Chapter

Flavonoids and Phenolic Acids as Potential Natural Antioxidants

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Abstract

For centuries, aromatic herbs and spices have been added to different foods to improve the flavor and organoleptic properties. The use of aromatic plants and spices in phytotherapy is mostly related to different activities of their essential oils, such as antimicrobial, spasmolytic, carminative, hepatoprotective, antiviral, and anticarcinogenic activities. Furthermore, many studies point to strong antioxidant activities of aromatic plants and their essential oils. Knowing that phenolic compounds are the most responsible for the antioxidant activity, the amount of total phenolic contents and content of flavonoids have also been determined. In order to examine the antioxidant properties of five different extracts of *Laurus nobilis* L. leaves, various assays which measure free radical scavenging ability were carried out: 1,1-diphenyl-2-picrylhydrazyl, hydroxyl, superoxide anion, nitric oxide and hydroxyl radical scavenger capacity test, and lipid peroxidation assay. In all of the tests, only the EtOAc extract showed a potent antioxidant effect.

Keywords: aromatic plants, flavonoids, phenolic acids, ROS, oxidative stress

1. Introduction

The history of medicinal herb usage dates back to the distant past, many centuries and civilizations ago. Plants have played an important role in many cultures in the treatment of various diseases, and floral fragrances have been used to refine the spirit and body, to attract partners, and to establish a psychophysical balance. The first written testimonials on the use of herbs for treatment are found in China. Emperor Kin-Nong knew about 100 medicinal plants in 3000 years BC. One of the oldest classical medical texts of ancient China is "Pent-Sao," which was written 2500 years BC and is composed of 52 books; of which, two books are dedicated to herbal remedies. In the nineteenth century, medicinal and exotic plants have become lucrative, as more and more people began growing plants in their homes. China, Japan, and South America were overwhelmed by collectors from plant companies who looked for tropical plants to meet the needs of society. This instigated scientific pharmacy and the start of chemical and physiological research on medicinal herbs. It can be said that the nineteenth century was the century of alkaloids, because hundreds were isolated from plants from all over the world. The beginning of the twentieth century threatened medicinal herbs to be completely thrown out of use. Thus, "medicines" that have been successfully used for thousands of years have become subject to mockery and disdain. The expulsion of medicinal herbs from therapy can be compared to the darkness of the Middle Ages that had ruled Europe.

In the last four decades, especially in the developed countries of Europe and America, scientists have shown increasing interest in plant research. It is estimated that today about 60% of the total world population in treatment relies on herbs and natural products that are thus recognized as an important source of drugs [1]. Phytochemistry studies a huge variety of organic substances that have been discovered and which accumulate in plants. Furthermore, phytochemistry is also defining the structure of these compounds, their biosynthesis, metabolism, natural distribution, and biological activities [2]. An important place among them is occupied by aromatic plants, whose aroma is associated with the presence of essential oils and complex mixtures of volatile compounds, dominated by mono- and sesquiterpenes. In addition to essential oils, aromatic plants are characterized by the presence of plant phenolic compounds, primarily coumarins and phenylpropanoids, that have been shown to possess multiple pharmacological activities. Investigations of these secondary biomolecules intensified when some commercial synthetic antioxidants were found to exhibit toxic, mutagenic, and carcinogenic effects [3]. It was also found that excessive production of oxygen radicals in the body initiates the oxidation and degradation of polyunsaturated fatty acids. It is known that free radicals attack the highly unsaturated fatty acid membrane systems and induce lipid peroxidation, which is a key process in many pathological conditions and one of the reactions that cause oxidative stress. Particularly, the biological membrane lipids in the spinal cord and brain are vulnerable, because they contain high levels of polyunsaturated fatty acids. Moreover, the brain contains significant amounts of transitional prooxidant metals and consumes a lot of oxygen. These features facilitate the formation of oxygen radicals involved in the processes of aging, Alzheimer's and Parkinson's disease, ischemic heart damage, arthritis, myocardial infarction, arteriosclerosis, and cancer. Phenolic antioxidants "stop" free oxygen radicals and free radicals formed from the substrate by donating hydrogen atoms or electrons. Many plant species and aromatic plants have been tested because of their antioxidant and antiradical activities [4].

The aim of this chapter was to show the antioxidant role of phenolic acids and flavonoids presented in aromatic plants and to assess their potential capacity as scavengers of different free radicals.

2. Oxygen as a toxic molecule

Atmospheric oxygen (O_2) is present as a biradical with two unpaired electrons, which have the same spin quantum number and are located opposite the orbited orbits. This electronic structure of molecular oxygen determines its chemical reactivity and allows the absorption of individual electrons, with the formation of numerous intermediate, partially reduced oxygen species that are commonly referred to as reactive oxygen species (ROS) [5, 6]. These reactive oxygen species are able to react with basic cellular structures and biomolecules [7] and are responsible for the emergence of many diseases and degenerative damage [8].

The normal concentration of free radicals in the body is very low. However, the effects are very disruptive, as the chain reaction allows one free radical to cause changes in thousands of molecules and damage DNA, RNA, and enzymes in cell membranes and leads to the formation of lipoxygenation products before being inactivated. Which part of the cell (proteins, nucleic acids, membrane lipids, cytosolic molecules) or the extracellular component (hyaluronic acid, collagen) will react with free radicals depends on the nature of the radical and the site of its formation (e.g., cytosolic membranes, mitochondria, endoplasmic reticulum, peroxisome, cell membranes). Due to the presence of molecular oxygen in aerobic

organisms and its ability to easily receive electrons, free radicals of oxygen origin start more reactions in the cell. The reactions responsible for their formation are respiration, processes of autoxidation of hydroquinone and catecholamine, reduced transition metals, some herbicides and drugs, as well as irradiation that causes water decomposition.

2.1 The role of ROS and RNOS in the onset of many diseases

Any disorder of oxygen species' regulation resulting from a disturbance in the balance between the formation of reactive oxygen metabolites and their elimination by the antioxidant protection system is the state of oxidative stress. In oxidative stress, the formation and accumulation of reactive metabolites are increased, resulting in oxidative processes of destruction of cellular components and genetic material.

2.1.1 Cardiovascular disease

ROS, RNOS, and LP are considered to be the major contributors to the etiology of atherosclerosis and various chronic disorders such as coronary disease, stroke, and ischemic dementia [9]. Antioxidants introduced through food can reduce the occurrence of cardiovascular diseases by inhibiting the production of free radicals and oxidative stress, protecting LDL from oxidation and aggregation, and inhibiting the synthesis of proinflammatory cytokines [10].

2.1.2 Neurodegenerative diseases

Oxidative stress often occurs in the brain, because although it represents only 2% of the body weight, the brain uses up to 20% of oxygen added. Also, the brain contains large amounts of polyunsaturated fatty acids subject to lipid peroxidation under conditions of high oxygen concentration [11, 12].

2.1.3 Carcinogenesis

Although there are insufficient facts to confirm that the presence of free radicals is necessary in the process of carcinogenesis, it is clear that they can lead to mutations, transformations, and cancers [13]. Regarding the development of cancer, the most important target for ROS is DNA. Carcinogenesis is the result of successive mutations in DNA molecules leading to uncontrolled growth and cell phenotypic modification. One of the first steps in this process is the direct interaction of electrophiles or free radicals with cellular DNA in which promutagen lesions develop. If no repair is performed, these lesions result in mutations in the next generation of cells [14]. An increased intake of antioxidants through diet or dietary supplements is associated with a reduction in the onset of cancer.

2.1.4 Aging

A reduced amount of free radicals or a reduction in the speed of their production postpones the aging process and a whole series of diseases related to the aging process [15]. A certain maximum life potential characterizes each animal species. There is a reciprocal correlation between the speed of oxygen consumption (and therefore the production of free radicals) and the maximum life potential. Some studies have shown that the aging process can be slowed by increased food intake

that increases antioxidant capacity (e.g., fruit and vegetables) or by supplemental intake of vitamins E, C, and β-carotene [16].

3. Antioxidant protection systems

The process of oxidative modification of proteins, carbohydrates, DNA, and lipids is a universal mechanism of damage to the cell, especially at the membrane level. On the other hand, the numerous roles of free radicals in physiological processes make their creation a mandatory precondition of life, which is why a protective system has been established during evolutionary development. The basic role of this protection system is to reduce the amount and uncontrolled creation of free radicals and their precursors in the cell.

From a functional point of view, the antioxidant protection of the organism includes three levels of action:

1. Antioxidant protection systems that prevent the endogenous formation of free radicals. This level of protection is ensured by the spatial separation of processes in which free radicals are formed.

2. Engagement of the system in conditions of normal and enhanced formation of free radicals. According to the nature and method of action, antioxidants are divided into two types:

   a. Enzymatic (superoxide-dismutase, catalase, xanthine oxidase, peroxidase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase). These enzymes make the so-called primary line of antioxidant protection.

   b. Nonenzymatic or the so-called secondary line of defense.

3. Enzymatic antioxidants involved in the reparation of oxidative damage of lipids, proteins, carbohydrates, and nucleic acids.

3.1 Phenolic compounds

During the evolution, the plants developed effective defense mechanisms against the harmful effects of visible, ultraviolet light and radiation and are a natural source of various antioxidants. Several thousands of biologically active secondary biomolecules of higher plants for phenolic compounds (vitamin E, flavonoids, biflavonols, benzophenones, xanthones, stilbene, quinones, betacities, phenolic acids, acetophenones, phenylpropanoids, coumarins, isocoumarins, chromones, phenols, and diterpenic alcohols) and different nitrogen compounds (alkaloids, amines, amino acids, and chlorophyll derivatives) have been shown to exhibit strong antioxidant activity, but antioxidant activity of essential oils of many spice plants is intense. Their significance is higher because it has been found that many synthetic antioxidants exhibit undesired effects after a prolonged use (e.g., some of them are withdrawn from the market as a possible carcinogen). These biomolecules exhibit their activity through various mechanisms: removing free radicals, binding metal ions, inhibiting enzymatic systems that produce free radical forms, increasing the concentration of biologically important endogenous antioxidants, and inducing the expression of a variety of genes responsible for the synthesis of enzymes that inhibit oxidative stress [14]. The term “herbal phenols”

encompasses a wide range of plant substances that form one of the most numerous classes of secondary biomolecules that have a common characteristic of an aromatic ring carrying one or more hydroxyl groups as substituents, including functional derivatives (esters, glycosides, etc.). However, this broad definition also includes some non-phenolic substances. For this reason, it is recommended to combine a definition that includes a chemical description and a biogenetic origin. In nature, there are two general biosynthetic pathways for the synthesis of plant phenols: (1) a polyacetate route and (2) a phenylpropanoid route with scrub acid as an intermediate. Some phenols are formed by a combination of these two times [17].

The efficiency of phenolic compounds in protection against oxidative stress depends on their reactivity in relation to toxic oxygen species and the reactivity of phenoxy radicals relative to critical biomolecules. Chemical or enzymatic oxidation of phenolic components of plant tissue results in a dark color which is of particular importance in food technology. Their susceptibility to oxidation allows their use in the protection of fats and oils.

Phenolic compounds also increase the activity of antioxidant enzymes, thus indirectly affecting the concentration of harmful oxygen radicals in the living cell. In high concentrations, radical reactions such as DNA damage, superoxide anion production, etc. can also be act as a prooxidant [18].

3.1.1 Phenolic acids

The term "phenolic acid" includes hydroxy and other functional derivatives of benzoic acid (C_6—C_1) and cinnamic acid (C_6—C_3) [19, 20]. **Figures 1** and **2** give the structures of the basic representatives of these acids.

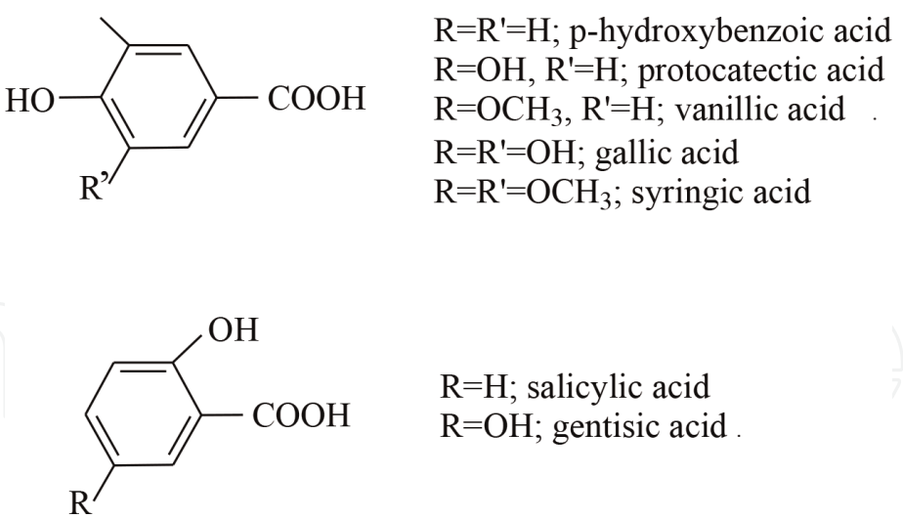


Figure 1.
Chemical compounds of basic benzoic acid derivatives.

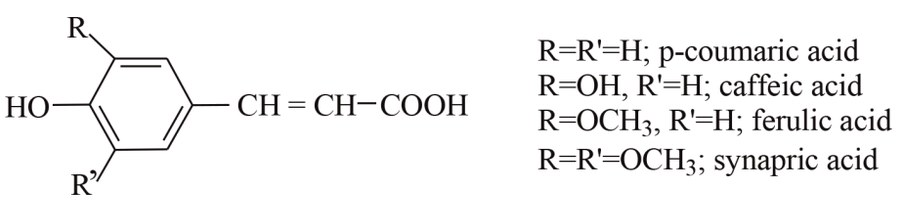


Figure 2.
Chemical formulas of basic derivatives of cinnamic acid.

Cinnamic acids, especially hydroxy-cinnamic acids, have the role of basic precursors in the biosynthesis of various plant phenols. The cinnamic acid and its derivatives are produced by condensation of the acidic acids with phosphoenolpyruvate to give the horizmic acid. Additional reactions of interconversion, decarboxylation, transamination, and disinfection lead to the formation of cinnamon (3-phenylpropenoic acid) and hydroxy-cinnamic acid. Subsequent reactions of hydroxylation, methoxylation, etc. produce cimetic acid derivatives such as p-coumaric acid (p-hydroxy cinnamic acid), β-acid (2,3-dihydroxy cinnamic acid), ferulic acid (2-methoxy-3-hydroxy cinnamic acid), and synapartoic acid (2,4-dimethoxy-3-hydroxy cinnamic acid).

The derivatives of cinnamic acid, in particular hydroxy-cinnamic acids, are the basis of the overall phenylpropanoid metabolism consisting of complex biochemical reactions which as a result supply the plant with important phenolic components [21].

3.1.2 Flavonoids

The term "flavonoids" was proposed by Geisman and Heinseiner [21] to describe all plant pigments having a C_6—C_3—C_6 skeleton, in which two benzene rings are linked via the C_3 unit. These natural products, varying in color from white to yellow, except anthocyanidins responsible for almost all pink and violet shades [20], are widely distributed in the plant kingdom with the exception of algae and fungi. So far, more than 4000 flavonoids have been found in plants, fruits, and vegetables [22]. The most common are seeds, citrus fruits, olive oil, tea, and red wine [23]. They are found in vacuoles, chloroplasts, and chromoplasts, in the form of glycosides, and in the extinct cells free of glycosides. The presence of OH groups directly linked to the carbon atoms of the benzene ring determines the antioxidant role of flavonoids, phenolic acids, and their esters. The expressed activity is shown by compounds with two hydroxyl groups, arranged as for catechol, and three hydroxyl groups arranged as in pyrogallol.

The structure of all flavonoids is based on the C_{15} skeleton of the chromatic structure for which the secondary ring (B) is attached (**Figure 3**) [24, 25].

Flavonoids are divided according to the substitution profile of the heterocyclic ring. In the classification of flavonoids, the oxidation state of the heterocyclic ring as well as the position of the secondary aromatic ring is taken into account. A total of about 12 subgroups of flavonoids are distinguished. The secondary (B) ring may be in position 2 (flavones, flavonols, dihydroflavonols, catechins, flavans, and

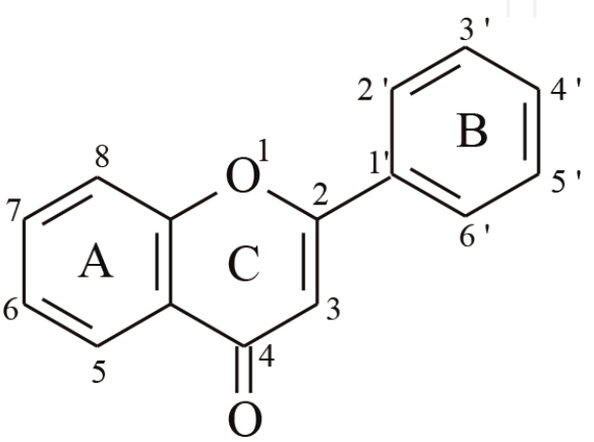


Figure 3.
Basic structure of flavonoids.

anthocyanidins), position 3 (isoflavonoids), or position 4 (4-phenyl-coumarins, neoflavonoids). In a few cases, the six-membered heterocyclic ring occurs in an open isomeric form (chalcones and dihydrochalcones) or is replaced by a five-membered ring.

The most widespread of all flavonoids are flavonols (3-hydroxyflavones) and flavones. The most commonly used flavonoids are quercetin, kaempferol, and myricetin. Quercetin is considered the most widespread component of all plant phenols. More than 100 glycosides of quercetin are known. Among flavonols there are about 200–300 known aglycons of these compounds [26].

3.1.3 The importance of phenolic compounds

Phenolic acids are important not only for ensuring the construction of lignin but also for regulating plant growth and disease resistance. Hydroxy-cinnamic acids are associated with the role of growth regulators and proteins in the development of certain diseases. In addition, it is possible that they are important for chloroplasts and the process of photosynthesis itself. Benzoic acid has been shown to inhibit photosynthesis in chloroplasts of spinach [17]. p-Coumaric acid is the most widespread compound among plant phenols. Furthermore, rosmarinic acid has antioxidant, anti-inflammatory, and antimicrobial effects. Its antioxidant effect is stronger than vitamin E. Rosemary acid prevents damage to cells caused by free radicals and reduces the risk of cancer and atherosclerosis. Unlike antihistamines, rosemary acid prevents the activation of immune system cells that cause swelling and fluid collection. It is used in the treatment of stomach ulcers, arthritis, cataracts, cancer, and bronchial asthma [27, 28]. Caffeic acid far exceeds other antioxidants because it reduces the production of α-toxin by more than 95%. It has been proven that high doses of coffee acids have a detrimental effect on the rats because they cause gastric papillomas. However, the combination of different antioxidants, including baconic acid, had a pronounced effect on the reduction of colon tumors in the same rats. The harmful effects of bicarbonate on human health are not known [29]. Calcium acid and its derivative caffeic acid phenethyl ester (CAPE) show a reduction in tumors and show anti-inflammatory and anticancer effects on ultraviolet-exposed skin, especially UVC and UVB rays [30]. Anticancer activity was observed in mice whose skin was treated with bee propolis and a papilloma-causing agent (TPA). CAPE significantly reduced the number of papillomas [31].

Flavonoids have a high ecological significance. They function as pigments that attract insect pollinators, not only as signal molecules for microorganisms that are useful for the plant but also as antimicrobial agents [32]. In this sense, yellow flavones and flavonols are particularly important. Because of the intense absorption of UV radiation, flavonoids protect the plant tissue from UV radiation, thereby influencing vital processes in chloroplasts.

In a pharmacological view, flavonoids show antiviral, antiallergic, antitumor, antibacterial, antifungal, and antithrombotic activity [33]. They act on blood vessels, namely, flavanones and catechins, that increase the resistance of the capillaries. They show an anti-inflammatory activity that depends on the structure of flavonoids [34]. The flavonoid anti-inflammatory activity was also confirmed by in vitro testing of the ability to inhibit lipoxygenase and cyclooxygenase [35]. Flavonoids eliminate pathological changes on capillaries and are used against diabetes, hypertension, and atherosclerosis. Flavonoids have been found to stimulate the secretion of bile and inhibit enzymes and enzymatic systems. Many flavonoids have antimicrobial and antiviral activity. A certain number of flavonoids show some cytotoxic activity. The common structural feature of cytotoxic flavonoids is trisubstituted ring A, methylation at position C4 [21].

For many flavonoids, high antioxidant activity has been demonstrated in various in vitro systems [36–38]. It has been shown that quercetin, rhamnetin, and isorhamnetin can reduce the amount of serum and liver cholesterol in addition to the in vivo antioxidant activity they show [39]. Flavonoids have been found to inhibit the activity of XOD and have the ability to capture superoxide radicals. Based on this, it is assumed that flavonoids can help in the treatment of gout and ischemia by reducing the amount of uric acid and superoxide anion of radicals in tissues [40]. Two flavonol glycoside-gallate esters showed inhibitory activity on human immunodeficiency virus-1 (HIV-1) integrase [41]. The HIV-1 integrase manages the process of incorporating viral DNA into the DNA of the host cell molecule, which is necessary for the virus to reproduce and produce virions. In this way, the inhibition of the given enzyme can be effective in anti-AIDS therapy. For example, quercetin has a beneficial effect on human health: it improves heart rate and reduces the risk of cancer. It has an anti-inflammatory and antiallergic effect. All of these effects are caused by a strong antioxidant effect of quercetin. Like many other flavonoids, quercetin inhibits the oxidation of LDL cholesterol, and its anti-inflammatory activity derives from inhibition of lipoxygenase enzyme and inhibition of inflammatory mediators. Quercetin also inhibits the release of histamine. Studies have shown that quercetin lowers the risk of prostate, uterine, breast, tissue, and colon cancer. It is presumed to reduce the production of uric acid by inhibiting XOD. It also shows NO inhibitory activity. Rutin has a strong antioxidant effect, as well as the ability to build chelates with metal ions (e.g., iron) and reduces Fenton's reaction in which harmful oxygen radicals are produced. It is supposed to stabilize vitamin C. If rutin is taken along with vitamin C, the activity of ascorbic acid increases. Rutin strengthens the capillaries, which helps people who easily bleed or get bruises. It prevents the formation of various edemas, which is an early symptom of a chronic vein disease. It has an anti-inflammatory effect. There are indications that rutin can inhibit some carcinogenic and precancerous conditions, prevent atherogenesis, and reduce the cytotoxicity of oxidized LDL cholesterol [22]. Furthermore, kaempferol prevents arteriosclerosis by inhibiting the oxidation of low-density lipoproteins and the formation of blood platelets. It has a role of a chemopreventive agent, which means it prevents the formation of cancer cells. Quercetin has a synergistic effect in reducing the proliferation of malignant cells, so treatment with quercetin and kaempferol combinations is more effective than their individual use [42]. In addition, tangeretin acts as an anticancer agent, and in in vitro studies, it has been shown to act against some forms of malignant cells. It strengthens the cell wall and protects it from attack. It also causes apoptosis of cells suffering from leukemia, while normal cells remain undamaged [43]. Tangeretin prevents tumor suppression of intercellular bonds when transmitting the signal [44]. In the G1 phase of the cell cycle, it "freezes" the cancer cells and prevents their replication. In short, in vitro studies have shown that tangerine exhibits antimutagenic, noniinvasive, and antiproliferative activity [45]. Animal studies have shown that tangeretin reduces cholesterol levels [46] and has a potentially protective effect from Parkinson's disease [47].

4. Lauraceae family

The Lauraceae family comprises over 2500 species, which occur within the subtropics and tropics of Eastern Asia and South and North America. Most species possess aromatic roots, stems, and fruits. One of the most well-known and most frequently used plants from this family is *Laurus nobilis* L., also called bay laurel.


Figure 4.
Laurus nobilis L [21].

L. nobilis is a species held in high esteem since ancient times. It was dedicated to Apollo, the ancient Greek god of light, and a symbol of peace and victory used to make wreaths for emperors, generals, and poets (**Figure 4**) [48].

4.1 *Laurus nobilis* L.

Laurel is a tree or a large bush of pyramidal shape with aromatic, constantly green leaves and shiny gray corn. It reaches a height of up to 5.5 m, but the cultivated form is usually lower (1–3 m). The leaves are elliptical, fairly thick, leathery, and shiny green. Clusters of tiny, yellow, single-polar flowers appear in the spring. Berries (fruit) (*Lauri Fructus*), when dry, are black and wrinkled and contain two oval fat seeds. Laurel is cultivated in several cultivated forms: spp. *aurea* with yellowish young leaves, spp. *angustifolia* with narrow leaves (often called Vrbolik laurel), and spp. *undulata* with corrugated leaf edges. Laurel is commercially grown for aromatic leaves in Turkey, Algeria, Morocco, Portugal, Spain, Italy, France, and Mexico [49, 50].

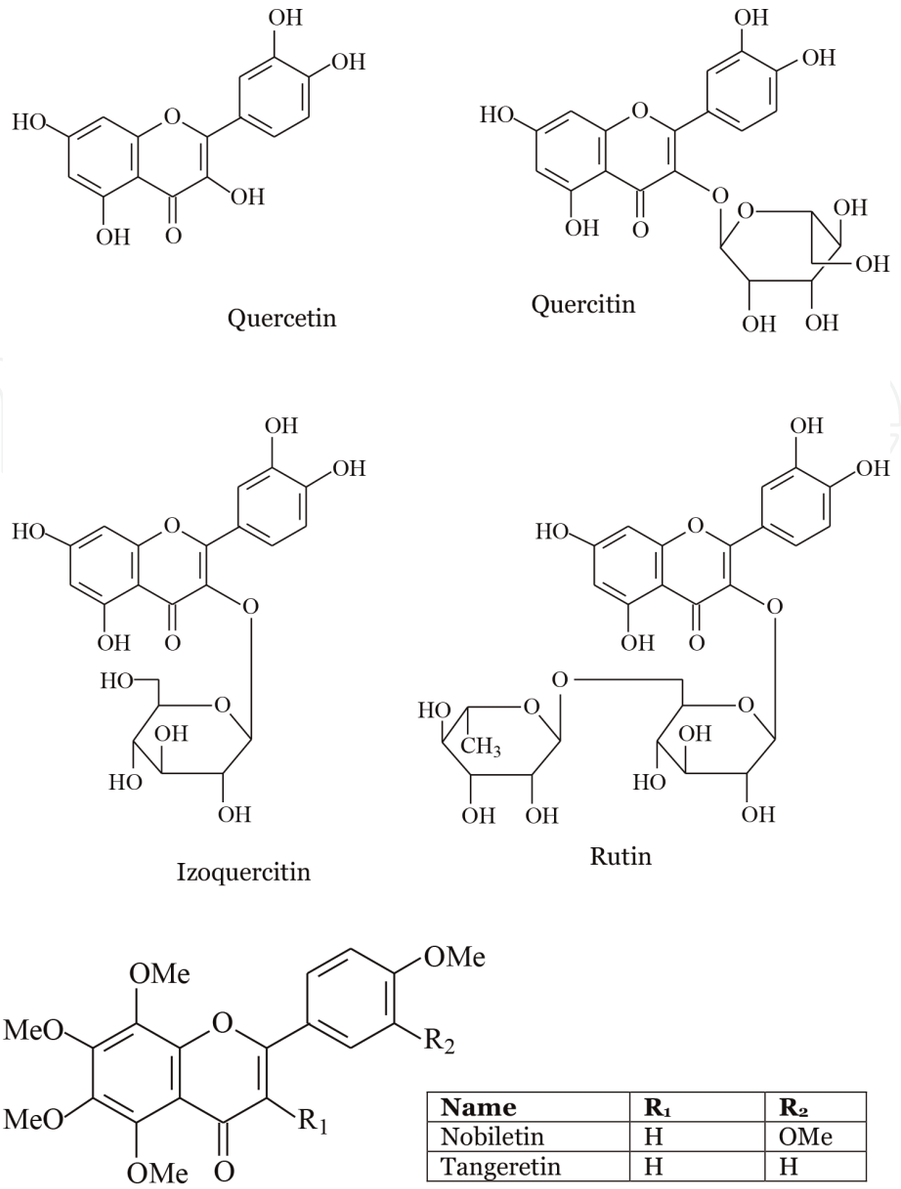
The distillation of laurel leaves produces green-yellow volatile oil that contains a high percentage of oxidized components. Essential oil leaf (0.8–3%) contains mainly 1,8-cineol (50%) and then eugenol, acetyleugenol, methyl eugenol, α- and β-pinene, felsenren, linalool, geraniol, and terpineol. Dried berries can extract green mass (melting point about 30°C) containing several percent essential oils (0.6–10%), depending on the conditions of breeding and storage. Berries contain both volatile and fixed oils. The others are known under the common name "laurel oil" (Oleum Lauri expressum, Oleum laurinum, and Oleum Lauri unguinosum). As essential ingredients, the oil contains laurosterin, glycerol ester with lauric acid, and sesquiterpenoid (the costume and dehydrocostus lactone), while the rest is made up of fats: triglycerides with lauric, myristic, and elastic acids. As with leaves, the aroma is mainly due to terpenes (cineol, terpineol, α- and β-pinene, citral) but also cinnamic acid and its methyl ester [51].

The main flavonoids in bay leaf are quercetin, kaempferol, rutin, and their derivatives (**Figure 5**).

Kaempferol appears in the form of four nonpolar glycosides (**Figure 6**) [52, 53].

Laurus nobilis is characterized by the presence of the other important plant phenolic substances such as phenolic acids (rosmarinic and caffeic acids) (**Figure 7**).

As a medicinal plant, bay leaves and fruits have been employed against rheumatism, skin rashes, and earaches. In addition, it has been used as a stomachic, astringent, carminative, diaphoretic, stimulant, emetic, emmenagogue, abortifacient,



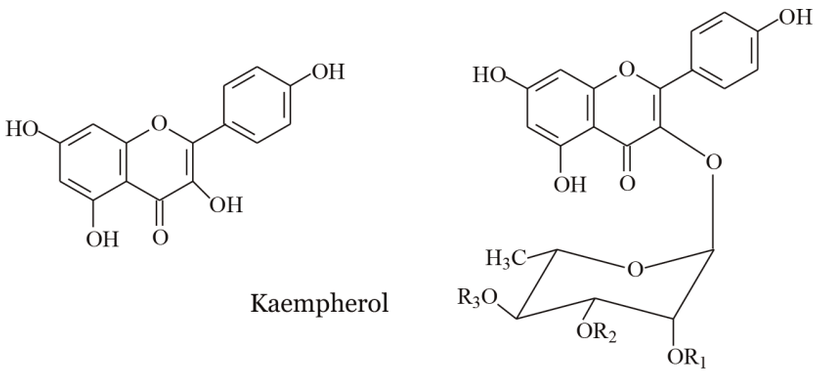
Name	R_1	R_2
Nobiletin	H	OMe
Tangeretin	H	H

Figure 5.
Structures of the main flavonoids present in L. nobilis [21].

and insect repellent. The essential oil is used by the cosmetic industry in creams, perfumes, and soaps.

Numerous investigations of qualitative composition of plant extracts have revealed the presence of high concentration of phenols in the extracts obtained using polar solvents [54]. The extracts that display the highest antioxidant activity have the highest concentration of phenols. Because of that, our research on laurel was recently extended to the comprehensive in vitro and in vivo studies of antioxidant activity of different extracts of leaves, to assess their potential capacity as scavengers of free radicals. Results of determination of total phenolic contents and total flavonoid contents in laurel leaf extracts are given in **Table 1**.

The amount of total phenolics in *L. nobilis* extracts ranged from 2.41 mg GAE/g d.w. (Et_2O extract) to 4.53 mg GAE/g d.w. (EtOAc extract). A significant amount of these compounds has also been observed in the n-BuOH extract (3.96 mg GAE/g d.e.). Furthermore, a considerable total flavonoid content was determined in the EtOAc and n-BuOH extracts. A little less amount of total flavonoids was determined in the $CHCl_3$ extract, while the smallest quantity of these compounds was found in the Et2O and H_2O extracts. HPLC-DAD analysis indicates a significant presence of flavonoids and phenolic in the EtOAc and n-BuOH extracts. Quercetin glycosides and flavonoids



Name	R_1	R_2	R_3
Kaempherol-3-O-α-L-(3",4"-di-E-p-kumaril)-ramnozid	H	E-p-kumaril	E-p-kumaril
Kaempherol-3-O-α-L-(2",4"-di-E-p-kumaril)-ramnozid	E-p-kumaril	H	E-p-kumaril
Kaempherol-3-O-α-L-(2",4"-di-Z-p-kumaril)-ramnozid	Z-p-kumaril	H	Z-p-kumaril
Kaempherol -3-O-α-L-(2"-E-p-kumaril)-ramnozid	E-p-kumaril	H	H

Figure 6.
Structures of kaempferol and its glucosides present in L. Nobilis [21].

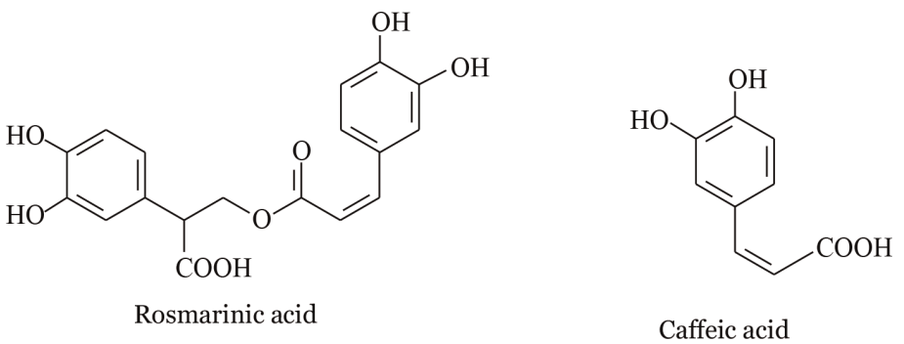


Figure 7.
Structures of two phenolic acids in L. nobilis.

Extracts	Et_2O	$CHCl_3$	EtOAc	n-BuOH	H_2O
Total phenolic content	2.41	2.85	4.53	3.96	3.20
Total flavonoid content	0.76	1.02	1.56	1.07	0.68

Table 1.
The amount of total phenolic contents (mg GAE/g d.w.) and content of total flavonoids (mg QE/g d.w.) in L. nobilis extracts.

(e.g., kaempferol-3-*O*-Glc) were detected in EtOAc extract. In addition, the presence of phenolic acids (such as caffeic acid) and flavonoids (rutin and kaempferol) was proven in the H_2O extract. The amount of flavonoids in extracts plays a significant role in their antioxidant capacity. Differences in flavonoid content between extracts and between plant organs can be explained by different numbers of secretory structures in various plant tissues [42, 55, 56].

It should be considered that the number of identified and quantified compounds in MeOH extract of *L. nobilis* L. has been expanded in the present work (**Table 2**).

The results indicate that the major bioactive compounds in *L. nobilis* extracts were kaempferol-3-O-glucoside, quercetin, and rutin. Phenolic acids were also

	Compounds	Extract
Phenolic acid	p-Hydroxybenzoic acid	38.46
	Protocatechuic acid	n.d.
	p-Coumaric acid	n.d.
	Vanillic acid	n.d.
	Gallic acid	n.d.
	Caffeic acid	16.18
	Quinic acid	n.d.
	Ferulic acid	n.d.
	Syringic acid	n.d.
	Chlorogenic acid	13.11
	Cinnamic acid	n.d.
Flavonoids	Apigenin	n.d.
	Naringenin	n.d.
	Luteolin	5.19
	Kaempferol	11.97
	Apigenin-7-O-β-glucoside	n.d.
	Luteolin-7-O-β-glucoside	n.d.
	Kaempferol-3-O-glucoside	56.15
	Quercetin-3-O-glucoside	31.18
	Rutin	17.44
	Quercetin	21.62
	Quercitrin	7.14

Table 2.
LC-MS-MS quantification of bioactive compounds presented in L. nobilis L. crude MeOH extract (μg/g d.w.).

observed in the high level, where the antioxidant, caffeic, and chlorogenic acids were found in the highest amount. Furthermore, p-hydroxybenzoic acid was also found in very high amount. The rest of the phenolic acids were not detected [57–59].

Furthermore, antioxidant activity was observed in the study of laurel leaf extracts in different solvents on the content of $DPPH^{\bullet}$, $O_2^{\bullet -}$, $NO^{\bullet}$, and $OH^{\bullet}$ radicals (**Table 3**).

The obtained results could point to strong quenching activities of flavonoids present in the leaves of laurel against DPPH radicals, and a high degree of correlation is observed between total phenol content and the ability of EtOAc extract to neutralize DPPH radicals. This is indicated by the fact that phenolic compounds

Extract	Et_2O	$CHCl_3$	EtOAc	n-BuOH	H_2O
DPPH radical	127.38	139.42	83.24	181.35	161.83
$O_2^{\bullet -}$ radical	327.60	429.43	163.57	288.64	486.32
NO radical	168.77	322.84	158.63	386.80	618.42
OH radical	442.84	241.18	121.84	213.36	187.65

Table 3.
IC50 values (μg/mL) of L. nobilis for different antioxidant assays.

play a key role in neutralizing free radical species which occurs by the mechanism of electron transfer. But, it can be supposed that such antiradical activity is also caused, besides flavonoids, by terpenoids, since nonpolar solvents also exhibited high antiradical potential. When investigating neutralization of $O_2^{\bullet-}$ and NO radicals, ethyl acetate extract has also exhibited the greatest ability of their scavenging. These results can be attributed to the presence of sesquiterpene lactones isolated from the plant that possess certain biological and pharmacological activity [60, 61]. Matsuda et al. [62] have also established that the methanolic extract from the leaves of *L. nobilis* was found to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages. It was concluded that seven sesquiterpene lactones (costunolide, dehydrocostus lactone, eremanthine, zaluzanin C, magnolialide, santamarine, and spirafolide) potently inhibited LPS-induced NO production. Inhibition of NO radicals with laurel extracts is very significant, having in mind the ability to neutralize the superoxide anion radicals as well. The common reaction between superoxide anion radical and nitrogen oxide radical yields a very reactive peroxynitrite anion ($ONOO^-$) which is very active in reaction of nitrification of phenols—e.g., nitrification of thyroxine causes enzyme dysfunctions, and increased amounts of 3-nitrothyrozine were found in various pathological states [63]. If formation of nitroderivatives of thyroxine is prevented, the occurrence of these diseases due to oxidative stress is reduced. Ethyl acetate extract of laurel leaves is especially suited in this process since it neutralizes both superoxide anion radical and NO radical. Obtained results can be related to the experiments in which the total amount of phenols and flavonoids were determined (**Table 1**), which show that ethyl acetate extract of laurel leaf contains the largest amounts of total phenolic content and total flavonoid content. The cellular damage resulting from hydroxyl radical is strongest among free radicals. Hydroxyl radical can be generated by biochemical reaction. Superoxide radical is converted by superoxide dismutase (SOD) to H_2O_2, which can subsequently produce extremely reactive $OH^{\bullet}$ radicals in the presence of transition metal ions such as iron and cooper [64, 65]. A good antioxidant potential of neutralization OH radical was shown by the EtOAc (IC_{50} = 121.84 μg/mL) and H_2O (IC_{50} = 187.65 μg/mL) extracts. Such a good antioxidant activity of H_2O and EtOAc extracts is expected, because it is known that the antioxidant activity of phenols is primarily a result of the ability of these compounds to act as donors of hydrogen atoms removing free radicals with the formation of less reactive phenoxyl radicals [66]. The increased stability of the formed phenoxyl radicals primarily attributed to electron delocalization and the existence of multiple resonant forms. Researching dependence of activity on the structure was found to have three structural features as important factors of radical removal potential and/or antioxidant potential of flavonoids: (1) *o*-dihydroxy function of ring B, which serves as the target of radicals; (2) 2,3-double bond in conjugation with 4-oxo function, which is responsible for electron delocalization of the ring B; and (3) the additional presence of 3- and 5-hydroxyl groups for the maximum radical scavenging potential [67]. The positive relationship between increased hydroxylation and increased antioxidant activity of flavonoids was found in different lipid systems, such as oil and liposome systems. Also, for phenolic acids and coumarins, it has been shown that vicinal diol groups are important for radical scavenging capacity and that methoxylation or glycosylation of o-hydroxy group in the coumarins and esterification of phenolic acids reduce the antioxidant activity of these compounds [68]. For example, it was determined that rosmarinic acid has stronger antioxidant effect than vitamin E. Rosmarinic acid prevents cell damage caused by free radicals and reduces the risk of cancer and atherosclerosis. In contrast to the histamines, rosmarinic acid prevents activation of the immune system cells that cause swelling and fluid collection [27, 69]. Furthermore, the action of

some flavonoids is based on their ability to chelate transition metal ions, thereby preventing the formation of radicals (initiators of LP), catching radical initiators of LP (ROS), scavenging lipid alkoxyl and lipid peroxyl radicals, and regenerating α-tocopherol by reduction of α-tocopherol radicals. Different metals have different binding affinities of the flavonoids. Thus, for example, iron has the highest binding affinity for 3-OH group of ring C, then catechol group ring B, and at the end of 5-OH group of ring A, while the copper ions bind to the first ring catechol group B [70]. Also, in the previous investigation, on *L. nobilis*, different groups of chemicals were isolated (luteolin, apigenin, alkaloids, monoterpene, and germacrane alcohols) [71].

5. Conclusions

One of the paradoxes of life on Earth is that, on the one hand, oxygen is necessary for the life of aerobic organisms. On the other hand, increased concentrations of oxygen and especially its reactive metabolites (reactive oxygen species) may lead to the development of numerous diseases. A major source of free radicals in biological systems is molecular oxygen (O_2). The results of our in vitro assays of examined five different extracts of *Laurus nobilis* leaves expressed significant protective effects on ROS (DPPH, $O_2^{\bullet -}$, NO, and OH radicals), which was found to be correlated to different compounds. HPLC-DAD analysis indicates a significant presence of flavonoids and phenolic in the EtOAc and n-BuOH extracts. Quercetin glycosides and flavonoids (e.g., kaempferol-3-*O*-Glc) were detected in EtOAc extract. In addition, the presence of phenolic acids (such as caffeic acid) and flavonoids (rutin and kaempferol) was proven in the H_2O extract. The amount of flavonoids in extracts plays a significant role in their antioxidant capacity, and it can be concluded that ethyl acetate proved to be the best solvent for extraction of plant material. Furthermore, it can be concluded that these extracts can be used in the preparation of various herbal medicines.

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Conflict of interest

The authors declare that there is no conflict of interest.

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