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Review

DPPH Radical Scavenging Assay

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Abstract: Today, there is an increasing interest in antioxidants, especially to prevent the known harmful effects of free radicals in human metabolism and their deterioration during processing and storage of fatty foods. In both cases, natural-source antioxidants are preferred over synthetic antioxidants. So, there has been a parallel increase in the use of assays to estimate antioxidant efficacy in human metabolism and food systems. Today, there are many bioanalytical methods that measure the antioxidant effect. Of these, the 1,1-diphenyl-2-picrylhydrazil (DPPH) removing assay is the most putative, popular, and commonly used method to determine antioxidant ability. In this review, a general approach to the DPPH radical scavenging assay has been taken. In this context, many studies, including attempts to adapt the DPPH radical scavenging method to different analytes, search for the highest antioxidant activity values, and optimize the method of measurement, have previously been performed. Therefore, it is highly important to introduce measures aimed at standardizing the conditions of the DPPH radical scavenging activity, including the various reaction media suitable for this assay. For this aim, the chemical and basic principles of DPPH free radical scavenging are defined and discussed in an outline. In addition, this study describes and defines the basic sections of DPPH free radical scavenging in food and biological systems. Additionally, some chemical, critical, and technical details of the DPPH free radical removal method are given. This is a simple assay in which the prospective compounds or herbal extracts are mixed with the DPPH solution and their absorbance is measured after a certain period. However, despite rapid advances in instrumental techniques and analysis, this method has not undergone extreme modification. This study presents detailed information about the DPPH method and an in-depth review of different developments.

Keywords: antioxidants; 1,1-diphenyl-2-picrylhydrazil; DPPH; antioxidant assay

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1. Introduction

1.1. Reactive Species (RS) and Oxidative Stress (OS)

Oxidation processes are essential for the survival of cells. Aerobic cellular respiration organisms provide energy from organic molecules such as glucose but also cause the formation of free radicals that cause cellular damage in metabolism [1]. A free radical contains an unpaired (free) electron with a quantum-mechanical property called spin. Such an entity typically has high reactivity because of its open shell structure [2]. However, today there are many free radicals that are stable under laboratory conditions, that is, in the air and at room temperature [3]. Free radicals are known to be mostly associated with oxidative stress [4,5]. Oxidative stress is a comparatively new concept that has been commonly used in the medical sciences recently [6,7]. It occurs when there is an excess of reactive oxygen species (ROS) produced by a cellular mitochondrion. It is inevitable that free radicals, which are known to cause many degenerative diseases such as carcinogenesis, acute inflammation, high blood pressure, diabetes, preeclampsia, acute renal failure, atherosclerosis, Alzheimer's disease and Parkinson's disorders, mutagenesis, aging, and cardiovascular disorders, are produced in biological systems [8,9]. There are many factors, including UV radiation and pollutants, that contribute to oxidative stress, which has a daily influence

on human health [10]. Cells metabolize oxygen, creating potentially harmful ROS. Under normal conditions, the rate and amplitude of oxidant formation are balanced by the rate at which they are removed [11]. However, disruption of the balance between antioxidants and pro-oxidants causes oxidative stress [12].

Recent extensive scientific research has classified reactive species (RS) and free radicals into three main categories: reactive nitrogen species (RNS), reactive oxygen species (ROS), and reactive sulfur species (RSS), composed of nitrogen, oxygen, and sulfur atoms, respectively [13–15]. Hydroxyl (HO·), superoxide anion (O_2^-), alkoxyl (RO·), nitric oxide (NO·), and peroxyl (ROO·) radicals are radicals. Nitrogen monoxide (NO), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), ozone (O_3), nitrous acid (HNO_2), nitrous oxide (N_2O), lipid hydroperoxide (LOOH), and hypochlorous acid (HOCl) are non-radical reactive species [16–18]. RS also occur in living organisms as part of their defense systems. Phagocytes such as monocytes, macrophages, or neutrophils defend themselves against foreign organisms by synthesizing large amounts of $O_2^{\cdot -}$ or NO· as part of their killing or defense mechanisms [19–24]. Antioxidant molecules inhibit oxidative processes and reduce the hazardous effects of RS. In this way, they are important in terms of health [25–29].

When free radicals occur excessively in the human body, they cause very serious negative effects in different tissues [30]. One of the most important complications related to this is the formation of lipid peroxidation in the plasma membrane. This event promotes RNS and ROS formation. Meanwhile, metals such as iron and copper enable Fenton and Haber–Weiss reactions and the formation of reactive species such as OH· [31–33]. In the presence of metal ions and oxygen, H_2O_2 can easily form OH· by the Fenton reaction [34]. In addition, the Haber–Weiss reaction produces OH· from $O_2^{\bullet -}$ and H_2O_2 catalyzed by iron ions. This impact was first suggested by Fritz Haber [35]. In later studies, it was known that both reactions constitute the main source of radicals and are the most important ones responsible for cellular damage [36–38].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \text{ (Fenton reaction)}$$
$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + H_2O + OH^{\bullet} \text{ (Haber–Weiss reaction)}$$

1.2. Antioxidants

Antioxidant molecules can be classified in different ways depending on their environment and the functions they perform [39–42]. An antioxidant is defined as a substance that can significantly delay or completely prevent the oxidation of substrate molecules, even at low concentrations [43]. They donate electrons to free radicals, rendering them harmless, and neutralize them by minimizing oxidative damage in biological processes [44–46]. Antioxidants prevent free radical formation by interfering with the free radical-mediated oxidative process at any of its three main stages: initiation, propagation, and termination [8,47,48]. The effectiveness of an antioxidant compound depends on different parameters and factors. The most important are the physical system state, temperature, structural properties, properties of the oxidation-sensitive substrate, concentration, synergistic effect, and presence of pro-oxidant compounds [49]. The chemical structure of an antioxidant molecule determines its intrinsic reactivity and antioxidant ability towards free radicals and other ROS [50]. In addition, the effectiveness of the antioxidant also depends on its concentration in the system and localization, such as interface distribution [51,52]. The reaction kinetics are another factor that plays an important role in the protective effect of the antioxidant in the long or short term. This includes the thermodynamics of the reaction between an antioxidant and a different oxidant, the reaction rate, and the antioxidant's ability to react. All of these parameters must be considered when testing the effectiveness of a particular antioxidant substance [53]. In this way, they maintain the balance between oxidants and antioxidants in metabolism [54]. In addition, antioxidants delay lipid peroxidation formation during storage and processing of foods, prevent the deterioration of drugs and food products, and extend the shelf life of products [55]. For this purpose, a wide variety of synthetic or natural antioxidants are often used to prevent food spoilage [56]. To

address this, the pharmaceutical industry has mainly used synthetic antioxidants to block or reduce the intracellular amounts of reactive oxygen or nitrogen species [57]. Of these, synthetic antioxidants are widely used because they can be found in high purity, have low costs, and are highly reactive even at low concentrations. However, some harmful effects have been reported [58].

Therefore, antioxidants of natural origin rather than synthetic antioxidants are preferred. There has been a parallel increase in methods used to estimate the efficacy of antioxidants [59]. The use of a free 1,1-diphenyl-2-picrylhydrazil radical (DPPH) is the most common method. Butylated hydroxytoluene (BHT), propyl gallate (PG), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) are the synthetic antioxidants that are most preferred by manufacturers, and therefore consumers have to use them despite their known negative effects. The chemical structures of the synthetic antioxidant molecules are given in Figure 1. These chemicals have been widely used as food additives for the prevention of oxidative deterioration in food and pharmaceutical products [60]. However, new studies have raised concerns regarding the safety of these synthetic compounds owing to unexpected consequences, particularly their inhibitory ability against numerous enzymes [61]. Due to the toxic effects of these synthetic additives, researchers are working hard to find new and alternative antioxidant substances with fewer side effects [62]. In this context, there is a considerably increasing trend to replace synthetic antioxidants with natural antioxidants, which have lower toxicity, high biodegradability, and safer methods of action [63].

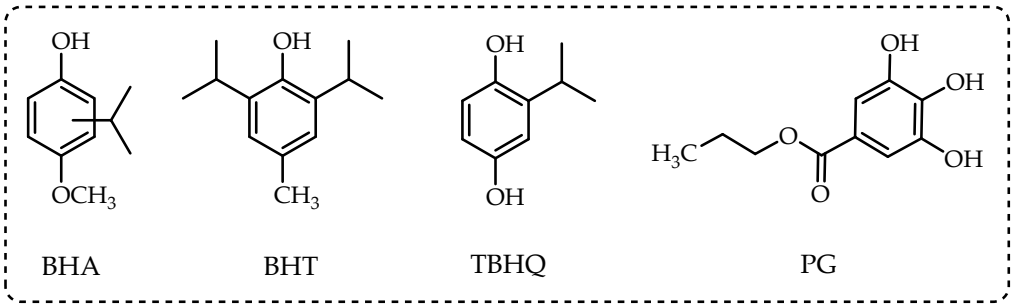

Figure 1. The chemical structures of the most putative and commonly used synthetic antioxidants.

In the case of long-term use of these synthetic antioxidants, it has been stated that they cause some health problems, including carcinogenesis, skin allergies, fatty liver, and gastrointestinal distress [64]. Therefore, conscious consumers are concerned about the negative effects of synthetic antioxidants and prefer natural antioxidants. The main and most accessible sources of these natural and safer antioxidants are fruits, vegetables, herbs, and spices [65]. For this purpose, plants such as tea, linden, cinnamon, cloves, fennel, anise, and rosemary are used as sources of natural antioxidants due to their rich tannin, catechin, theine, phenolic, and flavonoid contents [66]. Consumption of herbal products rich in phenolic content, which has an antioxidant effect, both reduces the risk of catching diseases and prevents the development of degenerative disorders [67]. However, the antioxidant capacity and quality of natural antioxidants and extracts depend not only on the natural source but also on the applied isolation and extraction processes [68].

2. Antioxidant Methods

Several studies have been performed recently on the oxidation process of free radicals and the general mechanism of action of antioxidants. This is because free radicals, although neutral, have a significant effect on the biological system [1,14]. In fact, some lipid derivative components, such as aldehyde, which can occur naturally during food processing and have adverse effects on human health, can easily occur as a result of the heat treatment of foods [51]. However, there are many antioxidant tests that directly measure the transfer of H atoms or electrons from antioxidants to free radicals [69]. The methods for measuring the

activities of antioxidants have recently made remarkable progress. Early methods measure the effectiveness of antioxidants on the formation of certain types of oxidation products and therefore rely on measuring lipid peroxidation. So far, different chemical methods have been used for the evaluation of antioxidant activity by specific methods, combining highly automated and sensitive detection technologies, such as removal activity against several types of ROS or free radicals, reducing potency and metal chelation, and others [70]. The concept of antioxidant capacity first emerged as a chemical concept, and later it was adapted to fields such as medicine, biology, food, and epidemiology [71]. It is very important to know the antioxidant profiles of these products in order to avoid loss of commercial and nutritional value during processing and preservation of foods and pharmaceutical products. Therefore, determining the potential antioxidant capacity of foods and pharmaceutical products requires the development of a fast and simple method [72]. Today, many different antioxidant procedures have been developed and used effectively [73]. In this context, the most commonly used methods are inhibition of autoxidation of emulsions of linoleic acid, the β-carotene bleaching method, total radical-trapping antioxidant parameter (TRAP) and oxygen radical absorbance capacity (ORAC) analyses [1], ferric (Fe^{3+}) and cupric (Cu^{2+}) ions reduction assays [74], DPPH·, N,N-dimethyl-p-phenylenediamine radicals ($DMPD^{\cdot+}$), 2,2-azinobis 3-ethylbenzthiazoline-6-sulfonic acid radicals ($ABTS^{\cdot+}$), superoxide anion radicals ($O_2\cdot^-$) removal experiments, and metal chelation tests [75]. As is known, most of these methods use similar principles and techniques. Measuring the ability of these antioxidant techniques is based on a suitable standard spectrophotometer measurement [76]. Antioxidant ability should not be tested with a single method; at least three different in vitro antioxidant methods must be performed together to determine antioxidant activity. A pure-only method does not reflect antioxidant activity. Given these, it is quite difficult to compare one method with another. Therefore, the methods to be used in analysis for research purposes should be carefully selected and applied [6]. Additionally, one of the most important objectives of this review is to detail the chemistry, mechanism, and application of the DPPH radical scavenging assay after giving some basic information about the antioxidant methods used to evaluate antioxidant properties. In recent years, researchers have focused on the DPPH radical scavenging method.

3. Radical Scavenging Methods

Despite the antioxidant defense mechanisms found in living things, especially humans, cell damage accelerates the aging process and plays an important role in the development of diseases. Tissue damage may occur as a result of the oxidative modification of biological macromolecules such as lipids, proteins, and DNA [77]. In order to understand and prevent these events, radical chain reactions in metabolism should be well understood.

Radical chain reactions are common mechanisms of lipid autoxidation and peroxidation. Radical scavenging agents can scavenge peroxide radicals to terminate radical chain reactions and improve the stability and quality of food products [14]. The radical-scavenging properties of antioxidants are the most important lipid oxidation inhibition mechanism. This method is an indispensable and standard test in antioxidant activity determination studies. Radical scavenging-based methods such as DPPH·, $DMPD^{+}$, $ABTS^{+}$, and $O_2{}^-$ are the most popular and putative spectrophotometric assays used for the determination of antioxidant activities of beverages, foods, and vegetable and fruit extracts. These chromogen radicals can react directly with antioxidant compounds. These assays are also commonly used because they are sensitive, simple, fast, and reproducible [1].

4. What Are DPPH Radicals?

The 1,1-diphenyl-2-picrylhydrazil (DPPH) radical was discovered 100 years ago by Goldschmidt and Renn in 1922 [78]. This method was developed by Blois [79] using a stable free radical, DPPH, to similarly determine antioxidant activity. The chemical structures of the 1,1-diphenyl-2-picrylhydrazil radical (DPPH·) are given in Figure 2. This assay is based on spectrophotometric measurements of the capacity of antioxidants to scavenge

DPPH radicals. Later, this test was developed in 1995 by Brand-Williams and his team and adopted by the vast majority of researchers. This antioxidant application was used very effectively by Gulcin's research group with a slight modification [80]. The single electron of the nitrogen atom in DPPH is reduced to the corresponding hydrazine by taking a hydrogen atom from the antioxidants. The DPPH· radical has a remarkably stable and intense color. Due to these two properties of the radical, its solution has been used intensively. This radical has been frequently used in polymer chemistry, especially in EPR spectroscopy, and in the evaluation of the antioxidant capacities of chemicals [80,81]. The use of this last feature in the evaluation of antioxidant capacities was first discovered by Blois in 1958 [79]. The stability of the radicals is due to the steric crowding on the first-order divalent N atom and the "push-pull" effect exerted by the second-order diphenylamino group, an electron donor, and picryl, an electron acceptor. This effect stabilizes the canonical structure considerably. EPR measures the spin densities at the two hydrazil N atoms, which are large and essentially equal. It has been reported that the two distinct bands seen in the UV-vis spectrum of DPPH· are produced by $\pi-\pi^*$ transitions, with the unpaired electron making a major contribution to the band in the visible region [82,83]. When a DPPH solution is mixed with a solution of a substance capable of donating a hydrogen atom, this violet color disappears, resulting in the reduced form of the DPPH radical (DPPH-H) [84]. The wider band is responsible for the deep violet color of the DPPH· solution. The formation of hydrazine (DPPH-H) induces the disappearance of the visible band as the color of the solution changes from violet to pale yellow as a result of radical reduction by hydrogen atom transfer from antioxidants, which are H donors. The color intensity of this reaction, known as the "DPPH test" in the literature, can be easily recorded by UV-vis spectroscopy. This method is widely used to evaluate the antioxidant capacity of pure antioxidant molecules, especially herbal extracts or phenolic compounds [85].

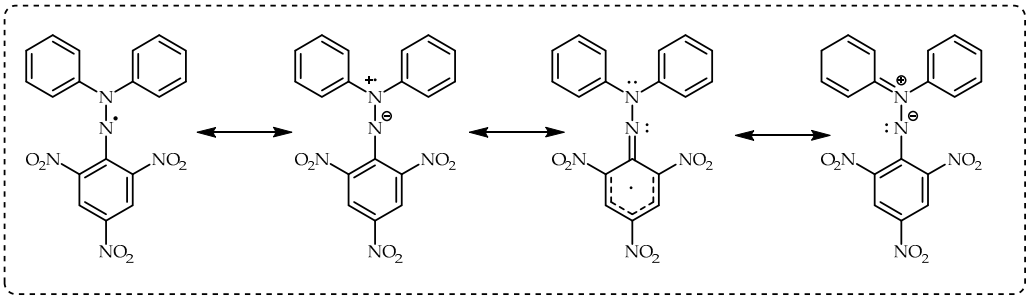

Figure 2. The chemical structures of a 1,1-diphenyl-2-picrylhydrazil radical (DPPH·).

DPPH remains a stable free radical thanks to the delocalization of the spare electron in the whole molecule (Figure 2). In this way, the DPPH radical does not dimerize like many other free radicals. Additionally, this electron delocalization causes a dark purple color to appear in the molecule and a maximum absorption of the ethanol solution at 517 nm [86]. Absorption is lost as the electron pairs are removed from the DPPH radical. The resulting color flare is dependent on the stoichiometry of the electron number. A concentrated solution of 0.5 mM colored alcohol also obeys Lambert-Beer's law [79].

While DPPH· is slightly soluble in nonpolar solvents, it dissolves quite well in different polar organic solvents. It is almost insoluble in water at room temperature. DPPH· selectively reacts with radicals and hydrogen atom donors at different reaction sites. Radicals usually attack the phenyl ring, while hydrogen donors react with the divalent nitrogen atom. The limited space around the nitrogen atom sterically inhibits the addition of bulky radicals to this region. Hydrogen atom donors can approach the nitrogen atom and release the hydrogens there with the formation of hydrazine (DPPH-H) [81]. The researchers purposefully used a methanolic DPPH radical solution (simple to use and available as ready-made radicals) to study the antioxidant ability of food and pharmaceutical ingredients by measuring the absorbance of radicals remaining in the reaction environment [80].

They measured residual DPPH radicals until they reached an equilibrium plateau. The simplicity of the procedure and the short reaction time made working with these radicals popular [79]. Molecular oxygen (O_2) does not react with DPPH·. However, in the presence of light, molecular oxygen reacts slightly with DPPH·. In addition, DPPH· solutions kept in the dark can remain stable for a long time. DPPH radicals do not dimer and exist in free monomeric form in alcohol solutions [87].

5. The Synthesis of DPPH Radicals

DPPH radicals are easily obtained by the oxidation of hydrazines with lead dioxide, lead tetraacetate, potassium permanganate, or silver oxide (Figure 3). These reactions are carried out in non-polar solvents such as benzene or dichloromethane. With simple filtration, the desired radical is obtained in quantitative yield. In this way, many hydrazyl permanent or stable free radicals containing carboxyl or sulfono groups are obtained from these derivatives, generally in a single step with high yield [3].

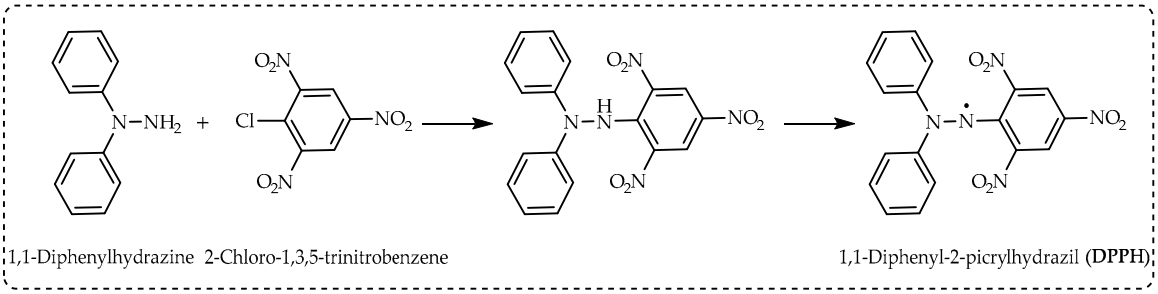

Figure 3. The synthesis route of 1,1-diphenyl-2-picrylhydrazil radicals (DPPH·).

6. Interactions of DPPH· with Phenols as H-Atom Donors

Some chemicals easily react with DPPH radicals by electron transfer or by donating H atoms. Especially phenolic compounds are the most reactive and important ones that react easily with DPPH·. Hydrogen atom abstraction contains reactions that form through electron transfer followed by or preceded by proton transfer and can be formally classified as Hydrogen atom transfer reactions. The DPPH· test therefore gives an estimate of the total content of reductants present in the solution in plant extracts. The antioxidant capabilities of phenolic compounds (ArOH) are quantified by the following reaction [1,14]:

$$\mathrm{ArOH} + \mathrm{ROO}^{\bullet} \rightarrow \mathrm{ArO}^{\bullet} + \mathrm{ROOH}$$

This radical scavenging reaction of phenolic compounds had great industrial and biological importance because it was used to reduce the oxidation rate of organic matter exposed to molecular oxygen in the air [88,89]. However, since peroxyl reacts very quickly with DPPH radicals, these reactions are difficult to monitor, and sophisticated devices are required. In contrast, the colored DPPH· radical is available and has much less reactivity than ROO· [90]. As seen in Figure 4, the best example of this situation is the electron-transfer reaction of cinnamic acids with DPPH radicals in alcoholic solutions [91].

The DPPH radical interaction of quercetin as an H-donor phenolic compound is a good example of this situation. Quercetin is a flavonoid widely found in plants and is a very important component of a regular diet [92]. In particular, the total flavonoid amount of plant extracts is given as the equivalent of this polyphenolic compound. Two-electron oxidation of quercetin yields quinomethide/quinone products, an intensely colored compound (Figure 4). Several tautomeric forms of quercetin can be found in solution, but the second tautomeric form shown in Figure 5 has been reported to be the most stable and abundant in solution. The interesting thing about these compounds is that their UV-vis spectrum and colors are coincidentally very similar to the DPPH radical. This was interpreted as a change in absorbance of 519 nm and a relatively different loss of DPPH radicals [93].

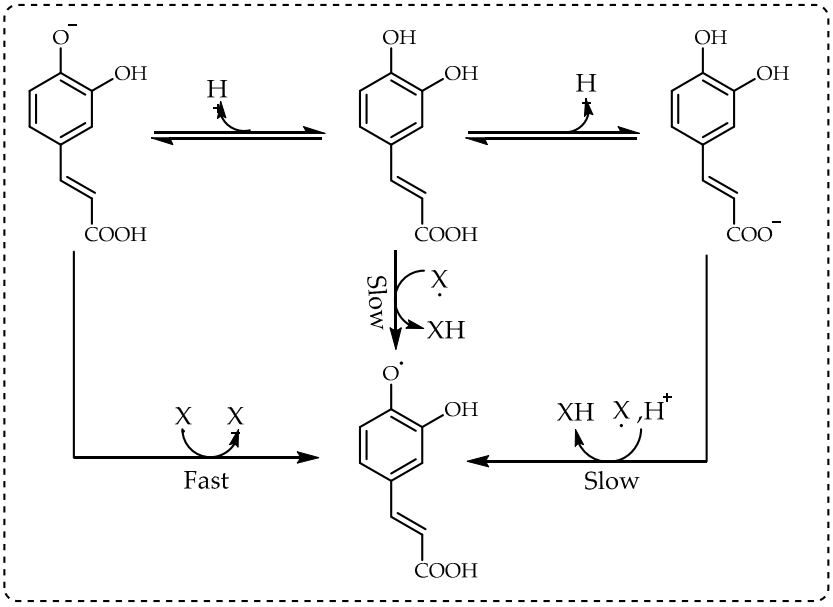

Figure 4. The mechanism between cinnamic acids and 1,1-diphenyl-2-picrylhydrazil (DPPH·) radicals.

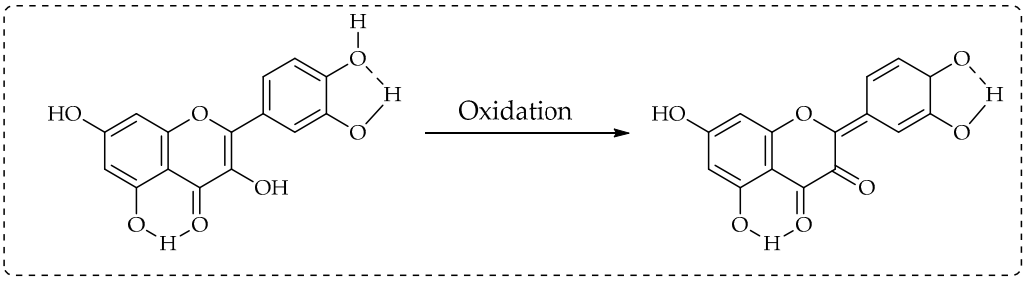

Figure 5. Enol and keto tautomeric forms of a quercetin molecule.

In radical scavenging studies, the antioxidant effects of phenolic compounds (Ar–OH) generally occur by two mechanisms, including hydrogen atom transfer (HAT) or single-electron transfer followed by proton transfer (SET-PT). However, in some cases, it may not be possible to separate these two mechanisms with clear boundaries [94]. In a HAT-based assay, an antioxidant molecule can quench free radicals through H-donation, while in a SET-based method, a potential antioxidant agent exhibits antioxidant ability by transferring an electron (e-) to reduce any compound, including radicals, metals, and carbonyls [95]. Recently, in addition to these two mechanisms, a third mechanism called the sequential proton loss electron transfer (SPLET) mechanism has been developed [14].

$$\mathrm{ArOH} \rightarrow \mathrm{ArO^-} + \mathrm{H^+}\,(\mathrm{HAT})$$
$$\mathrm{ArOH} \rightarrow \mathrm{ArO^\bullet} + \mathrm{H^\bullet}\,(\mathrm{SET-PT})$$
$$\mathrm{ArOH} \rightarrow \mathrm{ArO^{\bullet+}} + \mathrm{e^-}$$
$$\mathrm{ArO^{\bullet+}} \rightarrow \mathrm{ArO^\bullet} + \mathrm{H^+}$$
$$\mathrm{ArO^-} + \mathrm{ROO^\bullet} \rightarrow \mathrm{ArO^\bullet} + \mathrm{e^-}$$
$$\mathrm{ArOH} + \mathrm{ROO^\bullet} \rightarrow \mathrm{ArO^\bullet} + \mathrm{ROO^-}\ (\mathrm{SPLET})$$

The -OH group in the 7th position of flavonoids had great importance as the site of ionization and electron transfer, according to SPLET. This mechanism has been discovered recently [96,97]. In the first step, the reaction enthalpy corresponds to the proton affinity of the phenoxide anion (ArO^-), while in the second step, the phenoxy radical is formed by electron transfer from the phenoxide anion to ROO·. In terms of antioxidant effect, SPLET is similar to free radicals in the HAT mechanism. For example, the possible mechanism for

the reactions of quercetin and taxifolin (dihydroquercetin) with DPPH radicals is shown in Figures 6 and 7. Quercetin and taxifolin, as stable antioxidant flavonoids, can easily convert purple-colored DPPH radicals to yellow-colored DPPH-H. In addition, it has been reported that taxifolin, which is naturally bioactive, significantly inhibits some metabolic enzymes associated with some diseases [1,14].

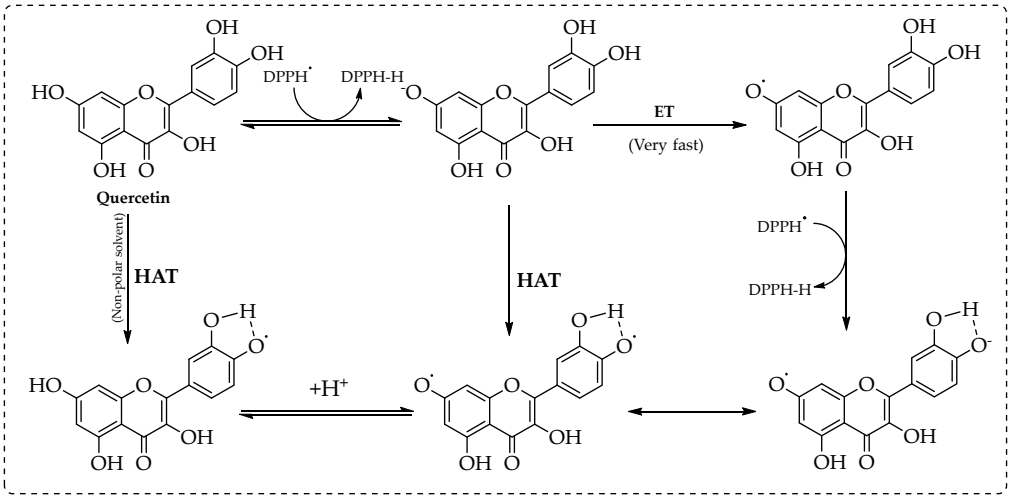

Figure 6. The reactions between DPPH free radicals and a quercetin molecule.

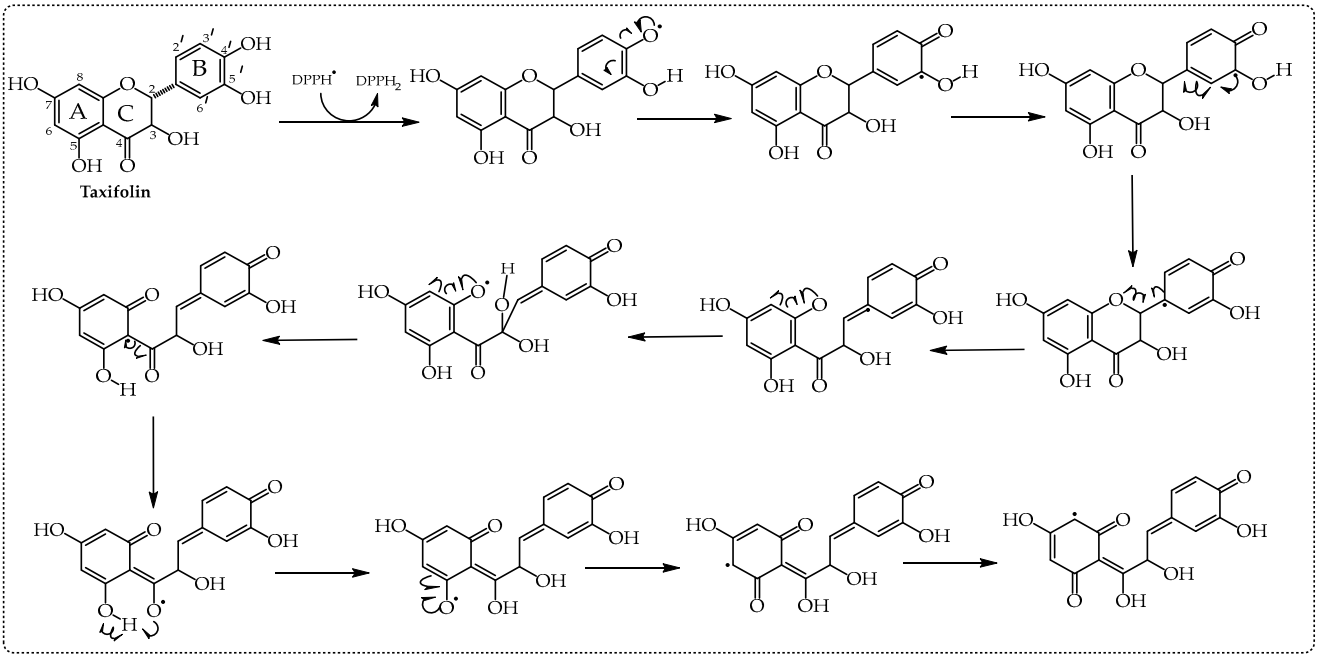

Figure 7. The reactions between taxifolin and DPPH free radicals.

These reactions are strongly and effectively accelerated due to the increased electron density in the A and C rings found in quercetin. Moreover, a fast electron transfer from the phenolate anion to the DPPH radicals is also an alternative route. The A ring in the quercetin molecule strongly attracts electrons, creating a positive effect on conjugation. On the other hand, the catechol moiety within the B ring constitutes the most likely site of deprotonation. Due to the existence of -OH groups in flavonoid molecules, many flavonoid compounds are present in the water phase of biological systems. The interactions of flavonoids with electron-deficient radicals can be accelerated by the SPLET mechanism to effectively minimize the generation and consequent accumulation of reactive oxygen species in the cells [98]. In addition, since the SET-PT and SPLET mechanisms

in the solvent environment are important, the effect of water on the three mechanisms also has significance. Additionally, Litwinienko and Ingold (2005) proposed a different mechanism for SPLET. Among organic solvents, methanol is the leading solvent that supports ionization. This mechanism is preferred in that phenols with low pKa react with electron-deficient radicals with relatively lower HAT activities and yield product molecules with low pKa [96]. In addition, SPLET formation in methanol and ethanol solutions was also reported by Foti et al. [91]. Many studies have shown that DPPH· reacts with phenolic acids. As a result of the suppression of the ionization of the phenolic hydroxyl group by the free carboxylic acid, the rate constants of the reactions for methyl esters of these acids are several times higher than for free acids. These experiments nicely confirm the effective role of ionization of phenolic compounds in the reaction of phenols with DPPH· in solvents that can promote ionization [97,98].

α-Tocopherol is a natural phenolic compound commonly added to food products as a preservative. Tocopherols show antioxidant activity by donating the hydrogen from the -OH group to DPPH·. The formation of an α-tocopherol radical is stabilized by the delocalization of the solitary electron on the structure of the aromatic ring (Figure 8). These compounds are highly lipophilic and more active in lipoproteins and membranes. The most crucial antioxidant effect is the inhibition of lipid peroxidation, which scavenges lipid peroxyl radicals, resulting in lipid hydroperoxides and the formation of a tocopheroxyl radical [14].

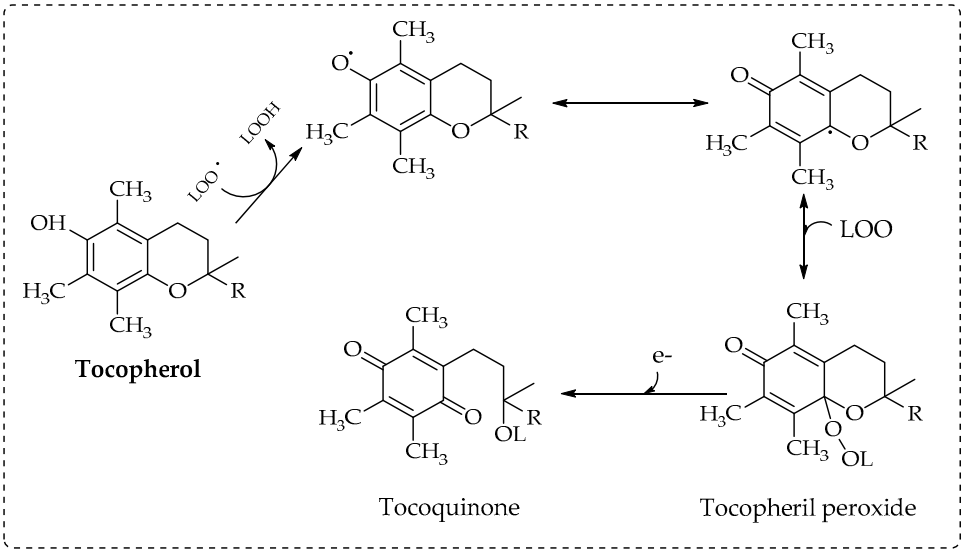

Figure 8. The reaction mechanism between DPPH free radicals and α-tocopherol as a commonly used food additive.

Similarly, the phenolic structure of usnic acid, a lichen metabolite, is highly suitable for interaction with DPPH·. After the mutual effect of usnic acid and DPPH radicals, DPPH radicals readily convert to DPPH-H by accepting an electron or hydrogen radical from usnic acid. This possible interaction pattern between usnic acid and DPPH· is shown in Figure 9. The phenolic group in usnic acid has two -OH units. The withdrawal of H atoms from phenolic -OH groups by a reactive radical can occur quite easily. It can remove three DPPH radicals due to the resonance structures that occur with the delocalization of electrons in the phenolic ring of usnic acid [99]. In this study, the interaction between usnic acid and DPPH· is documented and summarized in Figure 9. According to our best knowledge, a phenol group easily stabilizes radicals formed on the phenolic carbon with its resonance structures. The phenolic group in the usnic acid molecule has two hydroxyl groups. It is quite easy to withdraw H atoms from phenolic -OH groups. It can acquire a triradical one by inactivating three DPPH molecules using resonance structures, as shown in Figure 8.

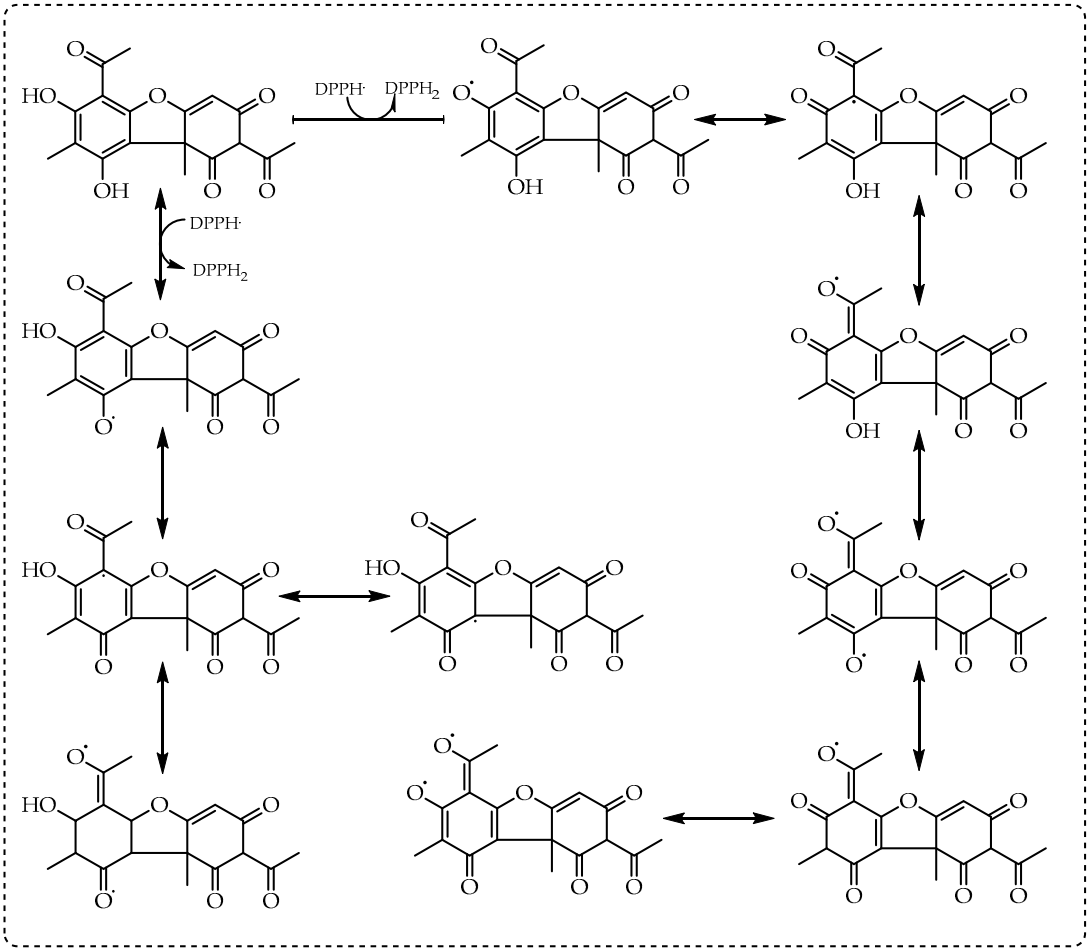

Figure 9. DPPH radical scavenging mechanism by usnic acid as a lichen metabolite.

Phenolic compounds with very electron-rich substituents are excellent H donors. Therefore, phenolic compounds have the ability to quickly extinguish all types of radicals, especially ROO radicals. In this context, the possible scavenging mechanisms of DPPH radicals in some phenolic compounds, which have a very rich biological activity spectrum and are recorded in the literature, have been predicted and clarified [14].

7. DPPH Radical Scavenging Assays

Radical chain reactions serve as a common mechanism for lipid peroxidation. Radical scavengers increase the stability and quality of food products by ending peroxidation chain reactions. For this purpose, radical scavenger molecules interact directly with peroxide radicals and scavenge them quickly [100]. Free radical scavenging has a known mechanism where antioxidants directly inhibit lipid peroxidation. This method is a standard, most widely used, and very fast and practical technique in antioxidant activity studies. Radical removal activity has great importance due to the hazardous effects of free radicals in foods and pharmaceutical systems. Many assays are used for the evaluation of the antioxidant activity of herbal extracts or phenolics. Different radicals and methods are used for antioxidant analyses and the determination of the final product of oxidation. $ABTS^+$, DPPH·, $DMPD^+$, or O_2^- radical removal methods are the most commonly used spectrophotometric methods for this purpose. When antioxidants are added to these radicals, color removal occurs with a mechanism that reverses the formation of DPPH·, $ABTS^+$, and $DMPD^+$ cations [1,14].

$$DPPH^{\bullet} + AH \rightarrow DPPH_2 + A^{\bullet}$$
$$ABTS^{\bullet+} + AH \rightarrow ABTS^{+} + A^{\bullet}$$
$$DMPD^{\bullet+} + AH \rightarrow DMPD^{+} + A^{\bullet}$$

These three radical scavenging methods are extremely fast, requiring no expensive reagents or sophisticated instruments. Preparing and analyzing a sample takes half an hour and requires very little labor. These methods, which have high sensitivity, are very easy to use. The analysis of antioxidant activity in many samples can be performed quickly and spontaneously [14].

7.1. Evaluation of DPPH Radical Scavenging

DPPH radical scavenging ability is determined mostly in organic solvents such as methanol or ethanol by measuring the absorbance drop at 517 nm [101]. The use of methanol is not preferred because of its toxic properties. Analyses were performed with a UV-vis spectrophotometer in 1 mL or 3 mL cuvettes. For this purpose, a stock solution of 10^{-3} M DPPH radicals in ethanol or methanol was freshly prepared before analysis. To prepare the DPPH solution, 3 mL of the stock solution was diluted to 50 mL with methanol in a volumetric flask and protected from light with aluminum foil. Absorbance values were set to 1.00 ± 0.200. Then, 3 mL of DPPH working solution was transferred to the 0.5 mL extract, mixed, and left in the dark for 30 min. The purple color disappears when an antioxidant agent is present in the reaction medium. A reference sample containing 0.5 mL of solvent was similarly prepared. A newly prepared DPPH radical solution shows maximal absorption at 517 nm. All analyses were carried out in 3 replicates, and absorbance was recorded at 517 nm. The blank is the reaction mixture that does not contain test compounds [1,102].

7.2. Evaluation of DPPH Radical Scavenging as TEAC

Another way to evaluate the results of DPPH's radical-removing ability is to express them as Trolox equivalent antioxidant capacity (TEAC). For this purpose, the radical-removing activity (RSA) of Trolox standard solution at different concentrations is determined. In this assay, Trolox, as a standard radical scavenger compound, is interpolated into a dose–response curve. Then, using these values (%RSA)—(Trolox; μM/L), a calibration curve is prepared. For herbal extracts or chemicals prepared at different concentrations, the TEAC (μM/L) was calculated, and Trolox equivalent antioxidant capacities were calculated using the linear regression equation obtained in the linearity range (0.01–0.05 μM/L) [14].

7.3. The Importance of the IC_{50} Value in DPPH Radical Scavenging Activity

Different antioxidant concentrations are used to determine the antioxidant concentration that scavenges 50% of the initial DPPH radicals in a specific but arbitrary time interval. This concentration was also referred to as "EC_{50}", short for "efficient concentration" or sometimes as "IC_{50}", short for "inhibitory concentration". Indeed, this EC_{50} designation has found appropriate scientific use in drug testing under a different name "LD_{50}". These terms became widely accepted as "IC_{50}" to indicate the practicality of antioxidant testing using DPPH radicals. The lower the IC_{50} values, the higher the DPPH radical-removing ability of the antioxidants. In this context, the IC_{50} value is widely used in biochemistry to compare the radical scavenging capacities of different antioxidants [103]. The IC_{50} values quantitatively describe the radical scavenging affinity. For all these reasons, the IC_{50} value is one of the most practical ways to evaluate DPPH radical scavenging affinities. The radical scavenging activity (RSA) of maca extracts was calculated using the following equation:

$$RSA(\%) = \left[\frac{(Ac - As)}{Ac}\right] \times 100 \text{ or } RSA(\%) = \left[1 - \frac{Ac}{As}\right] \times 100$$

where A_c is the absorbance at 517 nm of the control sample, and A_s is the absorbance at 517 nm that contains the test sample, including plant extracts or pure compounds. The IC_{50} was calculated from the graph plotting scavenging percentage against test sample concentration (µg/mL). DPPH radicals decrease significantly upon exposure to radical remover [104].

7.4. Scope of DPPH Radical Scavenging Applications

DPPH radical scavenging is a popular spectrophotometric method that has a wide application area and is used for determining the antioxidant capacity of beverages, pure substances, foods, and herbal extracts. This method is simple, sensitive, fast, and reproducible, making it the most convenient and common radical removal method for evaluating the antioxidant capacity of compounds and herbal extracts. For this purpose, the IC_{50} values of herbal extracts and pure compounds in recent studies on DPPH radical removal are given in Tables 1 and 2.

Table 1. Half maximal inhibition concentration (IC_{50}, µg/mL) of different antioxidant molecules.

Antioxidants	DPPH• Scavenging (IC_{50}, µg/mL)	References
Curcumin	34.86	[105]
Resveratrol	17.80	[106]
Eugenol	16.06	[107]
Coumestrol	25.95	[108]
Magnofluorine	10.58	[109]
Hederin	69.40	[110]
Hederasaponin	82.40	[110]
Hederacolchiside	73.50	[110]
Hederagenin	28.50	[111]
Morphine	56.82	[112]
Uric acid	17.80	[113]
Caffeic acid	10.64	[114]
Usnic acid	49.50	[109]
Tannic acid	23.65	[115]
Rosmarinic acid	3.07	[116]
L-Carnitine	58.90	[117]
L-Dopa	12.41	[18]
L-Tyrosine	43.86	[18]
L-Adrenaline	30.60	[118]
Propofol	16.23	[26]
Dipropofol	31.29	[24]
Silymarin	20.80	[119]
Cepharanthine	22.20	[43]
Fangchinoline	6.40	[43]
CAPE	3.30	[47]
Taxifoline	77.00	[120]
Cynarine	3.98	[32]
Olivetol	17.77	[121]
Nordihydroguaiaretic acid	6.60	[122]
(-)-Secoisolariciresinol	14.14	[122]
Secoisolariciresinol diglycoside	16.97	[122]
α-(-)-Conidendrin	23.29	[122]
Phillyrin	11.75	[111]
Pinoresinol-β-D-glycoside	19.60	[111]
Pinoresinol di-β-D-glycoside	26.52	[111]
Ligustroside	12.00	[123]
Oleuropein	57.50	[123]
Pelargonin	67.73	[63]
Silychristin	86.16	[63]
Callistephin	20.64	[63]
Oenin	16.72	[63]
Malvin	21.36	[63]
Arachidonoyl dopamine	84.10	[63]

Table 2. Half maximal DPPH radical scavenging concentration (IC_{50}, μg/mL) of water and ethanol extracts of different antioxidant plants.

Antioxidant Plants	DPPH• Scavenging (IC_{50}, μg/mL)		
	Water Extract	Ethanol Extract	References
Anise (*Pimpinella anisum*)	11.74	18.12	[124]
Fennel (*Foeniculum vulgare*)	263.21	343.41	[125]
Sage *(Salvia pilifera)*	30.95	28.92	[126]
Giant fennel (*Ferula orientalis*)	88.60	346.50	[127]
Thyme (*Thymus vulgaris*)	13.40	12.10	[59]
Clove (*Eugenia caryophylata*)	48.39	40.54	[128]
Lavender (*Lavandula stoechas*)	66.67	60.00	[128]
Black pepper (*Piper nigrum*)	68.18	78.13	[129]
Juniper (*Juniperus communis*)	22.27	23.81	[130]
Bay (*Laurus nobilis*)	38.46	32.60	[131]
Basil (*Ocimum basilicum*)	44.64	38.46	[132]
Lemon balm (*Melissa officinalis*)	31.40	202.70	[10]
Cauliflower (*Brassica oleracea*)	29.30	23.21	[133]
Liquorice (*Glycyrrhiza glabra*)	52.2	54.4	[134]
Cherry stem (*Cerasus avium*)	23.38	17.36	[135]
Galanga (*Alpinia officinarum*)	14.75	31.51	[136]
Ginger (*Zingiber offcinale*)	16.20	43.80	[137]
Flaxseed (*Linum usitatissimum*)	53.30	49.50	[138]
Cinnamon (*Cinnamomum verum*)	21.25	15.71	[139]
Pennyroyal (*Mentha pulegium*)	18.52	16.92	[140]
Avocado (*Folium perseae*)	601.00	240.40	[141]
Kınkor (*Ferulago stellata*)	57.80	34.70	[142]
Bindweed (*Convulvulus betonicifolia*)	346.50	77.00	[143]
Mint (*Cyclotrichium leucotrichum*)	28.85	23.74	[144]
Pomegranate (*Punica granatum*)	31.50	16.100	[145]
Achillea pseudoaleppica	25.57	23.24	[146]
Acantholimon caryophyllaceum	69.30	19.80	[147]
Salvia eriophora	9.94	9.21	[148]
Lecokia cretica	78.13	77.32	[149]
Stachys annua	8.90	7.80	[150]
Astragalus alopecurus	115.53	99.02	[151]
Verbascum speciousum	173.25	24.75	[152]
Cyclotrichium niveum	9.17	14.45	[153]
Nettle (*Urtica dioica*)	81.08	-	[154]
Cornelian cherry (*Cornus mas*)	91.77	-	[155]
Kiwifruit (*Actinidia deliciosa*)	83.40	-	[156]
Cranberries (*Vaccinium macrocarpon*)	86.63	-	[157]
Spearmint (*Mentha spicata*)	-	97.82	[158]
Yarrow (*Achillea cucullata*)		132.55	[70]
Sahlep *(Dactylorhiza osmanica*)	-	86.63	[159]

8. Limitations of the DPPH Assay

In DPPH removal activity, it is very important to convert the moles of DPPH lost by using the change in absorbance, Beer's law, and damping ε values of 10,900–12,500. Another important point is that this method does not detect reaction rates and ignores some crucial information in the reaction curves [160]. The reactions of DPPH and antioxidants appear to be complex when changes in absorbance are continuously monitored. Although the reaction curves were similar to $ABTS^{\bullet+}$ removal, there were some differences when comparing the studied phenolic compounds [85]. In DPPH radical scavenging, antioxidants can react with DPPH radicals by very fast electron transfer and slow hydrogen atom transfer. Although electron transfer is quite fast, it is slower than $ABTS^{\bullet+}$'s reactions with antioxidants due to the difficult accessibility of phenolic compounds to the radical site of the DPPH molecule. This barrier of access inhibits all reactions, particularly the hydrogen transfer, which is necessary for the formation of a hydrogen-bonded complex between the

α-C—H radical and the N lone pair required [161,162]. Complex molecules get in the way of each other more easily and block access to DPPH radicals at low concentrations and strongly block the reaction at high concentrations. Additionally, methyl alcohol, which is generally used as a solvent for the DPPH method, strongly binds H atoms and inhibits HAT processes [163–165]. However, when water is added to the reaction, it disrupts the bonding and facilitates hydrogen atom transfer. Any test compound with H-atom transfer capability will increase the reaction rate. In the same manner, electron transfer is pH-dependent, with speed increasing with pH and ionization degree, while HAT is pH-independent. The dominant mechanism of a test compound can be evaluated by reacting it with DPPH radicals in methanol and 50% methanol, where the water phase is buffered to a pH range from acidic to alkaline [85,166,167].

9. Conclusions

Antioxidant compounds play a vital role in reducing oxidative damage caused by ROS. The DPPH radical scavenging ability of the compounds used for this purpose can be extremely valuable for antioxidant profiles. DPPH radical removal is one of the most widely applied and used methods in food and pharmaceutical applications. As a result of the review, it has been reported that the DPPH method gives a better response for mostly phenolic compounds and then for compounds with limited polarity. In the case of polar and phenolic compounds, adding water to the reaction medium, that is, aqueous methanol, gives better results. When testing low-polarity compounds, ethyl acetate with a radical is suitable. All results show that DPPH reaction rates depend on the steric accessibility of the radical site rather than the chemical properties of the tested antioxidant compounds. The rate at which DPPH reacts with antioxidants depends on the varying ratios of mixed SET and HAT mechanisms. The reaction mechanisms of DPPH· scavenging and responses are modified by many environmental and experimental factors.

The interaction of antioxidants with DPPH radicals was less than the total activity of the individual compounds. It clearly shows that radical scavenging by extracts from mixtures is actually suppressed in the DPPH assay. Therefore, the assay does not fully demonstrate potential radical scavenging in cells or food, and steric interferences do not account for all synergisms or antagonisms in the test.

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