and drug ability.

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Author details

Sandra Gonçalves and Anabela Romano*

*Address all correspondence to: aromano@ualg.pt

Faculty of Sciences and Technology, MeditBio, University of Algarve, Campus de Gambelas, Faro, Portugal

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Chapter 7

Grape Seed Nutraceuticals for Disease Prevention: Current Status and Future Prospects

Anthony Ananga, James Obuya, Joel Ochieng and Violeta Tsolova

Additional information is available at the end of the chapter

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Abstract

Grapes (*Vitis* spp.) are consumed as fresh table fruits, raisins, and processed into wine, juice, jelly and other value-added products. Grapes contain bioactive secondary metabolites (polyphenols), such as proanthocyanins (oliogemeric flavonoids), flavonoids (catechin, epicatechin, and quercetin), and anthocyanins. They have non-flavonoids such as hydroxycinnamic acids (p-coumaric, cinnamic, caffeic, gentisic, ferulic, and vanillic acids), and hydroxybenzoic acids: trihydroxy stilbenes (resveratrol and polydatin). These phytochemicals are of economic importance to pharmaceutical, food and cosmetic industries. Nutraceuticals from grape seeds have potential cardioprotective, anti-cancer, antioxidant, anti-inflammatory, antiviral, neuroprotective, hepatoprotective and antimicrobial properties. Grape seed nutraceuticals have been re-invented in the past few years as a new paradigm in human medicine. In particular, nutraceuticals from grape seeds have been used in stopping wound bleeding, anti-inflammatory agents, pain relief, and anti-diarrhea. In addition, they can be used for the treatment of various human health conditions such as cancer, cholera, smallpox, and nausea as well as eye infections, skin, kidney, liver diseases, etc. Nowadays, consumers are demanding for healthy supplements and personal care products with natural ingredients. Therefore, the present review highlights recent developments and future opportunities of grape seed nutraceuticals for the prevention of human diseases.

Keywords: grape seeds, polyphenols, oxidation, nutraceuticals, human diseases

1. Introduction

Grape (*Vitis* spp.) is one of the most economically important fruit crops worldwide [1]. Grapevine has a rich diversity, as reflected by global variations between wines from different continents or adjacent vineyards. These differences can be attributed to geographical locations, diversity in

climate conditions, or by human interventions arising from breeding and other vineyard management practices. Grapes are consumed fresh as table fruits or raisins and can be processed into wine, jam, jelly, grape seed oil, vinegar, grape seed extracts (GSE) and other products [1, 2]. The quality of grape products depends on a wide range of factors such as variety, environmental conditions, viticultural practices and, more importantly, chemical properties of their secondary metabolites [1]. Secondary metabolites found in grape seeds include phytochemicals such as flavonoids, which are a group of natural polyphenols derived from phenylpropanoid pathway [2]. The stability of secondary metabolites may be impacted by external factors, such as environmental conditions (pH, light, temperature, etc.), due to the nature of physiological functions in growth and development of grapevines [3, 4]. Grape seeds contain phytochemicals such as alkaloids, terpenes, volatile oils, resins, glycosides, tannins, sterols, saponins, and phenolics. These polyphenols have important applications in pharmaceutical, agrochemical, food, and cosmetic industries [1, 5, 6]. More importantly, phenolic compounds are key determinants of wine quality such as aroma, color, and taste and are collectively referred to as organoleptic characteristics of grape products [7, 8]. For example, the quality of grape products is characterized by polyphenolic compounds such as flavonoids and anthocyanins. These natural molecules are generated through specific biosynthetic pathways in grapes.

Flavonoids are primarily located in the epidermal layer of berry skin and seeds in grapes [9]. They have biological attributes such as cytotoxicity and antioxidants, and their biochemical properties play important functions in the defense against biotic and abiotic stresses in grapevines [10, 11]. These phenolic-rich compounds are potential nutraceuticals with biological properties for treatment and/or prevention of various human diseases, whereby they can be used as antioxidants, anti-inflammatory, and antimicrobial agents. Application of nutraceuticals from grape seeds in human health is not new, because wine has been used for medicinal purposes since the medieval period. However, it has reemerged as an important field of human medicine and human nutrition in the past few years [12]. For instance, ethanol in "alcoholic" beverages has potential application in human health due to its properties in inhibition of platelet aggregation, eicosanoid biosynthesis, and an antioxidant for free radicals [13]. Hence, it is important to analyze and characterize grape seed products for biochemical composition, biological activity, and bioavailability of phytochemicals and investigate their correlation to human health.

2. History and establishment of grapes in North America for food and wine industries

Domesticated crops appeared after the Neolithic period (4000–1000 BC) and included grape varieties that were selected for wine production, because they were easier to propagate and had higher proportion of flesh compared to seeds. Wine production from grape has been part of human culture for nearly 6000 years, with the earliest evidence dating back to between 7000 and 5000 BC. Wine produced from grapes was used in dietary applications, medicine, and

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socioreligious activities. The earliest known winemaking facility, or at least its development, was considered to be in the Caucasus Mountains (4100–4000 BC), near the village of Areni in Southern Armenia. The region is considered as the origin of domesticated wine grapes (*Vitis vinifera*) [13]. However, the transfer of winemaking knowledge and technology has been somewhat linear, as it moved from Western Asia to Eastern Mediterranean region such as Egypt, Greece, and Southern Italy, before reaching the rest of Europe c.a. 2000 years ago. Today, for instance, wine grape is produced in every continent worldwide, and its chemical composition is profoundly influenced by factors such as improved enological techniques, region-specific grape cultivars, and local climatic conditions [13, 14]. The development of grape production and winemaking methods has been parallel to the overall technological advances, which came after Western civilization. There has been tremendous progress made in grape production, especially with respect to vine cultivation, vinification equipment, and winemaking practices such as fermentation, as compared with those used during the Neolithic era, including contemporary Egypt, ancient Greece, Western Asia, Ancient Israel, or the Roman Empire. These techniques plateaued around 200–400 AD and was followed by a period of between 1200 and 1400 years when the progress of wine technology slowed down and was generally restricted to monastic religious orders of Western Europe [15]. Wine production methods increased during the eighteenth century, and the phenomenon was likely due to positive changes in trade relations in Europe, which led to increased demand for vintage and age-worthy wines associated with higher quality [15].

Wine production began to expand during the seventeenth century, whereby North Americans became latecomers to join the viticulture industry after the arrival of the first European settlers. This was catapulted in part by the quest of the British government to produce its own wine in North America instead of France. The Franciscan missionaries planted the first large-scale vineyards in California c.a. 200 years ago and reestablished them shortly after the repeal of prohibition [16]. However, the "Great French Wine Blight" that originated from California vineyards in the 1850s almost wiped out the European grape production. Grape phylloxera, *Phylloxera vitifoliae*, was transported to Europe on infected California rootstocks, and the pandemic almost wiped out c.a. 2.5 million acres of vineyards in France alone. The tide turned around when vineyards in Europe were replanted with *V. vinifera* grafted on Phylloxera-resistant rootstocks, *V. labrusca*, a fox grape species native to the eastern North America [13].

With the rapid advancement of science, it was only natural that research on grapes with focus on value addition became such a huge subject worldwide. This led to increased demand for improved quality attributes such as wine color, flavor, and chemical composition of phenolic compounds as well as the growth of wine and grape associated-food industries. High demand for vintage and/or age-worthy wines led to increased research in wine and other grape products. The high demand for quality was driven by the fact that wine became an integral part of human lifestyle from various cultural backgrounds worldwide. For example, wine- and grape-related products were used during social events in many countries, whereas in a few other places, these products were used for either religious practices or medicinal purposes.

3. Grape seed polyphenols and their benefits to human health

Grape seeds contain polyphenol-rich phytochemicals such as proanthocyanidins (oligomeric flavonoids), flavonoids (catechin, epicatechin, and quercetin), and anthocyanins. They also contain non-flavonoids such as hydroxycinnamic acids (p-coumaric, cinnamic, caffeic, gentisic, ferulic, and vanillic acids) and hydroxybenzoic acids: trihydroxy stilbenes (resveratrol and polydatin) as well as vitamin E [12, 13, 17–19]. These secondary metabolites are responsible for blue, purple, and red colors in many plant tissues. Also, grape seeds are rich in proanthocyanidins, which are oligomeric anthocyanins [20, 21], and a few monomeric flavonoids. Previous studies indicated that these extracts could be used as supplements [22]. The polyphenols from grape seeds are potent antioxidants (**Figure 1**) that can protect the body from wide range of health conditions such as premature aging, numerous diseases, and decay [23]. During winemaking process, polyphenols from grape seeds can also infiltrate into wine products. Therefore, moderate wine consumption has been associated with reduced mortality from coronary heart disease, because it increases high-density lipoprotein (HDL) cholesterol content and, as a result, inhibits platelet aggregation [13]. The antioxidant effects of red wine and its major polyphenols have been demonstrated in many experimental systems spanning from *in vitro* studies (human low-density lipoprotein, liposomes, macrophages, cultured cells) to *in vivo* involving animal models as well as healthy human subjects [13].

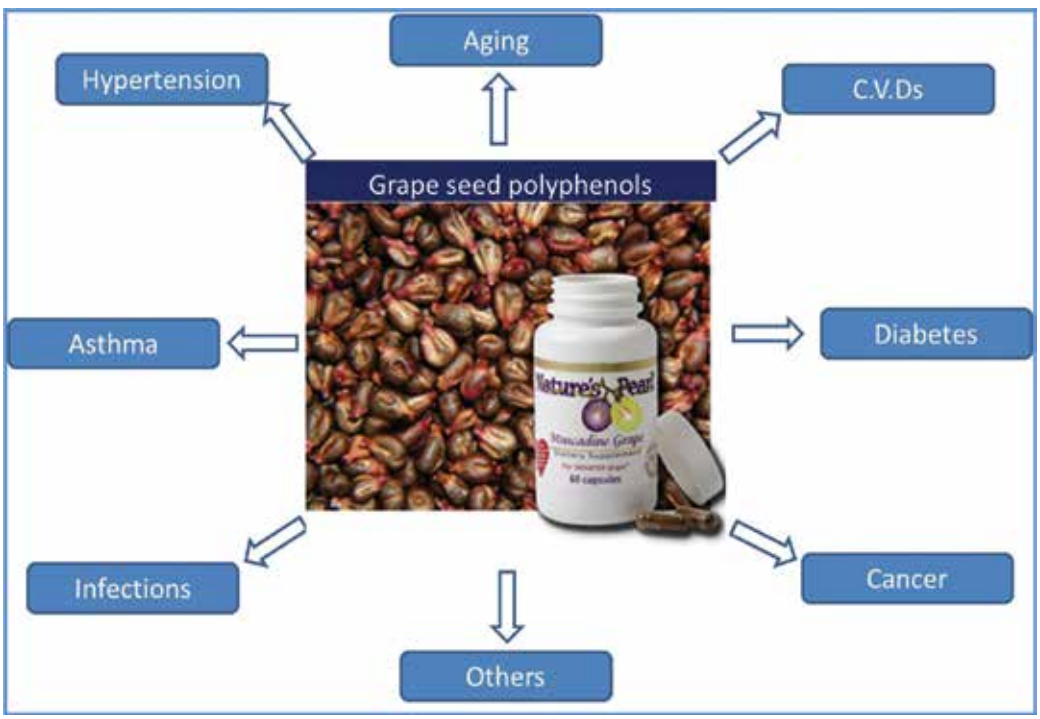

Figure 1. Muscadine grape seeds and their beneficial effects to human health.

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4. Chemical composition

Research in the chemical composition of grape and wine has advanced greatly in the past 30–40 years. Currently, more than 500 compounds have been identified from wine, out of which 160 of these phytochemicals are esters. Each individual compound may be either insignificant or has no role in human organoleptic (taste) perception, but collectively contribute to wine taste [13]. In grape seeds, the primary compounds such as water, ethanol, organic acids, sugars, and glycerol are present at high concentrations (>100 mg/L), and the rest are polyphenols. These polyphenols are particularly large and complex group of compounds, which are key determinants of quality of red wines. The identification of phenolic compounds in grapes and wine began in the late nineteenth century [15]. Previous studies indicated that grapes contain phytochemicals with antioxidant properties [16, 23].

Chemically, polyphenols are cyclic benzene compounds that contain one or more hydroxyl groups associated directly with the hydroxy-substituted benzene ring structure such as catechins, resveratrol, cyanidin (**Figure 2**), and proanthocyanidins, which are oligomers of resveratrol, catechin, and epicatechin.

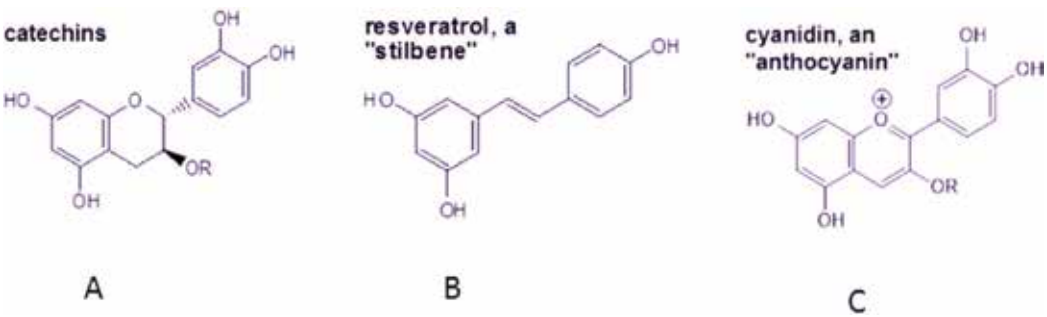

Figure 2. Structures of common healthy compounds found in grape seeds: (A) catechins, (B) resveratrol, and (C) cyanidin.

The two primary groups of polyphenols that occur in grape seeds and wine are flavonoids and non-flavonoids. Flavonoids have been characterized as molecules possessing two phenols, which are joined by a pyran (oxygen containing) carbon ring structure. They have a distinct C_6-C_3-C_3 three-ring structure. The most common flavonoids in wine are flavanols (flavan-3-ols), which are the building block of grape tannins; whereas anthocyanins are predominantly present in red wines. Flavan-3-ol monomers (catechin) are responsible for the bitterness in wine and are associated with astringency in wine. The major flavan-3-ol monomers found in grapes and wine include (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate [24]. Flavonoids are primarily from the skins, seeds, and stems of the fruit. Grape tannins, which are polymers of flavanols, are also known as condensed tannins or proanthocyanidins. Proanthocyanidins contribute to the complexity of wine taste and mouthfeel. Flavanol monomers and oligomers (links of two to four monomers) contribute to the bitterness, and their polymers contribute to astringency in wine. There are five anthocyanidins (cyanidin, peonidin, delphinidin, petunidin, and malvidin) in grapes. Anthocyanin with sugar bound

to the anthocyanidin moiety may be acylated (acid linked to the sixth position of the sugar) such as acetic, coumaric, and caffeic acids. Flavonols (kaempferol, quercetin, and myricetin) are present in grape seeds and wine and are esterified to sugars to form glycosides. Flavonols are important cofactors for color enhancement. They also act as a natural sunscreen in the skin of grape berries.

Non-flavonoids have either C_1-C_6 or C_3-C_6 structures, meaning that either one carbon or three carbons are attached to the primary benzene ring (six carbons). The majority of the non-flavonoids found in grapes are phenolic acids: hydroxycinnamic acids or hydroxycinnamates (esterified with tartaric acid: caftaric acid, coutaric acid, and fertaric acid), hydroxybenzoic acids (gallic acid, a hydrolysis product from grape or oak tannins), and stilbenes (resveratrol and piceid). They are predominantly present in pulp of grapes and are the major phenolic compounds in white wine [15]. Previous studies indicated that non-flavonoids are produced in the grape berry before veràison [25]. Polymerization of polyhydroxy flavan-3-ol units, (+)-catechin and (−)-epicatechin, and their gallate esters produces oligomers and polymers called proanthocyanidins. Resveratrol is mainly found in the grape skin, whereas proanthocyanidins are found in the seeds [26]. Previous studies indicated that these compounds are high in seed material [27] and are produced before veràison and change during fruit ripening [15]. Tannins on grapes protect wine against oxidation, stabilize wine color, and enhance mouthfeel [13]. Proanthocyanidins are members of a class of compounds described as anthocyanogens, leucoanthocyanidins, flavan-3,4-diols, condensed tannins, and tannins [15]. Proanthocyanidins are polymers of flavan-3-ol subunits, meaning that they have a wide range of molecular weight. These phenolic compounds mainly impact the astringency in red wines, and they have been extracted from the skin, seeds, and stems of the grape berry. Recent studies demonstrated that proanthocyanidins (flavonoids) are some of the major compounds present in grapes and wines.

Flavonoids are a class of secondary plant phenolics with significant antioxidant and chelating properties. For example, their cardioprotective characteristics stem from their inhibition of lipid peroxidation, chelation of redox-active metals, and attenuation of reactive oxygen species [28]. Primarily flavonoids occur in food polymers that are degraded to variable extents in the digestive tract. Although metabolism of these compounds has remained elusive, it has been established that their enteric absorption is correlated to reduction in reactive oxidative species in blood plasma [28]. The propensity of flavonoids to inhibit free radical activities is mediated by their chemical structure. However, their physical properties such as flavan nucleus, number, positions, and types of substitutions are considered to play a major role in their radical scavenging and chelating activity [28].

Structures of common anthocyanins were identified from *V. vinifera* and were determined wine in 1959. It was noted that malvidin-3-*O*-glucoside was a major grape anthocyanin and was present along with its acylated forms [15]. Similarly, it was noted that anthocyanins from *V. vinifera* showed different structural compositions to those isolated from non-*vinifera* species, because they were exclusively monoglucosides and those in non-*vinifera* species were present as 3,5-diglucosides [15]. Because of the unique hue manifestation of both grape and wine phenolics, several studies have been performed on anthocyanins as compared to other

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compounds [15]. These studies have focused on understanding the changes in anthocyanidins in respect to berry development [29], potential impact of cultural practices on production [30], and techniques for their extraction from wine and/or grape seed extracts [31].

5. Biological activities

Grape seed products are nutraceutical agents commonly consumed as a health/dietary supplement or are sold as nutrition supplements (100–500 mg). Grape seeds are rich sources of catechins and procyanidins, which are present in red wine and grape juice. Previous studies indicated that these compounds have shown potent antioxidant activities [32–34] and scavenging activity against free radicals [35–38]. The antioxidant capacity of grape seed extracts is considered to perform better compared with vitamins C and E [39]. It was reported that procyanidins inhibited platelet aggregation [40] and had also successfully inhibited the oxidation of low-density lipoprotein (LDL) as well as contributed to reduction in risks of heart disease associated with atherosclerosis [41]. In addition, procyanidins performed very well as anti-inflammatory [42, 43], antimutagens [44], anticancer [45–47], and antiviral [48] agents.

Grape seed extracts are potential antimicrobial for disease control [49] and can be used in food preservatives to ensure food safety [50]. Currently, there has been growing interest on the use of natural antibacterial compounds, such as herbal-based extracts for the preservation of foods, because they possess a unique characteristic flavor and have shown antioxidant and antimicrobial activities [51]. In general, grape seed extracts can be used as antibiotics, antidiarrhea, antiulcer, and anti-inflammatory agents [52, 53]. For example, the mechanism behind their antiulcer is considered to be due to their ability to protect the stomach wall from injuries caused by free radicals as well as the ability of procyanidins to bind other protein targets [53]. Flavonoids have shown success as antiulcer agents, because their suppressive effect depends on the presence of procyanidin oligomers. Previous studies indicated that procyanidins such as catechin oligomers significantly reduced gastric mucosal damage [53]. Furthermore, the binding ability of procyanidin oligomers to bind bovine serum albumin got strengthened in acidified solution. Thus, understanding the biosynthesis of these phenolic compounds may be important to efficiently manage their production as well as insure their bioavailability after wine production. However, it is important to further explore the *in vivo* potential activities of these secondary metabolites from grape seed extract, to determine their potential pharmacological applications in human medicine.

6. Health benefits

Grape seed extracts have potential to be used as nutraceuticals [46, 54]. For instance, red wine constitutes a reliable and rich source of biologically active phytochemicals, such as phenolic acids and polyphenols, whose individual and summated actions are believed to confer health benefits in humans. Epidemiologic studies revealed that individuals with the habit of daily

moderate wine consumption can experience significant reductions in ill-health conditions, including those leading to cardiovascular mortality as compared with individuals who either abstain from drinking or consume excess alcohol [55]. Moderate consumption of wine, for example, was implicated in reduced atherosclerosis cardiovascular heart disease in the French population [56]. Heart disease is lower among the French (who have a relatively high red wine intake) as compared with other Europeans, despite their propensity to consume foods known to be rich in cholesterol, which was referred to as the "French paradox" due to their low incidence of coronary heart disease. Previous studies determined that drinking one to two glasses of wine a day can help protect against heart disease. In addition, phenolics in grapes and red wines have been demonstrated to inhibit oxidation of human low-density lipoproteins (LDL) *in vitro* [57]. Hence, researchers are working toward understanding both molecular and nutritional basis for these anecdotal observations. Although the benefits of polyphenols from fruits and vegetables is gaining ground on how they are making significant contribution to human health, but consensus toward increasing the rate of wine consumption is developing quite slowly [55]. This could be due to the negative perception that a huge segment of the society has toward alcohol consumption.

Recently, plant polyphenols have generated increased attention due to their potent antioxidant properties and ability to prevent various diseases associated with oxidative stress [58]. This led to identification and development of phenolic compounds or extracts from different plants with health and related medical applications in the past few years. Medicinal and nutritional values of grapes have been known to exist for thousands of years. For example, several ancient Greek philosophers exalted the healing power of wine from grapes, and in places, such as Ancient Egypt, wine was mythologically considered as "The Gift of Osiris." They also used wine as a solvent for other medicinal products and in combination with other medicines ("polypharmacy"), and wine has been used in prescriptions dating back to between 3400 and 2550 BC [59]. More importantly, wine was at the core of Mediterranean civilization and was the basis of vast seaborne trade that contributed to the spread of Greek civilization beginning the sixth century, including western Asia Minor, southern Italy, Sicily, North Africa, southern France, and Spain [59]. Wine was held in high esteem by the Persians because of its fame as a cure, which was epitomized by the oldest desert proverb: "He that has health has hope; and he that has hope, has everything." Through the medieval period, the conversion of grapes into wine was considered as a gift from the gods, and the best wines were mainly preserved for ruling elite of the society, although the latter may have been associated with the high cost of wine. In addition, wine was used for medicinal purposes during the medieval period in areas such as ancient India in 2000 BC, ancient China in the past 5000 years, and ancient Rome after 146 BC as well as during the era of Biblical Jewish and Arabic period [59]. The rich history of wine provides an insight of its benefit to human health for several years. It is, therefore, the reason why modern science continues to explore benefits of grape seeds as nutraceuticals with the potential to revolutionize modern human medicine.

To date, various parts of grape are known to confer therapeutic benefits in humans. For example, grape leaves can be used to alleviate wound bleeding, inflammation, muscle pain, and diarrhea; whereas unripe grapefruits are recommended for the treatment of sore throat.

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Raisins (dried grape fruits) can be used to provide relief against constipation and are recommended for treatment of human diseases, including anemia in pregnant women. In addition, ripe and sweet grapes can be used for treatment of diseases such as cancer, cholera, smallpox, nausea, and eye infections as well as can provide relief against skin, kidney, and liver disorders [23]. Grape extracts are rich in bioflavonoids (procyanidins) and are some of the most commonly consumed dietary supplements in the United States due to their potential health benefits. Previous studies indicated that grape extracts are beneficial to human health, because they have showed some efficacy against several diseases such as prostate carcinoma, which causes prostate cancer in men; cardiovascular-related conditions such as hypertension; vascular fragility; and other health conditions such as allergies, hypercholesterolemia, LDL-cholesterol oxidation, and platelet aggregation disorder [60–63]. Other studies have showed that grape extracts can be used as therapeutic agents against diabetic cardiomyopathy [64].

Phenolic compounds such as catechin, quercetin, and resveratrol been known to promote the production of nitric oxide by vascular endothelium cells and, as a result, inhibit biosynthesis of thromboxane in platelets and leukotriene in neutrophils demonstrated to modulate biosynthesis and secretion of lipoproteins in animal models and human cell lines, and arrest tumorigenesis and subsequent carcinogenesis [13]. However, lack of statistical correlation between wine consumption and lower rates of atherosclerosis has made it difficult to resolve the key question of whether moderate consumption may likely lead to decreased atherosclerotic mortality [55]. However, some studies have suggested that there is a relationship between wine consumption and atherosclerotic mortality, which may likely be associated with its direct effect on lipoprotein metabolism.

Moderate ethanol intake is not generally contraindicated in diabetes, although diabetic patients have showed high risk for atherosclerotic cardiovascular disease [65], initiated from oxidative damage of glycosylated proteins and lipids [66–68]. Thus, diabetics as well as nondiabetics are likely to benefit from moderate ethanol intake, because it can help ameliorate risk factors associated with atherosclerotic heart disease that arise from lipoprotein metabolism. Although little information is available on the effects of polyphenols in wine and related grape seed products on diabetics, there may be confounding lifestyle variables associated with spontaneous wine consumption, such as reduced smoking, increased exercise frequency, and better dietary habits that mimic general recommendations given to diabetic patients [55].

7. Bioavailability

Some flavonoids found in wine, such as tannins, are in the form of polymers that may not readily break down under physiological conditions and may not be expected to be available for absorption [69]. Conversely, for non-tannin flavonoids in red wine, about half are present as glycosides, and the rest are present as aglycones (glycosyl group replaced by hydrogen). It has been shown that flavan-3-ols, flavonols, anthocyanins, and non-flavonoid stilbenes, which are

present in red wine and related grape products, can be absorbed in the gastrointestinal tract [55]. Previous studies reported that anthocyanin pigments, which are responsible for the red color of wine, were found in human urine [70] and human plasma [71] after wine consumption. Most glycosides that pass into the large intestine generally end up being hydrolyzed by fecal microflora, rendering them as free aglycones, which leads to their poor absorption in the small intestines. Resveratrol, a non-flavonoid trihydroxystilbene, which is a relatively minor component of red wine [72], is present at insignificant low levels in white wines [55, 73]. However, long-term wine consumption can increase tissue-specific resveratrol concentrations in the body [74].

Flavonoids can travel in the body while bound to plasma proteins [75, 76]. The metabolism of flavonoid glycosides to aglycones and specific glucuronides occurs in the intestinal tissue, which depends on the structure of the flavonoid [77]. The metabolism of esterified flavonoids to aglycones leads to increased lack of absorption in the small intestine. However, conjugated derivatives of quercetin have retained partial antioxidant activity, and it has been demonstrated that different combinations of these conjugates have potential additive effects [78]. Phenolic acids and polyphenols possess multiple hydroxyl groups and are subject to further metabolism by enzymes in the intestine, liver, and kidney [79, 80]. However, the main drawback is that other important nutraceuticals may not be fully absorbed in the human body. Besides, bioavailability under in vivo conditions needs to be resolved as well as their absorption in the gastrointestinal tract in order to keep them at sustainable pharmacokinetic levels in the blood system.

8. Safety concerns for application of grape seed products from American native grapes in foods and cosmetics

Today, consumer demand for health supplements as well as personal care products with natural and/or organic ingredients is promising. Consumption of a large amount of grapes and related products, such as wine, has contributed to the low risk of chronic diseases, such as coronary heart disease and certain cancers [81]. Grape seed extract, which is primarily a mixture of proanthocyanidins, has been shown to modulate a wide range of biological, pharmacological, and toxicological effects as well as cytoprotective functions. Previous studies investigated the ability of IH636 from grape seed proanthocyanidin extract (GSPE) for the prevention of acetaminophen (AAP)-induced nephrotoxicity, amiodarone (AMI)-induced lung toxicity, and doxorubicin (DOX)-induced cardiotoxicity in mice [82]. However, there are safety concerns on the use of these natural products in humans.

Probably one of the best-known properties of polyphenolic compounds, which may also be of safety concern, is their binding and precipitation of protein targets [52]. Most polyphenols have the capacity to bind proteins because of their high affinity for hydroxylation, that is, introduction of hydroxyl groups on other essential biological compounds. Conversely, low-molecular-weight phenols lack the ability to precipitate proteins, unless their oligomeric composition has at least three flavonol subunits, which may mainly be found on highly

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polymerized tannin molecules. Tannin-protein complexes arise from interactions between hydrogen molecules and hydrophobic moieties without the participation of their respective covalent or ionic bonds [52]. The safety of plant products may vary due to several factors such as geographical origin, growth conditions, and other production processes [83]. It is therefore important to conduct studies to characterize these phytochemicals from plant-based products, in order to investigate whether they are safe for use by humans.

To date, there are no conclusive studies on contraindication of grape products for either treatment or prevention of human diseases as well as their use in cosmetics. However, recent studies suggest that grape seed products may be safe for treatment of important human diseases. For example, in one particular study which involved the administration of grape seed extract (GSE) as a dietary admixture at levels of 0.02, 0.2, and 2% (w/w) to rats for 90 days, it was demonstrated that GSE showed no toxicity. Results indicated lack of toxicity, which supported the use of proanthocyanidin-rich grape seed extracts [22]. Similarly, in a different study that the administration of GSE IH636 to male and female Sprague-Dawley rats in the feed at levels of 0.5, 1.0, or 2.0% for 90 days, it was reported there were no significant toxicological effects [84]. Besides, Wren and colleagues found no significant changes in clinical signs, hematological parameters, organ weights, ophthalmology evaluations, or histopathology, and identical results were reported from a different study that was conducted on IGS BR rats, which were fed with dietary supplements of GSE at concentrations of 0, 0.63, 1.25, or 2.5% (w/w), which found lack of adverse effects on mouse models [85].

These studies pointed to lack of toxicity and supported the use of proanthocyanidin-rich extracts from grape seeds, except for personal care products, in which there has been some evidence for minimal side effects on the skin surface such as irritation, sensitization, phototoxicity, and immediate-type allergy [86].

9. Conclusions and future remarks

Phenolic compounds found in grape seeds have desirable biological activities, which are related to their antioxidant properties. These compounds have the ability to scavange for free radicals and inhibit non-desirable enzymatic activities, including the modulation of essential biosynthetic pathways for the metabolism of cellular and extracellular products such as membrane proteins and lipids. Moderate consumption of red wine, for example, can lead to increased plasma concentrations of HDL cholesterol and decreased adhesion of platelets, which may be beneficial to treating platelet aggregration in humans. Biosynthesis of pro-aggregatory eicosanoids such as thromboxane A_2 and synthesis of leukocytes by pro-inflammatory leukotrienes to inhibit the formation of atherosclerosis can reduce the risk of coronary arterial disease by promoting the relaxation of vascular smooth muscle [16, 87]. In addition, some polyphenols are capable of promoting the synthesis of prostacyclin (prostaglandin member of eicosanoids) and nitric oxide, which can lead to optimized blood flow through the arterial system [13].

Use of nutraceuticals from grape seeds for the mitigation of grape diseases for which biomarkers are known have not been validated. Although nutraceuticals have shown a great promise in the treatment and/or prevention of human diseases, consensus on their wide acceptance as alternative therapeutic agents is developing very slowly. Previous studies demonstrated that nutraceuticals found in grape seeds likely have the ability to modulate cellular metabolism and signaling, which is consistent with reduced coronary arterial disease [16, 87]. However, additional research is needed to address crucial important issues such as the mechanisms by which phytochemicals in grape seeds can be used as nutraceuticals, develop biomarkers for their role in disease prevention, and determine the appropriate dosage for their application in human health medicine.

There are still several gaps that need to be addressed. First, there should be a broad framework for research to understand the mechanism by which polyphenols from grape seed products confer dietary health benefits to humans. Second, additional clinical studies are needed to determine their pharmacokinetic properties in human disease prevention. Third, appropriate biomarkers or mechanistic end points should be developed to determine the interactions between diseases and polyphenols at cellular and subcellular levels. Lack of adequate clinical data on nutraceuticals from grape seed products precludes their use in human health. First, it is not known whether the benefit of red wine originates from the tendency to consume them during meal time, or their consumption together with other macronutrients improves their absorption in the gastrointestinal tract. Second, it is not known whether the ability of wine to scavenge for free radicals originates from its chemical properties as a biofuel, through its ability to cause oxidative stress, or using both mechanisms, which may suggest that it has multiple effects at the cellular or subcellular level.

The success of human genome project and recent developments in molecular biology has led to major revolution in biological sciences. Thus, the application nutraceuticals from grape seeds to human health need to be prioritized as one of the areas of current research. Additional research should focus on understanding potential effects of confounding factors that may be associated with the use of nutraceuticals from grape seeds. This is because preliminary data suggest that nutraceuticals from grape seeds have valuable applications in the prevention and/or treatment of several human diseases. Hence, there are unlimited opportunities presented by the application of polyphenols from grape seeds as nutraceuticals. Therefore, it is important to investigate not only their bioavailability and chemical composition but also to characterize their biological properties to determine their mechanisms as well as their synergistic properties and other components of human diet.

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Author details

Anthony Ananga[1, 2]*, James Obuya[1], Joel Ochieng[3] and Violeta Tsolova[1]

*Address all correspondence to: anthony.ananga@gmail.com

1 Center for Viticulture and Small Fruit Research, College of Agriculture and Food Science, Florida A&M University, Tallahassee, FL, USA

2 Food Science Program, College of Agriculture and Food Sciences, Florida A&M University, Tallahassee, FL, USA

3 Faculties of Agriculture and Veterinary Medicine, University of Nairobi, Nairobi, Kenya

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Chapter 8

Phenolic Compounds with Anti-virulence Properties

Naybi Muñoz-Cazares, Rodolfo García-Contreras,

Macrina Pérez-López and Israel Castillo-Juárez

Additional information is available at the end of the chapter

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Abstract

Natural products represent the major source of approved drugs and still play an important role in supplying chemical diversity as well as new structures for designing more efficient antimicrobials. They are also the basis for the discovery of new mechanisms of antibacterial action. In this regard, a large number of substances, mainly extracts from natural sources, have been obtained in order to identify their anti-virulence activity. In recent years, there is an increase in the study of anti-virulence natural product derivatives. Different targets have been proposed as a solution to the serious problem of bacterial antibiotic resistance. Inhibition of bacterial quorum-sensing systems has been one of the most studied; however, there are other mechanisms involved in virulence regulation, damage to the host and bacterial survival, which suggests that there are another good targets such as bacterial secretion systems, biofilm formation, two-component systems, flagellum, fimbriae, toxins and key enzymes. Within the natural products, the main anti-virulence compounds are phenolic in nature, so that the next chapter describes and analyzes the relationship between chemical structure and activity of the main phenolic compounds reported.

Keywords: anti-virulence, quorum sensing, antibiotic resistance, phytochemicals, antibiofilms

1. Introduction

Since their introduction in the middle forties, antibiotics had been extensively used for the treatment of infectious diseases, producing remarkable results and saving millions of lives worldwide [1]; nevertheless, bacteria are very dynamic organisms able to interchange genes by several mechanisms including conjugation, transformation and transfection via

bacteriophages [1]. In addition, they usually replicate at high rates and hence have the ability to evolve quickly and adapt to strong selective pressures; this combined with the self-prescription, inadequate prescription by some physicians (e.g., to treat viral diseases) and their improper use by patients who do not complete the recommended treatment scheme has derived in an alarming situation since to date antibiotic resistance (including multiresistance and panresistance) is a common trend in most of hospital-acquired infections and is becoming more common in community-acquired ones [2, 3]. In fact, the situation is so delicate that recently, the OMS warned that if the current trends are still observed, then by the year 2050 we will enter the post-antibiotic era and previously treatable infectious diseases will cause more deaths than other important diseases such as cancer [4].

Hence, the discovery of new antibiotics as well as the development of alternative approaches to combat bacterial infections is urgently needed [5]; among such new approaches are the inhibition of bacterial antibiotic resistance mechanisms, the utilization of non-antibiotic bactericide agents such as bacteriophages, the repurposing of clinically approved drugs, and the inhibition of bacterial virulence [5]. For the first approach, already successful examples can be found in the clinic; by instance, the co-utilization of clavulanic acid (an inhibitor of β-lactamases) and amoxicillin is commonly administrated [6]; and current research is focused on the utilization of broad spectrum anti-resistance compounds such as those inhibiting multidrug efflux pumps [7]. Regarding the second approach, it was recently demonstrated that some anticancer drugs such as 5-fluorouracil [8], mitomycin C [9] and cisplatin [10] have remarkable antibacterial properties, while bacteriophages had been used in east European countries for the treatment of diverse bacterial infections, and currently, its utilization in the occidental medicine is being proposed [11, 12]. Finally, targeting bacterial virulence instead of their viability is a concept that had derived in several publications, mostly centered in the inhibition of master virulence regulators such as quorum-sensing (QS) systems, which allow several Gram-negative and Gram-positive bacteria to coordinate the production of several virulence factors, once a high population density is reached (**Figure 1A**). Indeed, initially, it was claimed that this approach will be impervious to the generation of resistance since *in vitro* in rich media QS does not control metabolic processes linked to growth; nevertheless, in some conditions, QS inhibition can promote resistance [13–15] and not all clinical strains are sensitive toward current QS inhibitors [16]. However, since QS also regulates the stress response, it has been shown that QS-inhibited bacteria are more susceptible to the action of disinfectants, antibiotics and the immune system [17, 18], and hence, QS inhibition may be a valuable adjuvant therapy for recalcitrant bacterial infections [15].

As mentioned previously, QS is a master regulator of the production of several bacterial virulence factors, such as: exoproteases that degrade connective tissue such as elastase and alkaline protease (collagenase), phenazines that promote the generation of reactive oxygen species, siderophores that facilitate iron uptake, toxins that disrupt cellular processes and exopolysaccharides that form phagocytosis-resistant capsules and participate in the generation of the biofilm matrix [19] (**Figure 1C**).

Another key factor for the development of chronic infections and colonization of surfaces is the formation of biofilms, which is the main way the bacteria are found in nature [20].

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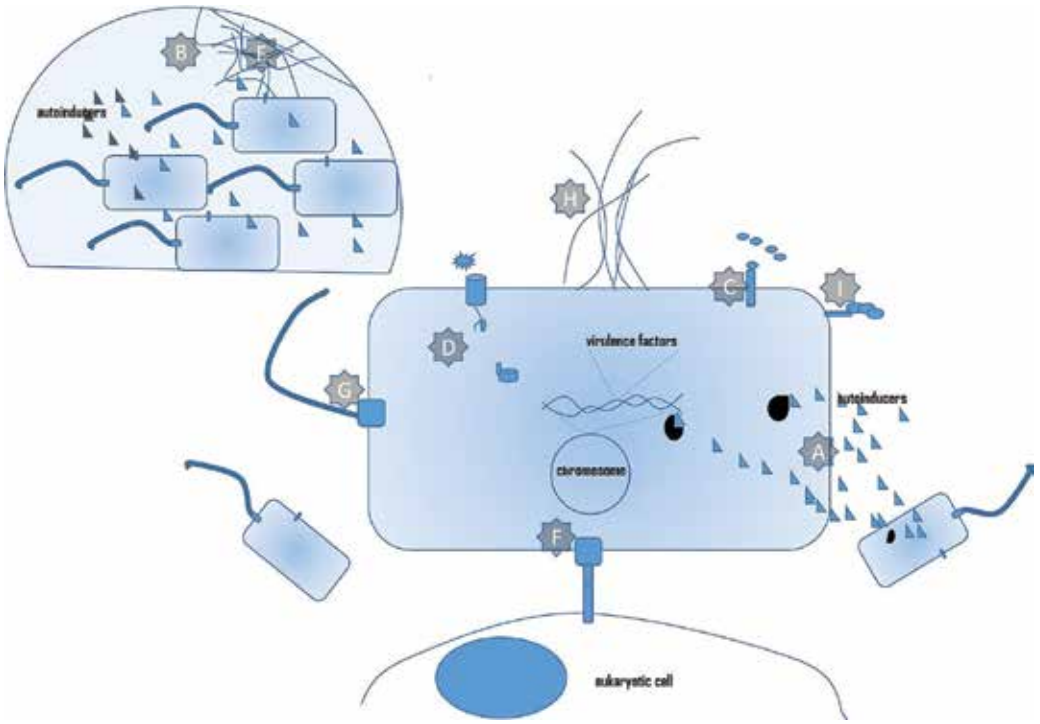

Figure 1. Main targets of anti-virulence of phenolic compounds. A: Quorum-sensing system, B: biofilm formation, C: toxins, D: two-component systems, E: curli fibers, F: bacterial type III secretion systems, G: flagellum, H: fimbriae, I: sortase enzymes.

These structures consist of multicellular communities enclosed in a matrix which makes them extremely resistant to antibacterial agents (**Figure 1B**) [21]. They also provide robust niches that allow the bacteria to protect themselves from environmental fluctuations and against the immune system, which drastically reduces the effectiveness of antimicrobial therapy [20].

Since for many pathogenic bacteria QS is the main regulator of expression of bacterial virulence factors [19], its disruption has been the main anti-virulence strategy investigated to date [19]. However, another alternative that has also been reported is the ***direct inhibition of*** individual ***virulence factors***, such as toxins, response regulators (two-component regulatory systems (TCS) and processes involved in the formation and maturation of structures such as the curli, the bacterial type III secretion system (T3SS), fimbriae and flagellum.

TCS are response regulators which are formed by a protein localized in the cytoplasmic membrane called histidine kinase sensory protein (HKSP), which acts as an environmental sensor that is activated in ATP-dependent way (**Figure 1D**) [22]. HKSP then activates a response regulator protein (RRP) found in the cytoplasm which is responsible for recognizing DNA sequences that modulate the expression of genes involved in various functions such as chemotaxis, porin expression and expression of virulence factors among others (**Figure 1D**) [22]. An important feature is that TCRs have not detected in mammalian cells, so there are a suitable specific target to treat bacterial infections [23].

The curli (**Figure 1E**) is the major protein component of the extracellular matrix and is mainly produced by enterobacteria to aid in the formation of three-dimensional structures such as biofilms [24]. Curli fibers belong to a growing class of fibers known as amyloid fibers, which are also involved in host cell adhesion and invasion, and are also strong inducers of host inflammatory response [24]. The structure and biogenesis of curli are unique among bacterial fibers and represent an excellent anti-virulence target [25].

The type III secretion system (T3SS) also known as the injectisome is a multiprotein apparatus that facilitates the secretion and translocation of toxins or effector proteins from the bacterial cytoplasm directly to eukaryotic cells (**Figure 1F**) [26, 27]. It is highly conserved in most Gram-negative pathogens, but its presence is not a necessary condition for bacterial survival *in vitro* [27].

Motility and recognition surfaces are key factors for the dispersal and colonization of new niches by bacteria [28]. For that, the flagellum and the fimbriae are target structures suitable for anti-virulence molecules [28, 29]. The flagella (**Figure 1G**) are multiprotein complexes based on flagellin, which rotate allowing bacterial displacement in aqueous media [29], while fimbriae (**Figure 1H**) are extracellular protein structures mainly constituted by pilin, which start in the plasma membrane, cross the cell wall and extend around the cell. These structures allow the adhesion of bacteria mainly to epithelial cells [30].

Another important virulence factors are the sortase enzymes (cysteine transpeptidases) (**Figure 1I**), which are used by Gram-positive bacteria to display proteins in cell surface, such as glycoproteins [30], and they can also attach to proteins in the cross-bridge peptide of the cell wall or link other proteins together to form pilin [31]. The phenomenon of protein deployment is essential for the development of virulence factors and promotes nutrient acquisition, adhesion and immune system evasion [30]. Because surface proteins play a fundamental role in microbial physiology and are frequently virulence factors, sortase enzymes are a very important target [31].

Reports related to the study of natural products as anti-virulence molecules had increased in the last decade. Their powerful attack against bacterial infections without promoting resistance and the elimination of antibiotic-resistant strains are the most attractive features of this kind of compounds. Among natural products with anti-virulence activity, those derived from plants with anti-QS and antibiofilm activity are the most common [32]. Phenolic compounds are secondary metabolites present in plants, which are crucial in many aspects of their lives, especially during the interactions with the environment, since they are used in the defense of plants against bacterial pathogens. Similarly, compounds of phenolic type are the major metabolites with anti-virulence properties described so far, and specifically, the flavonoids are the main representatives [33].

Most of the biologically active reported phenolic compounds have chemical structures with previously identified antimicrobial, antioxidant and anticancer activity. Similarly, for some of them their participation in the regulation of various physiological functions in plants and animals is well known. In recent years, the anti-virulence properties of phenolic compounds are being unravel, and most of the cases depend on the compound concentration and the bacterial

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system in which the phenolic compounds can exhibit bactericidal or anti-virulence effects. In the next chapter, we discuss studies of phenolic compounds derived mainly from plant species, starting with those that are better characterized and that have more anti-virulence reported properties. We focus on the relationship between their structures and their activity.

2. Phenolic compounds anti-virulence

2.1. Epigallocatechin gallate and related compounds

It is well documented that this kind of compounds has antimicrobial, antioxidant, anti-inflammatory, hypocholesterolemic and cancer-preventive properties [34, 35]. The *epigallocatechin gallate* (EGCG) (**Figure 2A**) is one of the flavonoids with the largest number of reports related to its antibiofilm activity; remarkably high compound doses can inhibit bacterial growth, but sublethal concentrations exhibit anti-virulence properties.

At the same concentration, *catechin* (**Figure 2B**) and EGCG inhibit the formation of biofilms of *P. aeruginosa;* however, only catechin do not affect the growth [36], so the presence of galloyl

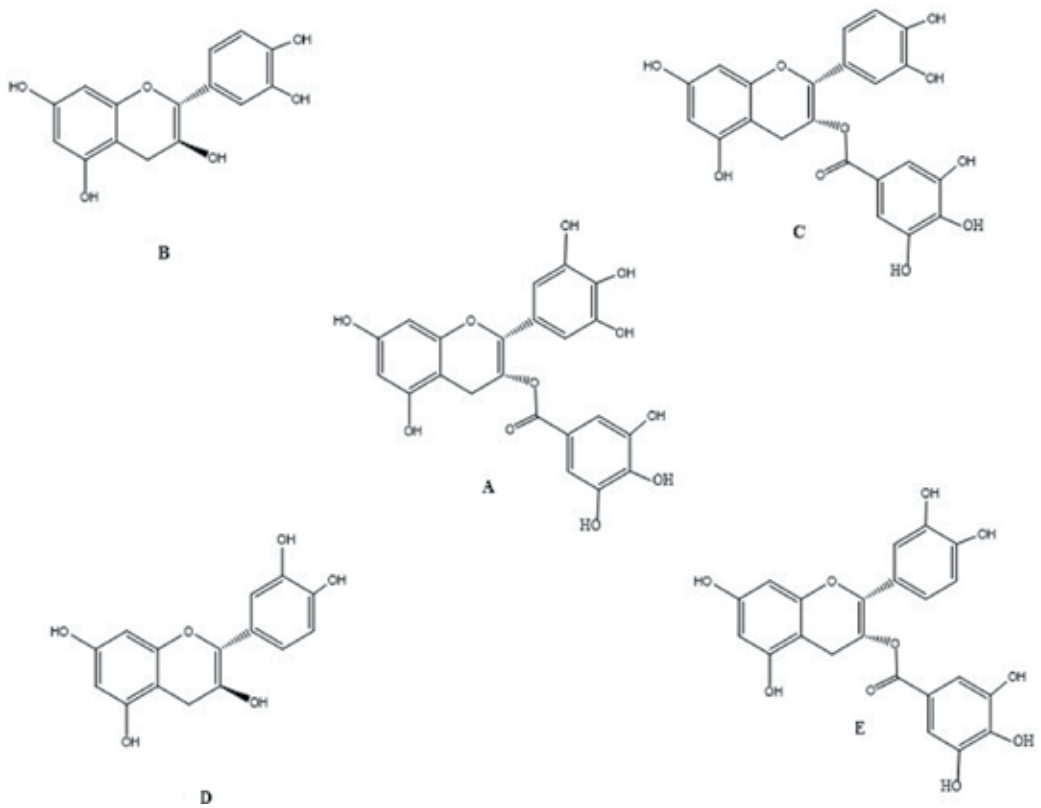

Figure 2. Epigallocatechin gallate and related compounds with anti-virulence properties. **A**: Epigallocatechin gallate, **B**: catechin, **C**: catechin-gallate, **D**: catechin-gallate, **E**: (−) epicatechin gallate.

group in EGCG seems to favor the bactericidal effect. In this regard, it is suggested that EGCG affect the viability because it binds to peptidoglycan, hence directly disrupting the integrity of the bacterial cell wall. Similarly, EGCG at concentrations that affect bacterial viability inhibit the biofilm of *Enterococcus faecalis*, an opportunistic pathogen implicated in urinary tract infections, endocarditis and root canal infections [37]. In this case, biofilm inhibition is attributed to a bactericidal effect, where the EGCG induces hydroxyl radicals that can damage DNA, proteins and lipids [37].

However, using sublethal concentrations, it has been found that EGCG significantly decreased the expression of virulence genes that regulate the expression of cytolysins, gelatinase and serine protease in *E. faecalis* [37]. It also inhibits biofilm formation of Staphylococcal isolates by interfering directly with polysaccharides of the glycocalyx [38]. Similarly, it inhibits swarming and biofilm formation of *Burkholderia cepacia* without affecting the growth, likely through QS inhibition [39].

EGCG and *catechin gallate* (**Figure 2C**) directly inhibit the anthrax lethal factor (LF) produced by *Bacillus anthracis*, which has a key role in the development of anthrax [40]. LF is a zinc metalloprotease that directly affects MAPK-signaling kinases, which are essential for transmitting signals in eukaryotes. EGCG and *catechin gallate* block the activity of LF, preventing MAPK-kinases cleavage and macrophages death [40]. In the case of EGCG, it also delays the death of mice exposed to the anthrax toxin [40]. It is noteworthy that although other catechins were evaluated, the presence of a galloyl group in the structure seems to be essential for this anti-virulence activity.

For the case of *catechin* (**Figure 2B**), it has also been reported that it inhibits the production of virulence factors regulated by QS in *P. aeruginosa*, such as pyocyanin and elastase [41]. Also, it was found to have a negative impact on the transcription of several genes involved in QS, such as those codifying proteins involved in the synthesis of autoinducer molecules [41].

Dental plaque is a complex biofilm that allows the survival and development of *Streptococcus mutans*. It has been reported that EGCG shows bactericidal activity against *S. mutans;* in addition, its antibiofilm activity is due to reducing the adherence of bacteria to surfaces by direct inhibition of glucosyltransferases [42], which are enzymes that synthesize polysaccharides [43, 44]. However, at sublethal concentrations, EGCG reduces biofilm by interfering with gene regulation, specifically by inhibiting the expression of the *gtf* genes (encoding glucosyltransferases), which are associated with adhesion and formation of biofilms [45]. Moreover, it represses genes encoding virulence factors associated with acidogenicity and aciduric, such as *ldh*, *eno*, dATP, Agud and the activity of the F_1F_0-ATPase and lactate dehydrogenase [42].

EGCG at sublethal concentrations also inhibits motility and biofilm formation of *Campylobacter jejuni*, a foodborne pathogen which is one of the main causes of gastrointestinal infections worldwide [46]. In this case, the mechanism involved in biofilm inhibition is related to QS inhibition [46].

It is worth noting that to date there are no studies to investigate its structure-activity relationship, so it is not yet known which parts of the structure are critical to their anti-virulence effects. However, for the (−) *epicatechin* (**Figure 2D**) which also possesses anti-QS activity

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against *Chromobacterium violaceum*, a Gram-negative bacteria with AHLs mediated QS [47]. The (−) *epicatechin gallate* (**Figure 2E**) at sublethal concentrations inhibits two of the major determinants of virulence in *S. aureus*, the α-toxin and the coagulase [48]. Furthermore, it has been shown that in combination with β-lactams, it is efficient to eliminate multiresistant strains of *S. aureus*. Although it has been observed that some synthetic analogs have better pharmacokinetic properties than the native (−) epicatechin gallate [49, 50].

2.2. Cinnamaldehyde and related compounds

Cinnamaldehyde (CN) (**Figure 3A**) is a major constituent of cinnamon essential oils and occurs naturally in the bark and leaves of cinnamon trees of the genus *Cinnamomum* [51]. The antimicrobial activity of this compound has been proven [52, 53], but new studies have explored their anti-virulence properties, and in contrast to another compounds, it is considered a nontoxic substance widely used in food and in the cosmetic industry and their use is generally recognized as safe [54].

In *P. aeruginosa*, the acylated homoserine lactones (AHLs) are their main autoinducer molecules (**Figure 1A**) and the CN can inhibit their synthesis as well as the production of the phen-

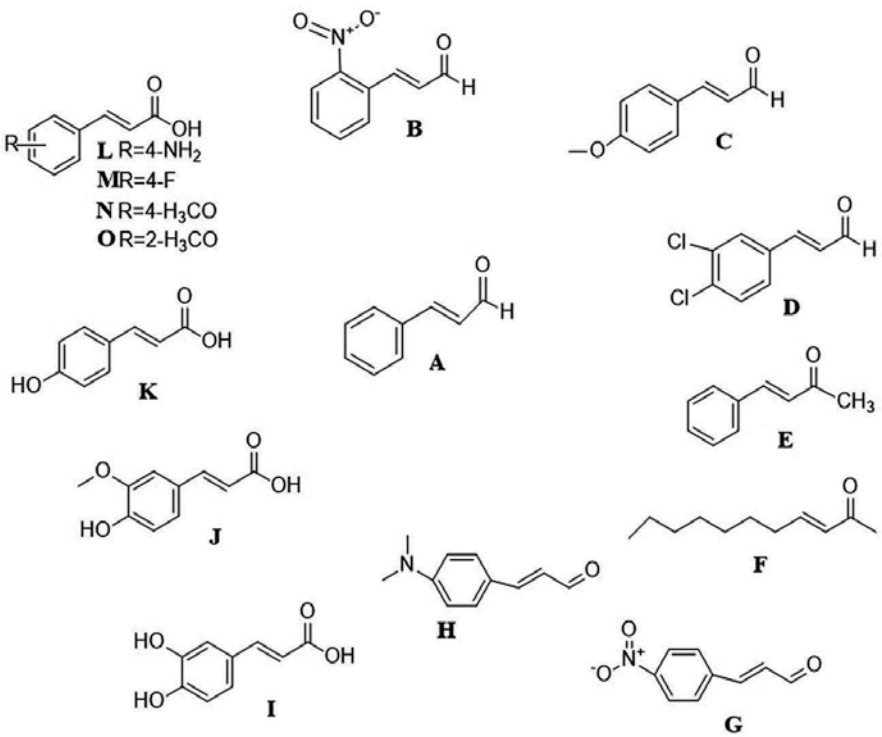

Figure 3. Cinnamaldehyde and related compounds with anti-virulence properties. **A**: Cinnamaldehyde, **B**: 2-nitro-cinnamaldehyde, **C**: 4-methoxy-cinnamaldehyde, **D**: 3,4-dichloro-cinnamaldehyde, **E**: (*E*)-4-phenyl-3-buten-2-one, **F**: (*E*)-3-decen-2-one, **G**: 4N-4-nitrocinnamaldehyde, **H**: 4D-4-dimethylaminocinnamaldehyde, **I**: caffeic acid, **J**: ferulic acid, **K**: *p*-coumaric acid, **L**: TS027, **M**: TS110, **N**: 4-methoxy-cinnamic acid, **O**: *trans-2-methoxy-cinnamic acid*.

azine, pyocyanin and swarming motility [55]. Remarkably, CN also has antitoxin production and anti-hemolytic activities [56]. Similarly, in *C. violaceum*, *Yersinia entrerolitica* and *Erwinia carotovora*, the concentration of AHLs was also reduced by CN and the mechanism proposed was the inhibition of synthesis or degradation transformation of the autoinducer [57].

The antibiofilm properties of CN have been widely documented; for example, in *P. aeruginosa* and in enterohemorrhagic *Escherichia coli*, this compound markedly abolished the biofilm formation in a dose-dependent manner by reducing the swarming motility and fimbriae production, respectively. In a previous report, it was shown that for the uropathogenic *E. coli*, CN prevented biofilm formation on plates and catheters, furthermore effectively inactivated preformed biofilms [54]. The mechanism proposed for the biofilm inhibition was related to the hydrophobicity of this compound, which helps to target lipids located in the bacterial cell membrane and mitochondria, increasing the membrane permeability, leading to the leakage of ions and other cell contents [54, 58]. The foodborne pathogen *Listeria monocytogenes* forms biofilm for persistence and survives in which CN has inhibitory effect on formation and inactivating mature biofilm by means of the down-regulated critical genes for biofilm formation in this bacteria [59].

In *Vibrio harveyi*, the autoinducer-2 (A2) is also blocked by CN in a concentration-dependent way by decreasing the binding ability of the autoinducer to its response regulator protein. Between cinnamaldehyde derivatives, the *2-nitro-cinnamaldehyde* (**Figure 3B**) was the most active compound yielding an inhibition of A2 similar to CN [60]. Similarly, the 2-nitro-cinnamaldehyde and *4-methoxy-cinnamaldehyde* (**Figure 3C**) inhibit pigment production and protease activity in *Vibrio anguillarum* [60]. The CN is an aromatic carboxylic acid, and its inhibitory was highly dependent on the substitution pattern of the aromatic ring. Replacement of the dimethylamine (Me_2N) substituent with a methoxy (MeO) or a nitro (NO_2) group enhanced the activity [60].

Various cinnamaldehyde analogs were also evaluated against *Vibrio* spp. The most active compounds were *2-nitro-cinnamaldehyde*, *3, 4-dichloro-cinnamaldehyde* (**Figure 3D**), *(E)-4-phenyl-3-buten-2-one* (**Figure 3E**) and *(E)-3-decen-2-one* (**Figure 3F**), which show inhibitory activity in A2, bioluminescence, pigment and protease production [61]. In this case, also the inhibitory effect of cinnamaldehyde analogs was dependent on the structure, and analogs in which the aromatic ring was replaced by an alkyl moiety, but which still contain the acrolein group, proved also to be active inhibitors [61]. In general, the inhibitory effect of cinnamaldehyde analogs is highly dependent on the nature and degree of substitution of the aromatic ring, and the substituents with electron-withdrawing properties increase its activity. The CN and their analogs furthermore proved to be active blockers of virulence in vivo in different models, suggesting that they may have potential for therapeutic applications in humans and animals [61].

The CN also has inhibitory activity on biofilm formation in a methicillin-resistant *Staphylococcus aureus* at dose-dependent manner and represses the expression of *sarA*, a gene implicated in the regulation of its biofilm [51]. In *Streptococcus pyogenes*, when the biofilm was treated with CN and their derivatives the *2-nitro-cinnamaldehyde* **(Figure 3B)**, *4N-4-nitrocinnamaldehyde* (**Figure 3G**) and *4D-4-dimethylaminocinnamaldehyde* (**Figure 3H**), the biomass, average thickness and

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colony size at substratum were decreased and the molecular docking shows sequence and structure similarity with the active site for QS inhibition [62].

Among the cinnamaldehyde-related molecules, the *caffeic acid* (CA) (**Figure 3I**) and *ferulic acid* (FA) (**Figure 3J**) have shown antibiofilm properties. CA is the first phenolic acid compound that has been reported to have inhibitory activity on biofilm formation in *Staphylococcus epidermis* by a mechanism that did not involve bacterial death [63]. The potential of FA to control biofilm formation has been demonstrated by the reduction in mass and metabolic activity in *Escherichia coli* and *Listeria monocytogenes* biofilms, and also this compound caused the total inhibition of motility in both bacteria and the colony spreading in *S. aureus;* a form of passive bacterial movement was also inhibited [64].

The QS inhibitory activity of CA and FA also was evaluated in *C. violaceum*, and the results revealed that the activity was mediated by their ability to modulate AHL activity and synthesis [47]. Other related compound the *p-coumaric acid* (**Figure 3K**) showed QS inhibition in reporter strains like *C. violaceum*, *Agrobacterium tumefaciens* and *Pseudomonas chlororaphis* [65]. In addition, it represses the expression of regulatory genes of the T3SS of the phytopathogenic bacteria *Dickeya dadantti*, and for this activity, its hydroxyl group on the phenyl ring and the double bond are important [66]. Some of their derivatives such as TS027 (**Figure 3L**) and TS110 (**Figure 3M**) also repress the expression of T3SS regulatory genes and inhibit T3 effector protein in *P. aeruginosa* without affecting its growth [67]. While the cinnamic acid and *4-methoxy-cinnamic acid* (**Figure 3N**) suppress the expression of T3SS in *Erwinia amylovora* [68], the *o-coumaric acid* (isomer of 3M) and *trans-2-methoxy-cinnamic acid* (**Figure 3O**) suppress translocation of two effector proteins of T3SS in *Xanthomonas oryzae* [69].

2.3. Coumarin and related compounds

The *coumarins* are compounds that have caused great interest for their pharmacological properties such as anti-inflammatory, antitumor, antioxidant and bactericidal activity [70]. Moreover, recently it has also documented that they possess anti-virulence properties. The *coumarin* (**Figure 4A**) and *umbelliferone* (**Figure 4B**) inhibit biofilm formation of *E. coli*, without affecting its growth. By a transcriptional analysis, it was identified that these phenols act by repressing genes related to curli production and motility, which causes a decrease in the production of fimbriae and swarming [71]. For these molecules, the hydroxylation of coumarin is an important determinant for their antibiofilm activity, since the position of hydroxyl groups as well as their number affects the antibiofilm compound activity [71].

Similarly, the presence of characteristic functional groups promotes the effective inhibition of virulence factors, as in the case of the furocoumarins [72], *dihydroxybergamottin* (**Figure 4C**) and *bergamottin* (**Figure 4D**), which exhibit anti-quorum-sensing effect on the AI-1 and AI-2 systems in *Vibrio harveyi*. Similarly, these furocoumarins inhibit biofilm formation of *E. coli*, *V. harveyi*, *Salmonella typhimurium* and *P. aeruginosa* without affecting bacterial growth. Although their mechanism of action is unknown, it is suggested that the presence of a furan residue could be acting as a competitive inhibitor for binding with the receptor protein of natural bacterial autoinducers [72].

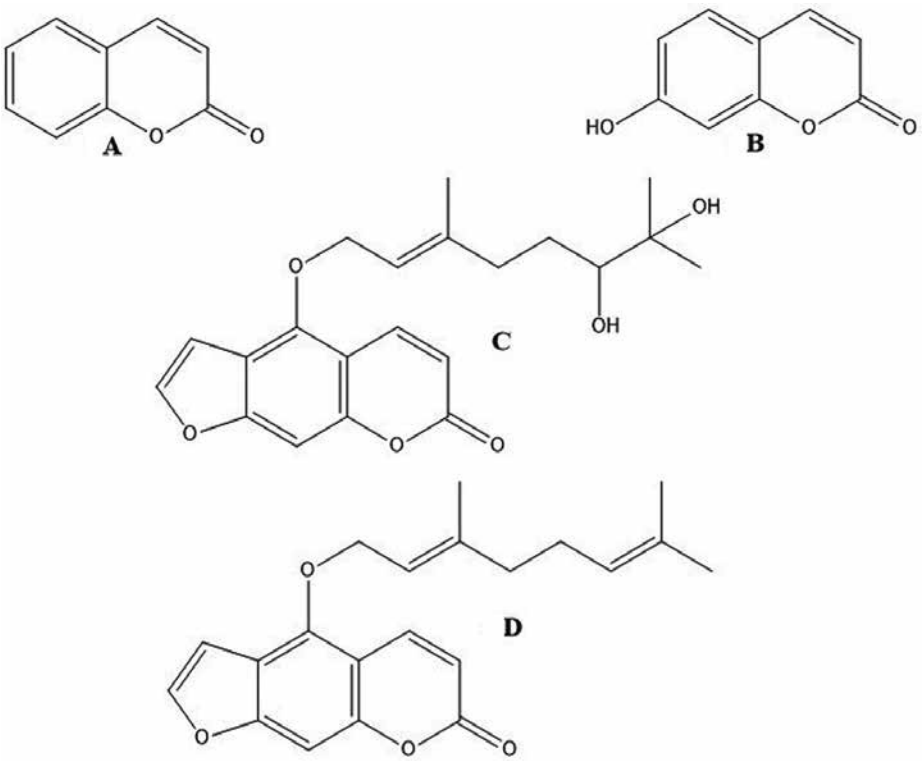

Figure 4. Coumarin and related compounds with anti-virulence properties. **A:** Coumarin, **B:** umbelliferone, **C:** dihydroxybergamottin, **D:** bergamottin.

2.4. Curcumin and related compounds

The major constituent of turmeric (*Curcuma longa* L.) roots/rhizomes is the *curcumin* (CUR) (**Figure 5A**), which is an active compound that showed an important antimicrobial activity [73, 74], but several studies also corroborate their inhibitory activity against virulence factors in pathogenic bacteria.

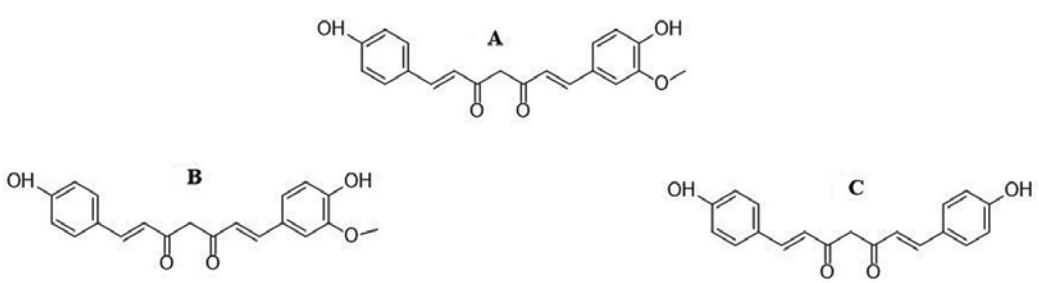

Figure 5. Curcumin and related compounds with anti-virulence properties. **A:** Curcumin, **B:** demethoxycurcumin, **C:** bisdemethoxycurcumin.

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The secretion of sortase A (SrtA) a surface protein in *S. aureus* involved in bacterial adhesion for pathogenesis was inhibited by CUR, and also on *in vivo* assays, this compound reduces the capacity of bacteria to adhere to surfaces in a dose-dependent manner [75]. The other derivatives present in turmeric extract are *demethoxycurcumin* (**Figure 5B**) and *bisdemethoxycurcumin* (**Figure 5C**), which show inhibitory activity of SrtA [75]. Similarly, in *Streptococcus mutans* CUR inhibited the activity of SrtA and other proteins implicated in bacterial adhesion reducing the biofilm formation in this bacteria [76, 77]. The diverse biological properties of CUR and its derivatives are attributed to the hydroxyl and phenol groups in the molecule [78], and structure-activity relationship studies suggest that a hydroxy group at the para-position is most critical for the expression of biological activity in these compounds [79].

The antibiofilm activity of CUR against uropathogens such as *E. coli*, *Proteus mirabilis* and *Serratia marcescens* was evaluated, and the results showed that their biofilm maturation was disturbed by a biomass reduction and by the interruption of swimming motility [80]. In clinical isolates of *Klebsiella pneumoniae*, the treatment with CUR was also effective for biofilm inhibition [81] as well in enterohemorrhagic *E. coli* [82]. In the same way, in *Vibrio* spp. the inhibitory effect on biofilm formation with the CUR treatment depends on the disruption of the maturation of biofilms and in the reduction of swimming and swarming motility. Further, this compound significantly represses other virulence factors like alginate and exopolysaccharide production and also inhibits bioluminescence. These inhibitory effects were also demonstrated on *in vivo* models in which CUR enhanced the survival rate of *Artemia nauplii* against *Vibrio harveyi* [83].

Diverse virulence factors in *P. aeruginosa* were inhibited by CUR, specifically the elastase, protease and pyocyanin production without affecting bacterial growth in a dose-dependent manner. The biofilm inhibition effect was demonstrated *in vivo* using *Arabidopsis thaliana*, where the treatment with CUR caused a reduction in the plant mortality by suppressing biofilm formation [84]. In the pathogenicity model using *Caenorhabditis elegans*, CUR demonstrate their anti-infective properties by reducing the nematode mortality [84]. Additionally, in *P. aeruginosa* and *C. violaceum*, CUR showed an anti-quorum sensing activity by inhibiting the production of acyl homoserine lactones [84].

2.5. Eugenol and related compounds

Eugenol (EG) (**Figure 6A**) is a major component of clove oil that possesses various biological properties [85], and their anti-virulence activity also has been evaluated. In pathogenic bacteria that secreted a broad spectrum of virulence factors that contribute to their pathogenicity,

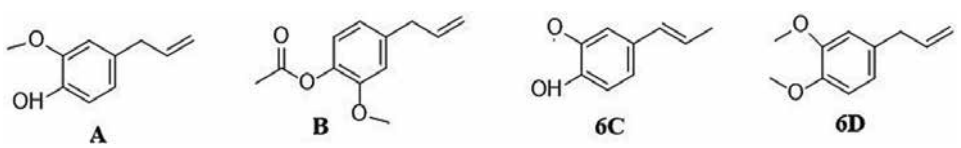

Figure 6. Eugenol and related compounds with anti-virulence properties. **A:** Eugenol, **B:** eugenyl acetate, **C:** isoeugenol, **D:** methyl eugenol.

EG showed inhibitory activity. For example, in the nosocomial pathogen *S. aureus*, the hemolysin, staphyloxanthin, toxic shock syndrome toxin 1 (TSST-1) and enterotoxins are the most important virulence factors that were remarkably affected by EG [85]. The expression of virulence-related genes (*sea*, *seb*, *tst* and *hla*) was also decreased after the treatment with this compound [85].

In a methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) S. aureus at subinhibitory concentration, EG eradicates pre-established biofilms and inhibited the colonization of this bacteria in a rat middle ear model, decreasing biofilm in biomass, cell viability and the expression of biofilm-related genes (icaD, sarA and seA), resulting in a low accumulation of polysaccharides and poorly adhesion of cells within biofilms [86]. The biofilm eradication effect of EG was mediated by two mechanisms: bacterial lysis within biofilms and by the disruption of cell-to-cell connections, hence dismantling the biofilm organization, which can be attributed to the hydrophobic and the lipophilic nature of their chemical structure [86].

The biofilm formation and biofilm-related genes in *L. monocytogenes* and *E. coli* also were inhibited by EG at dose-dependent manner [56, 59]. In *P. aeruginosa*, although EG was unable to inhibit biofilm formation, it markedly reduced the production of pyocyanin, fimbriae production, hemolytic activity and other QS-controlled virulence factors in this bacterium such as the pseudomonas quinolone signal (PQS) [56]. Other study showed that EG at subinhibitory concentrations has QS inhibitory activity in *P. aeruginosa* and *C. violaceum* [87].

Moreover, derivatives of EG *eugenyl acetate* (EA) (**Figure 6B**), *isoeugenol* (IE) (**Figure 6C**) and *methyl eugenol* (ME) (**Figure 6D**) showed anti-virulence properties against pathogenic bacteria. In *S. aureus*, EA inhibited the production of virulence factors like hemolysin and staphyloxanthin. Similarly, in *P. aeruginosa* the pyocyanin, pyoverdin and exoprotease production were significantly reduced after the treatment with EA, and it also exhibited QS inhibitory potential in *C. violaceum* [88]. The other derivatives, IE and ME, also presented QS inhibitory against *P. aeruginosa* and *C. violaceum* [89, 90], and in the case of *V. harveyi*, ME have anti-bioluminescence activity [90]. These anti-virulence properties can be attributable to the presence of numerous substituted aromatic molecules like in the case of other phenols [85].

2.6. Long-chain phenols

Long-chain phenols are a group of metabolites which have extensively studied antitumor, antimicrobial and antioxidant activities; they are also of great interest to the industry because they are used to manufacture different chemicals [91]. Also, different long-chain phenols reported have different anti-virulence properties.

Our research group identified a mixture of four *anacardic acids* (AA) capable of inhibiting QS in *C. violaceum* and also able to reduce the production of virulence factors such as pyocyanin, rhamnolipids and elastase activity in *P. aeruginosa* [92]. Similarly, another mixture of AA (**Figure 7A**) and one of *cardanols* (**Figure 7B**) was capable of inhibiting *P. aeruginosa* biofilms. Notably, although the antibiofilm mechanism is not known, the polymerization of the AA (**Figure 7C**) slightly potentiates the activity [36]. Similarly, the maximum antibiofilm activity observed for this phenol was around 80% inhibition, which is reduced to 50% by the

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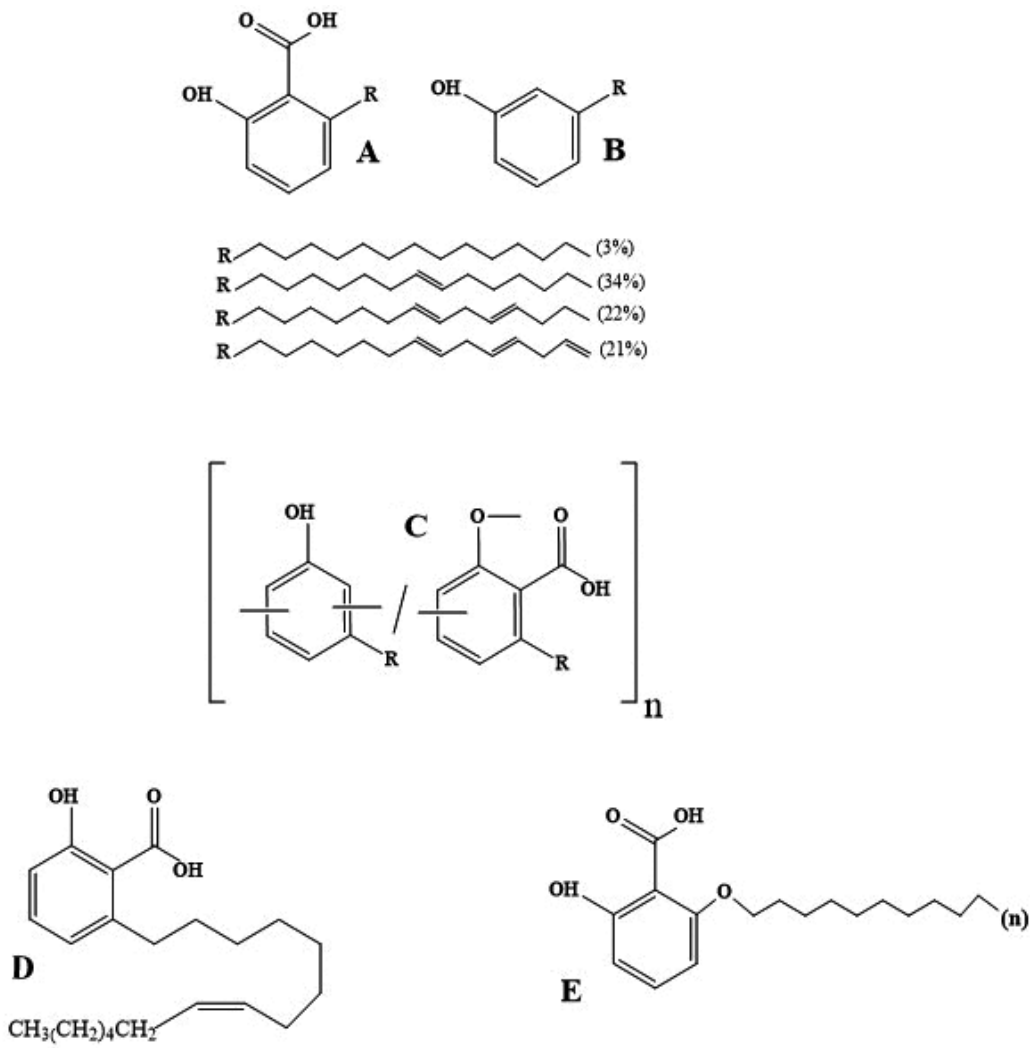

Figure 7. Long-chain phenols with anti-virulence properties. **A**: Anacardic acid mixture, **B**: cardanol mixture, **C**: polyanacardic acid, **D**: ginkgolic acids C15:1, **E**: 6-oxa isosteres of anacardic acids.

presence of a carboxyl group (salicylic acid) and only increases with the addition of an alkyl chain [36]. Hence, the incorporation of different types of alkyl chain in the meta-position of the salicylic acid seems to play a role in its activity, but this needs to be investigated in more detail.

Similarly, the antibiofilm activity of *gingolic acids* was reported, specifically the C15:1 (**Figure 7D**) abolished biofilm production without affecting bacterial viability, as well as reduced fimbriae production in enterohemorrhagic *E. coli* [93]. Transcriptomic analysis by DNA microarrays and qRT-PCR demonstrated that C15: 1 represses expression of genes involved in the synthesis of curli [93].

Furthermore, although mixtures of such compounds have shown anti-virulence activity, separation is laborious and costly, so their chemical syntheses become an attractive alternative. In this regard, AA synthetic (6-oxa isosteres) C: 11-C: 16 (**Figure 7E**) showed inhibition of TCS (KinA/SpoOF and NRII/NRI) [94]. Interestingly, AA with alkyl chains outside this range are not active [94]. Likewise, for this activity, the presence of the carboxyl group is important, as the C:12 and C:14 completely lose their effect, and the presence of phenolic OH partially restores it. Long-chain phenols are a group of natural products with great structural diversity, which represent an important potential source of molecules with anti-virulence activity.

2.7. Quercetin and related compounds

Various biological activities including anti-cancer, antibacterial, hepatoprotective, anti-inflammatory and antiviral activities have been attributed to flavonoids [95]; moreover, recent studies have shown that various flavonoids also have anti-virulence activity.

Flavonoids like *flavone* (**Figure 8A**), *quercetin* (**Figure 8B**), *apigenin* (**Figure 8C**) and *fisetin* (**Figure 8D**) decrease blood hemolysis induced by *S. aureus*. Specifically, for flavone it was elucidated that its activity is due the repression of the transcription of α-hemolysin genes (hla) and the global regulator gene (*Sae*) [96].

In addition, antibiofilm activity in *S. aureus* by *quercetin* (**Figure 8B**), *chrysin* (**Figure 8E**), *apigenin* (**Figure 8C**), *kaempferol* (**Figure 8F**) and *fisetin* (**Figure 8D**) has been reported where the number of hydroxyls is directly related to the increase in the activity [97], whereas *morin* (**Figure 8G**), *myricetin* (**Figure 8H**), *quercetin* (**Figure 8B**) and *kaempferol* (**Figure 8F**), having a hydroxyl group at C-2′ and C-4′ in ring B, inhibit SrtA and SrtB sortases of *S. aureus* more effectively [98].

The *myricetin* (**Figure 8H**) is a compound able to interact with listeriolysin O, a virulence factor of *Listeria monocytogenes* that is involved in the lysis of host cells. This interaction is related to the presence of the double bond in the molecule, specifically in the C1-C2 position in ring C [99]. This generates a complex that blocks the hemolytic activity of the listeriolysin as it prevents binding to cholesterol.

Furthermore, it has been shown that the *naringenin* (**Figure 8I**) have antibiofilm activity on *V. harveyi* and *E. coli;* however, this activity is compromised when sugar residues are incorporated [100]. In the case of V. harveyi, the naringenin also represses the expression of T3SS regulatory genes [100].

2.8. Resveratrol and related compounds

Resveratrol (RV) (**Figure 9A**) is a natural polyphenol and phytoalexin produced by plants in case of attacks by pathogens [101]. It is mainly found in the skin of grapes, some berries and red wine [102]. For its medical properties, it is recognized as a compound that provides multiple benefits to human health [103] and recent studies have demonstrated its anti-virulence potential.

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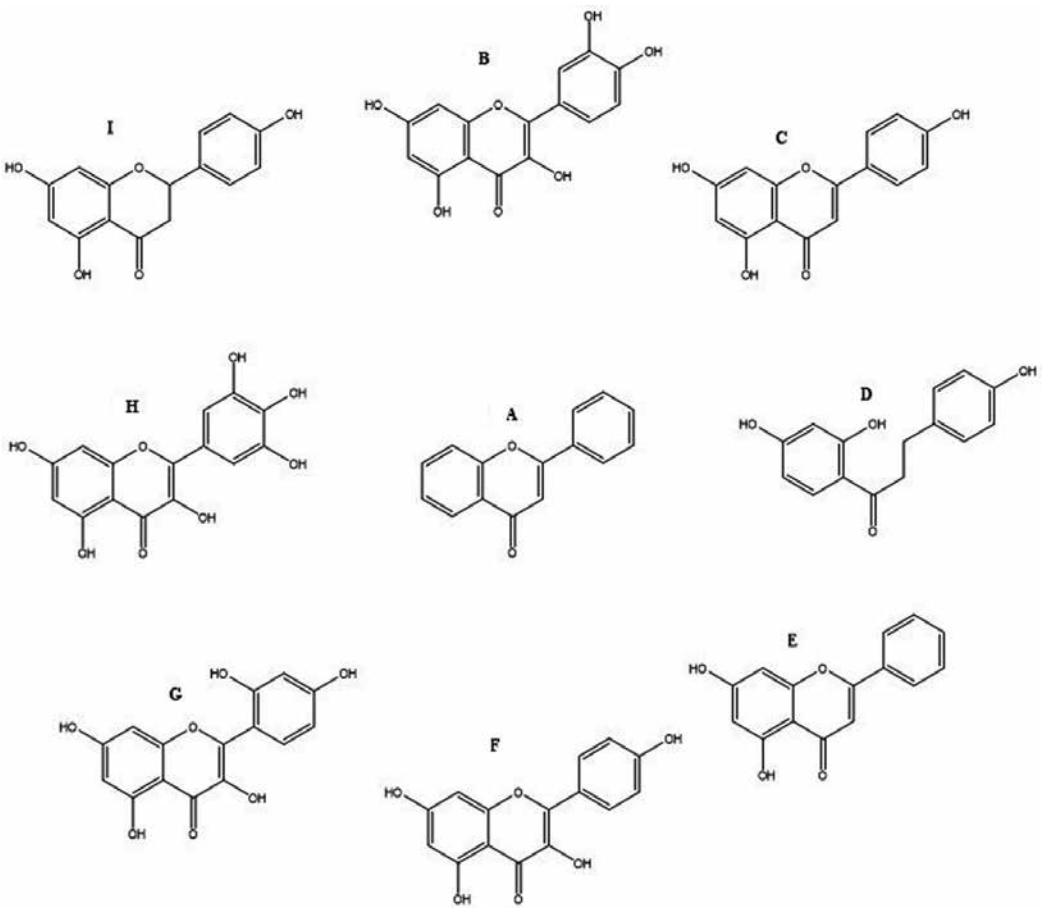

Figure 8. Quercetin and related compounds with anti-virulence properties. **A**: Flavone, **B**: quercetin, **C**: apigenin, **D**: fisetin, **E**: chrysin, **F**: kaempferol, **G**: morin, **H**: myricetin, **I**: naringenin.

Since plants produce RV, this metabolite was identified as the active compound with inhibitory activity against biofilm formation in *Propionibacterium acnes* from extracts of plants used in traditional Chinese medicine [104]. Also in *S. aureus*, the evaluation of different commercial red wines showed a dose-dependent inhibition of biofilm formation, hemolytic activity and increase in the survival of *Caenorhabditis elegans* exposed to the bacteria [97]. One of the major constituents of these red wines was RV, and similarly, it inhibited hemolysis in *S. aureus* [97]. In *Vibrio cholerae*, the biofilm formation has a prominent role in pathogenesis and RV was found to be a potent biofilm inhibitor at subinhibitory concentrations and showed binding affinity with the virulence activator AphB [102]. Furthermore, in the uropathogenic bacteria, *Proteus mirabilis* RV inhibited swarming motility, hemolysin and urease activity as well as the virulence factor expression at dose-dependent manner [101].

Compounds related to RV, the *oxyresveratrol* (**Figure 9B**), *dicinnamyl* (**Figure 9C**), *cis-stilbene* (**Figure 9D**) and *trans-stilbene* (**Figure 9E**) also were evaluated against *S. aureus* virulence.

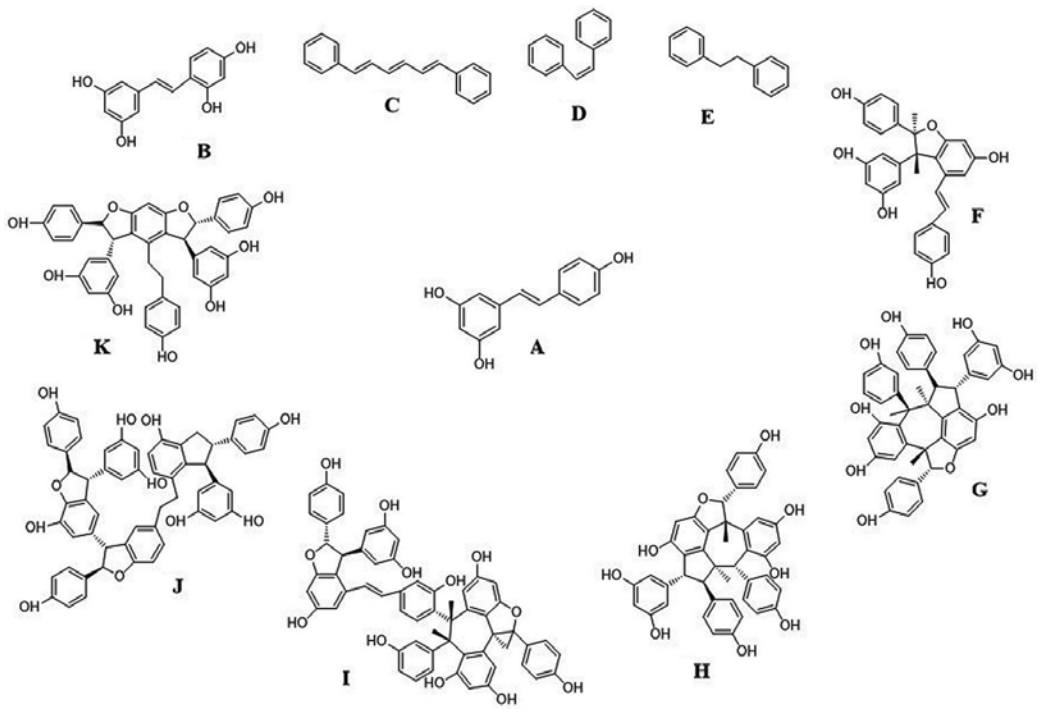

Figure 9. Resveratrol and related compounds with anti-virulence properties. **A**: Resveratrol, **B:** oxyresveratrol, **C:** dicinnamyl, **D:** *cis*-stilbene, **E:** *trans*-stilbene, **F:** ε-viniferin, **G:** suffruticosol A, **H:** suffruticosol B, **I:** vitisin A, **J:** vitisin B, and **K:** *trans*-gnetin.

Only, the *cis-stilbene* and *trans-stilbene* along with RV markedly inhibited the hemolytic activity by more than 80%, while *dicinnamyl*, *oxyresveratrol* and *trans-stilbene* have a significant biofilm inhibition effect [105]. The inhibitory activity of *trans-stilbene* was corroborated with the evidence that is able to repress the expression of the α-hemolysin gene (*hla*) and of genes implicated in adhesion (*icaA* and *icaD*) and with the attenuation of *S. aureus* virulence in the nematode *C. elegans* [105]. In enterohemorrhagic *E. coli*, the RV isolated from the extract of *Carex dimorpholepis* significantly reduced biofilm formation (up to 90%), expression of biofilm-related genes and swimming and swarming motilities, suggesting that this compound is a major antibiofilm component in this extract, corroborating its potential as therapeutic agent against *E. coli* [106].

The RV and its oligomers, namely *ε-viniferin* (**Figure 9F**), *suffruticosol A* (**Figure 9G**), *suffruticosol B* (**Figure 9H**), *vitisin A* (**Figure 9I**) and *vitisin B* (**Figure 9J**) isolated from different plant families, also have antibiofilm activities against *E. coli*. The qRT-PCR analyses showed that *ε-viniferin*, *suffruticosol B* and *vitisin B* repress the expression of genes involved in curli and fimbriae production [105]. Also, RV and *suffruticosol A*, *suffruticosol B*, *vitisin A* and *B* inhibit biofilm formation in *P. aeruginosa* at dose-dependent manner [106].

The oligomers *ε-viniferin* and *trans-gnetin* (**Figure 9K**) isolated from *Paeonia lactiflora* have inhibitory activity in neuraminidase activity, an enzyme involved in many pathological process in

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tropical human pathogens [107]. Furthermore, the *ε-viniferin* and RV isolated from *Carex pumila* extract also demonstrated significantly biofilm inhibition in *P. aeruginosa* and *E. coli* [108]. The anti-quorum sensing activity of RV also was demonstrated, in *C. violaceum*, since it reduces violacein production [57, 109]. In *Yersinia enterolitica* and *Erwinia amylovora*, it was one of the most active compounds that can reduce the concentration of the autoinducers due to degradation transformation or inhibition of synthesis [57].

2.9. Salicylic acid and related compounds

Salicylic acid (SA) (**Figure 10A**) is a phenolic compound synthesized by plants that play an important role in the regulation of various physiological processes [110, 111], and in recent years, their inhibitory activity against bacterial virulence has been reported.

Several studies have demonstrated that SA has inhibitory activity in the motility and production of extracellular virulence factors in the opportunistic pathogenic bacteria *P. aeruginosa*, and among those factors, pyocyanin was inhibited by approximately 80% by SA and decreased the elastase and exoprotease production [110]. Similarly, a subinhibitory concen-

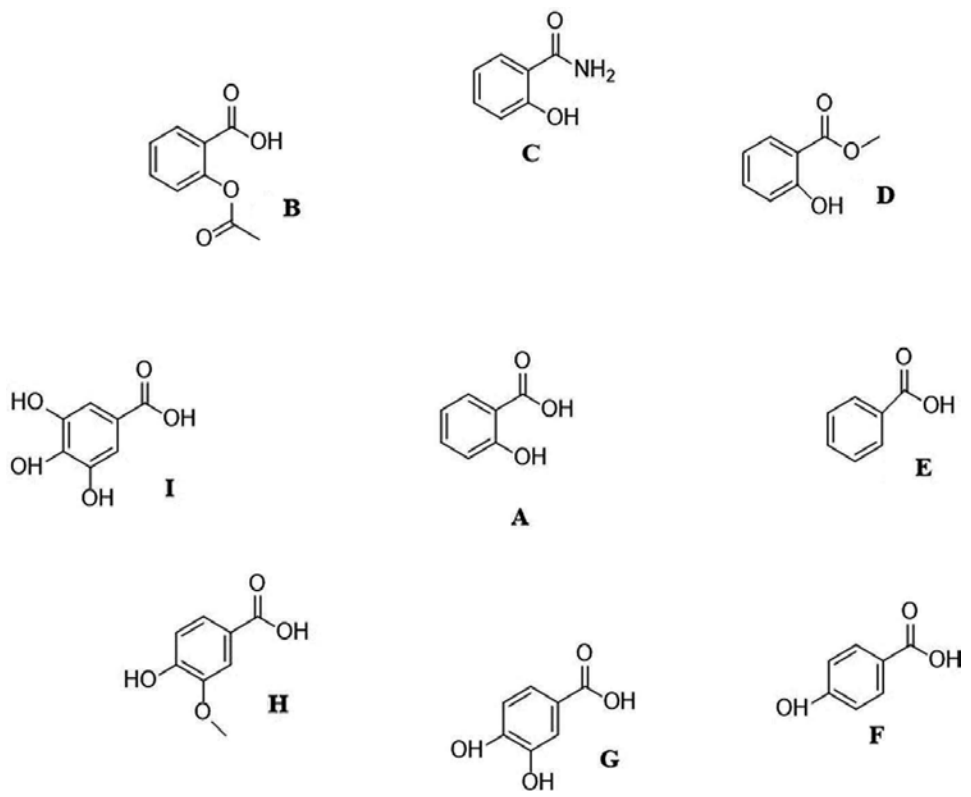

Figure 10. Salicylic acid and related compounds with anti-virulence properties. **A**: Salicylic acid, **B**: acetyl salicylic acid, **C**: salicylamide, **D**: methyl salicylate, **E**: benzoic acid, **F**: *p*-hydroxybenzoic acid, **G**: protocatechuic acid, **H**: vanillic acid, **I**: gallic acid.

tration of SA inhibited the twitching and swimming motility as well as the invasion and acute cytotoxicity of *P. aeruginosa* in corneal epithelial cells [112]. Some derivatives of SA, including *acetyl salicylic acid* (**Figure 10B**), *salicylamide* (**Figure 10C**), *methyl salicylate* (**Figure 10D**) and a precursor of SA, *benzoic acid* (**Figure 10E**), were evaluated, and the inhibition levels observed were comparable with those obtained with SA for the same virulence factors [110]. SA is a benzoic acid that possesses an aromatic ring bearing a hydroxyl group, and probably, one of these components of the structure is responsible for its anti-virulence activity.

The biofilm formation in *P. aeruginosa* was also inhibited by SA *in vitro* and *in vivo* decreasing the attachment and consequently the biofilm formation [36, 110]. Similarly, in other bacterial pathogenic species that form biofilms, SA has inhibitory activity; for example, in *Streptococus mutans*, the biofilm formation was highly decreased when the enzymes, glucosyl and fructosyl transferases, which synthetize extracellular polymeric substances, were inhibited by SA [113].

Compounds related to SA, the *p-hydroxybenzoic acid* (**Figure 10F**) and *protocatechuic acid* (**Figure 10G**) at growth subinhibitory concentrations have different modes of action on biofilm formation disruption in *Staphylococcus species* [114]. Also, for the bacteria *Helicobacter pylori* implicated in the development of peptic ulcer, duodenal ulcer and gastric cancer, which uses a urease enzyme for the basification of the stomach pH and hence the colonization of the gastric mucosa [115], the *protocatechuic acid* has an inhibitory effect of 40% in its urease activity [116]. *Vanillic acid* (4-hydroxy-3-methoxybenzaldehyde) (**Figure 10H**) also showed antibiofilm activity in *Aeromonas hydrophila* at all the concentrations used in the range of 0–0.250 mg/mL [117].

Other important hydroxy benzoic acid with a numerous reports of anti-virulence properties is *gallic acid* (GA) (**Figure 10I**), which shows inhibition in many virulence factors among bacteria. For example, in *S. aureus*, GA reduces the bacterial adhesion and biofilm formation as well as the production of α-hemolysin a virulence factor produced by the bacteria with hemolytic, cytotoxic, dermonecrotic and lethal properties [118] since its activity was inhibited in a dose-dependent manner by this compound [119]. Similarly, in *P. aeruginosa*, *E. coli* and *Listeria monocytogenes*, their biofilm formation was also inhibited by GA.

The inhibitory activity showed by these compounds may be related to some of their structural features, since different reports mentioned that in the active phenolic compounds, the basic skeleton remains the same, the basic skeleton remains same, but the number and positions of the hydroxyl groups on the aromatic ring and the type of substituents provide different biological properties [120–122]. Also, SA, *gallic acid* and *vanillic acid* have QS inhibitory activity by two different mechanisms: first, by affecting the synthesis of AHLs [55, 57, 123] and second, by interfering with the binding of short-chain AHLs to their receptor, especially in the case of vanillic acid [117].

3. Conclusion and future perspective

An important feature of the anti-virulence molecules is that they may be less prone to promote the emergence of resistance than conventional antibiotics. At the moment, phenolic compounds represent the largest number of natural products with anti-virulence-reported activity and whose main target has been the inhibition of QS and biofilms. However, it has

also been found that they can directly inhibit some of virulence factors such as sortases, curli, type III secretion system (T3SS), fimbriaes and two-component regulatory systems. It should be noted that most of the phenolic compounds represent structures already known, several of which have been subject to different pharmacological studies and some are even part of the international pharmacopeia and are active ingredients of herbal medicines.

Moreover, although QS is considered the main regulator of bacterial virulence, this is still part of a complex network of interconnected components including several environmental regulation systems and QS-independent virulence factors. Also, the direct *inhibition of virulence factors* and regulators of QS and TCS represents interesting options for achieving the implementation of this strategy. Thus, the correct design of anti-virulence therapies is very important [124, 125], and a feasible option is the combination of drugs with different action targets. In the same way, some challenges to overcome involve the evaluation of anti-virulence compounds in most bacterial systems, the corroboration *in vivo* in animal infection models and finally the evaluation of possible side effects on the populations of commensal and symbiotic bacteria.

Given the growing public health problem worldwide derived by the emergence of bacterial multiresistance to antibiotics, the development of suitable anti-virulence therapies is presented as a viable strategy to provide a solution to this problem; moreover, we are in the decisive years that will dictate the implementation of these kind of strategies, this is occurring in a period of resurgence of the interest in natural products activities in which phenolic compounds have a fundamental role.

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Author details

Naybi Muñoz-Cazares[1†], Rodolfo García-Contreras[2†], Macrina Pérez-López[1] and Israel Castillo-Juárez[1*]

*Address all correspondence to: israel.castillo@colpos.mx

1 Department of Botany, Postgraduate College, Texcoco, Mexico

2 Department of Microbiology and Parasitology, Faculty of Medicine, UNAM, Mexico City, Mexico

† Authors contributed equally to this work

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Chapter 9

Regulatory Mechanism of Skeletal Muscle Glucose Transport by Phenolic Acids

Tatsuro Egawa, Satoshi Tsuda, Rieko Oshima, Ayumi Goto, Xiao Ma, Katsumasa Goto and Tatsuya Hayashi

Additional information is available at the end of the chapter

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Abstract

Type 2 diabetes mellitus (T2DM) is one of the most severe public health problems in the world. In recent years, evidences show a commonness of utilization of alternative medicines such as phytomedicine for the treatment of T2DM. Phenolic acids are the most common compounds in non-flavonoid group of phenolic compounds and have been suggested to have a potential to lower the risk of T2DM. Skeletal muscle is the major organ that contributes to the pathophysiology of T2DM. Studies have shown that several phenolic acids (caffeic acid, chlorogenic acid, gallic acid, salicylic acid, *p*-coumaric acid, ferulic acid, sinapic acid) have antidiabetic effects, and these compounds have been implicated in the regulation of skeletal muscle glucose metabolism, especially glucose transport. Glucose transport is a major regulatory step for whole-body glucose disposal, and the glucose transport processes are regulated mainly through two different systems: insulin-dependent and insulin-independent mechanism. In this chapter, we reviewed recent experimental evidences linking phenolic acids to glucose metabolism focusing on insulin-dependent and insulin-independent glucose transport systems and the upstream signaling events in skeletal muscle.

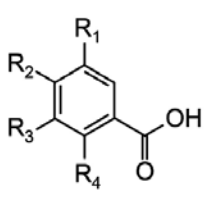
Keywords: glucose metabolism, 5′AMP-activated protein kinase, insulin, glucose transporter, phytomedicine, phytochemical

1. Introduction

Diabetes is one of the most rapidly increasing chronic diseases in the world. According to the International Diabetes Federation [1], there are now 415 million adults aged 20–79 with diabetes worldwide, and there will be 642 million people living with the disease by 2040. Type 2 diabetes mellitus (T2DM) is the most common type of diabetes, which is due primarily to lifestyle factors and genetics. Numerous lifestyle factors, including excessive caloric intake, physical inactivity, cigarette smoking, and generous consumption of alcohol, are considered to be important to the development of T2DM [2]. T2DM care is usually managed by a multidisciplinary healthcare approach, which includes a combination of dietary restriction, exercise, hypoglycemic agents, and/or insulin. In present times, evidences show a commonness of utilization of alternative medicines for the treatment of T2DM.

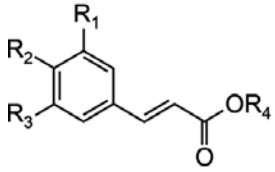
A traditional herbal medicine, also called as phytomedicine, has been used since ancient time in many regions in the world. Phytomedicine is a medicine mainly derived from whole leaves, roots, stems, and plant extracts for promoting health and treating illness [3]. Plants produce numerous diversity of chemicals known as secondary metabolites through evolved secondary biochemical pathways. These secondary metabolites serve as defense compounds against herbivores or infection and thereby enhance their ability to survive. These compounds are also helpful for humans to protect themselves against diseases and are called phytochemical. Each type of fruit or vegetable contain hundreds of phytochemical, and these phytochemicals exhibit multiple beneficial effects in the treatment of T2DM [4].

Phenolic acids, which are part of the secondary metabolites, belong to the family of phenolic compounds and are the most common compounds in non-flavonoid group. Phenolic acids are synthesized from the shikimic acid pathway from L-phenylalanine or L-tyrosine [5]. These compounds exist predominantly as hydroxybenzoic acids, which include gallic acid, salicylic acid, protocatechuic acid, vanillic acid, and gentisic acid and hydroxycinnamic



Benzoic acid derivatives

	R_1	R_2	R_3	R_4
Salicylic acid	H	H	H	OH
p-Hydroxybenzoic acid	H	OH	H	H
Gentisic acid	OH	H	H	OH
Protocatechuic acid	H	OH	OH	H
Gallic acid	OH	OH	OH	H
Vanillic acid	H	OH	OCH_3	H
Syringic acid	OCH_3	OH	OCH_3	H



Cinnamic acid derivatives

	R_1	R_2	R_3	R_4
p-Coumaric acid	H	OH	H	H
Caffeic acid	H	OH	OH	H
Ferulic acid	OCH_3	OH	H	H
Sinapic acid	OCH_3	OH	OCH_3	H
Chlorogenic acid	H	OH	OH	$C_7H_9O_5$

Figure 1. Chemical structures of benzoic acid and cinnamic acid derivatives.

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acids, which include *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, and chlorogenic acid (**Figure 1**). They are abundant in edible vegetable, fruits, and nuts and are the main contributors to the total polyphenol intake [6]. Although the beneficial role of phenolic acids in the lifestyle-related diseases is still controversial, reports have suggested the inverse relationship between high levels of phenolic acids intake and metabolic syndrome including T2DM [7, 8].

Because skeletal muscle is responsible for approximately 80% of insulin-mediated glucose utilization [9], it is considered that defects in insulin action on skeletal muscle are key contributors to the pathophysiology of T2DM. Studies have shown that several phenolic acids have antidiabetic effects [8], and these compounds have been implicated in the regulation of skeletal muscle glucose metabolism, especially glucose transport, a rate-limiting step for glucose utilization. However, the precise mechanism of how phenolic acids modulate glucose transport has not been firmly established. In this chapter, we provide recent experimental evidences linking phenolic acids to glucose transport and upstream signaling pathways in skeletal muscle.

2. Glucose transport in skeletal muscle

2.1. Glucose transport

Glucose transport is a major regulatory step for whole-body glucose disposal that occurs by a system of facilitated diffusion with glucose transporter (GLUT)-mediated process. GLUT is a protein of ~500 amino acids and is predicted to possess 12 transmembrane-spanning alpha helices and a single N-linked oligosaccharide. GLUT1, 3, 4, 5, 8, 10, 11, and 12 exist in mammalian skeletal muscle tissue, and especially, GLUT4 is the predominant glucose transporter isoform present in skeletal muscle. GLUT4 is present in intracellular vesicular pool in the basal non-stimulated state, and the translocation of GLUT4 from an intracellular location to the plasma membrane and T-tubules is a major determinant of acute regulation of glucose transport [10] (**Figure 2**). These glucose transport processes are regulated mainly through two different systems: insulin-dependent and insulin-independent mechanism.

2.2. Regulation of insulin-dependent glucose transport

Insulin is a peptide hormone produced by β cells of the pancreatic islets. Insulin consists of two polypeptide chains, the A and B chains, linked together by disulfide bonds. It is first synthesized as a single polypeptide called preproinsulin in pancreatic β-cells, and then it is cleaved to form a smaller protein, proinsulin. The conversion of proinsulin to insulin occurs through the combined action of the prohormone convertases [12].

The insulin receptor is a member of the ligand-activated receptor and tyrosine kinase family of transmembrane-signaling proteins that consists of two extracellular α subunits and two transmembrane β subunits connected by disulfide bridges [13]. Binding of insulin to the extracellular domain of the insulin receptor α subunit triggers tyrosine phosphorylation of the intracellular domain of the β subunit [14]. Following the autophosphorylation of the receptor,

the insulin receptor phosphorylates insulin receptor substrate (IRS)-1 on tyrosine residues. Tyrosine-phosphorylated IRS then binds to the Src homology 2 (SH2) domain-containing adaptor protein p85, a regulatory subunit of phosphatidylinositol-3 kinase (PI3K), resulting in activation of the catalytic p110 subunit of PI3K. This results in the generation of the critical second messenger PI3,4,5-triphosphate, which in turn triggers the activation of Akt. Recently, TBC1 domain family (TBC1D) member 1 (TBC1D1) and member 4 (TBC1D4) have been suggested to act as downstream mediators of Akt. TBC1D1 and TBC1D4 contain Rab GTPase-activating protein (GAP) domains that prevent GLUT4 translocation by inactivating Rab proteins. TBC1D1 and TBC1D4 dissociate from GLUT4 vesicles in the phosphorylated state and thereby facilitate GLUT4 translocation and glucose transport [15, 16] (**Figure 2**).

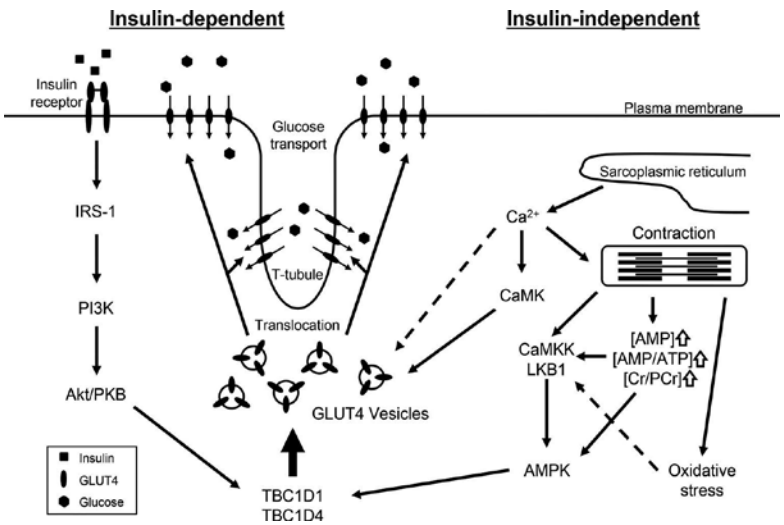

Figure 2. Molecular mechanism of stimulating insulin-dependent and insulin-independent glucose transport in skeletal muscle. This figure was adapted from Egawa et al. [11] with permission by the publisher. AMPK, 5′AMP-activated protein kinase; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; Cr, creatine; GLUT4, glucose transporter 4; IRS-1, insulin receptor substrate 1; LKB1, liver kinase B1; PCr, phosphocreatine; PKB, protein kinase B; PI3K, phosphatydilinositol-3 kinase; TBC1D1, TBC1 domain family member 1; TBC1D4, TBC1 domain family member 4.

2.3. Regulation of insulin-independent glucose transport

A serine/threonine protein kinase, 5′AMP-activated protein kinase (AMPK), is critical for insulin-independent glucose transport in the muscle through translocation of GLUT4. AMPK comprises a catalytic α subunit and the regulatory subunits β and γ [17] in a total of 12 possible heterotrimeric combinations of two α, two β, and three γ subunits [18]. In skeletal muscle, the predominant heterotrimeric complexes include $\alpha1/\beta2/\gamma1$, $\alpha2/\beta2/\gamma1$, and $\alpha2/\beta2/\gamma3$ [19]. The α subunit has a catalytic domain that contains the activating phosphorylation site (Thr^{172}) at the N-terminus, an auto-inhibitory domain, and a conserved C-terminal domain that interacts with β and γ subunits [20–24]. There are two distinct α isoforms ($\alpha1$ and $\alpha2$): $\alpha1$ is expressed

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ubiquitously, whereas $\alpha 2$ is dominant in the skeletal muscle, heart, and liver [25]. The regulatory β subunit contains a C-terminal region that interacts with α and γ subunits and a central region that binds glycogen [26]. The regulatory γ subunit contains binding sites of adenine nucleotides (adenosine monophosphate (AMP), adenosine diphosphate (ADP), or adenosine triphosphate (ATP)) [18].

AMPK typically works as a signaling intermediary in muscle cells by monitoring cellular energy status, such as AMP/ATP ratio and creatine/creatine phosphate (PCr) ratio [17]. Binding of AMP to the Bateman domains of the AMPK γ subunit leads the allosteric activation of AMPK and phosphorylation of the Thr^{172} residue of the α subunit, which is crucial for maximal kinase activity. The level of phosphorylation also depends on the balance of activities of upstream kinases including liver kinase B1 (LKB1) and Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) and protein phosphatases [24, 27]. The LKB1 complex is constitutively active but is not activated directly by AMP. The binding of AMP to AMPK induces a structural change that assists phosphorylation of AMPK by the LKB1 complex [28, 29]. On the other hand, CaMKK activates AMPK in response to increased intracellular Ca^{2+} levels independently of energy status [30–32].

AMPK is also activated without energy depletion by 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), a pharmacological activator of AMPK. When taken up into skeletal muscle, AICAR is converted by adenosine kinase to ZMP, a monophosphorylated derivative that mimics the effects of AMP on AMPK [17]. AICAR-induced activation of AMPK leads to insulin-independent stimulation of glucose transport in skeletal muscle [33, 34] accompanied by GLUT4 translocation to the plasma membrane [35]. Moreover, AICAR-stimulated glucose transport is abrogated completely in muscles from mice with muscle-specific expression of a dominant-negative (kinase dead) form of AMPK [36], indicating that increased AMPK activity is sufficient for the stimulation of glucose transport in skeletal muscle.

AICAR-stimulated glucose transport is not inhibited by a PI3K inhibitor wortmannin [33], and the increase in glucose transport induced by the combination of maximal AICAR and maximal insulin stimulation is partly additive [33]. Therefore, the underlying molecular signaling mechanisms regulating insulin-dependent and insulin-independent glucose transport have been considered to be distinct. In this regard, recent studies have revealed that AMPK promotes GLUT4 translocation likely through TBC1D1 and TCB1D4 [37]. In short, insulin-dependent and insulin-independent signaling of glucose transport systems seem to convergence at TBC1D1 and TBC1D4 (**Figure 2**).

3. Phenolic acids and glucose transport

3.1. Caffeic acid

Caffeic acid (3,4-dihydroxycinnamic acid) is the most frequently studied phenolic acids in diabetes research. A prospective investigation conducted in two cohorts of US women demonstrated that there was an inverse association between urinary excretion level of caffeic

acid and T2DM risk [38], indicating that dietary intake of caffeic acid may alleviate a development of T2DM. Indeed, several studies have shown the hypoglycemic action of caffeic acid. Intravenous injection of caffeic acid (0.5–5 mg/kg) into both streptozotocin (STZ)-induced diabetic rats and rats with insulin resistance exhibited an acute (<30 min) effect of lowering plasma glucose in a dose-dependent manner [39, 40]. Further, chronic (5–12 weeks) dietary supplementation with caffeic acid (0.02–2%) lowered blood glucose level in diabetic mice [41–43].

A previous work by us first demonstrated that incubation of isolated rat skeletal muscles with caffeic acid (0.1–1 mM) acutely (<30 min) enhanced AMPKα Thr^{172} phosphorylation [44, 45] (**Figure 3**). Phosphorylation of acetyl-CoA carboxylase (ACC) Ser^{79} exhibited parallel changes to AMPK phosphorylation. ACC is a major substrate of AMPK in skeletal muscle, and phosphorylation of ACC at Ser^{79} reflects the total AMPK activity [46–48]. Correspondingly, caffeic acid (1 mM, 30 min) stimulated insulin-independent glucose transport in skeletal muscle (**Figure 4**). Other researchers also have shown that caffeic acid enhanced insulin-independent glucose transport in isolated adipocytes [39] and cultured muscle cells [40]. Therefore, the stimulatory effect of caffeic acid on insulin-independent glucose transport may contribute to the hypoglycemic action, partly through AMPK-mediated mechanism.

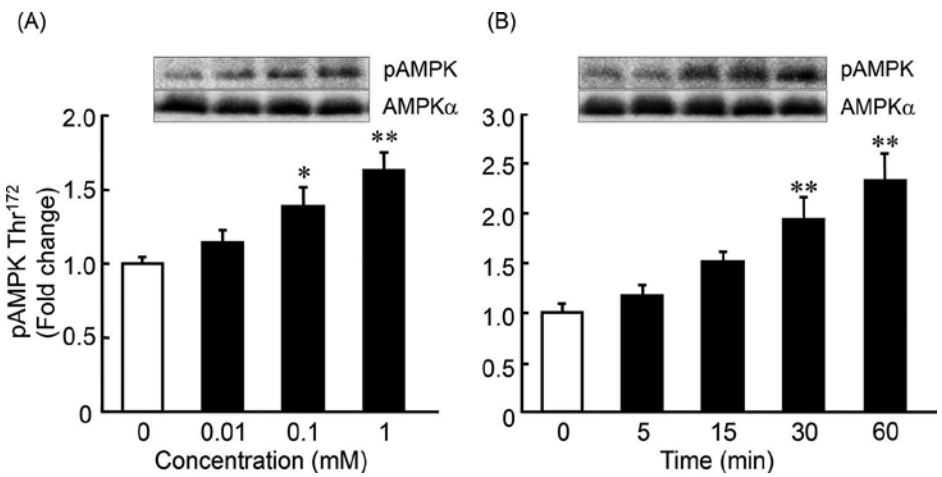

Figure 3. The effect of caffeic acid on phosphorylation status of AMPKα Thr^{172} in skeletal muscle. (A) Isolated epitrochlearis muscles were incubated with caffeic acid at indicated concentration for 30 min. (B) Isolated epitrochlearis muscles were incubated with caffeic acid (1 mM) at indicated time. Muscle lysates were then analyzed for phosphorylation of AMPKα Thr^{172} (pAMPK) by western blot analysis. Fold increases are expressed relative to the level of muscles in the non-stimulated group. Representative immunoblots are shown. Values are mean ± SE. *$P < 0.05$, **$P < 0.01$ vs. non-stimulated group. This figure was adapted from Tsuda et al. [44] with permission by the publisher.

The finding that caffeic acid enhances phosphorylation status of AMPKα Thr^{172} indicates that caffeic acid leads to covalent modification through upstream kinases. Since the binding of AMP to AMPK facilitates the phosphorylation of AMPK by the LKB1 complex [28], LKB1 is considered as a crucial AMPK kinase in response to energy depletion in skeletal muscle. When

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cellular ATP level is depleted, phosphate is transferred from PCr to ADP to reproduce ATP. Decreased PCr level leads to an increase in free ADP and thereby causes AMP accumulation through the reaction of adenylate kinase, and thus a reduction of PCr level indicates a cellular energy depletion. In our previous work [44], we observed that incubation of rat skeletal muscles with caffeic acid decreased PCr level, suggesting that LKB1 is a possible kinase to enhance the caffeic acid-induced AMPKα Thr^{172} phosphorylation.

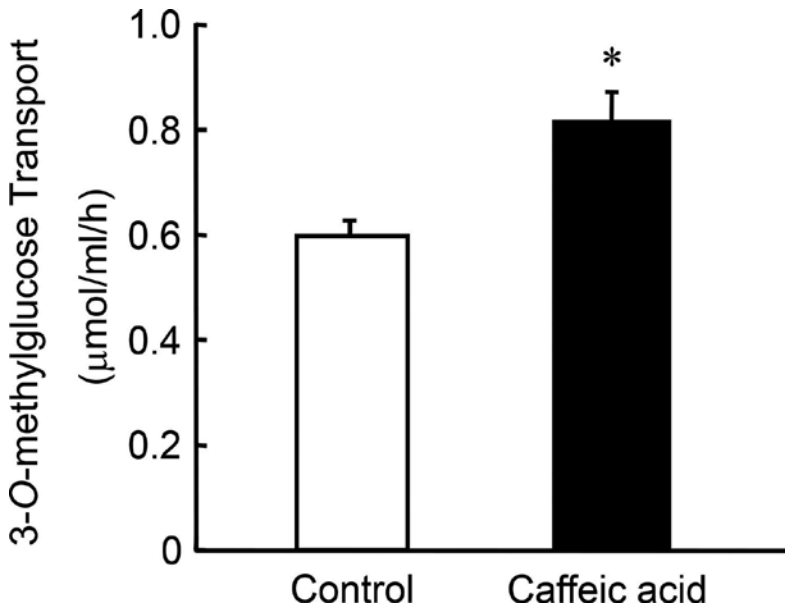

Figure 4. The effect of caffeic acid on insulin-independent glucose transport in rat skeletal muscles. Isolated epitrochlearis muscles were incubated in the absence (control) or presence of 1 mM caffeic acid for 30 min, and then glucose transport activity was measured using the glucose analog 3-O-methylglucose. Values are mean ± SE. *P < 0.05 vs. control. This figure was adapted from Tsuda et al. [44] with permission by the publisher.

Exercise (muscle contraction) is a strong stimulator for insulin-independent glucose transport. Due to the provision of energy for contracting muscle during exercise, AMP and ADP levels are rapidly increased in an intensity-dependent manner while ATP levels decline slightly. Since AMPK is a sensor of cellular energy status that is activated by AMP/ATP ratio, AMPK is activated during exercise in an intensity-dependent manner [49–52]. Thus, exercise can regulate insulin-independent glucose transport by a mechanism involving AMPK [33]. Recent work by us showed an interesting finding that muscle contraction and caffeine, which is the most widely consumed phytoactive substance in the world, synergistically stimulate insulin-independent glucose transport and AMPK Thr^{172} phosphorylation in skeletal muscle [45]. This result indicates the possibility that some phytochemicals enhance the maximal capacity of contraction-induced AMPK activity in skeletal muscle. In the point of view, we evaluated the effect of caffeic acid on contraction-stimulated AMPK activity in skeletal muscle. Maximal activation of AMPK by contraction was induced by 10 min tetanic contraction according to the protocol by Musi et al. [52]. AMPKα Thr^{172} phosphorylation was increased in response to caffeic

acid (1 mM, 30 min) stimulation; however, caffeic acid had no effect on the contraction-stimulated AMPKα Thr^{172} phosphorylation [45]. This finding suggests that caffeic acid has no capacity for enhancing contraction-induced AMPK activity.

It seems that caffeic acid stimulates insulin-dependent glucose transport at insulin resistance state in skeletal muscle. Insulin resistance is in which there are impaired biological and physiological responses to insulin in the tissue, and skeletal muscle insulin resistance is a major factor in the pathogenesis of T2DM. The underlying cellular mechanisms are yet unclear, but tumor necrosis factor α (TNFα), which is a member of the TNF ligand superfamily and a multifunctional cytokine, is implicated in the development of insulin resistance [53]. Activation of the TNF receptor results in stimulation of nuclear factor-κB (NF-κB) signaling via inhibitor κB kinase (IKK). IKK is the master regulator of NF-κB activation in response to inflammatory stimuli, and the IKK/NF-κB pathway is considered to be a core mechanism that causes insulin resistance in peripheral tissues including skeletal muscle [54, 55]. We demonstrated that, during insulin-stimulated condition, caffeine-induced insulin resistance which includes activation of IKK/NF-κB signaling and suppression of Akt Ser^{473} phosphorylation, which is required for the full activation of Akt, and insulin-dependent glucose transport, were alleviated by the treatment with caffeic acid in rat skeletal muscle [56]. Hence, caffeic acid may have an ability to improve insulin resistance state that is induced by activation of IKK/NF-κB signaling. Notably, caffeic acid does not stimulate insulin signaling pathway in normal state because we have shown that incubation of isolated rat skeletal muscle with caffeic acid had no effect on stimulating Akt Ser^{473} phosphorylation in the basal condition [44, 45].

3.2. Chlorogenic acid

Chlorogenic acid is the ester of caffeic acid and (−)-quinic acid and has been implicated in reducing the risk of T2DM. In animal study, treatment of chlorogenic acid (250 mg/kg) acutely (<30 min) lowered blood glucose concentration during glucose tolerance test in diabetic db/db mice [57, 58]. Furthermore, repeated (2–12 weeks) treatment of chlorogenic acid (80–250 mg/kg/day) improved fasting blood glucose concentration, HOMA-IR index (fasting insulin [μU/ml]×fasting glucose [mmol/l]/22.5), blood glucose concentration during glucose or insulin tolerance test in db/db mice [58, 59], and high-fat diet-induced diabetic mice [60]. Intervention with lower doses of chlorogenic acid (5 mg/kg/day) also improved the peak blood glucose concentration during glucose tolerance test in Zucker (fa/fa) rats although fasting blood glucose concentration did not change [61]. In human study, chlorogenic acid ingestion (1 g) reduced blood glucose concentration during oral glucose tolerance test in overweight men [62]. Thus, the accumulated evidences strongly suggest that chlorogenic acid has a hypoglycemic effect, but the cellular mechanism of action is not fully understood yet.

Stimulatory effect of chlorogenic acid on skeletal muscle glucose transport was firstly reported by Prabhakar and Doble [63]. They revealed that incubation with chlorogenic acid (25 μM) stimulated insulin-independent glucose transport within 3 h in differentiated L6 skeletal muscle cells. Subsequently, Ong et al. [57] demonstrated that incubation of isolated skeletal muscle from db/db mice and L6 skeletal muscle cells with chlorogenic acid (1–10 mM) for 1–24 h enhanced insulin-independent glucose transport. They also showed that

chlorogenic acid-stimulated glucose transport was inhibited by the pretreatment with compound C, an AMPK inhibitor, but not wortmannin, a PI3K inhibitor. These findings suggest that chlorogenic acid stimulates skeletal muscle glucose transport via insulin-independent and AMPK-dependent mechanism.

The previous work by us investigated the acute effect of chlorogenic acid on AMPKα Thr^{172} phosphorylation status in rat skeletal muscle [44] and showed that incubation with chlorogenic acid (<1 mM, <60 min) had no effect on AMPKα Thr^{172} phosphorylation in isolated rat skeletal muscle. In contrast, Ong et al. [57] demonstrated that chlorogenic acid had an ability to enhancing AMPK activity in L6 skeletal muscle cells in dose-dependent (1–10 mM) and time-dependent (1–24 h) manners. These findings suggest that chlorogenic acid directly acts skeletal muscle and stimulates AMPK, and that relatively higher concentration of chlorogenic acid (>1 mM) and/or longer stimulation period (>60 min) is needed to stimulate skeletal muscle AMPK.

Adiponectin is an adipokine that has been recognized as a key regulator of glucose metabolism. Binding of adiponectin to adiponectin receptor AdipoR1 induces Ca^{2+} influx and leads to the activation of CaMKK/AMPK signaling in skeletal muscle [64]. A study showed that AMPKα Thr^{172} phosphorylation and ACC Ser^{79} phosphorylation were upregulated in response to chronic (2 weeks) administration of chlorogenic acid (250 mg/kg/day) in skeletal muscle of db/db mice [58]. In addition, the treatment also increased CaMKK expression in skeletal muscle. More recently, Jin et al. [59] showed that treatment with chlorogenic acid (80 mg/kg/day) for 12 weeks increased AMPKα Thr^{172} phosphorylation as well as AdipoR1 expression in skeletal muscle of db/db mice. Collectively, chronic treatment of chlorogenic acid may act as an antidiabetic agent through stimulating adiponectin-AMPK signaling because AMPK induces a variety of metabolic changes toward antidiabetic property: promoting glucose transport [33, 34, 36, 65], GLUT4 expression [66–68], fatty acid oxidation [49, 69, 70], mitochondrial biogenesis [71, 72], insulin sensitivity [73, 74], and fiber-type shift toward the slower and more oxidative phenotype [75].

Notably, chlorogenic acid is hydrolyzed by intestinal microflora into various aromatic acid metabolites including caffeic and quinic acids [76]. Additionally, it is reported that absorption rate of caffeic acid in the small intestine of humans is 95% but chlorogenic acid is 33% [77]. These observations suggest that the health-promoting effects of chlorogenic acid might be attributed to the actions of chlorogenic acid-derived caffeic acid. In this context, the stimulatory effect of oral intake of chlorogenic acid as well as caffeic acid at physiological doses on AMPK activation and AMPK-related metabolic events, including glucose transport in skeletal muscle, must be confirmed.

3.3. Gallic acid

Gallic acid (3,4,5-trihydroxybenzoic acid) is known to have a variety of cellular functions including beneficial effects on T2DM. Chronic treatment (4–16 weeks) with gallic acid (25–100 mg/kg/day) produced significant decrease in elevated fasting serum glucose level in STZ-induced diabetic rats [78], in high-fat diet-induced diabetic mice [79], or in high-fat diet/STZ-induced diabetic rats [80, 81]. Four weeks of treatment with gallic acid (10–30 mg/kg/day) in

high-fructose diet-induced diabetic rats also ameliorates hyperglycemia and HOMA-IR index and improved glucose clearance during oral glucose tolerance test [82].

A study reported that treatment with gallic acid (10 μM) for 30 min induces GLUT4 translocation and insulin-independent glucose transport in 3T3-L1 adipocytes [83]. We found that a water-soluble Pu-erh tea extract which contained 9.11% gallic acid stimulated Akt Ser^{473} phosphorylation in a dose- and time-dependent manner with a concomitant increase in insulin-independent glucose transport in isolated rat skeletal muscle [84]. By contract, the Pu-erh tea extract did not change the phosphorylation status of AMPKα Thr^{172}. Correspondingly, incubation of isolated rat skeletal muscle with gallic acid (820 μM) for 30 min robustly stimulated Akt Ser^{473} phosphorylation without affecting AMPK phosphorylation [84] (**Figure 5**). These findings indicate that gallic acid stimulates glucose transport via enhancing insulin signaling transduction in the absence of insulin and raise the possibility that gallic acid can be an insulin-mimetic agent.

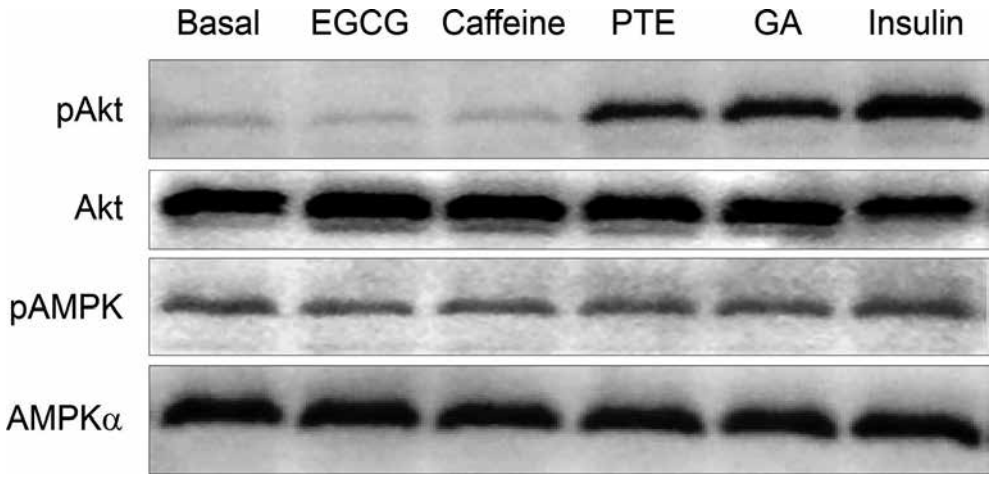

Figure 5. The effect of gallic acid (GA) on phosphorylation status of Akt Ser^{473} and AMPKα Thr^{172} in skeletal muscle. Isolated epitrochlearis muscles were incubated in the absence (Basal) or presence of epigallocatechin gallate (EGCG) (2.2 μM), caffeine (150 μM), Pu-erh tea hot-water extract (PTE) (1.5 mg/mL), GA (820 μM), or insulin (1 μM) for 30 min. The concentrations of GA, caffeine, and EGCG were adjusted to the concentration of each constituent to the level corresponding to 1.5 mg/mL of PTE. Muscle lysates were then analyzed for phosphorylation of Akt Ser^{473} (pAkt) and AMPKα Thr^{172} (pAMPK) by western blot analysis. Representative immunoblots are shown. This figure was adapted from Ma et al. with permission by the publisher.

3.4. Salicylic acid

Salicylic acid (salicylate or 2-hydroxybenzoic acid) is one of the oldest drugs in clinical practice. Salicylate has been used for treating pain, fever, and inflammation, but recent evidences have accumulated the effectiveness of treating T2DM. Over 100 years ago, Ebstein [85] and Williamson [86] showed that high doses of sodium salicylate (5–7.5 g/day) reduced glucosuria in diabetic patients. After that, additional trials have been reported similar effects that the treatment of sodium salicylate improved glucose homeostasis [87–94]. A recent meta-analysis

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of salicylates, including sodium salicylate, aspirin (acetylsalicylate), and salsalate (2-[2-hydroxybenzoyl]oxybenzoic acid), for T2DM showed that any doses of salicylates reduce glycated hemoglobin (HbA1c) level and that high doses of sodium salicylate (>3000 mg/day) improve fasting plasma glucose level [95].

The mechanism of antidiabetic action of salicylate might be attributed to the stimulation of both insulin-dependent and insulin-independent glucose transport. Kim et al. [96] demonstrated that infusion of lipid into tail vain of rats for 5 h impaired insulin-dependent glucose transport in skeletal muscle, whereas the impairment was attenuated by concomitant infusion of sodium salicylate (7 mg/kg/h). In that situation, the decreases in insulin-dependent glucose transport in skeletal muscle were associated with the reduction of tyrosine phosphorylation of IRS-1 and PI3K activity [96]. Salicylate is a known inhibitor of IKK/NF-κB signaling. Kim et al. [96] also revealed that the defects of insulin-dependent glucose transport with lipid infusion were not induced in IKK-β knockout mice. Overall, these results indicate that salicylate may protect the defects of fat-induced insulin resistance in skeletal muscle by preserving insulin signaling transduction via the inhibition of IKK/NF-κB signaling.

Recent work by us first showed that the treatment of sodium salicylate (5 mM, 30 min) stimulated insulin-independent glucose transport in rat-isolated skeletal muscles [97]. The stimulation of insulin-independent glucose transport by sodium salicylate may be explained by the activation of AMPK. A study found that sodium salicylate (>1 mM) activates AMPK in human embryonic kidney cells directly by binding to AMPK (1–10 mM) and indirectly by energy depletion (>10 mM) [98]. In addition, we showed that incubation of isolated rat skeletal muscles with sodium salicylate (>5 mM) increased AMPKα Thr^{172} phosphorylation and AMPK activity accompanied by the reduction of energy status (ATP, PCr, and glycogen) [97]. The depletion of energy levels in response to sodium salicylate stimulation was also observed in Drosophila tissue culture (SL2) cells [99] and neutrophils [100]. Inhibition of oxidative phosphorylation by sodium salicylate was suggested to cause to energy depletion [101]. These findings suggest that salicylate stimulates AMPK via both energy-dependent and energy-independent processes in skeletal muscle. It seems that CaMKK signaling is not involved in salicylate-induced AMPK activation because the CaMKK inhibitor STO-609 had no effect on responses to salicylate [98].

3.5. *p*-Coumaric acid

p-Coumaric acid (4-hydroxycinnamic acid) is the precursor of caffeic acid and has potential to reduce the risk of T2DM. Some studies showed that chronic (30–45 days) treatment with *p*-coumaric acid improved fasting blood glucose and HbA1c levels in STZ-induced diabetic rats [102–104]. In addition, a study demonstrated that *p*-coumaric acid stimulated insulin-independent glucose transport and AMPKα Thr^{172} phosphorylation in L6 skeletal muscle cells and that the upregulation of glucose transport was partially attenuated by concomitant treatment with AMPK inhibitor compound C [105]. This finding indicates that *p*-coumaric acid stimulates insulin-independent glucose transport via AMPK-activation in skeletal muscle.

3.6. Ferulic acid

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is derived from the biosynthesis of caffeic acid and has antidiabetic effects. Chronic treatment with ferulic acid showed a hypoglycemic effect in diabetic mice [106–108]. A study reported that ferulic acid stimulated insulin-independent glucose transport in L6 skeletal muscle cells in a dose-dependent (<50 μM) and time-dependent (<5 h) manners [63]. In contrast, another study showed that treatment with ferulic acid (250–500 μM) inhibited insulin-independent glucose transport in L6 skeletal muscle cells [109]. Therefore, further studies are needed to clear the effect of ferulic acid on glucose transport system.

3.7. Sinapic acid

Sinapic acid (sinapinic acid or 4-hydroxy-3,5-dimethoxycinnamic acid) is known to have an anti-inflammatory action through NF-κB inactivation [110]. Inflammation links with the progress of T2DM, and thus, it is indicated the merit of sinapic acid in the treatment of T2DM. Indeed, a single administration of sinapic acid (10–30 mg/kg) dose-dependently reduced the hyperglycemia of STZ-induced diabetic rats [111, 112]. Further, sinapic acid (0.1–10 μM) stimulated enhanced insulin-independent glucose transport in isolated rat skeletal muscle and L6 skeletal muscle cells [112]. Repeated treatment with sinapic acid (25 mg/kg) for 3 days increased the gene expression of GLUT4 in skeletal muscle of STZ-induced diabetic rats [112]. Considering that AMPK promotes GLUT4 expression [66–68], sinapic acid-induced stimulation of glucose transport and GLUT4 expression may be mediated by AMPK activation.

4. Conclusion

Phytomedicine is becoming to be an important medical treatment, and thus it is necessary to understand the molecular mechanism underlying the effectiveness of phytochemicals on health promotion. In this chapter, we reviewed the relationship between phenolic acids and T2DM focusing on skeletal muscle glucose transport systems. Among many phenolic acids, it has been reported that caffeic acid, chlorogenic acid, gallic acid, salicylic acid, *p*-coumaric acid, and sinapic acid stimulate glucose transport in skeletal muscle (**Table 1**). AMPK appears to be involved in these glucose utilization processes. Caffeic acid, chlorogenic acid, salicylic acid, and *p*-coumaric acid seem to have capacity for stimulating AMPK activity, thereby enhancing insulin-independent glucose transport. On the other hand, gallic acid has no effect on AMPK activity but stimulates insulin signaling without insulin. Caffeic acid and salicylic acid may also enhance insulin sensitivity by suppressing IKK/NF-κB signaling.

Physical exercise is a powerful tool that promotes good health, and it reduces the risk of T2DM. Skeletal muscle AMPK is considered to be a candidate therapeutic target molecule in T2DM since AMPK is activated by physical exercise. If skeletal muscle AMPK could be activated by alternative approaches including phytochemicals, it would benefit people who are unable to engage in physical exercise. As described above, caffeic acid has no capacity for enhancing contraction-induced AMPK activity. This finding suggests that caffeic acid may not strengthen

the exercise benefit but simultaneously means that caffeic acid and contraction have a common mechanism to stimulating insulin-independent glucose transport through AMPK. Therefore, caffeic acid has a potential as an exercise-mimetic stimulator for glucose transport systems. Thus, we expect that some kinds of phytochemicals have potential to act as preventive and therapeutic agents for T2DM.

Phenolic acids	Insulin-dependent glucose transport	Insulin-independent glucose transport	Molecular responses
Caffeic acid	↑ (insulin resistance state)	↑	AMPK activity ↑, Energy status ↓, NF-κB activity ↓
Chlorogenic acid	—	↑	AMPK activity ↑ (>1 mM, >60 min) AMPK expression ↑, CaMKK expression ↑
Gallic acid	—	↑	Akt activity ↑, AMPK activity →
Salicylic acid	↑ (lipid infused state)	↑	Insulin-stimulated IRS-1 tyrosine phosphorylation ↑, Insulin-stimulated PI3K activity ↑ NF-κB activity ↓, AMPK activity ↑ Energy status ↓, CaMKK activity →
p-Coumaric acid	—	↑	AMPK activity ↑
Ferulic acid	—	↑	—
Synapic acid	—	↑	GLUT4 gene expression ↑

AMPK, 5′AMP-activated protein kinase; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; GLUT4, glucose transporter 4; IRS-1, insulin receptor substrate 1; NF-κB, nuclear factor-κB; PI3K, phosphatydilinositol-3 kinase; ↑, increase; ↓, decrease; →, no change; —, no study.

Table 1. Summary of the effect of phenolic acids on skeletal muscle glucose transport.

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Author details

Tatsuro Egawa[1,2*], Satoshi Tsuda[1], Rieko Oshima[1], Ayumi Goto[1], Xiao Ma[3], Katsumasa Goto[2] and Tatsuya Hayashi[1]

*Address all correspondence to: tatsuro.egawa@gmail.com

1 Laboratory of Sports and Exercise Medicine, Graduate School of Human and Environmental Studies, Kyoto University, Kyoto, Japan

2 Department of Physiology, Graduate School of Health Sciences, Toyohashi SOZO University, Toyohashi, Aichi, Japan

3 Key Laboratory of Puer Tea Science, Ministry of Education, Yunnan Agricultural University, Kunming, Yunnan, China

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Chapter 10

Health Benefits of Phenolic Compounds Against Cancers

Abdelkader Basli, Nassim Belkacem and
Iman Amrani

Additional information is available at the end of the chapter

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Abstract

Phenolic compounds are the biggest group of phytochemicals, and many of them have been found in plant-based foods. Polyphenol-rich diets have been linked to many health benefits including cancer. The potential anti-carcinogenic mechanisms of action that have been so far identified for phenolic compounds, as well as the feasibility reports occurred *in vivo*. In general terms, under the oxidative stress, polyphenols could act in those cellular mechanisms by participating in the modulation of the redox status and on multiple key elements in intracellular signal transduction pathways related to cell proliferation, differentiation, apoptosis, inflammation, angiogenesis and metastasis. A protective role of polyphenols against carcinogenesis is supported by many studies carried out on animal models and different mechanisms of action have been proposed to explain such protective effects. Studies performed in animals have demonstrated that phenolic components can prevent and/or slow down the initiation-progression of different types of cancers. They act through the regulation of cell signal transduction and gene expression and exhibit either up or down regulation of genes controlling tumor development.

Keywords: phenolic compounds, carcinogenesis, apoptosis induction, tumor metastasis and angiogenesis

1. Introduction

Cancer is a broad term used to describe a large group of disorders characterized by an uncontrolled growth of abnormal cells. These cells have the ability to escape surveillance by the immune system, to multiply indefinitely, to invade nearby tissues and to spread to distant sites of the body forming metastases [1]. Most cancers fall into one of the four main groups: carcinomas, sarcomas, leukemias or lymphomas. Carcinomas are cancers of epithelial origin. They represent approximately 90% of human malignancies. Sarcomas, which are rare

in humans, refer to solid tumors deriving from connective tissues, such as muscle, bone, cartilage and fibrous tissue. Cancers arising from the blood cells precursors and from cells of the immune system are called leukemias and lymphomas, respectively. Together, these two account for about 8% of human malignancies. Cancers can further be classified according to the topography of the primary tumor into several types, such as colon cancer, breast cancer, lung cancer, etc. [2].

1.1. Worldwide cancer incidence and mortality

Cancer ranks among the leading causes of morbidity in the world. According to GLOBOCAN 2012, the latest online database produced by the International Agency for Research on Cancer (IARC): 14.1 million new cancer cases occurred in 2012 worldwide. About 8 million (57%) were in economically developing countries, in which about 82% of the world's population reside [3]. The global incidence of cancer is expected to increase to 22.2 million by 2030 (an increase of 57% from 2012), based only on projected demographic changes and unchanged cancer incidence rates [4]. The most common malignancy worldwide is lung cancer accounting for 1.8 million new cases in 2012, followed by breast cancer (1.7 million new cases), colorectal cancer (1.4 million new cases), prostate cancer (1.1 million new cases), stomach cancer (951,000 new cases) and liver cancer (782,000 new cases) [5].

The overall age standardized cancer incidence rate in 2012 was almost 25% higher in men than in women, with rates of 205 and 165 per 100,000, respectively [3]. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervix and stomach cancer are the most common among women [5].

In terms of mortality, cancer is the second most common cause of death worldwide after cardiovascular diseases. The total number of cancer deaths in 2012 was 8.2 million, of these 2.9 million (35%) occurred in economically developed countries and 5.3 million (65%) in less developed countries [3]. Lung cancer remains the leading cause of death worldwide with 1.6 million deaths in 2012, followed by liver cancer (745,000 deaths), stomach cancer (723,000 deaths), colorectal cancer (694,000 deaths) and breast cancer (522,000 deaths) [5].

1.2. Cancer development process and prevention

Cancer is a multifactorial disease; many exogenous factors (such as poor diet, tobacco smoking, chemicals, radiation and infectious organisms) and endogenous factors (such as inherited mutations, hormones and immune conditions) contribute to its aetiology [6–8]. These factors may act together or in sequence to trigger and/or promote cancer development. The latter, also known as “carcinogenesis” or “tumorigenesis”, is a complex multistep process resulting from the progressive accumulation and functional cooperation of genetic and epigenetic alterations that eventually allow cells to break free from the tight network of regulation systems that maintain the homeostatic balance between proliferation and programmed cell death [1].

The genetic alterations can be the result of endogenous processes, such as errors in DNA replication, intrinsic chemical instabilities of certain DNA bases or attacks by free radicals gen-

erated during metabolism. DNA damage can also result from interactions with exogenous agents, such as radiation and chemical carcinogens. Under normal conditions, human cells have the ability to overcome these alterations thanks to DNA repair genes, apoptosis and cell buffer systems. Whenever these cell protection mechanisms are constitutionally altered or the DNA attack overtake the capacities of a normal cell, permanent mutations occur. These mutations could activate genes involved in cell growth and proliferation (oncogenes) or inactivate genes involved in cell senescence and apoptosis (tumour suppressor genes). If the permanent mutations occur in DNA repair genes as well, they will facilitate the acquisition of additional mutations [9, 10].

Cancers are also a consequence of epigenetic alterations, which are by definition, persistent and heritable changes in gene expression that result from modifications of chromatin structure without modification of the cell's DNA sequence. This can occur with DNA methylation and histone modifications [11]. This type of alterations along with the genetic ones lead to the transformation of a normal cell into a neoplastic cell with six essential physiological dysfunctions that collectively dictate its malignant growth: self-sufficiency in growth signals, insensitivity to growth inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis [1].

The multistage nature of the carcinogenesis process, the presence of precursor lesions at the intermediate stages between normal and malignant cells, the slow growth of tumors and, the long latency, generally for decades before the diagnosis is established indicate that the carcinogenic process could be blocked or delayed and that the development of invasive cancers could be prevented [12].

Many scientists are focusing their researches on finding new strategies for cancer prevention. One strategy with promising potential is "chemoprevention" that has been defined by Sporn in 1976 as "the use of natural, synthetic or biological agents to reverse, suppress or prevent either the initial phases of carcinogenesis or the progression of premalignant cells to invasive disease" [13, 14].

Several epidemiologic studies suggest that regular consumption of fruits and vegetables significantly reduces the risk of different cancers. The beneficial effects of this type of diet are in part attributed to their content of phenolic compounds, which have shown promising antitumour properties in both *in vitro* and *in vivo* studies [15–17].

2. Phenolic compounds

2.1. Classification

Phenolic compounds, widely distributed secondary metabolites in plants, form a group of molecules with highly diversified chemical structures. They can be classified according to their carbon skeleton into the following main classes: simple phenols, phenolic acids, flavonoids, tannins, lignans, lignins, curcuminoids, coumarins and stilbenes as shown in **Figure 1**.

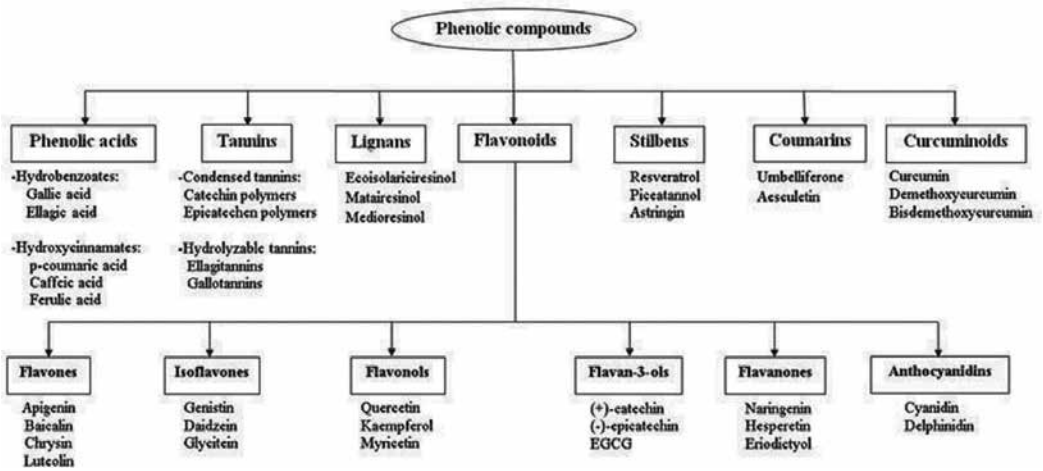

Figure 1. Main classes of phenolic compounds.

Phenolic acids include hydroxybenzoates (C6–C1) and hydroxycinammates (C6–C3). Hydroxybenzoic acids are represented mainly by gallic and ellagic acids, whereas the major hydroxycinnamic acids are caffeic and ferulic acids.

Flavonoids are the largest group of phenolic compounds containing two aromatic rings linked by a three atoms of carbon bridge (C6–C3–C6). They include mainly flavones, isoflavones, flavonols, flavans, flavanones and anthocyanidins [18]. Rutin, quercetin-3-O-rutinoside, is the glycoside of the flavonol quercetin. Epigallocatechin-3-gallate (EGCG), a type of the flavanol catechin, is the ester of epigallocatechin and gallic acid. Silymarin is a flavonolignan composed mainly by silybin (A and B), isosilybin (A and B), silychristin (A and B) and silydianin [19].

Tannins are divided into two different chemical groups, hydrolysable tannins that are polymers of gallic or ellagic acids and condensed tannins that are polymers of catechins or epicatechins.

Curcumin (C6–C7–C6) is a diferuloylmethane belonging to the group of curcuminoids [20].

2.2. Food sources of phenolic compounds

Phenolic compounds are widespread in food. Fruits and vegetables, such as apples, cherries, oranges, citrus, grapes, berries, peaches, cereals and tomatoes are particularly rich in polyphenols as shown in **Table 1**.

2.3. Phenolic compounds as antioxydants

Phenolic compounds have received increasing interest in the human health due to their benefit effects against several diseases like cancers attributed in particular to their antioxidant activity [29, 30]. Multiple investigations support that oxidative stress plays a key role in the cancer occurrence and other health problems induced by the excess production of the reactive

oxygen species (ROS) that includes many radicals, such as superoxide (O-2), hydroxyl (OH—), hydroperoxyl (OOH—), peroxyl (ROO—), alkoxyl (RO—), nitric oxide (NO—) and peroxynitrite anion (ONOO—). The ROS may cause oxidative damage to vital biomolecules, such as DNA, lipids and proteins [31].

Phenolic compound	Carbon skeleton	Food source	References
Phenolic acids Gallic acid Ellagic acid Hydroxybenzoates *p*-Hydroxybenzoic acid protocatechuic acid Vanillic acid Syringic acids	C6–C1	Berries, particularly raspberries, strawberries, and blackberries, grape juice and cereals	[19–21]
Hydroxycinammates *p*-Coumaric, caffeic, ferulic acids Hydroxycinnamic derivatives Chlorogenic acid Curcumin	C6–C3	Blueberry, cherry, sweet pear, apples (chlorogenic acid), orange, potato, grape fruit, coffee beans, plum, tomatoes, grape, wheat bran, kiwis, cereal grains (ferulic acid), apricots, carrots, cereals, citrus fruits, oilseeds, peaches and spinach	[20–23]
Flavonols Quercetin Kaempferol Myricetin Isorhamnetin	C6–C3–C6	Onions *Allium cepa,* apples, plums, cranberries, strawberries, grapes, kale, broccoli, celery stalks, tomatoes, buckwheat, endive, leeks, lettuce, olive, pepper, red wine, green tea and grape juice	[19–27]
Flavones Apigenin Luteolin	C6–C3–C6	Celery, parsley, artichoke, green olive, sweet peppers, onion, garlic, chamomile tea, Thai chili, citrus fruits, celery and spinach	[19–21, 23, 24, 26]
Flavan-3-ols (+)-Catechin (-)-Epicatechin	C6–C3–C6	Tea, apricots, sour cherries, grapes and blackberries, apples, peaches nectarines, barley (cereal), plums, nuts, red wine and chocolate	[19–27]
Flavanones Naringenin Hesperetin Eriodictyol Minor compounds Sakuranetin Isosakuranetin	C6–C3–C6	Citrus fruits: orange, lemons, grapes and tomatoes (Naringenin)	[19, 21, 23, 24, 26, 27]
Anthocyanidins Cyanidin Delphinidin Petunidin Peonidin Pelargonidin Malvidin	C6–C3–C6	The most widespread anthocyanidin in fruits is cyanidin-3 glucoside Grapes, blueberry, red onions, blood oranges and red wine Blackcurrant, blackberry, and elderberry (only cyanide)	[19, 21, 23, 24, 26, 27]
Isoflavones Genistin Daidzein Glycitein	C6–C3–C6	Soybeans and soy products are almost the sole dietary source of isoflavones Found also in small amounts in chickpeas	[19–24, 26, 27]

Phenolic compound	Carbon skeleton	Food source	References
Stilbenes Resveratrol	C6–C2–C6	Red wine and peanuts Also found in berries, red cabbage, spinach, grapes, berries, plums and pine nuts	[19, 21–23]
Piceatannol Astringin		Brazilian red wines	[19]
Lignan Secoisolariciresinol Matairesinol Medioresinol Pinoresinol Lariciresinol	$(C_6–C_3)_2$	Flaxseed is the richest source Buckwheat, sesame seed, rye and wheat	[21–23]
Tannins Condensed tannins Catechin polymers Epicatechin polymers	$(C6–C3–C6)_n$	Lentils, pear, grapes, peaches, plums, mangosteens, pears, red and white wine and apple juice	[22, 30]
Hydrolyzable tannins Ellagitannins Punicalagin Casuarictin		Strawberries, blackberries, raspberries, walnuts, pecans pomegranate bark, leaf and the fruit husk	[20, 21, 23, 28]
Gallotannins		Mangoes	
Coumarins Umbelliferone Aesculetin	C6–C3	Carrots, celery, citrus fruits, parsley and parsnips	[21]

Table 1. Food sources of some phenolic compounds.

Phenolic compounds may suppress ROS formation by different mechanisms, such as inhibiting some enzymes like xanthine oxidase responsible for superoxide ion production; chelating trace elements like metals, such as free iron and copper ions involved in the formation of radicals and scavenging radical species by hydrogen donation. The antioxidant capacity is related to the number and the position of hydroxyl groups in the phenolic compound [24, 25].

3. Polyphenols in prevention of cancer

Natural polyphenols are naturally occurring compounds found largely in the fruits, vegetables and are the most antioxidants in human diets, and their radical scavenging activities are related to substitution of hydroxyl groups in the aromatic rings of phenolic. They have been considered powerful antioxidants *in vitro* and proved to be more potent antioxidants than Vitamin C and E and carotenoids [32]. Phenolic compounds are also capable of scavenging free superoxide radicals, reducing the risk of cancer and protecting biological systems against the harmful effects of oxidative processes on macromolecules, such as carbohydrates, proteins, lipids and DNA [33]. It was found that in addition to their primary antioxidant activity, this group of compounds displays a wide variety of biological functions which are

mainly related to modulation of carcinogenesis. Furthermore, prevention of cancer is one of the most documented biological properties of the polyphenols. The effects of polyphenols on human cancer cell lines are protection and reduction of the number of tumors or their growth [34]. Mechanisms of anti-cancer effects of polyphenols, found in fruits, vegetables and spices representing parts of daily nutrition, have been considered. These compounds may be the basis for development of cancer preventive preparations. Several studies in extracts or isolated polyphenols from different plant food reported in a number of cancer cell lines including different evolutionary stages of cancer. Extracts prepared from blackberry, raspberry, blueberry, cranberry, strawberry as well as the isolated polyphenols from strawberry mainly like anthocyanins, kaempferol, quercetin, esters of coumaric acid and ellagic acid, have nutraceutical properties against tumor growth and cancer. They have revealed to be more effective to inhibit the growth of human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT-116) and prostate (LNCaP, DU-145) tumor cell lines in a dose-dependent manner with various sensitivity between cell lines [35, 36]. Many studies have focused on the antioxidative effects of phenolic compounds and it is suggested that its potential physiological effects for the protection and treatment of cancer and cardiovascular diseases come from its antioxidant activity. According to Ref. [37], phenolic compounds can block carcinogenesis initiation by inactivation of exogenous or endogenous genotoxic molecules including reactive oxygen species. Another mechanism consists in inhibition of activity and synthesis of carcinogen-metabolizing enzymes. Polyphenols activate phase I enzymes (cytochrome P450) to detoxify molecules procancérogènes [38, 39]. Many polyphenols, such as quercetin, catechins, isoflavones, lignans, flavanones, ellagic acid, red wine polyphenols, resveratrol or curcumin, showed protective effects in some cancerous models by different mechanisms. All the mechanisms of action of phenolic compounds against cancer are summarized in **Table 2**.

Dietary polyphenols	Protective effects and mechanisms	Conditions	Level
Hydroxytyrosol	Inhibiting cell proliferation	In human promyelocytic	*In vitro*
	Inducing apoptosis by arresting the cells in the G0/G1 phase with a concomitant decrease in the cell percentage in the S and G2/M phases		
Resveratrol	Inhibiting cell proliferation and down regulating telomerase activity	In human colon tumor cells	*In vitro*
	Inducing apoptosis mediated by p53-dependent pathway	In HepG2 cells	*In vitro*
	Inhibiting cell proliferation by interfering with an estrogen receptor-α-associated PI3K pathway	In estrogen-responsive MCF-7 human breast cancer cells	*In vitro*
	Suppressing COX-2 expression by blocking the activation of MAPKs and AP-1	In dorsal skin of female ICR mice	*In vitro*
	Decreasing the expression of COX-1, COX-2, c-myc, c-fos,c-jun, transforming growth factor-β-1 and TNF-α	In mouse skin	*Ex vivo*
	Inhibiting oncogenic disease through the inhibition of protein kinase CKII activity	In HeLa cell lysates	*In vitro*

Dietary polyphenols	Protective effects and mechanisms	Conditions	Level
	Inhibiting the Ca(2+)-dependent activities of PKCα and PKCβI	On the activities of PKC isozymes	*In vitro*
	Inhibiting nitrobenzene(NB)-DNA adducts	In male Kunming mice adducts	*In vivo*
Chlorogenic acid	Inhibiting the formation of DNA single strand breaks	In supercoiled pBR322 DNA	*In vitro*
Quercetin Luteolin	Blocking EGFR tyrosine kinase activity	In MiaPaCa-2 cancer cells	*In vitro*
Myricetin Apigenin Quercetin Kaempferol	Inhibiting human CYP1A1 activities Inhibiting the formation of diolepoxide 2(DE2) and B[a]P activation	On 7-ethoxyresorufin *o*-deethylation	*In vitro*
Silymarin Hesperetin Quercetin Daidzein	Interacting with *p*-glycoprotein and modulating the activity of ATP-binding cassette transporter, breast cancer resistance protein (BCRP/ABCG2)	In two separate BCRP-overexpressing cell lines	*In vitro*
EGCG	Inhibiting telomerase	In human cancer cells	*In vitro*
		In nude mice models	*In vivo*

Table 2. Anti-mutagenic/anti-carcinogenic properties of polyphenols [40].

3.1. Natural polyphenols and apoptosis targeting in cancer cells

In chemoprevention, suppression of cell proliferation and induction of differentiation and apoptosis are important strategies, with the induction of programmed cell death currently considered as one relevant target in a preventive track. Apoptosis (programmed cell death) is the process by which cells trigger their self-destruction in response to a signal. It is defined by a set of characteristic morphological features such cell shrinkage, chromatin condensation and DNA fragmentation due to endonuclease activation, cell budding and apoptotic body formation and loss of the membrane integrity [41]. Programmed cell death plays an important role in the maintenance of biological cells and systems. Apoptosis can be triggered through two main pathways: extrinsic and intrinsic. Extrinsic factors could act in the activation of cell surface receptors, such as tumor necrosis factor (TNF)-alpha that leads to the induction of caspase-8. Intrinsic pathways involved internal cell signaling primarily through the mitochondria. Regulation system of apoptosis are also induced in the mitochondria on the intrinsic pathway by several families of proteins, including small mitochondrial-derived activator of caspases (SMACs), inhibitor of apoptosis proteins (IAPs) and the B-cell lymphoma 2 protein (Bcl-2) family, as well as membrane polarity and integrity [42]. Other key molecule in apoptosis regulation is the transcription factor p53. The main role of p53 is the protection against genomic instability and tumorigenesis. Functionally promotes survival by activating checkpoints and facilitating damage repair, sustained proliferation blocking and apoptosis [43]. Many dietary phenolic compounds, including quercetin, EGCG [(-)-epigallocatechin-3-gallate], apigenin, chrysin, silymarin, curcumin, ellagic acid and resveratrol, may block carcinogenesis through induction of apoptosis. They may induce apoptosis via multiple mechanisms.

In vitro studies show that EGCG, curcumin or resveratrol sensitize LNCaP prostate cancer cells to TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis through modulation of the extrinsic apoptotic pathway [44, 45]. Furthermore, apoptosis intrinsic pathway could be triggered by phenolic compounds. Resveratrol induces apoptosis through the intrinsic pathway in prostate cancer-derived cell lines by activating caspases-9/3 and by changing the Bax/Bcl-2 ratio [46]. Apoptosis may be also induced through activation of proapoptotic proteins (e.g. caspases, proapoptotic Bcl-2 family members) and/or inhibition of antiapoptotic proteins (e.g. Bcl-2, Bcl-xL and survivin) [41]. Interestingly, a synergistic effect has been reported to induce apoptosis by combination of drugs and/or natural phenolics. In this line, (-)-epicatechin (EC) showed a major synergistic effect on the induction of apoptosis in gastric cancer MKN-45 cells treated with epigallocatechin-3-gallate [47]. Similarly, the combination of curcumin with (-)-epicatechin increased the inhibition of cell growth as compared to curcumin or EC alone, as well as the apoptosis rate and the expression of related genes to the programmed cell death, such as growth arrest DNA damage 153/45 (GADD153/45) in PC-9 cells [48]. Hexameric procyanidins inhibited the deoxycholic acid (DOC)-induced cytotoxicity and partly delayed the DOC-induced Caco-2 cell apoptosis [49]. In the same way, curcumin suppresses Caco-2 proliferation partially via activation of the mitochondrial apoptotic pathway and cell cycle retardation [50].

3.2. Antiproliferative effect

Suppression of cell proliferation and induction of differentiation and apoptosis are relevant strategies in preventive approaches. Deregulated cell cycle and resistance to apoptosis are hallmarks of cancer. The activity of the transcription factor nuclear factor-kappa B (NF-κB), responsible for the activation of many genes involved in cell proliferation, is closely linked to the redox status of cells. Indeed, NF-κB is part of a family of dimeric proteins (p50/p65). In the absence of stimulation, NF-kB is localized in the cytoplasm and is associated with its natural inhibitor IκB (inhibitor of NF-κB). ROS production (H_2O_2, superoxide anion and the hydroxyl radical) induces phosphorylation of IκB causing its ubiquitination and degradation by the proteasome. NF-κB is activated and translocated into the nucleus. At this stage, many genes (about 200) will be active [51]. Moreover, a number of human cancers, including breast cancer, non-small cell lung carcinoma, thyroid cancer, T- or B-lymphocyte leukemia, melanoma, colon cancer, bladder cancer and several virally induced tumors have been characterized by constitutive NF-κB activity and the inhibition of NF-κB abrogates tumor cell proliferation [52, 53]. Indeed, it has been postulated that some natural plant product anticancer effects are due to its capacity to inactivate NF-κB-dependent signaling. Many phenolic compounds including resveratrol [54], curcumin [55] and (-)-epigallocatechin-3-gallate [56] inhibit IKK-mediated IκB phosphorylation by stimulating the retention of NF-κB in the cytosol and its subsequent inactivation. Other studies demonstrate the ability of flavonoids as NF-κB inhibitors and their role in preventing NF-κB signaling pathway-mediated disorders. It is identified that apigen, quercetin, kaempferol, rutin is a potent inhibitor of NF-κB, which may perform a pivotal function in the regulation of cell growth, apoptosis and the regulation of the cell cycle [57–59]. Overall, the results indicated that flavonoids suppress the activation of NF-κB and NF-κB-regulated gene expression, leading to enhancement of apoptosis. This provides the molecular basis for the ability of polyphenols to act as an anticancer.

3.3. Effects on angiogenesis and metastasis

Angiogenesis, the formation and growth of new blood vessels from preexisting microvasculature, is a key stage in tumor growth, invasion and metastasis [41], many proteins have been identified in humans as activators of angiogenesis, among them, fibroblast growth factor (FGF), interleukin 8 (IL-8), the platelet-derived epidermal growth factor, transforming growth factor α (TGFα), the vascular endothelial growth factor (VEGF) and small molecules, such as adenosine, prostaglandin E and tetrahydrofolate (THF) [60]. According to many *in vitro* studies, VEGF and FGF-β appear to be the most important factors responsible for tumor growth and are produced by many types of cancer cells as well as normal cells [61]. Polyphenols can act as suppressing agents and inhibit the formation and growth of tumors from initiated cells; they inhibit cell proliferation *in vitro* [62]. Moreover, polyphenols, such as those of green tea, can also inhibit angiogenesis and, therefore, limit the growth of the tumors [63] or prevent tumor invasion through inhibition of the matrix metalloproteinases [64]. (+)-Catechin-inhibited tumour-specific angiogenesis by regulating the production of pro- and anti-angiogenic factors, such as pro-inflammatory cytokines, nitric oxide, VEGF, IL-2 and tissue inhibitor of metalloproteinase-1 [65]. Several studies report a selective effect of phenolic compounds in inhibiting angiogenesis in cancer cells. Thus curcumin, baicalin and resveratrol can also inhibit the angiogenic factor VEGF in tumor cells in culture [66–68]. On the other hand, quercetin inhibits angiogenesis through multiple mechanisms, including interaction with the cox-2 and lipoxygenase-5 enzymes, EGFR, the HER2 intracellular signalling pathway and the NF-κB nuclear transcription protein [69]; furthermore, it has been shown that proanthocyanidins added to mice with tumor xenografts reduced VEGF secretion, which resulted in reduced intratumoral microvasculature [70]. Previous studies reported that the chemical modification of (-)-epicatechin by its acylation improved the anti-cancer and anti-angiogenic activities of this flavanol [71].

The tissue invasion and metastasis formation require that tumor cells acquire the ability to migrate to other tissue and to invade them. This involves changing some cellular functions (cell adhesion) and the modification of the expression of certain genes, such as those encoding metalloproteinases degrading the extracellular matrix (MMP) or molecules adhesion [72]. Because metastasis occurs through a multistep process, dietary polyphenols have also been reported to interfere with cancer cell adhesion and movement processes through various mechanisms. Polyphenols, such as curcumin, apigenin, quercetin and catechin, have been reported to be chemopreventive through their anti-proliferative, anti-metastatic and/or anti-invasive properties [73, 74]. Resveratrol has been shown to inhibit cell migration/invasion and metastasis in several types of cancer, including breast cancer [75]. In addition, it was reported that Interleukin 6 (IL-6) and its major effector, the signal transducer and activator of transcription 3 (STAT3), are part of an important inflammation-associated pathway in malignancies [76] and metastasis [77] in different types of cancer. Resveratrol might be a potential agent chemosensitization on several types of cancer. This ability would be explained by the regulation of many signaling molecules including drug transporters, cell proliferation regulators, members of the NF-κB signal transducer and activator of transcription 3 (STAT3) signaling pathways [78]. CD44 and CD54 played an important role in tumor metastasis by the mutual adhesion

and interaction between cancer cells and vascular endothelial cells [79]. Tea polyphenols, known as catechins, have effects on cancer prevention, inhibition and anti-metastasis. Recent studies reported their role in the blockage of adhesion of lung carcinoma cell lines to endothelial cells is related to CD44 and CD54. The mechanism of tea polyphenol prevention of human lung carcinoma metastasis might be through inhibiting adhesion molecule expression to block cancer cell adhesion [80]. In breast cancer, curcumin exerts a strong anti-invasive effect on estrogen receptor (ER)-negative MDAMB231 cells through the down regulation of nuclear factor κB and activator protein-1 (NF-kB/AP 1) transcription factors dependent MMP-1 and -2 expression, the up regulation of TIMP-1 (metallopeptidase inhibitor-1), and the inhibition of VEGF and b-FGF [81]. In addition, caffeic acid is a widespread phenolic acid exerts an effective inhibition of the *in vitro* invasion of PC3 cells in prostate cancer [82]. Quercetin has also been widely investigated for its potential to inhibit both cellular migration and the invasion of cancer cells. Mechanistically, quercetin may inhibit cellular migration and invasion through the deactivation of matrix metalloproteinases-2 (MMP-2) and/or matrix metalloproteinases-9 (MMP-9) [83]. Recent study showed that polyphenol enrichment of a blueberry preparation by fermentation increases its chemopreventive potential by protecting mice against tumor development, inhibiting the formation of cancer stem cells and reducing lung metastasis [84]. Indeed, the cytoprotective and anticancer action of dietary in-taken natural polyphenols has for long been attributed only to their direct radical scavenging activities. Quercetin has been reported to possess anticancer property against benzo-pyrene-induced lung carcinogenesis in mice, an effect attributed to its free radical scavenging activity [85]. The anti-carcinogenic effects of resveratrol appear also to be closely associated with its antioxidant activity [86].

4. Conclusion

Cancer has become in the recent decades one of the leading causes of death worldwide. The search for effective prevention has become a priority for the basic and clinical science. Polyphenols have been proposed as alternative therapy and shown effective in cancer treatment especially when consumed in synergistic mixtures. It has been already demonstrated that polyphenols are able to exert differential effects on tumor cells. Their action can be attributed not only to their ability to act as antioxidants but also to their ability to interact with basic cellular mechanisms. Polyphenols, such as resveratrol, EGCG, curcumin and quercetin, have been shown to promote extrinsic and intrinsic apoptosis induction in different types of cancers (e.g. colon, lung, prostate, breast, melanoma or leukemia). Others studies performed in animals reported that phenolic components can prevent and/or slow down the initiation-progression of different types of cancers, such as cancer of prostate, liver, colon, leukemia, etc. Polyphenols can also act as suppressing agents, and inhibit the formation and growth of tumors from initiated cells; they inhibit cell proliferation *in vitro.* However, the exact mechanisms of actions are not fully understood and many properties remain unclear, require further consideration. These experimental and hypothetical data evince the need to perform further studies to understand the differential mechanisms of the polyphenols on cancer cells, which could contribute to find selective targets in cancer treatment.

Author details

Abdelkader Basli[1]*, Nassim Belkacem[2] and Iman Amrani[3]

*Address all correspondence to: mbasli@yahoo.fr

1 Research Laboratory of Biochemistry, Biophysics, Biomathematics and Scientometrics "L3BS" Faculty of Natural Sciences and Life, University of Bejaia, Bejaia, Algeria

2 Plant Biotechnology Laboratory and Ethnobotany, Faculty of Natural Sciences and Life, University of Bejaia, Bejaia, Algeria

3 Laboratory of Constitutional and Acquired Genetic Diseases, Faculty of Medicine, University of Batna 2, Algeria

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Chapter 11

Health Status Improved by *Aronia Melanocarpa* Polyphenolic Extract

Manuela Ciocoiu, Laurentiu Badescu and Magda Badescu

Additional information is available at the end of the chapter

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Abstract

This chapter focuses on certain natural polyphenolic extracts from *Aronia melanocarpa* (Michx.) Elliott and also on their effects in insulin-dependent diabetes mellitus. The phenolic profile of berries ethanolic extract was characterized by HPLC/DAD/ESI-MS. HPLC/DAD/ESI-MS allowed identification of five phenolic compounds: chlorogenic acid, kuromanin, rutin, hyperoside, and quercetin. The results reveal that the glycosylated hemoglobin values are much higher in the diabetic group (DM) and they are significantly lower in the group protected by polyphenols (DM+P). It is found that due to the polyphenolic protection of the rats from the DM+P, the atherogen risk is preserved at normal limits. The serous activity of glutathione-peroxidase (GSH-Px) and superoxide-dismutase (SOD) has significantly lower values in the diabetic group as compared to the group protected by polyphenols. Renal function indicators like creatinine and blood-urea nitrogen (BUN) were also elevated in the streptozotocin diabetic rats when compared with control rats. When compared with the diabetic group the elevated levels of BUN was significantly ($p < 0.001$) reduced in animals treated with natural polyphenols. Through the hypoglycemiant, hypolipemiant, and antioxidant effects, *A. melanocarpa* represents a possible dietary adjunct for the treatment of diabetes and a potential source of active agents for the prevention of microvascular diabetes complications.

Keywords: Aronia melanocarpa, hypoglycemiant, hypolipemiant, antioxidant effects, renal function indicators

1. Introduction

Numerous population-based observational studies revealed that consumption of polyphenol-rich foods, principally fruits and vegetables, is beneficial to health, reducing mortality rates

and the incidence of the major diseases of modern civilization, cancer and cardiovascular disease [1, 2].

Antioxidants have been extensively investigated because of their ability to promote disease prevention and health maintenance by suppressing oxidative stress. From epidemiological and dietary intervention studies, it appears, however, that exogenous antioxidants at physiologic (nutritional) doses play an important role in the maintenance or re-establishment of redox homeostasis, an essential state in maintaining healthy biological systems [3, 4].

Experimental studies relating to the chemistry of *Aronia melanocarpa* refer to its berries being a rich source of pharmacologically relevant compounds. Polyphenols, especially anthocyanins and procyanidins, make up the main group of biologically active constituents in black chokeberry fruits [5].

It is known that natural polyphenols possesses complex biological properties such as antioxidant, antiinflammatory, cardioprotective, and antiplatelet activities [6–8]. Recently, interest has been focused on plant-based natural antioxidants such as tannins, polyphenols, and flavonoids to reduce the negative effect of oxidative stress and free radicals in diabetes patients and to prevent the destruction of β-cells [9]. The modulation of the enzymatic and nonenzymatic processes in diabetes mellitus, experimentally, via natural polyphenols, emphasizes the role of these vegetal extracts in metabolic diseases.

The chapter focuses on certain natural polyphenolic extracts from *A. melanocarpa* (Michx.) Elliott and also on their effects in insulin-dependent diabetes mellitus. *A. melanocarpa*, sometimes called black chokeberry, belongs to the Rosaceae family. Numerous health-promoting activities, such as antioxidative, cardioprotective, antidiabetic, antiinflammatory, antibacterian, antiviral, and immunomodulatory, have been demonstrated for *A. melanocarpa* extracts by both *in vitro* and *in vivo* studies [10–13].

2. The biochemical and morphological modifications of *A. melanocarpa* extract on experimental diabetes model

2.1. The biochemical modifications of *A. melanocarpa* extract on experimental diabetes model

Ripe berries of *A. melanocarpa* Elliott (Rosaceae, black chokeberry) were sampled in Botanical Garden, Iasi, Romania. Berries ethanolic extract contained 24.87 ± 0.54 mg total phenolics/g and 4.46 ± 0.06 μmol anthocyanin/g. Total phenolics quantification was performed by Folin-Ciocalteu method. The phenolic profile of berries ethanolic extract was characterized by HPLC/DAD/ESI-MS. HPLC/DAD/ESI-MS allowed identification of five phenolic compounds in berries ethanolic extract: chlorogenic acid, kuromanin, rutin, hyperoside, and quercetin (**Figure 1**). (+)-Catechin hydrate, chlorogenic acid, caffeic acid, rutin trihydrate, hyperoside, quercetin dihydrate, and kuromanin chloride were used as standards. Main phenolic constituents were identified by comparison of their retention times and mass spectral data to those of authentic standards.

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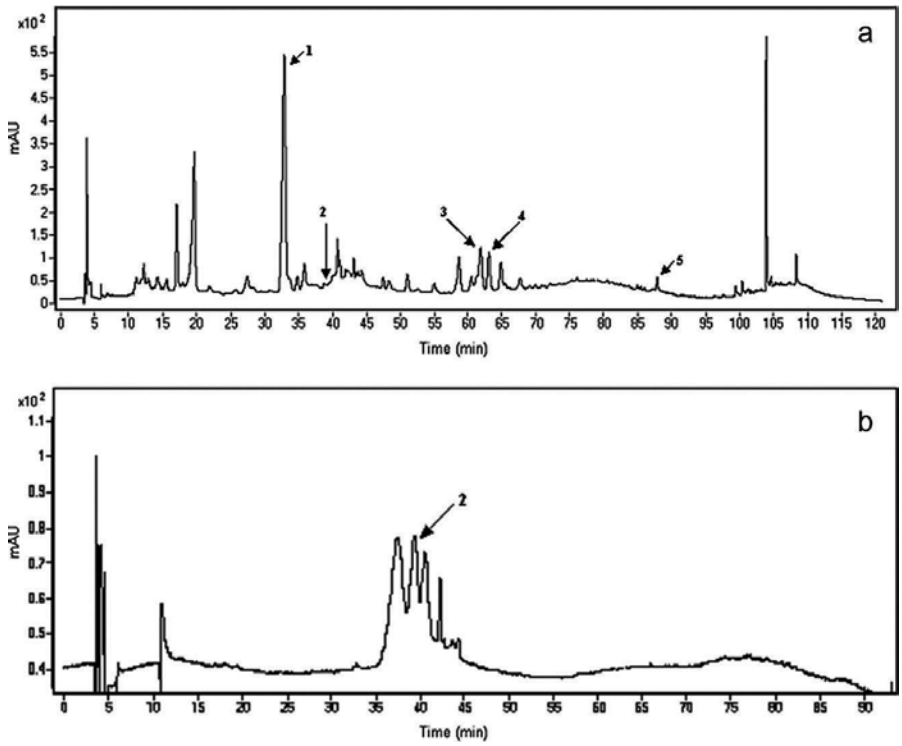

Figure 1. HPLC-DAD chromatograms of ethanolic extract of black chokeberry fruits. (a) Detection at 280 nm; (b) detection at 515 nm (1—chlorogenic acid, 2—kuromanin, 3—rutin+unknown compound, 4—hyperoside, 5—quercetin).

Chlorogenic acid has the capacity of radical trapping and singlet oxygen removal, and may prevent LDL oxidization and oxidative injury to nucleic acids [14, 15] and is reported to have effects associated with the prevention of diabetes. Chlorogenic acid inhibits α-glycosidase activity and postprandial elevation of blood glucose levels in sucrose- and maltose-treated rats [16].

α-Glucosidase, a membrane-bound enzyme located at the small intestine epithelium, is essential in carbohydrate digestion, catalyzing glucose cleavage from disaccharides and oligosaccharides. The onset of diabetes may be prevented by controlling postprandial hyperglycemia, inhibiting α-glucosidase, and α-amylase, which delays carbohydrate digestion to absorbable monosaccharide. Specialized literature shows that anthocyanins potentially inhibit intestinal α-glucosidase. Adisakwattana et al. [17] reports that cyanidin 3-rutinoside uses the same mechanism in delaying carbohydrate absorption.

All the requirements regarding the use of laboratory animals and biological preparations issued by the International Society of Pain Study and the European Council Committee (86/609/EEC) were followed during the experimental study, and it has been approved by the professional ethics committee of Grigore T. Popa University of Medicine and Pharmacy of Iasi (9803/12.09.2006).

The diabetes was obtained through the administration of STZ [2-deoxi-2(3-methyl-nitrozo-ureido)-p-glucopyranoza] in a single dose of 60 mg/kg body mass, 1% solution intraperitoneal (i.p.), after fasting for 18 hours.

The animals were kept in normal microclimate conditions. The clinical state of the animals was observed daily, the water and food ingestion, diuresis, glycosuria, and the possible presence of ketone bodies. The diet consisted of carbohydrates 59.12%, raw proteins 21.10%, raw lipids 5.08%, raw fibers 4%, minerals 5.14%, and humidity of 7.98%.

The research was performed on Wistar white rats, with an average weight of 250–280 g, which were divided into four groups of 10, namely: W Group = control, normal animals, that did not receive natural polyphenols; P Group = rats that were administered natural polyphenols 0.050 g/kg body every 2 days (by tube-feeding), for a period of 16 weeks; DM Group = rats with diabetes induced through streptozotocin (STZ) injection, 3 weeks after the beginning of the experiment; DM+P Group = rats that were administered a polyphenolic preparation for 3 weeks before and 13 weeks after the induction of diabetes mellitus.

The dry polyphenol extract was diluted in DMSO, 100 mL polyphenolic solution containing 840 mg natural polyphenols, 95 mL distilled water, and 5 mL DMSO. The experiment used *A. melanocarpa* active therapeutic doses, well-determined fractions of DL50 on an experimental model of diabetes mellitus. The dose representing 1/20 of DL50 was chosen, as it is the smallest dose that determined the pharmacodynamic effect that is being researched, without producing significant toxic effects. Polyphenols are able to penetrate tissues, particularly those in which they are metabolized, but their ability to accumulate within specific target tissues needs to be further investigated.

Mean hyperglycemia in rats suffering from diabetes mellitus and without polyphenolic protection increased progressively. This study indicates that streptozotocin-induced diabetes and subsequent glycemia level increase were reduced by the simultaneous administration of natural polyphenols. The hyperglycemia of the DM+P group was insignificantly reduced ($p < 0.05$) in comparison to the DM group. Thus, the polyphenol administration did not offer protection against the disease installation. The glycaemia evolution was reduced insignificantly (with only 29.5% at the diabetic rats with polyphenolic protection in comparison to the diabetic rats without protection).

Hyperglycemia activates glycolytic intermediates associated with injurious mechanisms such as hexosamine, polyol pathways, and advanced glycation end products formation. It had been reported that digestive enzymes such as lipase, α-amylase, and α-glucosidase were inhibited by proanthocyanidins and tannins in young chicks, which decreased the digestibility of protein, starch, and lipid [18].

The antidiabetic potential of *A. melanocarpa* may result from decreased mucosal maltase and sucrase activities in the small intestine. Yet other mechanisms may be involved, namely, stimulation of glucose uptake, increased insulin secretion, or reduction of oxidative stress [19, 20].

Specialized literature proposes multiple molecular mechanisms to explain hyperglycemia-induced diabetic complications. Such mechanisms include: increased polyol pathway,

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activation of the diacylglycerol (DAG)/protein kinase C (PKC) pathway, increased oxidative stress, formation and action of increased advanced glycation end products (AGE), and increased hexosamine pathway. Hyperglycemia is known to inhibit endogenous protective factors in vascular tissues, such as insulin, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and activated protein C (APC). These are crucial in maintaining vascular homeostasis and neutralizing hyperglycemia-induced toxic factors, such as oxidative stress, AGE, or activation of nuclear factor-jB (NF-jB) and prevent and delay the evolution of diabetic complications [21].

In diabetes mellitus, the prolonged excess of plasma glucose determines a glycosylation proportional with the severity and duration of the hyperglycemia, affecting numerous proteins: hemoglobin, albumin, lipoproteins, collagen, etc. [22]. The results reveal that the glycosylated hemoglobin values are much higher in the diabetic group (DM) and they are significantly lower in the group protected by polyphenols (DM+P). The photometric dosing of total hemoglobin and the colorimetric measurement of glycosylated hemoglobin (HbA_{1c}) were performed. Glycosylated hemoglobin values, considerably high in the diabetic group, diminish significantly in the protected group (**Table 1**).

The exploration of the lipid profile included the measurement by photocolorimetry, in the serum obtained after separation, of the concentration of total cholesterol (Ch-T), of triglycerides (TG) [23], of total lipids (TL) [the method with sulfovaniline], of high-density lipoproteins (HDL) [24], of low-density lipoproteins (LDL) [according to the Friedewald formula] for all the animals included in the experiment. The lipid profile obtained after the biochemical determinations is given in **Table 1**.

The delivery of plant polyphenols extracted from *A. melanocarpa* fruit significantly improves the dyslipidemia triggered by diabetes mellitus and the microangiopathic lesions. Following the perturbation of the lipid metabolism in the diabetic rats, atherogen risk has significantly increased values in comparison to the rats from the witness group (**Table 1**).

Experimental groups	W	P	DM	DM+P
Ch-T (mg/dL)	72.33 ± 4.81	66 ± 2.32	93.5 ± 7.89***	66.38 ± 3.02$^{\#\#\#}$
TG (mg/dL)	87.64 ± 6.88	70.38 ± 8.27*	149 ± 17.46***	92.83 ± 29.76$^{\#\#\#}$
HDL (mg/dL)	33.17 ± 4.24	31.17 ± 3.58	18.66 ± 5.33***	26.17 ± 3.01$^{\#\#}$
LDL (mg/dL)	22.07 ± 4.69	19.17 ± 3.81	44.43 ± 6.83***	25.24 ± 9.42$^{\#\#\#}$
Total Hb. (g/100 mL)	13.12 ± 1.13	13.66 ± 1.04	17.05 ± 2.48**	14.22 ± 1.85$^{\#\#}$
HbA_1c (% from Hb)	1.73 ± 0.04	1.61 ± 0.10	33.63 ± 5.68***	24.72 ± 2.21$^{\#\#}$
Adhesivity index	1.27 ± 0.04	1.46 ± 0.04	2.09 ± 0.07***	1.79 ± 0.08$^{\#\#}$

Note: Values are mean ± SEM. Statistical analyses *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. W group. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ vs. DM group.

Table 1. Lipid profile, total hemoglobin, glycosylated hemoglobin, and adhesivity index values in the studied groups.

Chlorogenic acid administered to golden hamsters enhanced expression of PPARα in liver and total cholesterol, LDL, HDL, and glucose. Insulin levels in blood were reportedly lower than in the placebo group [25], showing that chlorogenic acid affects lipid metabolism through the activation of PPARα in liver.

A simple and reliable method for the evaluation of the atherogen risk is the calculation of the HDL-cholesterol/total cholesterol ratio. The calculus of the atherogenity index (AI) has shown a 57.14% AI decrease at the protected diabetics (DM+P) in comparison to the unprotected ones. The AI experienced an increase of 170.8% in DM group compared to W group and a 16.08% increase in DM+P group compared to W group. Taking into account the variability coefficient (%), the environments are representative for the respective series (medium dispersion). Depending on the values of the significance threshold (p), the statistical analysis shows insignificant differences ($p > 0.05$) for P and DM+P in comparison to W, and very strong significant differences ($p < 0.001$) for DM in comparison to W, and strong significant differences for DM+P in comparison to DM.

Following the perturbation of the lipid metabolism in the diabetic rats (DM), AI has significantly increased values in comparison to the rats from the witness group (W) and even from DM+P. It is found that due to the polyphenolic protection of the rats from the DM+P, the AI is preserved at normal limits. The administration of vegetal polyphenols in healthy rats (P) did not cause a significant change of AI. As a consequence, the administration of natural polyphenols determines a significant increase of HDL and a significant decrease of LDL in experimental diabetes mellitus.

Rovatti's method was employed to determine the adhesivity index, which is the ratio between the initial platelet number and the platelet number after thrombocyte adhesion to glass. Platelets were counted before and after thrombocyte adhesion to glass, using the visual EDTA solution method.

Diabetic platelet adhesion is considerably lowered by the polyphenols extracted from *A. melanocarpa*, as compared to the diabetic group. Statistical analysis reveals highly significant differences ($p < 0.001$) between DM and control groups, and significant differences between the DM+P and the DM groups. Platelets of nondiabetic yet hypercholesterolic rats had reportedly normal sensitivity to ADP, which shows that glycemia influences platelet activity by membrane protein glycation, both directly and indirectly (**Table 1**).

Understanding the mechanism through which the natural polyphenols have effects on the functionality of the endothelium cells, including on the membrane sensitivity and intracellular signaling, could represent a new way of therapeutically approaching the chronic metabolic diseases.

Insulin secretion, insulin resistance, and homeostasis are important factors in the onset of diabetes, a disease associated with pancreatic ß-cell dysfunction. Oxidative stress is thought to contribute to this dysfunction and antioxidant effects on diabetes onset were investigated. It is well-known that *A. melanocarpa* offers a strong antiradical activity resulting from high amount of natural antioxidants, especially polyphenols [26, 27]. The natural polyphenols compounds do reduce the lipids peroxides, do neutralize the lipid peroxil radicals, and do inhibit the LDL oxidation at the inner level of the blood vessels.

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The aim of the study was to assess the effectiveness of natural polyphenols supplementation on the antioxidant defense mechanisms of diabetic rats, in order to reduce damage caused by the peroxidation of membranes and other cell components. Reactive oxygen species oxidized GSH to GSSG, leading to a decrease in GSH and an increase in GSSG concentrations. Long-time oxidative stress can consume antioxidants, and reduce SOD, CAT, and glutathione-peroxidase (GSH-Px) levels in experimental diabetes.

The serous activity of GSH-Px and superoxide-dismutase (SOD) has significantly lower values in the diabetic group as compared to the group protected by polyphenols. The serum activity of SOD diminished by 18.10% in the DM group as compared to the W group. In the DM+P group, the serum activity of SOD reached normal values again (**Table 2**).

Group	SOD (U/gHb)	CAT (μmol/gHb)	GSH (γ/mL)	GSH-Px (μmol/gHb)
I – W	14.6 ± 1.03	8.5 ± 1.50	3.24 ± 0.29	63.6 ± 9.93
II – P	14.8 ± 1.07	8.0 ± 1.25	3.04 ± 0.49	62.8 ± 8.75
III – DM	9.7 ± 0.72	6.6 ± 1.37	2.06 ± 0.04	46.8 ± 4.9
IV – DM+P	16.4 ± 0.10	7.8 ± 1.75	2.36 ± 0.10	53.7 ± 15.9

Table 2. The effect of *A. melanocarpa* on antioxidant activity in normal and diabetic rats.

Polyphenols act as free radicals scavengers by donating hydrogen atoms or electrons from phenolic hydroxyls. This is the main mechanism by which polyphenols scavenge many reactive oxygen species (ROS) (i.e., superoxide anion radical, hydroxyl radical).

Oxidative stress is diminished in diabetic rats enjoying natural polyphenolic protection as compared to the rats in the control group. The plant polyphenols referred to increase the antioxidant *in vivo* action of the serum and provide protection against excessive oxygen-free radicals generated by the oxidative stress.

In the diabetic group, the SOD and GSH levels in the blood were significantly ($p < 0.001$) decreased when compared with normal group (**Figure 2**). In the diabetic group the GSH-Px level was significantly ($p < 0.001$) decreased when compared with the normal group. There was no significant change in the CAT values.

Our findings revealed that the antioxidant activity of catalase (CAT) in the liver decreased by 69.90% in the DM group as compared to the W group. The antioxidant activity of CAT increased significantly, i.e., by 97.46% ($p < 0.001$), in the animals in the DM+P group, unlike the same activity in the animals included in the DM group. This phenomenon is due to the polyphenol protection by direct mechanism of microsomal hepatic membranes and proteins against the destructive action of ROS.

The oxidative stress causes the SOD activity in the liver homogenate to decrease by 42.85% in the DM group against the W group. The DM+P group enjoys the normalization of the antioxidant activity of SOD, which increases by 75.26% as compared to the DM group (**Figure 3**).

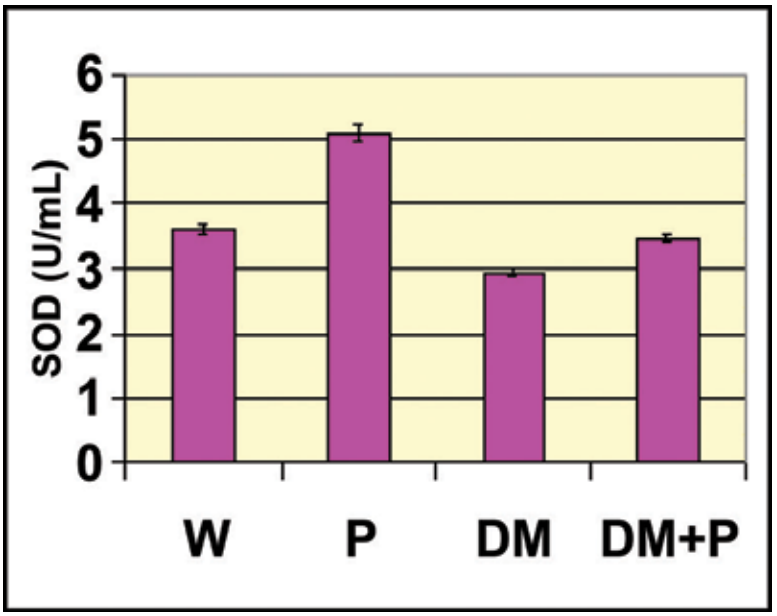

Figure 2. SOD blood activity in white Wistar rats.

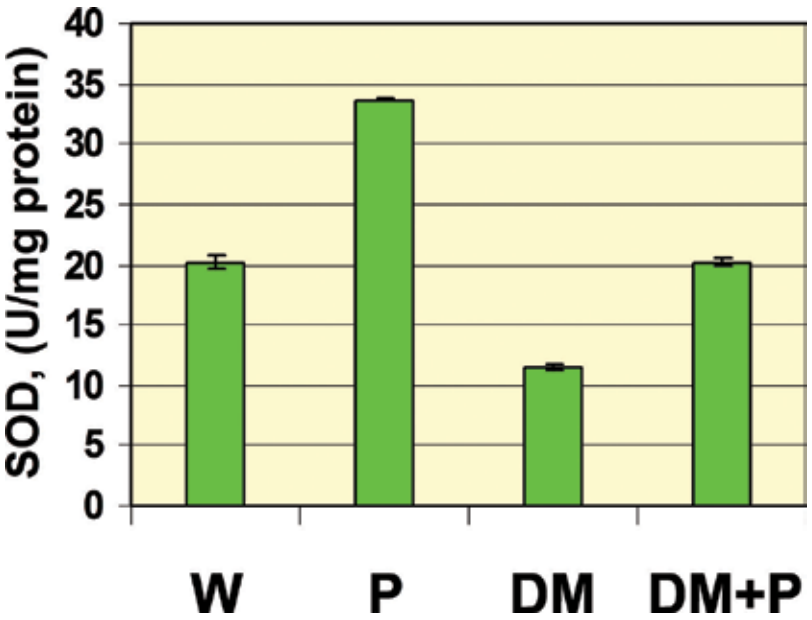

Figure 3. SOD hepatic activity in white Wistar rats.

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The analysis of our experimental findings in relation to the enzymatic systems involved in the antioxidant defense in the hepatic tissue and in the serum enabled us to conclude that the antioxidant defense increased significantly ($p < 0.001$) when diabetes mellitus was present.

The fact that the therapy with natural polyphenols administered to the diabetic group caused the recovery of the thiol groups up to normal limits in the liver tissue is suggestive of the protective effect that polyphenols have on reduced glutathione (GSH) as compared to the excessive peroxides developed in streptozotocin-induced diabetes. As a consequence of oxidative stress intensification, GSH has significantly lower values in the rats included in the DM group, than in those included in the W and DM+P groups, respectively. The decrease of GSH levels could be the result of decreased synthesis, or increased degradation of GSH by oxidative stress in diabetes. The GSH value in the liver increased by 66.22% in the DM+P group as compared to the DM group.

Polyphenols are secreted via the biliary route into the duodenum, where they undergo the action of bacterial enzymes, especially β-glucuronidase, in intestine distal segments, after which they may be reabsorbed, conducing to a longer presence of polyphenols within the body [28].

Liver function markers like *glutamate pyruvate transaminase* (GPT) and *alkaline phosphatase* (ALP) in serum were significantly ($p < 0.001$) elevated in STZ-induced diabetes when compared with normal rats. Animals treated with polyphenols showed significant ($p < 0.001$) reduction in the elevated level of ALP and GPT compared with diabetic rats (**Table 3**).

Group	GPT (U/mg prot)	ALP (KA/dL)
I – W	141.0 ± 11.92	33.1 ± 0.87
II – P	140.2 ± 10.86	32.7 ± 0.83
III – DM	307.1 ± 19.67	46.4 ± 1.78
IV – DM+P	225.8 ± 6.40	38.1 ± 0.68

Table 3. Liver function markers—GPT and ALP in the studied groups.

Aronia juice had a hepatoprotective effect in rats after acute exposure to carbon tetrachloride (CCl4) [29]. The liver cytotoxicity from CCl4 depends on its metabolism by cytochrome P450 in the presence of highly reactive trichloromethyl-free radicals. CCl3 radical reacts with oxygen and initiates lipid peroxidation, which results eventually in cell death. *Aronia* juice prevented the increase of lipid peroxidation induced by CCl4 as measured by the malondialdehyde content in rat liver and plasma.

The liver expresses batteries of cytoprotective genes that confer cellular resistance to oxidative stress and xenobiotic toxins, and protection against other stress-related diseases. These genes are mainly regulated by nuclear factor erythroid 2-related factor 2 (Nrf2), making this transcription factor a target for small molecule discovery to treat such diseases. Polyphenols may induce cellular defense genes by derepressing Nrf2 inhibition by Keap1 (Kelch ECH associating protein). This ability to derepress Nrf2 and reactivate its target genes may underlie the protection conferred by polyphenols against oxidative stress-related diseases [30].

Nrf2 is key to regulating GSH levels by upregulating GSH synthetic and regenerative enzymes, as well as enzymes using GSH as a cofactor [31]. Nrf2, which is a redox-sensitive transcription factor, is a highly protective factor, regulating multiple genes encoding antioxidant proteins and phase II detoxifying enzymes, thus regulating the physiological response to oxidative and electrophilic stress. Literature shows that natural compounds, including polyphenols, target Nrf2 and consequently can suppress oxidative stress and inflammation, and activate the antioxidant/electrophilic response element (ARE/ERE)-related cytoprotective genes [32].

Nrf2 is the transcription factor controlling ERE, which is normally bound to the sensory protein Keap1. Bound to an inducer, such as phytochemicals (i.e., curcumin), some polyphenols or sulforaphane release Nrf2, which activates ERE and the proteins it regulates [33].

ROS and oxidative stress increase as a result of diabetes, reacting with proteins-forming AGEs. AGEs interact with their receptors (RAGE) and activate the nuclear transcription factor kaba B (NF-κB) and its controlled genes like IL-6. AGE/RAGE interactions have been also shown to induce vascular oxidative stress through the activation of NADPH oxidase [34]. Blockade of the AGE/RAGE interaction by soluble RAGE has been shown to suppress atherosclerosis and neointimal formation [35] and nephropathy in diabetic animals [36].

Renal function indicators like creatinine and blood-urea nitrogen (BUN) were also elevated in the streptozotocin diabetic rats when compared with control rats. When compared with the diabetic group the elevated levels of BUN was significantly ($p < 0.001$) reduced in animals treated with natural polyphenols (**Table 4**).

Group	Creatinine (mg/dL prot)	BUN (mg/dL)
I – W	0.9 ± 0.13	9.4 ± 0.28
II – P	0.8 ± 0.13	9.1 ± 0.18
III – DM	2.3 ± 0.14	20.1 ± 0.97
IV – DM+P	1.9 ± 0.25	13.5 ± 0.39

Table 4. Renal function indicators—creatinine and blood urea nitrogen (BUN) in the studied groups.

When isolated rat kidney mitochondria were treated with quercetin, various changes consistent with access of quercetin to the interior of the mitochondria were observed, including increased mitochondrial membrane permeability and oxygen consumption, but decreased membrane potential and oxidative phosphorylation [37]. It thus appears that mitochondria would be easily able to absorb significant concentrations of polyphenols, provided the intracellular concentrations around them were high enough.

Mitochondrial adaptation, rather than antioxidant capacity, is emerging as the primary mode of action of the health benefits of dietary polyphenols. Possible mechanisms of action for polyphenols are direct activation of the components of the mitochondrial-biogenesis signaling pathway (e.g., sirtuin 1 and PPARγ); direct activation of the ERE via binding to its regulatory

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protein Keap1; stimulation of glycolysis and glucose uptake, which increases the supply of nutrients to the mitochondria; and stimulation of NO synthesis, which is a known signal for mitochondrial-biogenesis [31].

2.2. Morphological aspects related with polyphenols administration at the pancreas, kidney, and liver level in experimental diabetes mellitus

The morphological examination of the pancreas performed within the study showed a decrease in the number and volume of the Lagerhans islands (atrophy), as well as the decrease of the beta cells in the DM group. There was a major disturbance of the endocrine pancreas, with sclerosis and intense interstitial infiltrate. The inflammatory infiltrate was produced intra- and pericanalicular (**Figures 4** and **5**).

Atrophy was also revealed in the diabetic group under polyphenolic protection (DM+P). Nevertheless, the changes are much more discrete (**Figure 6**).

Diabetic nephropathy is a major cause of end-stage renal disease worldwide. Glomerular filtration rate progressively declines, associated to glomerular hyperfiltration, glomerular, and tubular epithelial hypertrophy, increased urinary albumin excretion, increased basement

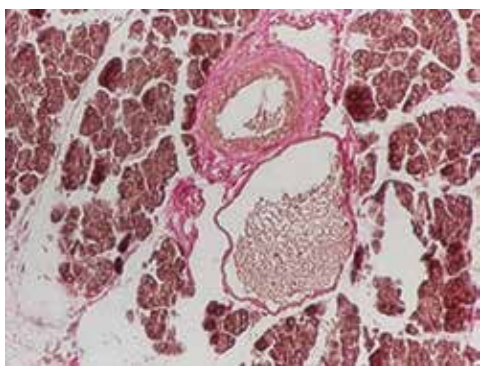

Figure 4. Pancreas, DM group: interstitial edema, perivascular, and interstitial fibrosis, vascular stasis (col.VG × 10).

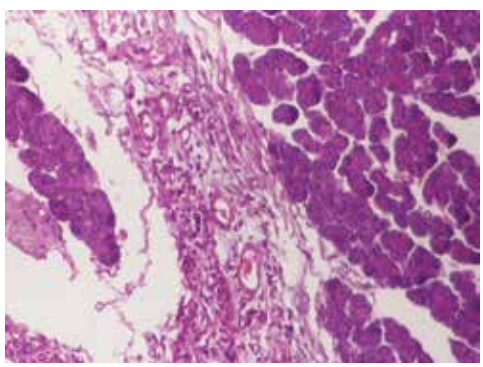

Figure 5. Pancreas, DM group: interstitial edema, fibrosis, dilated arterioles, and inflammatory infiltrate (col.HE × 10).

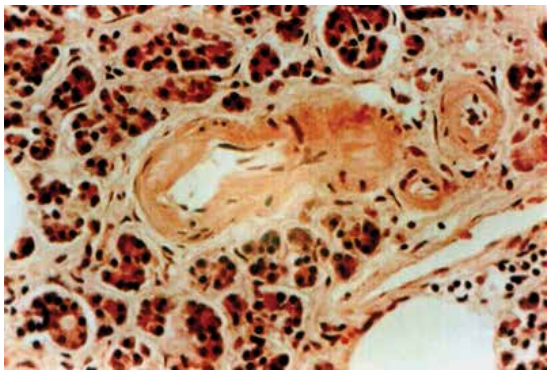

Figure 6. Pancreas, DM+P group: insulitis, edema, and a low level of fibrosis (col. HE × 10).

membrane thickness, and mesangial expansion with the accumulation of extracellular matrix proteins (ECM) [38].

The mechanisms which determined the production of renal alterations in the group with streptozotocin-induced diabetes could be: the formation of the nonenzymatic glycation products modifies the content in sialic acid of the glomerular filter with the alteration of the electric barrier; the accumulation of glycosphingolipids and glycosylceramides in the kidneys, as they represent secondary paths for glucose metabolizing process. There is a correlation between the accumulation of these substances in the kidneys and the renal hypertrophy in DM; and dyslipidemia secondary to the chronic hyperglycemia.

IL-6 has a strong association with the development of glomerular basement membrane thickening as well as possible relationships with increased endothelial permeability and mesangial cell proliferation. This reduction in the levels of IL-6 could play a major role in the attenuation of the progression of diabetic nephropathy and subsequently the significant reduction of the serum urea and creatinine in proanthocyanidin-treated group [39].

The histological images in the rat liver reveal granulo-vacuolar dystrophic lesions and inflammatory infiltrate in the portal spaces in the diabetic group, as well as glycogen spoliation in the pericentrolobular areas. In the animals included in the DM+P group, the degenerative and inflammatory phenomena are associated with the regeneration phenomena, which are probably stimulated by the administration of natural polyphenols (**Figures 7** and **8**).

The biologically active properties of flavonoids are closely related to their antioxidant and antiinflammatory capacity. Supplementing the alimentation with polyphenols may ameliorate the evolution of diabetes through: free radicals scavenger effect, reducing the glycation of proteins, and inhibiting the NMDA receptors (it may be considered as inhibitory of these receptors) [40–42].

Several studies and experimental research demonstrate that vegetal polyphenols increase the antioxidant capacity of the serum, *in vivo*, they have antiradical effect on oxygen-free radicals, produced in excess, under conditions of oxidative stress [43, 44].

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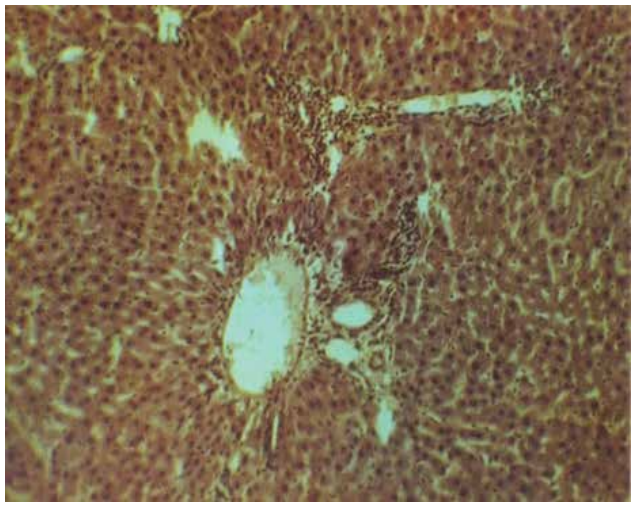

Figure 7. Liver, DM group. Distrophic granulo-vacuolare lesions and inflammatory infiltrate in the porte spaces.

The delivery of plant polyphenols extracted from *A. melanocarpa* fruit significantly improves the dyslipidemia triggered by diabetes mellitus and the microangiopathic lesions. Due to the health-promoting effects of *A. melanocarpa* extracts, it may constitute a valuable dietary supplement for people with risk factors of diabetes mellitus and cardiovascular diseases.

The biochemical parameters revealed the undeniable kidney protection achieved by polyphenolic extract delivery. The vascular protection effects of natural polyphenols on experimentally induced diabetes mellitus depend both on the administered dose of polyphenols, and on the length of their administration.

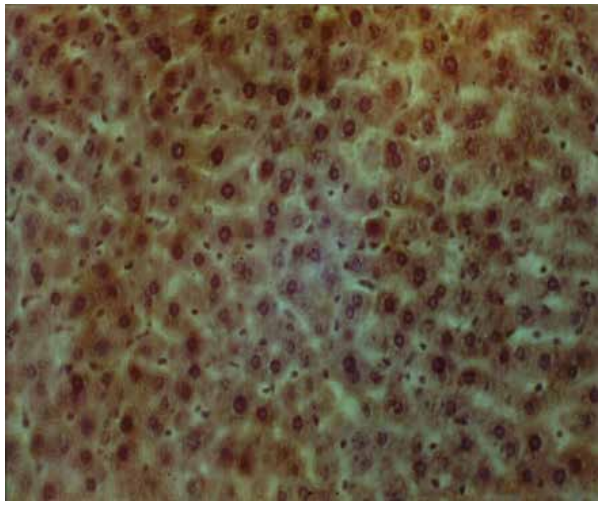

Figure 8. Liver, DM+P group. Degenerative and inflammatory phenomena, associated with regenerative phenomena, probably stimulated by the administration of natural polyphenols.

3. Conclusions

The low antioxidant enzyme levels found in the experimentally induced diabetes rats support the benefits of adding antioxidant supplements to the ordinary food intake. Through the hypoglycemiant, hypolipemiant, and antioxidant effects, *A. melanocarpa* represents a possible dietary adjunct for the treatment of diabetes and a potential source of active agents for the prevention of microvascular diabetes complications.

Author details

Manuela Ciocoiu[1]*, Laurentiu Badescu[2] and Magda Badescu[1]

*Address all correspondence to: mciocoiu2003@yahoo.com

1 Department of Pathophysiology, University of Medicine and Pharmacy "Grigore T. Popa", Iasi, Romania

2 Department of Cell and Molecular Biology, University of Medicine and Pharmacy "Grigore T. Popa", Iasi, Romania

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Phenolic compounds comprise a broad class of natural products formed mainly by plants, but also microorganisms and marine organisms that have the capacity to form them. Nowadays the interest in these compounds has increased mainly due to their diverse chemical structure and wide biological activity valuable in the prevention of some chronic or degenerative diseases. The functional foods are a rich source of these phytochemicals, and this is the starting point for this book, which shows the state of the art of the phenolic compounds and their biological activity. This book integrates eleven chapters that show the state of the art of diverse biological activity of the phenolic compounds, present in some crops or fruits.

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