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Review

Analysis of Natural Antioxidants Using Electron Paramagnetic Resonance (EPR) Spectroscopy

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Abstract: Electron Paramagnetic Resonance (EPR), also known as Electron Spin Resonance (ESR), is a modern spectroscopic technique that is used for the analysis of materials with unpaired electrons. EPR spectroscopy, focusing on electron spins, uses magnetic fields and microwaves to obtain chemical and structural information about the environment around an unpaired electron, providing detailed information on the structure and bonding of paramagnetic species. This spectroscopic technique has been used in the study of natural products, especially for evaluating free-radical-scavenging properties. EPR spectroscopy is expected to continue providing unique and complementary information about natural products, enhancing insights into their chemical properties, biological activities, and potential therapeutic applications. This review article critically evaluates recently published literature on the application of EPR in the assessment of antioxidant properties of various natural products, by retrieving relevant information from online databases, e.g., Google Scholar, Web of Science and PubMed.

Keywords: Electron Paramagnetic Resonance (EPR); antioxidant; free-radical scavenger; natural products; assay

1. Introduction

Electron Paramagnetic Resonance (EPR) is a spectroscopic technique used to study materials with unpaired electrons, and this technique is also known as Electron Spin Resonance (ESR) spectroscopy [1–3]. It is somewhat analogous to Nuclear Magnetic Resonance (NMR), but it focuses on electron spins rather than nuclear spins. EPR spectroscopy uses magnetic fields and microwaves to obtain chemical and structural information about the environment around an unpaired electron, providing detailed information on the structure and bonding of paramagnetic species [2]. An unpaired electron has a spin and an associated magnetic moment, causing it to behave like a tiny bar magnet. When this unpaired electron is placed in an external magnetic field, it aligns either parallel (lower energy state) or antiparallel (higher energy state) to the direction of the magnetic field, creating two distinct energy levels, known as the Zeeman effect. It can be noted that the Zeeman effect, first observed by Pieter Zeeman in 1896, is the splitting of a spectral line into multiple components when an atom is placed in a static magnetic field. This splitting is caused by the interaction between the external magnetic field and the magnetic dipole moments of the electrons in the atom, which are associated with their orbital motion and spin.

In EPR spectroscopy, the sample is typically exposed to a fixed frequency of microwave radiation, while the magnetic field strength is varied [1–3]. When the energy difference between the two spin states matches the energy of the microwaves, the electrons absorb the radiation and transition to the higher energy state, producing a detectable signal, known as resonance. The resulting spectrum, usually presented as the first derivative of the absorption, provides information about the local environment of the unpaired electron. So, there are three key steps in any EPR spectroscopy operation: placing the sample with unpaired electrons in a magnetic field, exciting the electrons by using microwaves, and causing their spins to transition between energy states, and the detection of

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absorption of microwave energy, providing a spectrum that displays information about unpaired electrons and their environment. Therefore, a typical EPR spectrometer consists of a main magnet to generate a strong magnetic field, a microwave bridge (including a klystron) to produce microwaves, a microwave cavity (known as the sample cavity) to hold the sample, and a detector to measure the absorbed microwave signal. Modulation coils are sometimes added to enhance sensitivity, and the accessories like cryostats allow for low-temperature experiments, a sample holder to hold the sample in the microwave, a temperature control to measure temperature-dependent phenomena, appropriate software and computation to run the experiments and to analyze and process data, and a gradient coil in EPR imaging to create spatial resolution, requiring very stringent designs. A schematic presentation of an EPR spectrometer is shown in Figure 1.

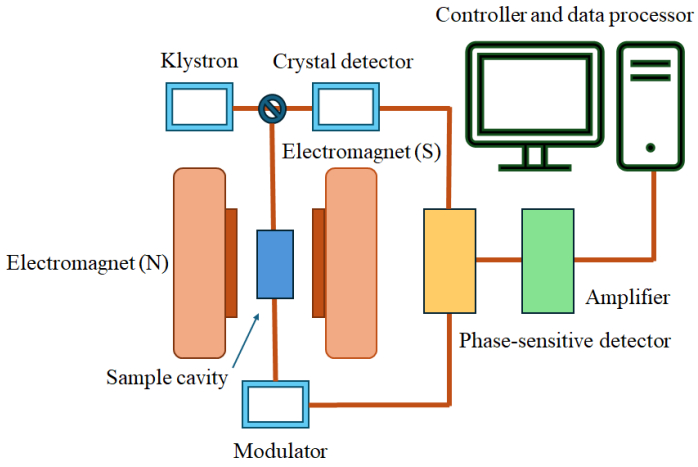

Figure 1. A schematic presentation of an EPR spectrometer.

Valuable information about the paramagnetic species and their surroundings can be obtained from EPR spectra [1–3], including the g-value, which is specific to the electronic structure of the radical or metal ion and its environment; hyperfine coupling, which is the interactions between the unpaired electron and nearby magnetic atomic nuclei that cause the main signal to split into multiple lines, offering structural information; signal intensity, which is proportional to the concentration of the paramagnetic species; and, line shape and width providing insights into molecular motion, polarity, and reaction kinetics. EPR spectroscopy has been extensively used in chemistry to study reaction intermediates (e.g., radicals produced during electrochemistry), in biology to investigate biological processes such as photosynthesis and to detect radicals in biological systems, in medicine to assess oxidative stress and associated diseases, in material science to study structural transformations and charge compensation mechanisms in various materials (e.g., carbon-based electrodes), in medical imaging to obtain images targeting tumour hypoxia, in archaeology and geology to obtain information on dating and dosimetry, in quantum computing to control electron spin qubits in quantum information research, and in natural products research to assess free-radical-scavenging properties of natural products.

Natural antioxidants are compounds, e.g., anthocyanins, carotenoids, flavonoids and other phenolics, found in various natural sources, e.g., plants and microbes, which help protect the body from oxidative stress-related damage, often caused by free-radicals [4]. One of the key mechanisms by which an antioxidant can exert its antioxidant activity is through scavenging of the free-radicals, which are species with one or more unpaired electrons in its outer shell, formed from molecules via the breakage of a chemical bond such that each fragment keeps one electron, by cleavage of a radical to give another radical and, also via redox reactions [5,6]. Examples of free-radicals include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$). Biological free-radicals are highly unstable molecules because of the availability of electrons to react with various organic substrates such as DNA, lipids and proteins [5]. This free-radical-scavenging aspect of natural antioxidants makes EPR spectroscopy a suitable tool for evaluating the antioxidant potential of natural products [7–9]. This is possible by measuring changes in the intensity of the EPR spectrum that results from the interaction of the stable radical, e.g., 1,1-diphenyl-2-picrylhydrazyl (DPPH), with natural antioxidants found in natural matrices. Using EPR spectroscopy, it is possible to determine antioxidant properties either by directly measuring stable radicals [7] or by using spin trapping [8] to stabilize short-lived, reactive radicals for detection. As EPR can identify specific radical types and quantify antioxidant capacity, it has now emerged as a powerful tool for assessing the quality and stability (often affected by oxidative damage) of natural products in cosmetics, food, pharmaceuticals, and other industries.

This review article critically appraises the most recently published literature (January–November 2025) on the application of EPR in assessing antioxidant properties of natural products. The well-recognized online databases like Google Scholar, Web of Science and PubMed were used to retrieve relevant information using the keywords EPR, antioxidant, free-radical-scavenger, natural products and phytochemical. Only the papers that describe the use of EPR spectroscopy in assessing the antioxidant property or free-radical-scavenging property of natural products are included in this review.

2. Analysis of Natural Antioxidants by EPR Spectroscopy

Many natural products, especially various phytochemicals, possess significant antioxidant properties; for example, carotenoids like β-carotene, phenolics like flavonoids (e.g., kaempferol and quercetin), polyphenolics like catechin-derived compounds and tannins (e.g., epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate), are well-known natural antioxidants. Usually, the antioxidant activity of natural products is assessed by various in vitro chemical assays like 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) test, cupric reducing antioxidant power (CUPRAC) test, DPPH scavenging assay, Folin–Ciocalteu test, ferric reducing antioxidant power (FRAP) test, hydroxyl radical antioxidant capacity (HORAC) test, oxygen radical absorption capacity (ORAC) test, total oxyradical scavenging capacity (TOSC) test and total peroxyl radical trapping antioxidant parameter (TRAP) [10,11], but the use of EPR spectroscopy in the measurement of radical-scavenging antioxidant capacity of natural products has become a popular choice over the last few decades because of its simplicity, low cost and environmentally-friendly aspect. The use of EPR spectroscopy in the analysis of antioxidant potential of various natural products, reported most recently, is summarized in Table 1.

Horse Chestnut (*Aesculus hippocastanum* L.) from the family Sapindaceae is an ornamental tree with some medicinal values. The antiradical capacity of non-irradiated and irradiated horse chestnut seed extracts (from peeled seeds and shell seeds) was evaluated by EPR spectroscopy using DPPH stable radicals [12]. The experimental protocol was as follows: the EPR spectra were recorded as a first derivative of the absorption signal on a JEOL JES-FA 100 EPR spectrometer equipped with a standard TE_{011} cylindrical resonator operating in the X-band (9.3 GHz). The measurements were conducted at r.t. The solid samples were placed in a quartz standard EPR tube (i.d. 4 mm) and placed in the EPR sample cavity (Figure 1). Three independent measurements were performed for every solid sample, including a procedure where 'inserting–removing–inserting' of the sample was conducted in the sample cavity of the EPR spectrometer. Before and after each series of measurements under the same conditions, a reference sample, magnetic Mn diluted in MgO (an internal standard for the above-mentioned spectrometer), was analyzed in the same way as those used for the sample measurements, to normalize the signal intensity of the samples and to minimize the error resulting from any instability in the spectrometer. The estimation of DPPH free-radical-scavenging property of the extracts comprised the addition of a specified amount of the extract to a 1 mL 0.002 M DPPH solution (ethanol or acetone) and allowing them to react, after which, the mixture was transferred into a capillary tube, placed inside the sample cavity of the EPR spectrometer. The percent DPPH radical-scavenging activity of the extracts was determined using the following equation: scavenged DPPH radicals (%) = $[(I_0 - I)/I_0] \times 100$, where "$I_0$" was the intensity of the second DPPH peak of the control samples. The same procedure was used to prepare the DPPH control, which did not contain any extract sample. The "I" was the intensity of the second peak of the same EPR spectrum after the addition of the measured extract. Trolox was used as the positive control. The EPR spectrum exhibited a typical stable signal for irradiated cellulose-containing substances, with the samples irradiated with a dose of 1 kGy, 45 d after treatment, whereas those irradiated with 5 and 10 kGy, showed a signal even 250 d later. The free radical-scavenging activity increased in shell seeds, while it decreased in peeled seed extracts after irradiation, depending on the dosage, which corresponded to the total phenolic content.

Astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione) (Figure 2), a carotenoid, is a red pigment found naturally in microalgae, phytoplankton, salmon and lobster, and is well known for its strong antioxidant property, because of which this compound is linked to various health benefits, including heart and brain health, and protection against UV damage [13,14]. The interaction of astaxanthin with iron salts Fe^{2+} and Fe^{3+} in organic solvents was studied by EPR spectroscopy [13]. The EPR spectra were obtained at r.t. on a Varian E-4 X-band spectrometer (9.15 GHz), which was coupled to a personal computer. A strong pitch sample was used as the standard. A solution of Fe^{3+} in methanol was added to the astaxanthin solution (30 μM) to a final concentration of 0.05 μM (methanol content 0.1%). Samples (*ca.* 200 μL) were placed in a quartz ampule (i.d. 4 mm). Measurements were conducted at a microwave power of 2 to 10 mW using a modulation amplitude of 10 G. The samples were incubated for 3 d and the EPR spectrum was recorded periodically. The Origin 2016 software package was used for the Data analysis, graphing, and spectral processing. The appearance of Fe^{3+}, astaxanthin oxidation and degradation occurred in methanol and dichloromethane. The formation of an intermediate product with an absorption maximum at 876 nm

was recorded spectrophotometrically. EPR spectroscopy identified the radical nature of the intermediate product. The radical product, formed in the reaction with Fe^{3+}, was assumed to be the radical cation of astaxanthin. At the same time, no changes were detected when astaxanthin was incubated with Fe^{2+}. An EPR-assisted assessment of antioxidant potential and oxidative stress modulation of the oil extract of *Geranium macrorrhizum* L. (family: Geraniaceae) in gentamicin-induced nephrotoxicity has recently been reported [15]. To assess the renal antioxidant status, the activities of specific antioxidant enzymes, indicators of lipid and DNA peroxidation and renal functional damage were assessed using standard commercial kits, ELISA (enzyme-linked immunosorbent assay) and EPR spectroscopy, conducted at r.t. using a Bruker BioSpin GmbH, equipped with a standard resonator. The EPR analysis was conducted utilizing WIN-EPR SimFonia 1.2/6130860 software, version V2.00Rev.03 (2017). Spectral processing was accomplished using Bruker WIN-EPR SimFonia 1.2/6130860 software after double integration; the results were presented in arbitrary units. The analysis of the results was carried out with Statistica 8.0 software, and the results were displayed as the mean, including the standard error as a range. All EPR analyses were conducted using fivefold measurement in the recorded spectra with the following characteristics: 3503–3515 G center field; 6.42–20.00 mW microwave power; 5–10 G modulation per sample and 1–5 scans per sample. This study demonstrated that *G. macrorrhizum* oil could serve as a potent natural complex antioxidant and anti-inflammatory agent suitable for complementary therapy against gentamicin-induced nephrotoxicity.

Green tea polyphenols, e.g., epigallocatechin gallate (EGCg) (Figure 1), are potent natural antioxidants [16,17]. The bioactivities of plant polyphenols include their inclination to tightly bind protein, to function as antioxidants and to bind metals. Fu et al. [17] studied the binding or reaction of EGCg with Cu(II)-serum albumin using a combination of fluorescence, UV-Vis and EPR spectroscopy, while green tea extract and/or ethylenediaminetetraacetic acid (EDTA) were incorporated into the expeller-pressed high oleic soybean oil oleogel and their antioxidative activity on the oleogel oxidation was investigated by EPR spectroscopy [18]. In the EGCg and Cu(II)-serum albumin binding study [17], the EPR data were collected in MOPS [3-(*N*-morpholino)propanesulfonic acid)], a buffer that quenches the EPR signal of Cu(II)-aquo complexes. The BSA (bovine serum albumin) stock solution (2.4 mM) was prepared in 200 mM MOPs buffer at pH 7.4. Stock solutions (40 mM) of EGCg and $CuCl_2$ were prepared in nanopore water. MOPs containing 13.5% glycerol were prepared by adding the desired amount of glycerol to 200 mM MOPs buffer. The solution of histidine-chelated copper (Cu(II)-His_2) contained 40 mM $CuCl_2$ dissolved in 80 mM L-histidine-HCl. The reaction mixtures containing 0.5 mM BSA, 10% glycerol, and up to three equivalents of copper relative to BSA were prepared by mixing the BSA stock solution with glycerol-MOPs, adding the required amount of $CuCl_2$ stock solution, followed by diluting to 1.0 mL with MOPs buffer. The desired amount of EGCg stock solution was added to BSA or the BSA–copper complex; the sample was transferred to a 4 mm EPR tube to take the EPR spectrum at 5 K within 4 h. The EPR experiments were performed on a Bruker ELEXSYS E580 X- and Q-Band EPR spectrometer, the attenuation was adjusted to 11 dB (11 mW), modulation amplitude to 7.2 G, receiver gain to 60 dB, time constant to 40.90, conversion time 40.90, sweep time 41.94, centre field 2600 G and sweep width 5000 G, 10 scans. EPR spectra were simulated using Easyspin v. 5.2.35 and MATLAB R2021a. The pepper function of EasySpin was used to fit all spectra obtained in water/glycerol mixtures at 5 K. In the EPR experiment involving green tea extract and/or EDTA incorporated into the expeller-pressed high oleic soybean oil oleogel and assessment of their antioxidative activity on the oleogel oxidation [18], an EPR spectrometer fitted with an ER 4122SGHQ resonator was used to track the formation of free-radicals in the oleogel during the storage study. In this study, *N*-*tert*-butyl-α-phenylnitrone (PBN) was used as the spin trapper and added to the oleogel samples at 1.0% during the final 5 min of the heating process at the time of the preparation of oleogel to avoid overheating of PBN. The PBN-infused oleogel was transferred into a sealed test tube and incubated at 50 °C for 30 d, and free-radicals were measured every five days using EPR. Before testing, the oleogels were taken out of the incubator and heated in a water bath (70 °C) for 20 min to facilitate the formation of spin adducts. The oleogel samples were added to the EPR spectroscopy using a micropipette. The oleogel was screened using the following parameters: center field 3500 G, sweep width 200 G, sweep time 83.89 s, microwave frequency 9.83 GHz, microwave power 5 mW, modulation amplitude 1.0 G, modulation frequency 100 kHz, and two accumulated scans. The quantification of free-radicals was achieved by double integration of the peak area obtained from the examination of the PBN adduct formed in oleogel using EPR. Any signals with a signal-to-noise ratio below 10 were recorded as 0. Infusions of Ginkgo biloba, ginseng, Yerba Mate, and green tea (useful in the treatment of Alzheimer's disease) [19] were studied for their free-radical-scavenging properties using X-band (microwave radiation frequency of 9.3 GHz) EPR spectroscopy and UV-Vis spectrophotometry. Yerba Mate appeared to be the strongest antioxidant extract. The model DPPH free-radicals were used and the magnitude and changes with time of EPR and UV-Vis spectra of DPPH by the tested extracts were measured. It was observed that EPR and UV-Vis lines of DPPH free-radicals decreased with increasing time of interactions of the extracts with DPPH, and after reaching the minimum value,

they did not change with time. In this experiment, EPR spectra of DPPH stable free-radicals were recorded with a microwave power of 2.2 mW using an EPR spectrometer from Radiopan and a numerical data acquisition system, which was the Rapid Scan Unit from Jagmar. The magnetic field modulation was 100 kHz; sweep time 0.1 s, sweep range 10 mT, and field modulation amplitude 0.1 mT, were used. An MCM 101 microwave radiation frequency meter and an NMR magnetometer, from EPRAD, were used.

Naturally occurring flavonoids are well known for their significant antioxidant (free-radical-scavenging) property [20]. A direct kinetic EPR spectroscopic method with pulse injection of reagents was used to determine the antiradical [scavenging of *tert*-butyl peroxyl radical (*tert*-butylOO·] activity of three flavonoids morin, quercetin and rutin, measured at −32 °C and compared with the results obtained using the widely used oxygen radical absorbance capacity (ORAC) method. Trolox was used as the positive control. The kinetic EPR method with pulse reagent injection allowed effective and highly accurate determination of the antiperoxyl radical capacities of these flavonoids with multiple active centers [21]. An EPR-B spectrometer operating at a frequency of 9.468 GHz with a modulation frequency of 100 kHz, with additional EPR instrument settings of microwave power at 3.2 mW, modulation amplitude at 2 G and time constant at 41 ms, was used. Similarly, a concentration-dependent radical-scavenging activity of alfaalfa flavonoids was determined by EPR spectroscopy, where these flavonoids significantly reduced EPR signals for HO center dot and O_2 center dot- by 42% and 54%, respectively [22]. Similarly, the antioxidant effect of the flavonoid acacetin was determined by EPR spectroscopy [23], revealing that the antioxidant effect, DNA-binding, and transport of acacetin (Figure 2) were influenced by the presence of redox-active Cu(II) ion. The equimolar addition of acacetin to the model Fenton-like system containing Cu(II) did not have a noticeable effect on the concentration of hydroxyl radicals produced, but in excess, the formation of (OH)-O-center dot decreased, as proved by EPR spin trapping.

Assessment of the antioxidant property of melanin purified from the fungus *Scolecobasidium musae* was performed by EPR spectroscopy [24]. It can be noted that melanin is a natural pigment that gives colour to skin, hair, and eyes, and its primary function is to protect the body from harmful ultraviolet (UV) radiation [14,25]. Produced by specialized cells called melanocytes, the amount and type of melanin determine an individual's pigmentation, with higher concentrations leading to darker shades. It also plays a role in other bodily functions, such as immune response and inflammation reduction.

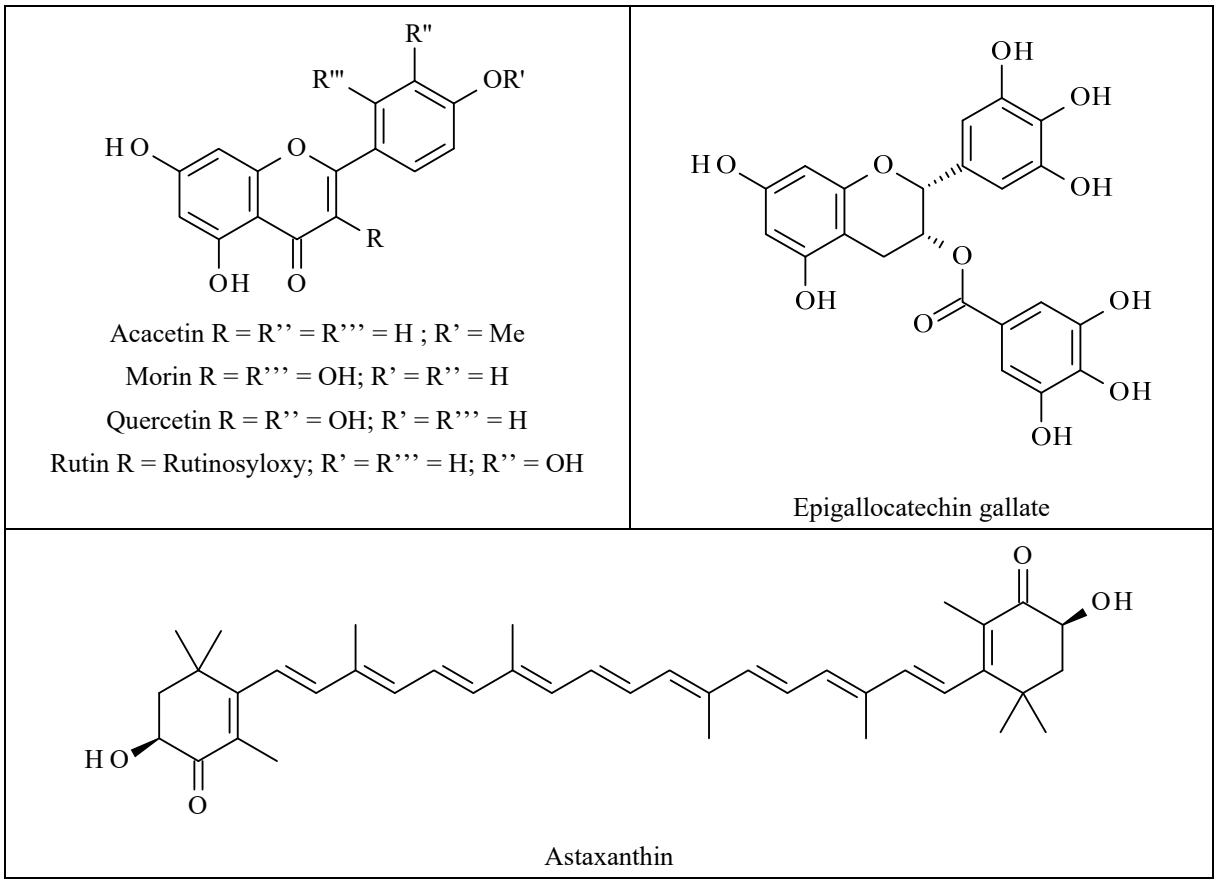

Figure 2. Examples of some natural antioxidants analyzed by EPR spectroscopy.

The effect of pasteurization on the antioxidant property of pineapple juice [*Ananas comosus* (L.) Merr.] processed in an inert atmosphere was evaluated by EPR spectroscopy [26]. Before the experiments, solid material from the pineapple juices was removed using a centrifuge operating at 10,000× *g* and 5 °C for 10 min. Supernatants were stored at 7 ± 1 °C in the dark between the experiments. All EPR experiments were performed in triplicate using a portable Bruker X-band EPR spectrometer e-scan with accessories. The ability of pineapple juices to scavenge $ABTS^{\bullet+}$ [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation] and TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) free-radicals was investigated. Trolox was used as a positive control. It was observed that total phenolic content expressed as gallic acid equivalent decreased, albeit not significantly, after pasteurization and during storage.

The antioxidant and redox-modulatory activities of the hydroethanolic extract of prokupac grape pomace were evaluated through EPR spectroscopy, where a strong scavenging activity against superoxide anion and hydroxyl radicals, with inhibition efficiencies of 84.37% and 81.81%, respectively, was observed, while the activity against the DPPH radical was lower (17.75%) [27]. In this experiment, 1 μL of extract (resuspended in 50% EtOH, 1 mg/mL) was added to 29 μL of a 210 μM DPPH solution in ethanol. After a 2 min incubation, the EPR signal was recorded using a Bruker ELEXSYS-II E540 spectrometer operating in the X-band range, with the following parameters: magnetic field centre at 3500 G, microwave power 10 mW, microwave frequency 9.85 GHz, modulation frequency 100 kHz, and modulation amplitude 1 G. A control measurement was performed by adding the same volume of pure solvent instead of the extract. The antioxidant activity (AA) of the extract was calculated using the following equation: $AA = (Ic - Ia)Ic \cdot 100\%$, where Ic is the double integral value of the control spectrum, and Ia is the corresponding value for the extract-treated sample, both derived from the EPR signal. Similarly, EPR spectroscopy was employed in assessing the antioxidant activity of the ultrasound-assisted extraction product of prokupac grape skins [28].

Quan et al. [29] conducted an EPR-driven assessment of the antioxidant activity of the herbal medicine *Pyrrosia lingua* (Thunb.) Farw., which is a traditional Chinese herbal medicine with the characteristics of clearing heat and draining dampness, is commonly used in the clinical treatment of kidney stones, urinary infections, prostate cancer, diabetes and other diseases, by measuring its scavenging activity against hydroxyl radicals. Using the DPPH radical as a scavenging model, the *P. lingua* extract (0.6 mg/mL) scavenged 95.1% of 0.5 mmol/L DPPH radicals, showing a quantitative linear relationship between the DPPH radical concentration and the double integral area under the characteristic peaks of the EPR spectra, with a coefficient of determination $R^2 = 0.9986$. After heating 100 g of the plant material in 2 L of deionized water for 2 h at 90 °C, the aqueous phase was collected and then concentrated using a rotary evaporator to obtain approximately 6.5 g of extract. A glass capillary tube (i.d. 0.9–1.0 mm) containing the sample (50 mL) was sealed with rubber putty and placed into a standard EPR quartz tube (inner diameter: 4 mm). The tube was then placed in the resonance cavity (sample cavity) of the EPR spectrometer. The settings used for EPR spectroscopy were as follows: central magnetic field, 337.70 mT; sweep width, 15 mT; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.2 mT; sweep time, 60 s; and test temperature, 25 °C. The standard curve of the DPPH concentration versus EPR signal intensity was constructed as follows: DPPH was serially diluted in ethanol to prepare standard samples with a concentration range of 0.025 to 1.00 mmol/L (mM), which were analyzed by EPR spectroscopy. The area under the characteristic peaks of DPPH at different concentrations was calculated by double integration of the corrected EPR spectra using Bruker ESRStudio (version 1.90.1) software and used to construct the standard curve of DPPH concentration against EPR signal intensity. A similar process was followed for constructing the standard curve of the DMPO-OH· concentration against EPR signal intensity for the hydroxyl radical (•OH) scavenging assay using EPR. The double integral of the characteristic peak in EPR spectra displayed a linear and quantitative relationship with the concentration of DPPH radicals and the concentration of the DMPO-OH• radical was quantitatively related to the amplitude of the characteristic peaks in the EPR spectra [29].

Royal jelly is a gelatinous substance secreted by worker bees to feed larvae and exclusively the queen bee [30]. It contains considerable amounts of amino acids, fatty acids, proteins, and vitamins, and is used in traditional medicine, dietary supplements, and beauty products. The effects of gamma radiation on free-radicals, antioxidant activity, phenolic and flavonoid content in royal jelly were investigated by EPR spectroscopy [31]. Free-radical generation during irradiation, their stability and free-radical-scavenging activity were determined by EPR. EPR kinetic study of the stability of gamma-induced free radicals in royal jelly showed exponential decay with time and identification of gamma sterilization was possible for 5 months after irradiation. In this study, non-irradiated and irradiated by 1, 5 and 10 kGy royal jelly samples (1 g) were suspended in distilled water (10 mL). The suspensions were extracted for 2 h at r.t. without air access on a magnetic stirrer. After filtration, supernatants were used in the EPR experiments. The EPR spectra were obtained at r.t. as a first derivative of the absorption signal on JEOL JES-FA 100 EPR spectrometer, operating in the X–band, and equipped with a standard TE011 cylindrical

resonator. The samples were put in quartz tubes and were fixed in the cavity center. The effect of irradiation treatment of royal jelly samples on free radical scavenging activity was estimated using DPPH. The experiment was performed by adding a portion (0.2, 0.25, 0.3, 0.35 and 0.4 mL) of the royal jelly extracts to a 1 mL 0.002 M DPPH ethanol solution. After approximately 50 min of incubation of the royal jelly mixture, it was transferred into a capillary tube, which was located inside a standard EPR quartz tube, and was placed in the EPR cavity. The experiment was repeated at least three times for each sample. The percentage of DPPH radicals scavenged was calculated using the equation shown earlier. Trolox was used as the positive control.

Sambucus nigra-lyophilized fruit extract was assessed for its antioxidant activity using EPR spectroscopy [32]. The reactive oxygen species (ROS) and reactive nitrogen species (RNS) levels, as well as 5-MSL-protein oxidation, after *Sambucus nigra*-lyophilized fruit extract treatment, were measured by EPR spectroscopy. The antioxidant-protective effect of *S. nigra* extract (120 mg/kg) was evident from the reduced amounts of malondialdehyde (MDA), ROS, and RNS and increased activation of endogenous enzymes. Similarly, the resistance to free-radical formation at elevated temperatures, defining the oxidative stability of tomato (*Solanum lycopersicum* L.) seed oil, was investigated by EPR spectroscopy and DSC (differential scanning calorimetry) [33]. This study provided useful information about the composition and antioxidant properties of tomato seed oil, which is crucial for future industrial production and consumption.

EPR spectroscopic studies involving natural antioxidants from terrestrial and marine animals, plants and microbes, published recently (Table 1), have demonstrated the feasibility and compatibility of this technique with other in vitro antioxidant assays, particularly free-radical-scavenging assays. However, one should bear in mind some limitations of EPR spectroscopic methods, including their inability to deal with instability, low concentration and the short lifespan of many free-radicals, the need for exogenous probes, the production of artefacts when using spin traps and challenges with quantification and interpretation in complex environments, albeit these are particularly pertinent to experiments conducted in biological systems.

Table 1. Natural antioxidants assessed by EPR spectroscopy (2021–2025).

Natural Antioxidants	Description of Work	References
Aesculus hippocastanum L. seeds	EPR-spectroscopy-based evaluation of antiradical capacity of Horse Chestnut (*Aesculus hippocastanum* L.) seeds using DPPH stable radicals. The EPR spectrum showed a typical stable signal for irradiated cellulose-containing substances. It was observed with the samples irradiated with a dose of 1 kGy, 45 d after treatment, whereas for samples irradiated by 5 and 10 kGy, it was even found 250 d later. The free radical-scavenging activity increased in shell seeds, while it decreased in peeled seed extracts after irradiation, depending on the dosage, which corresponds to the total phenolic content.	[12]
Astaxanthin	The presence of Fe^{3+}, astaxanthin oxidation and degradation occurred in methanol and dichloromethane. The formation of an intermediate product with an absorption maximum at 876 nm was recorded spectrophotometrically. EPR spectroscopy proved the radical nature of the intermediate product. The radical product formed in the reaction with Fe^{3+} was assumed to be the radical cation of astaxanthin. At the same time, no changes were detected when astaxanthin was incubated with Fe^{2+}.	[13]
Geranium macrorrhizum L. oil	EPR-assisted assessment of antioxidant potential and oxidative stress modulation of *Geranium macrorrhizum* L. oil extract in gentamicin-induced nephrotoxicity. To assess the renal antioxidant status, the activities of specific antioxidant enzymes, indicators of lipid and DNA peroxidation and renal functional damage were examined using standard commercial kits, ELISA and EPR spectroscopy.	[15]
Green tea polyphenol	The binding or reaction of EGCg with Cu(II)-serum albumin was studied using fluorescence, UV-Vis and EPR spectroscopy.	[17]
	Green tea extract and/or ethylenediaminetetraacetic acid (EDTA) were incorporated into the expeller-pressed high oleic soybean oil oleogel and their antioxidative activity on the oleogel oxidation was investigated by EPR spectroscopy.	[18]
Infusions of Ginkgo biloba, ginseng, Yerba Mate, and green tea	EPR spectroscopy was used to determine the scavenging of free radicals by these plant extracts that are useful in the treatment of Alzheimer's disease. Yerba Mate was found to be the extract that displayed the strongest antioxidant properties.	[19]

Table 1. *Cont.*

Natural Antioxidants	Description of Work	References
Flavonoids	A direct kinetic EPR spectroscopic method with pulse injection of reagents was used to determine the antiradical activity of morin, quercetin and rutin, measured at −32 °C and compared with the results obtained using the widely used oxygen radical absorbance capacity (ORAC) method. The kinetic EPR method with pulse reagent injection allowed effective and highly accurate determination of the anti-peroxyl radical capacities of these flavonoids with multiple active centers.	[21]
	A concentration-dependent radical-scavenging activity of alfaalfa flavonoids was determined by EPR spectroscopy, where these flavonoids significantly reduced EPR signals for HO center dot and O_2 center dot- by 42% and 54%, respectively.	[22]
	The antioxidant effect of the flavonoid acacetin was assessed by EPR spectroscopy. It was found that the antioxidant effect, DNA-binding, and transport of acacetin were influenced by the presence of redox-active Cu(II) ion. The equimolar addition of acacetin to the model Fenton-like system containing Cu(II) did not have a noticeable effect on the concentration of hydroxyl radicals produced, but in excess, the formation of (OH)-O-center dot decreased, as proved by EPR spin trapping.	[23]
Melanin	Assessment of antioxidant property of melanin purified from the fungus *Scolecobasidium musae* by EPR spectroscopy.	[24]
Pineapple juice	The effect of pasteurization on the antioxidant property of pineapple juice processed in an inert atmosphere was evaluated by EPR spectroscopy.	[26]
Prokupac grape pomace	The antioxidant and redox-modulatory activities of the hydroethanolic extract of prokupac grape pomace were evaluated through EPR spectroscopy, where a strong scavenging activity against superoxide anion (O_2 center dot-) and hydroxyl radicals (center dot OH), with inhibition efficiencies of 84.37% and 81.81%, respectively, was observed, while the activity against the DPPH radical was lower (17.75%).	[27]
Prokupac grape skins	EPR-based evaluation of the antioxidant activity of ultrasound-assisted extraction product of prokupac grape skins. Polyphenolic compounds in the extract were responsible for the activity.	[28]
Pyrrosia lingua (Thunb.) Farw.	EPR-driven assessment of antioxidant activity of herbal medicine *Pyrrosia lingua* (Thunb.) Farw. by measuring its scavenging activity against (OH)-O-center dot radicals. Using the DPPH radical as a scavenging model, the *P. lingua* extract (0.6 mg/mL) scavenged 95.1% of 0.5 mmol/L DPPH radicals, showing a quantitative linear relationship between the DPPH radical concentration and the double integral area under the characteristic peaks of the EPR spectra, with a coefficient of determination R2 = 0.9986.	[29]
Royal jelly	The effects of gamma radiation on free radicals, antioxidant activity, phenolic and flavonoid content in royal jelly were investigated by EPR spectroscopy. Free radical generation during irradiation, their stability and free-radical-scavenging activity were determined by EPR. EPR kinetic study of the stability of gamma-induced free radicals in royal jelly showed exponential decay with time and identification of gamma sterilization was possible for 5 months after irradiation.	[31]
Sambucus nigra-lyophilized fruit extract	The reactive oxygen species (ROS) and reactive nitrogen species (RNS) levels, as well as 5-MSL-protein oxidation, after *Sambucus nigra*-lyophilized fruit extract treatment, were measured by EPR spectroscopy. The antioxidant-protective effect of *S. nigra* extract (120 mg/kg) was evident from the reduced malondialdehyde (MDA), ROS, and RNS and increased activation of endogenous enzymes.	[32]
Tomato seed oil	The resistance to free-radical formation at elevated temperatures, defining the oxidative stability of tomato seed oil, was investigated by EPR spectroscopy and DSC (differential scanning calorimetry). This study provided useful information about the composition and antioxidant properties of tomato seed oil, which is crucial for future industrial production and consumption.	[33]

3. Future Perspectives

EPR spectroscopy has significant prospects in natural products research, primarily for the detection and study of free-radicals and paramagnetic metal ions in natural compounds and biological systems [34]. As demonstrated above, many natural products, particularly polyphenols found in fruits and vegetables, exhibit potent antioxidant properties, which can be assessed by EPR spectroscopy, a sensitive and specific method for direct detection and quantification of the free-radicals involved in the mechanisms by which these natural products scavenge ROS and RNS. EPR spectroscopy can help elucidate the molecular mechanisms of natural products that operate via radical pathways. For example, it has been used to study how certain natural products generate ROS as part of an anticancer or antibacterial strategy, or how they protect against oxidative stress in inflammation and infection.

In addition to work related to antioxidant natural products, advanced EPR techniques, such as pulsed EPR [35], can provide detailed structural and dynamic information about the environment of spin-labelled natural products or intrinsic paramagnetic centers within complex biological matrices like cell membranes. It can be noted that pulsed EPR can be defined as a technique that uses short pulse lengths to measure electron spin relaxation times, allowing access to information about weakly coupled nuclei, weakly coupled electron spins, and spin dynamics, which are not obtainable through continuous wave methods.

EPR can play a pivotal role in the phytofood industry, for example, micro-ESR (EPR) [36] is used for testing free-radicals in oils and fats to avoid rancidity, ensuring the quality and shelf-life of products derived from natural sources. Micro-ESR (EPR) refers to a class of compact, portable, and user-friendly spectroscopic instruments designed for studying paramagnetic materials, containing unpaired electrons; these smaller spectrometers are often used in educational settings for teaching purposes, and in industrial and research applications such as analyzing the stability of lubricants or the shelf life of beer, with some being used for continuous, real-time monitoring.

Recent advances in low-field and in vivo EPR [37,38] technology allow researchers to study free-radicals directly within living organisms or tissues, enabling the mapping of oxidative stress distribution in disease states and the monitoring of drug delivery systems based on natural products. Natural products can serve as building blocks or photosensitizers for nanomedicines. EPR spectroscopy is used to characterize these nanocarriers, evaluate drug localization, and monitor the generation of singlet oxygen or other radicals within tumor cells, particularly for applications utilizing the enhanced permeability and retention effect [39] in cancer therapy. EPR can help understand the complex metabolic and biosynthetic pathways of secondary metabolites in plants that involve a paramagnetic intermediate, which is a short-lived, high-energy molecule formed during a chemical reaction that contains one or more unpaired electrons, making it paramagnetic [40,41].

In summary, the prospects of EPR spectroscopy in natural products research will primarily focus on advanced applications in antioxidant evaluation, drug mechanism elucidation, and quality control through the use of advanced instrumentation and new methodologies. The current trend of using EPR for assessing the free-radical-scavenging property of natural extracts will continue and be refined. Direct and specific detection of free-radicals by EPR spectroscopy makes it the best-suited technique for such research, offering high accuracy even with opaque or colored samples. Advances in the development of new EPR probes with narrow line widths will address the sensitivity limitations, making in vivo EPR imaging more practical for monitoring the distribution and behaviour of natural product-based drug delivery systems within living organisms.

4. Conclusions

Electron Paramagnetic Resonance (EPR) is a spectroscopic technique used to study materials with unpaired electrons, and has been proven to be a cost-effective, robust, user-friendly and environmentally friendly green technique for the study of natural antioxidants, which are free-radical-scavengers. With the recent technological developments and remarkable advances in computation techniques, EPR technology has evolved over the years to become more affordable and versatile, making it a method of choice for relevant natural products studies. EPR spectroscopy is expected to continue to provide unique and complementary information about natural products, enhancing insights into their chemical properties, biological activities, and potential therapeutic applications. The versatility and unique capabilities of EPR spectroscopy for directly studying paramagnetic species make it a powerful and evolving tool in the future of natural products research.

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Use of AI and AI-Assisted Technologies

AI or AI-assisted technologies have not been used.

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