Journal of Natural Products Discovery

ISSN 2755-1997, 2022, Volume 1, Issue 3

Review

APPLICATIONS OF ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY IN NATURAL PRODUCT RESEARCH

Alistair J. Fielding[1]

1. Centre for Natural Product Discovery (CNPD), School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, James Parsons Building, Byrom Street, Liverpool L3 3AF, United Kingdom.

D.O.I. 10.24377/jnpd.article725

Received 2022-09-20

Accepted 2022-12-22

Published 2022-12-30

Keywords:
Electron paramagnetic resonance

Spectroscopy,

Natural products

Author's Main Contact:

a.j.fielding@ljmu.ac.uk

©2022 by the authors.
Licensee Liverpool John Moores Open Access, Liverpool, United Kingdom.
This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution.

Abstract

Background: Applications of electron paramagnetic resonance (EPR) spectroscopy to natural product research have proven useful to understand the properties of natural product extracts and their intrinsic compounds. There are few reviews on this subject written as an initial introduction.
Aims: To provide an explanatory review of EPR spectroscopy applied to natural products research.
Methods: The search for this review was carried out in the following databases: SciFinder, Google Scholar, PubMed, MEDLINE, and Science Direct, with particular emphasis on publications from the last decade.
Results: The articles chosen typically used EPR to provide qualitative and quantitative data of radicals or the action of radicals in natural product research. Studies include the measurement of radical scavenging capacity, singlet oxygen detection and direct detection of semiquinone radicals in extracts. Other applications include the study of autooxidation, and photochemical properties.
Conclusion: The current trend for use of EPR in natural products research is by far in radical scavenging capacity. A fast array of experimental methods and reaction chemistry are available to tailor for biological context, which is a great advantage of the versatility of EPR. Previous ground-breaking use in detection of semiquinone radicals in extracts has waned, however, given the prevalence for quinones in plants, and cheaper bench top EPR instruments, there must be an opportunity for this to progress further. The current generation of EPR instruments offers new opportunities to probe natural product molecules and extracts with greater resolution.

INTRODUCTION

Electron paramagnetic resonance (EPR), also called electron spin resonance (ESR), spectroscopy has been used in natural product research since the 1960s (Rex, 1960) and, although not widespread, has provided insightful characterization on many types of samples. EPR spectroscopy is the study of species containing unpaired electrons by observing the magnetic fields at which they come into resonance on application of electromagnetic radiation. In this review, recent applications of EPR in natural product research will be covered. The focus is the EPR experiment and how it has been applied and what has been learnt, as a result the review is restricted to certain examples rather than a complete survey of publications. The review is particularly aimed at the natural products community and the general reader.

A previous review by Ahmed *et al.* has given a brief overview of the use of continuous-wave (CW) EPR in natural product free radical research (Ahmed *et al.*, 2020). There are also reviews of the EPR spectroscopy of various classes of natural product compound such as phenolic compounds (Bors *et al.*, 1998) and quinones and quinols (Pedersen, 2002). The range of the compounds considered to be "natural products" is broad and in this review it is restricted to extracts and isolated compounds. It will not include the vast array of EPR literature on the photosystems (Britt *et al.*, 2004; Un *et al.*, 2001), irradiated food (Aleksieva *et al.*, 2018), seeds (Barbana *et al.*, 2013), shells (Kavetskyy *et al.*, 2022), and clays (Götze *et al.*, 2002).

A brief description of EPR theory will be given in brief, aimed at the non-specialist, as well as direction towards further reading material. Through EPR spectroscopy, information such as the gross chemical structure and the detailed conformation of a radical can be obtained and unpaired electron populations at various positions in delocalised radicals may also be deduced. By measuring relative and absolute radical concentrations and by determining radical lifetimes, it is possible to obtain detailed information concerning the reaction mechanisms of free radicals. The sensitivity of the technique is such that radicals in the nM-μM concentration range are routinely detected, although signal strength is highly dependent on spectral width and temperature.

Principles of EPR spectroscopy

The isolated electron has an intrinsic "spin" angular momentum and, because it is also a charged particle, it possesses a magnetic moment. When placed in an externally-applied magnetic field there are two observable spin states it can adopt. The electronic magnetic moment can be aligned parallel (m_s = -1/2, the β spin state) or antiparallel (m_s = +1/2, the α spin state) to the applied field and the difference in energy between these two states is given by Eqn. 1, in which *g* is a proportionality constant (known as the *g* value),

$$\Delta E = g\mu_B B \qquad \textbf{(Eqn. 1)}$$

μ_B is the Bohr magneton, and *B* is the strength of the applied magnetic field (or, more precisely, the magnetic flux density) (see Figure 1). Initially, there is a Boltzmann distribution of unpaired electrons between the two spin states, with more electrons in the lower energy level (m_s = -1/2). The ratio of the numbers of electrons in the two energy states at thermal equilibrium is given by Eqn. 2, in which N_β

$$N_\alpha/N_\beta = \exp(-\Delta E/kT) \qquad \textbf{(Eqn. 2)}$$

and N_α are the numbers in the lower and higher energy states, respectively, and *k* is Boltzmann's constant. On supplying the sample of radicals with electromagnetic radiation at a fixed frequency υ, resonance will occur at an applied magnetic field *B* such that the resonance condition (Eqn. 3) is satisfied.

$$h\upsilon = g\mu_B B \qquad \textbf{(Eqn. 3)}$$

The Boltzmann factor (N_α/N_β) and the lifetimes of transient radicals both increase with decreasing temperature and so, in general, lower temperatures lead to stronger EPR signals. The act of observing an EPR spectrum tends to equalise the populations of the two spin states and could rapidly lead to saturation (i.e. $N_\beta = N_\alpha$) and cause loss of the signal. However, various mechanisms of relaxation exist which tend to restore the system to Boltzmann equilibrium and permit the EPR spectrum to be observed continuously. In practice, an EPR spectrometer uses a magnetic field of approximately 330 mT (3300 G) and the corresponding value of υ is *ca*. 9.2 GHz, in the X band microwave region of the electromagnetic spectrum. It is usually arranged so that the frequency is kept constant, and the magnetic field is varied to achieve resonance. This is called the continuous-wave (CW) experiment. The applied field is modulated by

application of a small magnetic field oscillating at 100 kHz; the amplitude of this modulation field is generally 0.5-4 G. The output from the detector is thus also oscillating at the modulation frequency and this output is applied to a phase-sensitive amplifier that only amplifies signals oscillating in-phase with and at the same frequency as the modulation field. If the modulation amplitude is small in comparison with the linewidth, the output from the phase-sensitive amplifier will be the first-derivative of the normal output with respect to applied field. The net effect of field modulation coupled with phase-sensitive detection is noise reduction and enhanced resolution. Multiple scans are typically used to improve signal-to-noise. Conditions are often adjusted so that spectra can be recorded in a matter of minutes.

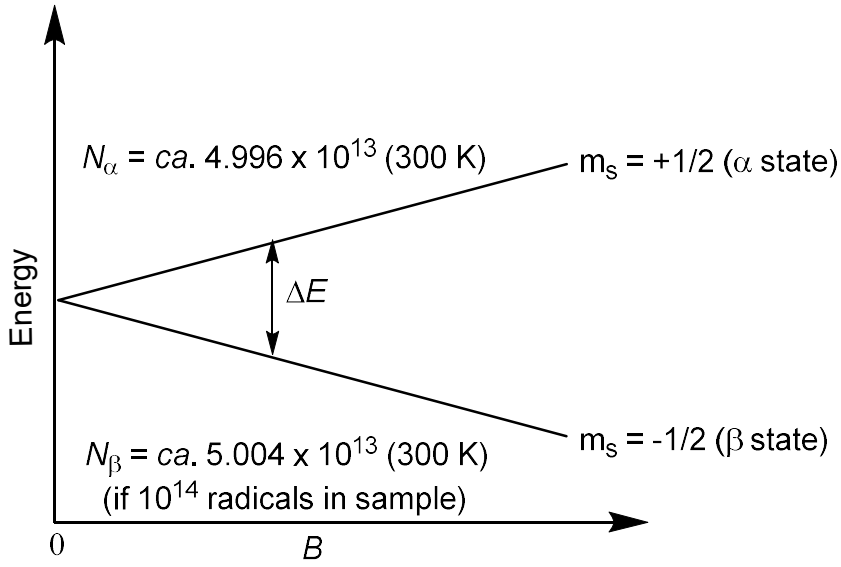

Figure 1. Diagram showing the energies and state populations for unpaired electrons in an applied magnetic field.

Characteristics of EPR spectra

An EPR spectrum from a radical in fluid solution is characterised by three basic parameters: the *g* value, the linewidths, and the hyperfine splitting constants.

g value

The *g* value, sometimes called *g* factor, is characteristic of the radical type and reflects the variable amount of orbital magnetism possessed by the unpaired electron, in addition to its spin magnetism. In a free atom, an unpaired electron may have orbital angular momentum in addition to its spin angular momentum, but when this electron is in a polyatomic radical its orbital motion is usually quenched by the "ligand field" strength (Δ). However, spin-orbit coupling can restore a small amount of orbital magnetism to the electron and this causes it to have an effective magnetic moment marginally different from that of the free electron which, in turn, causes the *g* value to deviate from the spin-only value (*g* 2.00232). The spin-orbit constant (ζ**)** for an atom increases rapidly with increasing atomic number and thus *g* is dependent on the nature of the atoms with which the unpaired electron is associated. For example, a purely carbon centred alkyl radical (*g* 2.0026) can be distinguished readily from an α-alkoxyallyl radical (*g* 2.0033), in which the unpaired electron is delocalised between carbon and oxygen (Wertz & Bolton, 1993). The sign of δg is dependent on the detailed electronic configuration and orbital energies of the radical, and is positive for most types of organic radicals, although it can be negative especially for certain radicals e.g. acyl radicals. The magnitude of δg depends on the size of (ζ/Δ), although the major variation between radicals is in ζ rather than Δ.

Line-widths and dynamic EPR spectroscopy

Chemical and physical processes that lead to exchange of the unpaired electron between different radical sites can give rise to line shape effects in the EPR spectra. Such processes include hindered rotation

around bonds, tumbling of the radical in a viscous liquid, interactions with other paramagnetic species and chemical reactions (e.g. acid-base equilibria and electron-transfer reactions) (Wertz & Bolton, 1993). Dynamic EPR spectroscopy refers to the study of these exchange processes undergone by radicals on the EPR time-scale (10^{-6}-10^{-9} s). If the processes are slow with respect to this time-scale, lines may be assignable to distinct species, while if the rate of exchange is fast, a weighted average spectrum consisting of sharp lines will be seen. However, if exchange takes place at an intermediate rate on the EPR time-scale, line-broadening will occur and these line shape effects can be analysed to estimate the rate constant for the exchange process.

Hyperfine splitting constants

The most useful information derivable from an EPR spectrum is obtained from the hyperfine splitting, which usually enables identification of the radical and also its detailed structure to be determined. The origin of the observed splittings is the interaction between the unpaired electron and the magnetic moments of neighbouring magnetic nuclei within the radical. The interaction with n equivalent nuclei of spin ***I*** results in (2n*I* + 1) lines and the separation between each of these lines is (to first-order) equal to the hyperfine splitting constant. Since ^{12}C has no magnetic moment, proton hyperfine couplings dominate EPR spectra of neutral and ionic hydrocarbon radicals. The interactions of the unpaired electron with n equivalent protons (or other *I* = 1/2 nuclei) gives rise to signal splitting into (n +1) lines and, furthermore, the relative intensities of these lines are given by the coefficients of the binomial expansion of $(1+x)^n$, which can be found readily from Pascal's triangle. Although the natural abundance of ^{13}C (*I* = 1 /2) is only *ca*. 1.1 %, several other common elements have magnetic isotopes that are present in high abundance. These include ^{10}B (*I* = 3) *ca*. 19.8%, ^{11}B (*I* = 3/2) *ca*. 80.2% and ^{14}N (*I* = 1) *ca*. 99.6%.

Quantitative EPR

The area of the EPR signal, obtained through double integration, is linearly related to concentration of the free radical. Careful comparison to standards measured under the same conditions, or normalised for conditions, leads to absolute concentrations. Ideally, standard samples should be of the same chemical type, phase, volume, and concentration, to minimize error. A full explanation of the considerations can be found in the book by Eaton *et al.* (Eaton, Eaton, Barr & Weber, 2010).

Pulsed EPR

If the magnitudes of some magnetic couplings are small, these interactions are unresolved in the CW line width. Pulsed EPR can be used to disentangle this information, which can provide crucial information about structure and identity. A further advantage is the direct detection of relaxation times. In pulse EPR the spectrum is recorded by exciting a large frequency range simultaneously with a high-power microwave pulse of given frequency υ at a constant magnetic field *B*. A wide variety of experiments can be performed by using multiple pulses, often used to measure hyperfine and election-electron couplings (Schweiger & Jeschke, 2001).

High field EPR

In practice, for spectral over-lapping spin species, and for large spin systems, the standard X band EPR experiment reaches its limits of resolution, with information on magnetic parameters and molecular orientations hidden under, non-descript, broad lines. By going to higher magnetic fields and microwave frequencies the field-dependent spin interactions are separated from the field-independent ones. For example, *g* factor resolution is increased in relation to the hyperfine couplings. The key advantage is enhanced spectral resolution. The most common bands for EPR are: L band (1-2 GHz), S band (2-4 GHz), Q band (34 GHz) and W band (94 GHz). High field EPR can be practiced in CW, and pulsed modes (Savitsky & Möbius, 2009).

Further reading

The readers interested in a deeper knowledge understanding of the field of EPR are advised to other review articles (Eaton & Eaton, 2004; Hagen, 2006; Roessler & Salvadori, 2018). Older textbooks may be daunting

as a first introduction but in recent time a generation of easily readable texts has been published (Brustolon & Giamello, 2009; Chechik, Carter & Murphy, 2016; Goldfarb & Stoll, 2018).

RESULTS AND DISCUSSIONS

Radical scavenging activity

Natural products contain a large group of antioxidant compounds that inhibit the chain reaction of oxidation, acting as hydrogen donors or acceptors of free radicals. Many different methods are used to understand antioxidant activity (AA) offering complementary information (Munteanu & Apetrei, 2021). Experiments involving the peroxyl radical (or hydroperoxyl) have particular relevance to AA as they predominate in lipid oxidation in biological systems (Amorati & Valgimigli, 2018). EPR experiments have been designed to show specific reactivity with different radicals providing information on intrinsic antioxidant potential with minimal environmental interference. There are several experimental approaches using EPR that measure reactivity with radicals.

Intensity reduction of a stable radical

Figure 2 shows several stable radicals that have been used as intensity standards, including the 1,1‑diphenyl-2-picrylhydrazyl (DPPH), 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPOL) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation. The double-integrated EPR signals of the stable radicals in an antioxidant-radical mixture and control are measured after the same reaction time (typically 3 minutes).

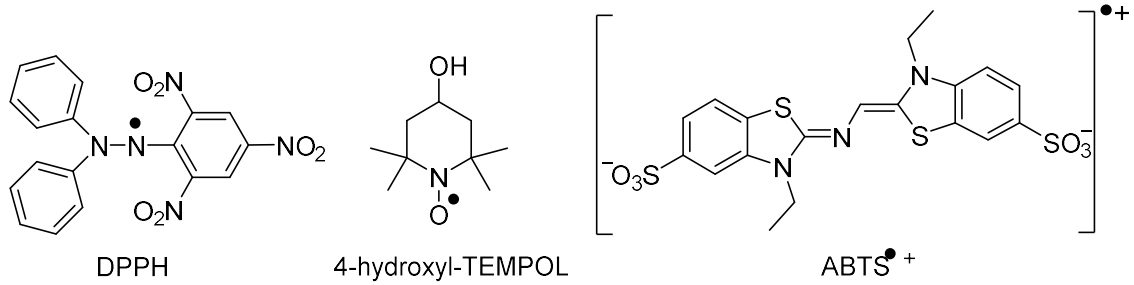

Figure 2. Chemical structures of stable radicals commonly used to measure radical scavenging capacity.

The results are expressed as stable radical scavenging percentage (Eqn 4.).

$$I\,(\%) = 100 \times (S_{SR} - S_{sample})/S_{SR} \quad \textbf{(Eqn 4.)}$$

where I correspond to EPR signal reduction, and S_{SR} and S_{sample} correspond, respectively, to the EPR signals for solution containing the stable radical in the absence (control) and presence of the extract (sample).

Spectrophotometric assays are regularly employed using DPPH (Brand-Williams, Cuvelier, & Berset, 1995), and made through a decrease of signal intensity at 517 nm. The mechanism is generally thought to be electron transfer with proton loss, in which the DPPH radical accepts an electron followed by proton transfer from antioxidant compounds. Other authors have postulated that a marginal mechanism of HAT (hydrogen atom transfer) may occur depending on the reaction medium and physicochemical properties of the compounds under study (Amarowicz, 2019; Magalhães *et al.*, 2008). The prime disadvantage of this method is spectral interferences, such as from the solvent used and the presence of undesired coloured compounds in the samples. The EPR method provides a route without such interference.

Recently, Barroso *et al.* (Barroso *et al.*, 2019), have investigated the *in vitro* antioxidant properties of golden grass (GG), a grass-like herb (*Syngonanthus nitens*), using the DPPH/EPR method on methanolic extracts. The EPR spectrum of DPPH radical shows a five-line pattern, due to the interaction of the two equivalent ^{14}N (I = 1) nuclei with the unpaired electron ($2nI + 1 = 5$), in which the depletion of signal intensity is proportional to the radical scavenging capacity of compounds present in the extracts (Figure 3). The time

to reach the endpoint of DPPH-EPR test depends on the medium and the properties of the molecules under study. The kinetics of reaction between DPPH and GG extract was determined. The kinetics followed a biexponential decay, and this behaviour was attributed to different flavonoids acting together as antioxidants.

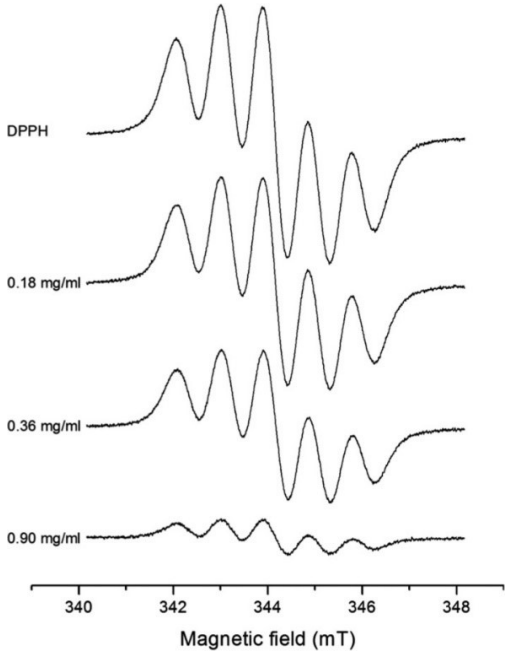

Figure 3. The effect of different concentrations of golden grass on the EPR spectra of DPPH. Adapted from (Barroso *et al.*, 2019). (Creative Commons — Attribution 4.0 International — CC BY 4.0)

Further examples of the use of the EPR/DPPH method include hydroxycinnamic and hydroxybenzoic systems (Mura *et al.*, 2014), extracts of *Lavandula angustifolia* and *Lavandula x intermedia* Cultivars (Dobros *et al.*, 2022), kale flavonoids extracts (Chen *et al.*, 2022), and an investigation on the effect of temperature and ultraviolet irradiation on free radical scavenging activity of simvastatin (Zdybel *et al.*, 2022).

The ABTS radical cation-based assays (Re *et al.*, 1999) are among the most abundant antioxidant capacity spectrophotometric assays. Acsova *et al.* have recently made an *in vitro* study of the antioxidant efficacy of natural ubiquinol compared to synthetic references (Acsova *et al.*, 2021). The ABTS radical cation scavenging ability of test substances was evaluated by EPR spectroscopy and expressed as Trolox equivalent antioxidant capacity $(TEAC)_{EPR}$. High correlation between EPR spectroscopy and UV-VIS spectrophotometry was found even in quantifying the antioxidant capacity of different types of compounds and substrates. $(TEAC)_{EPR}$ values were significantly lower compared to $(TEAC)_{UV}$ and this was attributed to spectral UV interference and differences in concentrations. Overall, results obtained by the EPR study demonstrated a co-antioxidant effect that ubiquinol can increase the natural antioxidant activity of α-tocopherol at certain combinations of concentration.

Targeted radical generation with spin trapping

EPR can be used to test the reactivity of extracts and compounds with reactive oxygen species (ROS) of short lifetime, radical traps are used to generate stable radical species. Figure 4 shows some common spin traps that are used alongside a generalised reaction scheme. The radicals add to the nitrone group to form a stable adduct. The hyperfine values of the adducts are used to identify the radical products. Different radical species are used as reactive species depending on the biochemistry being investigated.

Hydroxyl radicals are generated using the Fenton reaction (Eqn 5.) (Fenton, 1894). Similarly, to the previous method the results are expressed as stable radical scavenging percentage in the presence of the substance under study.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-} \qquad \textbf{(Eqn 5.)}$$

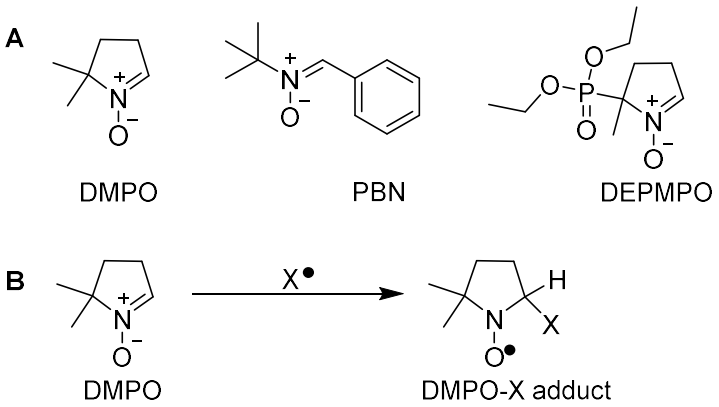

Figure 4. (A) Chemical structures of commonly used spin traps. (B) Generalised nitrone spin trap reaction equation.

In order to generate •OH free of transition metal ions that can be readily chelated, the Haber–Weiss (Eqn 6.) reaction has been used (Haber & Weiss, 1932):

$$^{\bullet}O_2^- + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2 \qquad \textbf{(Eqn 6.)}$$

The key advantage of these methods is specificity combined with direct detection, allowing unambiguous assignment of the species measured. The method has further advantages of versatility, measuring both solid and liquid samples irrespective of how well they absorb light.

Recently, a study (Giordano *et al.*, 2022) has been made on polyphenolic composition and AA of different extracts of *Argylia radiata* vitroplants (artificially propagated plants that are grown *in vitro*) and natural roots. Hydroxyl radicals were generated by a non-catalytic Fenton reaction, and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was used as a spin trap. The extracts of *Argylia* vitroplant in the three solvents used decreased the intensity of the (DMPO/OH) spin adduct signal, which implied a decrease in the OH radical concentration. Figure 5 shows a representative spectrum of (DMPO/OH) spin adduct with the four-line pattern arising from coupling to the ^{14}N nucleus and a ^{1}H nucleus. The radical scavenging values indicated that the ethanol extract of stem and leave showed a higher hydroxyl radical scavenging capacity, followed by the corresponding methanol extract. The antioxidant capacity studied by this methodology correlated to that obtained using oxygen radical antioxidant capacity – Fluorescein (ORAC-FL), since both methods involve the transfer of the hydrogen atom.

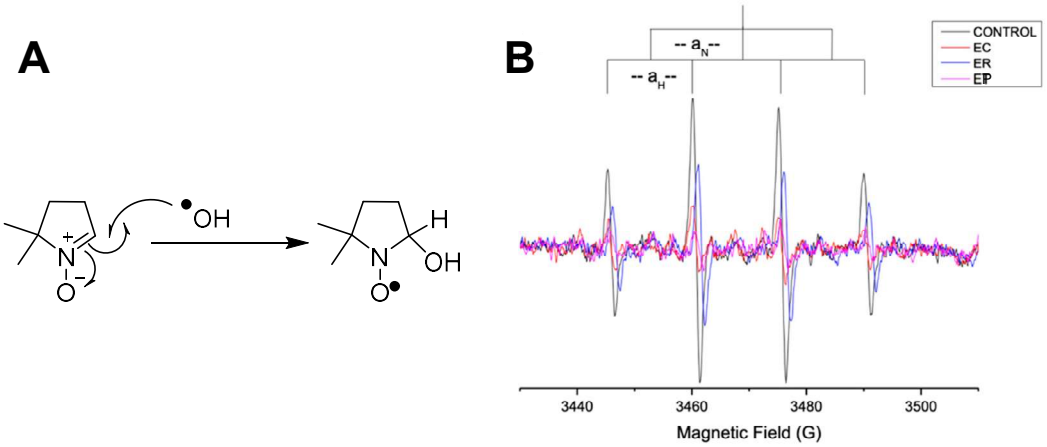

Figure 5. (A) DMPO-OH spin adduct formation reaction. (B) EPR Spectra of *A. radiata* ethanolic extracts (EC, ethanol-callus; ER, ethanol root; EP ethanol-plant. Adapted from (Giordano *et al.*, 2022). (Creative Commons — Attribution 4.0 International — CC BY 4.0)

A recent study by Sanna *et al.* has highlighted the importance of the medium in different Fenton systems for measurement of the scavenging activity of hydroxyl radicals (Sanna *et al.*, 2022). Fe/phosphate buffer, Fe/quinolinic acid, Fe/phosphate buffer/quinolinic acid, and the thermal degradation of peroxydisulfate were used to produce hydroxyl radicals; the hydroxyl radical scavenging activity of plant extracts (ginger, blueberry juices and green tea infusion) and chemical compounds (epigallocatechin gallate and gallic acid) was estimated by EPR spin trapping with DMPO. Phosphate buffer was used to mimic the physiological pH of cellular systems, while quinolinic acid (pyridine-2,3-dicarboxylic acid) facilitated the experimental procedure by hindering the spontaneous oxidation of Fe^{2+}. The EC_{50} [the concentration of chemical compounds or plant extracts which halves the intensity of the (DMPO/OH) adduct] values were determined in all the systems. The results showed that, for both the chemical compounds and the plant extracts, there was not a well-defined order for the EC_{50} values determined in the four hydroxyl radical generating systems. The interactions of phosphate buffer and quinolinic acid with the antioxidants and with potential iron-coordinating ligands present in the plant extracts justified the observed differences.

Spasic *et al.* have studied the effects of equimolar concentrations of glucose, fructose and mannitol and three phosphorylated forms of fructose (fructose-1-phosphate (F1P); fructose-6-phosphate (F6P); and fructose-1,6-bis(phosphate) (F16BP)) on OH radical production *via* the Fenton reaction. EPR spectroscopy using spin-trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) was applied to detect radical production (Spasojevic *et al.*, 2009). They found that the percentage inhibition of OH radical formation decreased in the order F16BP > F1P > F6P > fructose > mannitol = glucose. As ketoses can sequester redox-active iron thus preventing the Fenton reaction, the Haber–Weiss-like system was also employed to generate OH radical, so that the effect of iron sequestration could be distinguished from direct OH radical scavenging. In the latter system, the rank order of OH radical scavenging activity was F16BP > F1P > F6P > fructose = mannitol = glucose. The results clearly demonstrated that intracellular phosphorylated forms of fructose have more scavenging properties than fructose or glucose.

Almajano *et al.* used a variation of the Fenton reaction to study the reactivity of the methoxy radical ($CH_3O^{\cdot}$) combined with the EPR spin trapping to measure the radical scavenging activity of white tea and its major catechin components, epicatechin , epicatechin-3-gallate, allocatechin, and epigallocatechin-3-gallate (Almajano *et al.*, 2014) Alkoxy radicals are considered among ROS species in biological processes as critical mediators in several serious human diseases (Gaschler *et al.*, 2017). In the experiments the reduction of H_2O_2 with $FeSO_4$ was conducted in MeOH, under these conditions it was assumed a nonradical process was the dominant mechanism in the Fenton reaction leading to formation of Fe^{IV} oxide and its oxidant action in MeOH causing the generation of the methoxy radical in two consecutive one-electron sets. DMPO was used as the spin trap. Ferulic acid was used as standard and radical scavenging activity was reported as equivalents of ferulic acid in grams per litre per gram of white tea. The values obtained with the spin trap method show a much greater difference in antioxidant activity among the polyphenols than the other methods, although the order of activity was the same. The authors make the point that an advantage of the spin method is that it works with ROS, the true mediators of human diseases, whereas Trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) methods work with “atypical” and more stable radicals.

Azman *et al.* have successfully employed the same method to evaluate the antioxidant activity of *Betula pendula* (BP) leaves extract and its effects on model foods (Azman *et al.*, 2017) Decay graphs indicated the exponential relationship of the decrease in signal in the spectrum as the concentration of BP extracts increased. The study confirmed that the scavenging activity of the BP extracts containing polyphenol constituents could be measured by the decrease of the intensity of the spectral bands of the (DMPO/CH_3O) spin adduct EPR spectrum.

The EPR spin trapping method can also be applied to lipid peroxidation. Xenakis *et al.* have examined olive oil and olive mill wastewaters (OMW) as direct scavengers of free radicals using EPR spin trapping and a photometric procedure based on the formation of the *N*,*N*-dimethyl-*p*-phenylenediamine coloured radical cation ($DMPD^{+\bullet}$) (Xenakis *et al.*, 2005). In the EPR study, free radicals were generated by oxidizing the lipid moiety of low-density lipoprotein or lecithin micelles by Cu^{2+}. The nitrone compound, α-phenyl-t-butylnitrone (PBN), trapped these radicals to form stable lipid-PBN spin adducts. The formation of the three-line EPR signal was inhibited in the presence of compounds with known antioxidant activity or of polar extracts with potent antioxidant activity. The antioxidant capacity was quantified by measuring the percentage of inhibition of the EPR spectra of PBN spin adducts formed during the oxidation of lecithin micelles in the

presence of increasing concentrations of Trolox. The extraction procedures of olive oil seemed to affect olive oil content in antioxidants and, consequently, the corresponding antioxidant capacity. Tannin-rich polar extracts isolated from the OMW were much more potent in scavenging free radical formation than polyphenol-rich polar extracts from olive oil.

Influence of metals on AA

The chemical implications and considerations on techniques used to assess the *in vitro* AA of coordination compounds has recently been extensively reviewed by Marchi *et al.* (Marchi, *et al*., 2022). EPR techniques are shown as direct and practical alternatives to colorimetric methods with the advantage of not having the results influenced by the colour of the metal complex.

Phenolic Plant Antioxidants

Phenolics are ubiquitous in the plant kingdom and are the most abundant secondary metabolites of plants. Plant polyphenols have accentuated attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative stress associated diseases such as cancer (Dai *et al*., 2010).

Much work has been carried out to characterise using EPR radicals derived from isolated phenols, defined as phenoxyl radicals, semiquinones or generally aroxyl radicals. Many methods are employed to generate the phenoxyl radicals: i) strongly alkaline solutions. ii) Oxidation by Ce^{4+} salts in acidic conditions. iii) Enzymatic catalysis employing either horseradish peroxidase/hydrogen peroxide or tyrosinase under *in situ* conditions. Phenoxyl radicals derived monophenols have been observed in curcuminoids, hydroxycoumarins and isoquinolines. Polyphenol aroxyl radicals have been found in catechols, hydroquinones, hydroxy-anthraquinones, flavonoids, tannins, marchantins (Bors *et al.*, 1998). The EPR spectra are rich in hyperfine patterns, which are crucial for the correct identification of primary radicals. *g* values are used to work out if the radicals are carbon or oxygen centred.

Semiquinone radical from extracts

The importance of semiquinones lies as key intermediates in redox reactions. They are broadly distributed in in respiring organisms. Several quinones are of pharmacological interest. They form a major class of cytotoxins, used in the fight against cancers (Pereyra *et al*., 2019). Semiquinone radicals can be used in elucidating biogenesis of natural occurring compounds and as markers in chemotaxonomic studies. EPR is used as a tool to identify and electronically characterize the properties of the semiquinone as intermediates along mechanistic biological pathways. Important information can be obtained, for example, knowledge of spin densities calculated from hyperfine splitting coupling constants can help understand electron transfer (O'Malley, 2001) and nucleophilic substitution reactions (Epiotis, 1973).

There is a stupendous array of work by Pedersen, forming a library of hyperfine splitting constants for semiquinone radicals to aid identification. The semiquinone radicals were generated by extracting samples (typically fresh leaves) with ethanol and then mixed with alkaline water or performed in neutral buffered water solutions, with the requirement of a circulating solution for continuous access of oxygen. The semiquinone radical derived from the oxidation of quinones, of course, generates an EPR signal with characteristic hyperfine patterns. The relative stability the semiquinone radicals lends itself to study by EPR. Figure 6. displays an example of the rich EPR hyperfine patterns that have been observed. The 24-line spectrum is derived from a radical of a 3,4-dihydroxyphenylethoxy glycoside, obtained from an ethanolic extract of *Conandron ramondioides*.

EPR can be applied to the detection and identification of ortho and para quinols or quinones in plant extracts, without prior isolation (Pedersen, 1978). The radicals of compounds lacking an ortho or a para dihydroxy grouping, e.g. resorcinols, monohydric phenols or phloroglucinols are not observed due to their short lifetimes. Alcohol extracts of sixteen *Pyrus* species gave an EPR spectrum with five equidistant lines of relative intensity 1:4:6:4:1. Both the hyperfine splitting constant (2.36 Gauss) and the *g* value (2.00471) were identical to the values of the semiquinone obtained from an authentic sample of hydroquinone. A standard curve was used to estimate the amount of hydroquinone. Similarly, in the same work, the quinones, plumbagin in *Drosera* and *Ceratostigma*, and hydrojuglone in *Juglandaceae*, were identified. The study was also easily able to detect and distinguish esters of phenolic acids e.g. chlorogenic and rosmarinic acids. The results were obtained in as little as 50 µl of sample and the lower limit of detection with confidence in

identification was in the µg range. Disadvantages were discussed which include the potential role of alkali to cause diamagnetic products, the unknown role of contaminants, and the masking of weaker signals.

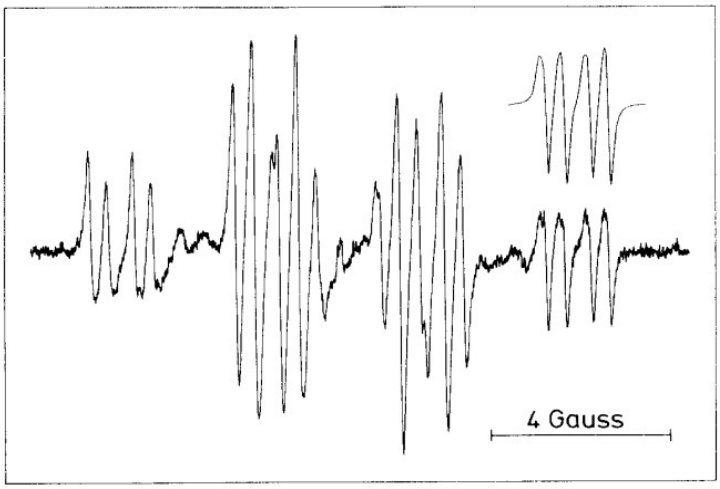

Figure 6. EPR spectra of the semiquinone radicals of acteoside and conandroside obtained simultaneously from crude alcoholic leaf extracts of *Conandron ramondioides*. Inset: Simulation of the outmost eight lines (2 x 4) to the right. Reprinted by permission from Elsevier (Kvist *et al*., 1986).

The ease by which quinones and quinols are generated as semiquinones makes the EPR technique suitable in screening programs of large number of plants, easily extended to various plant parts, roots; leaves; stems; etc. Some studies have focused on screening medicinal plants for their content of quinones/quinols, Mouhajir *et al.* surveyed forty-nine Moroccan medicinal plant species comprising forty-five genera of twenty-seven families demonstrating how the technique is highly applicable to chemotaxonomic studies (Mouhajir *et al*., 2001). The technique acts a complimentary tool to nuclear magnetic resonance (NMR) spectroscopy and liquid-chromatography mass spectrometry (LC-MS) and needs known spectral references. Again, only quinones and compounds with an ortho or a para dihydroxy grouping, i.e., oxidizable phenolics, can be immediately observed by the technique. Furthermore, compounds without oxidizable phenolics cannot be observed using this technique.

Mouhajir *et al.* have also worked on single plant studies such as the amount of thymoquinone in seeds of *Nigella satia* used in Moroccan folk medicine (Mouhajir *et al*.,1999). A further study investigates the formation of catechol from the phenolic glycoside, salicortin, in trembling aspen and the role it plays in plant-herbivore interactions (Haruta *et al*., 2001).

Spectral simulation has been employed to identify multiple overlapping radical species. For example, studies in the crude leaf extracts of *Chimaphila umbellata* gave a multicomponent EPR spectrum and simulation and subtraction was used to extract three separate semiquinone spectra of three compounds thought to be intermediates on the reaction pathway leading to chimaphilin. Further examples can be found in a handbook by Pederson (Pedersen, 2018).

Autoxidation

EPR is the most sensitive method to detect ROS radicals derived from natural products. EPR spin trapping has been used to study the autoxidation mechanism of flavonoids. Canada *et al*. studied flavonols by EPR during redox cycling and oxygen consumption experiments (Canada *et al*., 1990). Quercetin produced a characteristic DMPO-OH radical, dependent on pH and iron level. Myricetin autooxidation yielded a semiquinone signal which upon the addition of iron, converted to a DMPO-OH signal detected at a pH of 7.5. In a microsome-NADPH system, quercetin produced an increase in oxygen utilization and with EPR, an ethanol-derived radical signal which could be completely suppressed by catalase, indicating the dependence of the signal on hydrogen peroxide. These studies demonstrated that the extracellular production of active oxygen species by dietary flavonols is not likely to occur *in vivo* but the potential for

intracellular redox cycling may have toxicologic significance. Flavonoids have also been shown to autoxidize in aqueous buffer solutions at pH 7.5 to generate the hydroxyl radical and hydrogen peroxide.

Yao *et al.* have investigated the instability of flavonoids in air-saturated alkaline solutions to investigate the considered unreliable general synthesis of bioflavonoid–metal complexes (Yao *et al*., 2020). Dihydromyricetin (DHM) and its analogues were observed with DMPO to form the superoxide anion radical adduct. This validated a proposition that DHM after deprotonation by base forms its electron-rich phenolate, which undergoes a redox reaction with dissolved oxygen. The DHM analogues myricetin quercetin, daidzein, genistein, chrysin, baicalein, rutin, and kaempferol as potential bioflavonoid–metal complex ligands were also dissolved in air-saturated alkaline solutions and observed by EPR `to generate superoxide radical anion at different capacities. These differences are possibly associated with the number and location of the multi-hydroxyl moieties attached to their molecular skeleton or their configurations. Therefore, the general synthetic procedure for bioflavonoid–metal complexes using a transition metal ion and air-saturated alkaline solution was shown to require improvement.

Singlet oxygen detection

The generation of reactive oxygen species in thylakoids from senescing flag leaves of the barley varieties *Lomerit* and *Carina* (Krieger-Liszkay *et al*., 2015) have been investigated using EPR spin trapping. EPR measurements were performed with specific spin traps to discriminate between singlet oxygen and reactive oxygen intermediates. The results showed that the generation of reactive oxygen intermediates increased in both varieties during senescence. Singlet oxygen increased only in the variety *cv. Lomerit* while it remained constant at a low level in the variety *cv. Carina*. The study showed that during senescence the production of individual reactive oxygen species varied in different barley varieties. 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TEMPD-HCl) was used for singlet oxygen detection (Moan *et al*., 1979). A stable nitroxide radical is generated when $^1O_2$ reacts with the sterically hindered amine 2,2,6,6,-tetramethylpiperidin. Figure 7 shows some representative spectra of the 1:1:1 triplet arising from coupling of the unpaired electron to a sole ^{14}N nucleus. The advantages of this approach are stated as being highly sensitive and specific in comparison with other techniques such as semiquantitative DAB (3,3-diaminobenzidine) staining for H_2O_2 or SOSG (singlet oxygen sensor green) fluorescence for $^1O_2$ detection (Gollmer *et al.*, 2011). The scope and limitations of the method have been investigated by Nardi *et al*. (Nardi *et al*., 2014).

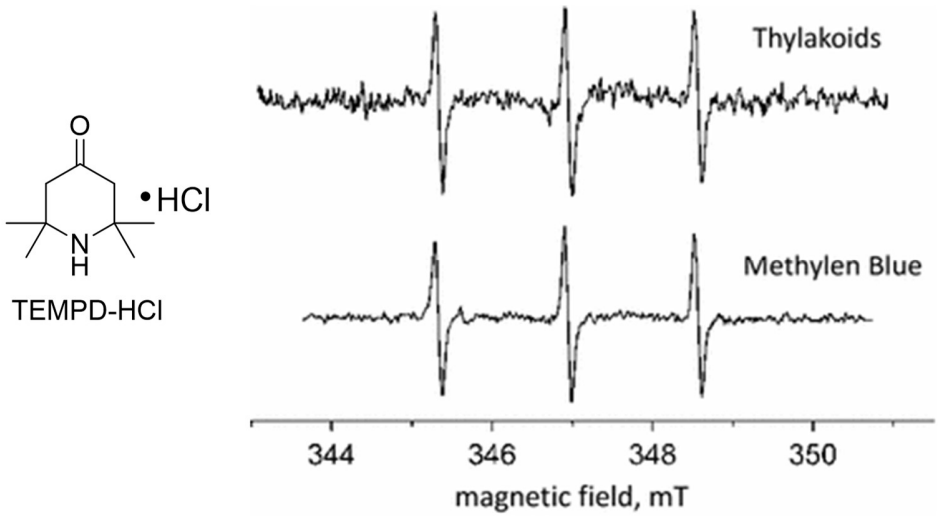

Figure 7. Singlet oxygen detection by EPR spectroscopy using TEMPD-HCl as spin probe. Shown is a typical spectrum recorded after 2 min illumination with red light (RG 630) of 500 µmol quanta m^{-2} s^{-1}. The measurement was performed with thylakoids isolated from flag leaves of *cv. Lomerit* in a medium state of senescence. As a control, singlet oxygen was generated by illuminating methylene blue with the same light intensity. Reprinted by permission from Springer (Krieger-Liszkay *et al*., 2015).

Photoexcitation

Natural products that absorb strongly in the UV/vis have been studied using EPR using *in situ* photoexcitation. Colourants obtained from natural sources such as plants, insects, molluscs or lichens have been particularly investigated in this way.

Machatova *et al.* (Machatova *et al.*, 2016) have performed a spectroscopic study on 9,10-anthraquinone derivatives [purpurin, alizarin, carminic acid, and 2-(hydroxymethyl)-9,10-anthraquinone)] in dimethylsulfoxide in the presence of triethylamine (TEA) to bring information on their protonation/deprotonation equilibria in aprotic solvent. The formation of paramagnetic intermediates upon irradiation of 9,10-anthraquinone derivatives was monitored *in situ* by CW EPR spectroscopy. The UVA excitation (λ_{max} = 365 nm) of purpurin and carminic acid resulted in the generation of low-intensity EPR signals. However, the addition of TEA, caused the deprotonation of both hydroxyanthraquinones, and led to the increase of EPR signal intensity and narrow signals with unresolved hyperfine couplings and *g* values about 2.0054 were detected. UVA irradiation of DMSO solution of alizarin, caused the generation of a stable EPR signal, and its intensity was also enhanced significantly upon TEA addition. The hyperfine splitting could be attributed to a semiquinone-like radical species. Complementary, quantum chemical calculations enabled the identification of the individual protonated/deprotonated tautomeric forms present in the experimental systems. UV photoexcitation of hydroxyanthraquinones (HAQ) led to the generation of reactive radical species and singlet oxygen, detected by *in situ* EPR spectroscopy (spin trapping, nitroxide radical elimination, oxidation of sterically hindered amines). The changes in the electronic absorption spectra upon photoexcitation, linked with the ability of the studied HAQ to generate reactive oxygen species upon exposure, confirm a substantial effect of the substituent character and position on the overall photochemical behaviour of the HAQ significantly influenced by the actual experimental conditions (solvent, pH).

The melanins are a class of functional biomacromolecule found throughout nature in diverse roles including pigmentation, free radical scavenging, hard radiation protection, and even high-adhesive–strength structural components. Melanin is implicated in the development of deadly melanoma cancers of the eyes and skin and therefore the photoreactivity of these pigments is thus a matter of considerable interest. CW EPR has shed light on its spin properties, and a persistent, stable free radical appears to be a central feature of melanin physiochemistry (Meredith *et al*., 2006). Recently, using a novel *in situ* photoinduced EPR technique with simultaneous electrical measurements, the distinct photoreactivity of the two different radical species has been elucidated. The production of the semiquinone was found to be light- and water-driven, explaining electrical properties and revealing biologically relevant photoreactivity (Mostert *et al*. 2018).

CONCLUSION

The current trend for use of EPR in natural products research is by far in radical scavenging capacity. A fast array of experimental methods and reaction chemistry are available to tailor for biological context, which is a great advantage of the versatility of EPR. Previous ground-breaking use in detection of semiquinone radicals in extracts has waned, possibly because the original studies were so comprehensive, however, given the prevalence for quinones in plants, and cheaper bench top EPR instruments, there must be an opportunity for this to progress further.

The current generation of EPR instruments offers new opportunities to probe natural product molecules and extracts with greater resolution. Recent years has seen the development of rapid scan EPR techniques, for improved signal-to-noise, into commercial instrumentation, which has been applied to semiquinones (Eaton *et al*., 2015), and spin trapping experiments (Mitchell *et al.*, 2013). Furthermore, the use of high-field and pulsed EPR provides the opportunity for more detailed screening of extracts containing multiple paramagnetic signals. These EPR methods could be applied to understand the coordination environment between natural products and metal ions. Likewise, there is scope for using EPR to understand redox properties on interaction with metals. Photochemical EPR could also be implied to increase the time resolution for observation of transient radical generation. Overall, EPR has much to offer natural product discovery.

ACKNOWLEDGMENTS

The author is grateful to Dr Jose M Prieto-Garcia for suggestions of source material.

REFERENCES

Acsova, A., Hojerova, J., Tobolkova, B., Martiniakova, S. (2021). Antioxidant Efficacy of Natural Ubiquinol Compared to Synthetic References - In Vitro Study. ChemistrySelect, 6(18), 4495-4505.

AbouZid, S., Ahmed, H. (2020). Chapter 13 - Brief review on applications of continuous-wave electron paramagnetic resonance spectroscopy in natural product free radical research (Vol. 66): Elsevier, 355-369.

Aleksieva, K.I., Yordanov, N.D. (2018). Various approaches in EPR identification of gamma-irradiated plant foodstuffs: A review. Food Res Int, 105, 1019-1028.

Amarowicz, R., Pegg R.B. (2019). Functional Food Ingredients from Plants. Series title: Advances in Food and Nutrition Research, Editor: Toldra, Academic Press.

Amorati, R., Valgimigli, L. (2018). Methods To Measure the Antioxidant Activity of Phytochemicals and Plant Extracts. Journal of Agricultural and Food Chemistry, 66(13), 3324-3329.

Azman, N.A.M., Peiró, S., Fajarí, L., Julià, L., Almajano, M.P. (2014). Radical Scavenging of White Tea and Its Flavonoid Constituents by Electron Paramagnetic Resonance (EPR) Spectroscopy. Journal of Agricultural and Food Chemistry, 62(25), 5743-5748.

Azman, N.A.M., Skowyra, M., Muhammad, K., Gallego, M. G., Almajano, M.P. (2017). Evaluation of the antioxidant activity of Betula pendula leaves extract and its effects on model foods. Pharmaceutical Biology, 55(1), 912-919.

Barbana, V., Guedes, C., Di Mauro, E. (2013). Characterization of paramagnetic species in seeds by electron paramagnetic resonance (EPR). Renewable Energy and Power Quality Journal, 1(11), 346-348.

Barroso, R.P, Berlim, L.S., Ito, A.S., Costa-Filho, A.J. (2019). In vitro antioxidant properties of golden grass (*Syngonanthus nitens*) by electron paramagnetic resonance. Food Sci Nutr. 194, 394-400.

Bors, W., Michel, C., Stettmaier, K. (1998). EPR Spectroscopy of Phenolic Plant Antioxidants. In T. Özben (Ed.), Free Radicals, Oxidative Stress, and Antioxidants: Pathological and Physiological Significance (pp. 363-374). Boston, MA: Springer US.

Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. LWT - Food Science and Technology, 28(1), 25-30.

Britt, R.D., Campbell, K.A., Peloquin, J.M., Gilchrist, M. L., Aznar, C.P., Dicus, M.M., Robblee, J., Messinger, J. (2004). Recent pulsed EPR studies of the Photosystem II oxygen-evolving complex: implications as to water oxidation mechanisms. Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1655, 158-171.

Brustolon, M., Giamello, G. Editors (2009). Electron paramagnetic resonance: A practitioner's toolkit. Hoboken, N.J., Wiley.

Canada, A.T, Giannella, E., Nguyen, T.D., Mason, R.P. (1990). The production of reactive oxygen species by dietary flavonols, 9(5), 441-449.

Chechik, V., Carter, E., Murphy, D. (2016). Electron paramagnetic resonance. Oxford Univ Press.

Chen, X., Wang, H., Huang, X., Xia, S., Chen, C., Nie, Q., Nie, S. (2022). Efficient enrichment of total flavonoids from kale (Brassica oleracea L. var. acephala L.) extracts by NKA-9 resin and antioxidant activities of flavonoids extract in vitro. Food Chemistry, 374, 131508.

Dai, J., Mumper, R. J. (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. Molecules, 15(10), 7313-7352.

Dobros, N., Zawada, K., Paradowska, K. (2022). Phytochemical Profile and Antioxidant Activity of Lavandula angustifolia and Lavandula x intermedia Cultivars Extracted with Different Methods. Antioxidants, 11(4), 711.

Eaton, G.R., Eaton S.S., Barr, D.P., Weber, R.T. (2010). Quantitative EPR : A Practitioners Guide. Vienna: Springer Vienna.

Eaton, S. S., Eaton, G. R. (2015). Chapter One - Rapid-Scan EPR of Nitroxide Spin Labels and Semiquinones. In P. Z. Qin & K. Warncke (Eds.), Methods in Enzymology (Vol. 563, pp. 3-21): Academic Press.

Eaton, S. S., Eaton, G.R. (2004). Chapter 12, Electron Paramagnetic Resonance in Analytical Instrumentation Handbook (3rd ed.). (J. Cazes Ed. 3rd ed.). Boca Raton: CRC Press.

Epiotis, N.D. (1973). Simple Correlation of Hyperfine Splitting Constants of Radical Ions and Orientation in Aromatic Substitution-Reactions. Journal of the American Chemical Society, 95(10), 3188-3193.

Fenton, H.J.H. (1894). LXXIII.—Oxidation of tartaric acid in presence of iron. Journal of the Chemical Society, Transactions, 65(0), 899-910.

Gaschler, M.M., Stockwell, B. R. (2017). Lipid peroxidation in cell death. Biochemical and Biophysical Research Communications, 482(3), 419-425.

Giordano, A., Morales-Tapia, P., Moncada-Basualto, M., Pozo-Martinez, J., Olea-Azar, C., Nesic, A., Cabrera-Barjas, G. (2022). Polyphenolic Composition and Antioxidant Activity (ORAC, EPR and Cellular) of Different Extracts of Argylia radiata Vitroplants and Natural Roots. Molecules, 27(3).

Goldfarb, D., Stoll, S. Editors. (2018) EPR Spectroscopy: Fundamentals and Methods (eMagRes Books): Wiley.

Gollmer, A., Arnbjerg, J., Blaikie, F. H., Pedersen, B. W., Breitenbach, T., Daasbjerg, K., . . . Ogilby, P. R. (2011). Singlet Oxygen Sensor Green (R): Photochemical Behavior in Solution and in a Mammalian Cell. Photochemistry and Photobiology, 87(3), 671-679.

Götze, J., Plötze, M., Th, G., Neuser, R. D., Richter, D. K. (2002). Cathodoluminescence (CL) and electron paramagnetic resonance (EPR) studies of clay minerals. Mineralogy and Petrology, 76(3-4), 195-212.

Haber, F., Weiss, J. (1932). Über die Katalyse des Hydroperoxydes. Naturwissenschaften, 20(51), 948-950.

Hagen, W.R. (2006). EPR spectroscopy as a probe of metal centres in biological systems. Dalton Transactions(37), 4415-4434.

Haruta, M., Pedersen, J. A., & Constabel, C. P. (2001). Polyphenol oxidase and herbivore defense in trembling aspen (Populus tremuloides): cDNA cloning, expression, and potential substrates. Physiologia Plantarum, 112(4), 552-558.

Krieger-Liszkay, A., Trösch, M., Krupinska, K. (2015). Generation of reactive oxygen species in thylakoids from senescing flag leaves of the barley varieties Lomerit and Carina. Planta, 241(6), 1497-1508.

Kvist, L.P., Pedersen, J.A., (1986). Distribution and taxonomic implications of some phenolics in the family gesneriaceae determined by EPR spectroscopy, 14(4), 385-405.

Machatova, Z., Barbierikova, Z., Poliak, P., Jancovicova, V., Lukes, V., Brezova, V. (2016). Study of natural anthraquinone colorants by EPR and UV/vis spectroscopy. Dyes and Pigments, 132, 79-93.

Magalhães, L.M., Segundo, M.A., Reis, S., Lima, J.L. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. Anal Chim Acta, 613(1), 1-19.

Marchi, R.C., Campos, I.A.S., Santana, V.T., Carlos, R.M. (2022). Chemical implications and considerations on techniques used to assess the in vitro antioxidant activity of coordination compounds. Coordination Chemistry Reviews, 451, 214275.

Meredith, P., Sarna, T. (2006). The physical and chemical properties of eumelanin. Pigment Cell Research, 19(6), 572-594.

Mitchell, Deborah G., Rosen, Gerald M., Tseitlin, M., Symmes, B., Eaton, S. S., Eaton, G.R. (2013). Use of Rapid-Scan EPR to Improve Detection Sensitivity for Spin-Trapped Radicals. Biophysical Journal, 105(2), 338-342.

Moan, J., Wold, E. (1979). Detection of singlet oxygen production by ESR. Nature, 279(5712), 450-451.

Mostert, A.B., Rienecker, S.B., Noble, C., Hanson, G. R., Meredith, P. (2018). The photoreactive free radical in eumelanin. Science Advances, 4(3).

Mouhajir, F., Pedersen, J. A., Rejdali, M., Towers, G.H.N. (1999). Antimicrobial thymohydroquinones of Moroccan Nigella sativa seeds detected by electron spin resonance. Pharmaceutical Biology, 37(5), 391-395.

Mouhajir, F., Pedersen, J.A., Rejdali, M., Towers, G.H.N. (2001). Phenolics in Moroccan medicinal plant species as studied by electron spin resonance spectroscopy. Pharmaceutical Biology, 39(5), 391-398.

Munteanu, I.G., Apetrei, C. (2021). Analytical Methods Used in Determining Antioxidant Activity: A Review. International Journal of Molecular Sciences, 22(7).

Mura, F., Silva, T., Castro, C., Borges, F., Zuñiga, M.C., Morales, J., Olea-Azar, C. (2014). New insights into the antioxidant activity of hydroxycinnamic and hydroxybenzoic systems: Spectroscopic, electrochemistry, and cellular studies. Free Radical Research, 48(12), 1473-1484.

Nardi, G., Manet, I., Monti S., Miranda, M.A., Lhiaubet-Vallet, V., (2014). Scope and limitations of the TEMPO/EPR method for singlet oxygen detection: the misleading role of electron transfer. Free Radical Biology and Medicine, 77, 64-70.

O'Malley, P.J. (2001). Electronic structure studies of quinones and semiquinones: Accurate calculation of spin densities and electron paramagnetic resonance parameters. Antioxidants and Redox Signaling, 3(5), 825-838.

Papadimitriou, V., Maridakis, G.A., Sotiroudis, T.G., Xenakis, A. (2005). Antioxidant activity of polar extracts from olive oil and olive mill wastewaters: an EPR and photometric study. European Journal of Lipid Science and Technology, 107(7-8), 513-520.

Pedersen, J. A. (1978). Naturally Occurring Quinols and Quinones Studied as Semiquinones by Electron-Spin Resonance. Phytochemistry, 17(4), 775-778.

Pedersen, J. A. (2002). On the application of electron paramagnetic resonance in the study of naturally occurring quinones and quinols. Spectrochimica Acta Part a-Molecular and Biomolecular Spectroscopy, 58(6), 1257-1270.

Pedersen, J. A. (2018). CRC handbook of EPR spectra from quinones and quinols: CRC press.

Pereyra, C.E., Dantas, R.F., Ferreira, S.B., Gomes, L.P., Silva-Jr, F.P. (2019). The diverse mechanisms and anticancer potential of naphthoquinones. Cancer Cell International, 19(1), 207.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine, 26(9), 1231-1237.

Rex, R. W. (1960). Electron Paramagnetic Resonance Studies of Stable Free Radicals in Lignins and Humic Acids. Nature, 188(4757), 1185-1186.

Roessler, M. M., Salvadori, E. (2018). Principles and applications of EPR spectroscopy in the chemical sciences. Chemical Society Reviews, 47(8), 2534-2553.

Sanna, D., Fadda, A. (2022). Role of the Hydroxyl Radical-Generating System in the Estimation of the Antioxidant Activity of Plant Extracts by Electron Paramagnetic Resonance (EPR). Molecules, 27(14).

Savitsky, A., Möbius, K. (2009). High-field EPR. Photosynthesis Research, 102(2), 311-333.

Schweiger, A., Jeschke, G. (2001). Principles of Pulse Electron Paramagnetic Resonance. Oxford: Oxford University Press.

Spasojevic, I., Mojovic, M., Blagojevic, D., Spasic, S.D., Jones, D.R., Nikolic-Kokic, A., Spasic, M.B. (2009). Relevance of the capacity of phosphorylated fructose to scavenge the hydroxyl radical. Carbohydrate Research, 344(1), 80-84.

Kavetskyy, T.S, Soloviev, V.N, Khalilov, R.I., Serezhenkov, V.A., Pan'kiv, L.I., Pan'kiv, I.S., Nasibova, A.N., Stakhiv, V.I., Ivasivka, A.S., Starchevskyy, M.K., Pavlovskyy, Y.V., Bondaruk, Y.V., Dyachok, D.A., Bodnar, L.V., Voloshanska, S.Y. (2022). EPR study of self-organized magnetic nanoparticles in biomaterials. Semiconductor Physics, Quantum Electronics & Optoelectronics, 25(2), 146-156.

Un, S., Dorlet, P., Rutherford, A. W. (2001). A high-field EPR tour of radicals in photosystems I and II. Applied Magnetic Resonance, 21(3), 341-361.

Wertz, J.E., Bolton, J.R. (1993). Electron Spin Resonance: Elementary Theory and Practical Applications. New York: Wiley Interscience.

Yao, Y. Y., Zhang, M., He, L.B., Wang, Y. Y., Chen, S.X. (2020). Evaluation of General Synthesis Procedures for Bioflavonoid-Metal Complexes in Air-Saturated Alkaline Solutions (vol 8, 589, 2020). Frontiers in Chemistry, 8.

Zdybel, M., Pilawa, B., Chodurek, E. (2022). Effect of temperature and ultraviolet irradiation on free radical scavenging activity of simvastatin. Spectroscopy Letters, 55(1), 35-43.