J Sci Food Agric 85:265–272 (2005)
DOI: 10.1002/jsfa.1950

# Free-radical scavenging activity of wormwood (*Artemisia absinthium* L) extracts

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**Abstract: In an effort to discover new antioxidant natural compounds, wormwood (*Artemisia absinthium* L) an aromatic-bitter herb, was screened. The sequential extraction was realized with five solvents of different polarities (70% methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol). The antioxidative activity was tested by measuring their ability to scavenge stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and reactive hydroxyl radical during the Fenton reaction trapped by 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), using electron spin resonance (ESR) spectroscopy. Results demonstrated that the antiradical and antioxidative activity depend on the type and concentration of applied extracts and increased in the order ethyl acetate > methanol > *n*-butanol > chloroform > petroleum ether > remaining water extracts. The investigation showed that the antiradical activity increased with increasing concentration of all extracts. The high contents of total phenolic compounds (25.6 mg g$^{-1}$) and total flavonoids (13.06 mg g$^{-1}$) indicated that these compounds contribute to the antiradical and antioxidative activity. In a model system, the formation of *o*-semiquinone radicals from quercetin and chlorogenic acid was obtained to prove the mechanism (hydrogen donating and/or one-electron reduction) of free-radical scavenging activity.**


**Keywords:** wormwood extracts; DPPH; hydroxyl radical; polyphenols; radical scavenging; ESR; spin trapping

## INTRODUCTION

Antioxidants are important species which possess the ability to protect the organism from damage caused by free radical-induced oxidative stress.[1–4] Highly reactive free radicals, especially oxygen radicals, which are formed by exogenous chemicals or endogenous metabolic processes in the human body or in food systems, are capable of oxidizing biomolecules, resulting in tissue damage.[5–8] Oxidative damage to protein, DNA and lipid is associated with chronic degenerative diseases including cataracts, cancers and coronary heart disease.[9,10] A variety of free radical-scavenging antioxidants exist within the body, some of which are derived from dietary sources.[11,12]

However, concern about the safety of the commonly used synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary butylated hydroxyquinone[13,14] had led to increasing interest in naturally occurring alternatives which occur in plants as secondary metabolites.

There is currently much interest in the antioxidant role of flavonoids and other polyphenols found in tea, wine, fruit, vegetables, herbs and spices.[15–20] These plant-derived polyphenols provide a prolonged and balanced dose of antioxidants beneficial to human health.

One approach to assessing antioxidative activity is to examine directly free radical production and its inhibition by antioxidant by using highly sensitive electron spin resonance (ESR) spectroscopy. Owing to the unpaired electron in the outer orbital, free radicals are paramagnetic species and, when in sufficient quantity, are directly detectable and measurable by ESR spectroscopy.[21,22] ESR spectroscopy is the only analytical technique that directly measures free radicals. ESR relies on the absorption of microwave energy (which arises from the promotion of the unpaired electron to a higher energy level) when the samples are placed in a variable magnetic field. The position in the magnetic field of this absorption (*g factor*) and the structure, number and splittings (*hyperfine coupling constants*) between the absorption bands (which are usually recorded as their first derivatives) give valuable information as to the nature and structure of the radical(s) present in the system.

Wormwood (*Artemisia absinthium* L) is an aromatic-bitter herb, which has been used as a medicine from ancient times. It has traditionally been used

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Contract/grant sponsor: Ministry of Science, Technologies and Development of the Republic of Serbia; contract/grant number: 1862
(*Received 15 January 2003; accepted 6 August 2004*)
Published online 15 October 2004

 

as anthelmintic, choleretic, antiseptic, balsamic, depurative, digestive, diuretic, emmenagogue and in treating leukaemia and sclerosis. Extracts of wormwood have been used as a muscle relaxer that is occasionally added to liniments and as a mild sedative to treat anxieties.[23–25]

Wormwood is the aromatic spice, widely employed as a flavouring agent in wine and other alcoholic beverages. Also, to a lesser extent it is used in soft drinks and some foods, especially confectionery and desserts. Thujone has achieved notoriety as the neurotoxic agent in wormwood oil from *Artemisia absinthium* (Compositae/Asteraceae), used in preparation of the drink absinthe, now banned in most countries.

In this paper, the free-radical scavenging activity of methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol and remaining water extracts of wormwood (*Artemisia absinthium* L) on stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and reactive hydroxyl radical formed in the Fenton reaction was investigated by ESR spectroscopy.

## MATERIAL AND METHODS

### Chemicals

Methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol, *tert*-butanol, acetic acid and formic acid were obtained from Merck (Hohenbrunn, Munich, Germany). DPPH, 5,5-dimethyl-1-pyroline-N-oxide (DMPO), standards of flavonoids (rutin and quercetin) and phenolic acid (chlorogenic, salicylic, syringic, vanillic and *p*-coumaric acids) were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Plant material, herb of wormwood (*Artemisia absinthium* L) was collected in the region of Zlatibor (Serbia) and dried in air. All other chemicals were of analytical reagent grade.

### Extraction

The extracts were obtained during successive extraction by using solvents (methanol, petroleum ether, chloroform, ethyl acetate and *n*-butanol) with different polarities. Figure 1 shows the sheme for the preparation of all extracts, the free-radical scavenging activity of which was investigated.

The yields of extracts (g) were: methanol, $0.2589 \pm 0.0095$; petroleum ether, $0.0177 \pm 0.0006$; chloroform, $0.0355 \pm 0.0012$; ethyl acetate, $0.1001 \pm 0.0068$; *n*-butanol, $0.3474 \pm 0.0240$; and remaining water, $0.5278 \pm 0.0360$.

### Determination of total phenolic compounds

Total phenolic content was determined spectrophotometrically using the Folin–Ciocalteu method.[26] The

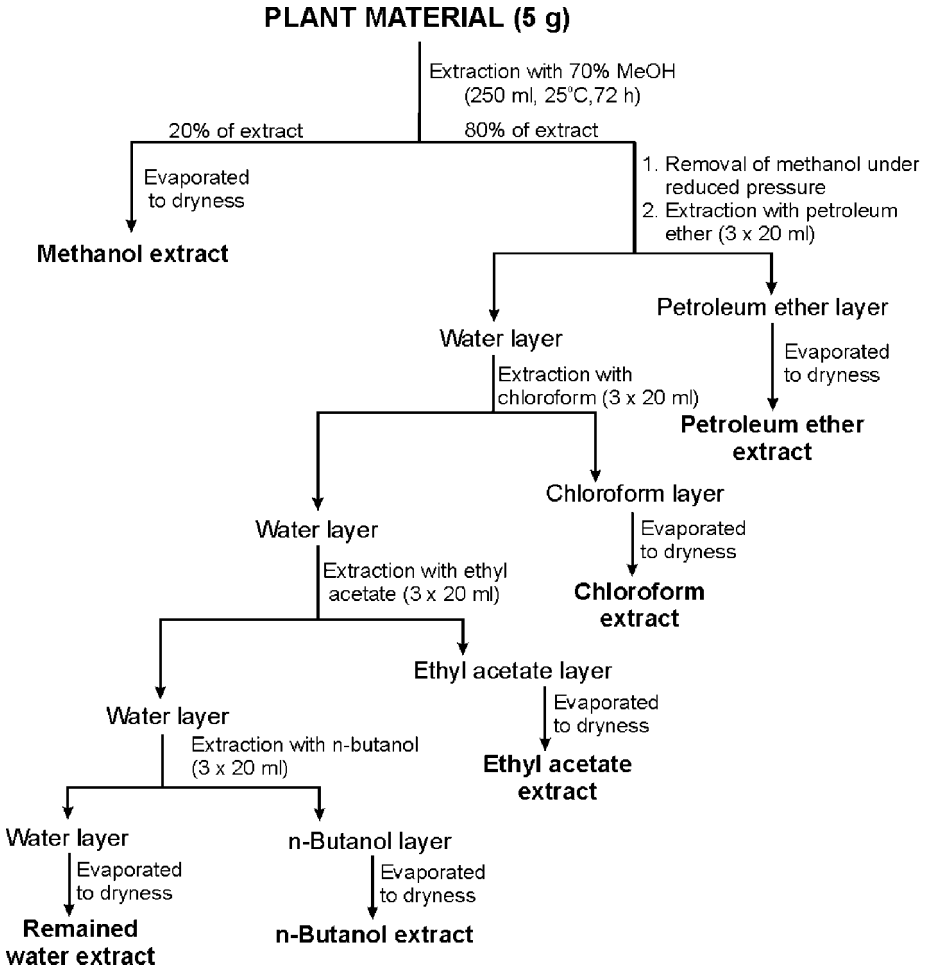


**Figure 1.** Sheme for preparation of extracts.

concentration was calculated using chlorogenic acid as standard and the results were expressed as milligrams chlorogenic acid equivalents per gram dry weight.

### Total flavonoids

Total flavonoids (expressed as mg rutin per g dry weight) were estimated according to Markham.[27]

### Thin-layer chromatography (TLC)

The qualitative composition of each extract was examined using TLC, along with reference substances. TLC was performed on 20 × 20 cm plates precoated with microcrystalline cellulose (Camag, Muttanez, Switzerland). A volume of 1 µl of 1% methanolic solutions of standards and investigated extracts was spotted on the plates. Spots were observed under UV light at 366 nm and sprayed with DPPH reagent. One-dimensional TLC analysis was performed with ethyl acetate:formic acid:acetic acid:water in volume ratio 100:11:11:26 as mobile phase.

Mobile phases for two-dimensional TLC analysis were: (1) *tert*-butanol:acetic acid:water in volume ratio 3:1:1; and (2) 15% acetic acid.

### DPPH radicals generation and detection

DPPH radicals were prepared in methanol to the final concentration of $1.8 \times 10^{-4}$ mM. Different concentrations of investigated extracts (0.005–3.250 mg ml$^{-1}$) were added and mixed with DPPH solution. After that the mixture was stirred for 2 min and transferred to a quartz flat cell ER-160FT.

The ESR spectra were recorded on an ESR spectrometer Bruker 300E (Rheinstetten, Germany) under the following conditions: field modulation, 100 kHz; modulation amplitude, 0.256 G; receiver gain, $2 \times 10^4$; time constant, 40.96 ms; conversion time, 327.68 ms; centre field, 3440.00 G; sweep width, 100.00 G; *x*-band frequency, 9.64 GHz; power, 20 mW; and temperature, 23 °C.

### Hydroxyl radicals generation and detection

As hydroxyl free radicals are highly reactive, with relatively short half-lives, the concentrations found in natural systems are usually inadequate for direct detection by ESR spectroscopy. Spin-trapping is a chemical reaction that provides an approach to help overcome this problem.[28]

The Fenton reaction was conducted by mixing 0.2 ml 0.3 M DMPO, 0.2 ml 10 mM $H_2O_2$ and 0.2 ml 10 mM $Fe^{2+}$ (blank). The influence of different types of extracts on the formation and stabilization of hydroxyl radicals was investigated by adding the methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol and the remaining water extracts in the Fenton reaction system at the range of concentrations 0.005–3.250 mg ml$^{-1}$. ESR spectra were recorded after 5 min, with the following spectrometer settings: field modulation, 100 kHz; modulation amplitude, 0.512 G; receiver gain, $2 \times 10^5$; time constant, 81.92 ms; conversion time, 163.84 ms; centre field, 3440.00 G; sweep width, 100.00 G; *x*-band frequency, 9.64 GHz; power, 20 mW; and temperature, 23 °C.

Magnetic field scanning was calibrated using Fremy's salt (peroxylamine disulphonate). Splitting constants were calculated from computer-generated second derivatives of the spectra after optimizing signal-to-noise ratios, and were verified by computer simulations.

### Detection of oxygen-centred free radicals of flavonoids generated in alkaline solution

The oxygen-centred free radicals of phenolic acids and flavonoids were determined in the reaction system containing 5 ml 5 M NaOH plus 5 mg ml$^{-1}$ of chlorogenic acid and quercetin, respectively. ESR measurement conditions were: field modulation, 100 kHz; modulation amplitude, 0.144 G; receiver gain, $2.5 \times 10^5$; time constant, 81.92 ms; conversion time, 163.84 ms; centre field, 3440.00 G; sweep width, 100.00 G; *x*-band frequency, 9.64 GHz; power, 0.632 mW; and temperature, 23 °C.

### Statistical analysis

All measurements were performed at least in triplicate, unless specified otherwise, and presented as mean ± SD. Significance of differences with respect to the control group was evaluated using the Student's *t*-test ($p < 0.05$) as significant.

## RESULTS AND DISCUSSION

The successive extraction procedure resulted in different amounts of extracts of wormwood. The total phenolic and flavonoid contents in plant were 25.6 and 13.06 mg g$^{-1}$, respectively.

Because of their chemical structures, the flavonoids such as quercetin, rutin and other flavonoid glycosides and phenolic acids such as chlorogenic, syringic, coumaric, salicylic and vanillic acids (detected by TLC, results are not shown) are probably involved in the mechanism of free-radical scavenging activity.[29] HPLC chromatographic data showed the presence of some other flavonoid glycosides (isoquercitrin, quercitin-3-*O*-β-D-glucoside, quercitin-3-*O*-rhamnoglucoside, isorhamnetin-3-*O*-rhamnoglucoside, isorhamnetin-3-glucoside),[30] which possess antioxidative activity too. Wormwood also contains thujone, anabsin, absinthin, arabsin, artabin, artemetin, artenisetin, arthamarin, bisabolen, cadinene, pinene, sabinene, β-carotene, etc.[31] These compounds are generally distributed in the investigated extracts as a function of polarity: phenolic acids, aglycon or monoglycosyl flavanoids and monomers or polymers of procyanidins in the ethyl acetate fraction—the most polar compounds such as triglycosyl flavanoids, galloyl tannins and procyanidin polymers in the water fraction.[32] Polyphenolic compounds were generally not found in the chloroform and petroleum ether fractions (only very small quantities could be found in the chloroform).

The chemical reactivity of herb extracts and organic compounds that react as antioxidants has been characterized using a model or *in vitro* systems based on the scavenging of reactive oxygen species or stable free radicals.

In this paper the stable DPPH radicals have been used to investigate the wormwood antiradical activity.[33] The ESR spectrum of stable DPPH free radical is readily recognized in blank probe by its five lines of relative intensities 1:2:3:2:1 and hyperfine splitting constant $a_N = 9.03$ G (Fig 2(a)).

No change in the shapes of ESR spectra, in all examined cases, was detected, but the relative intensity of ESR signals, corresponding to the concentration of formed DPPH radicals, was reduced with higher concentrations of the added wormwood extracts.

The antiradical activity (AA) of different concentrations of methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol and remaining water extracts of wormwood (*Artemisia absinthium* L) on DPPH radicals is presented in Fig 3. The AA value of the extract was defined as:

$$AA(\%) = 100 \cdot (h_o - h_x)/h_o$$

where $h_o$ is the height of the second peak in the ESR spectrum of DPPH free radicals of the blank and $h_x$ is the height of the second peak in the ESR spectrum of DPPH free radicals of the probe. Values for a given concentration are not significantly different at the level of 0.05.

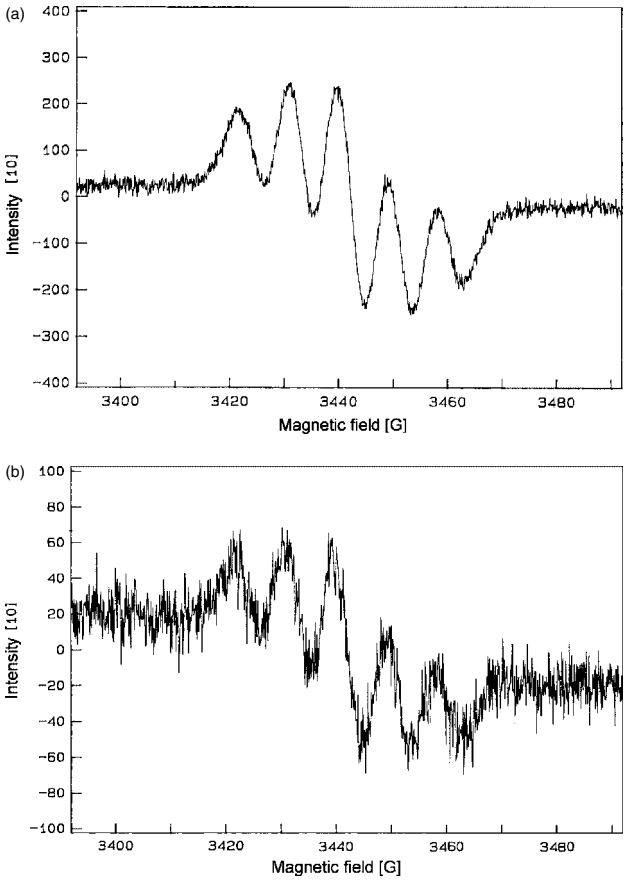


**Figure 2.** ESR spectra of DPPH radicals: (a) with no addition of extracts (blank). The final concentration of DPPH radicals was $1.8 \times 10^{-4}$ mM; (b) same as blank but with 0.060 mg ml$^{-1}$ of ethyl acetate extract of wormwood.

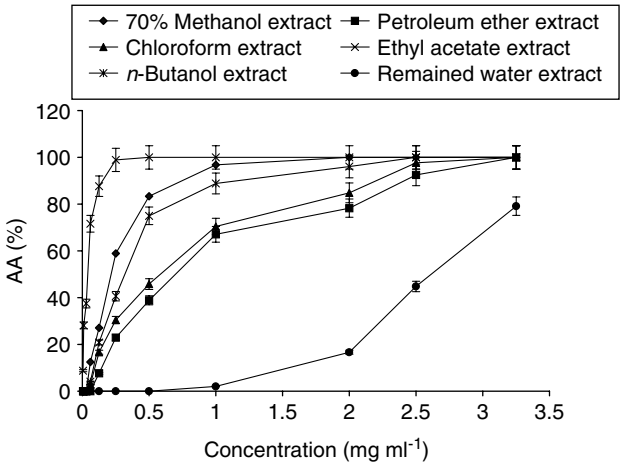


**Figure 3.** The antiradical activity (AA) of different concentrations of methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol and remaining water extracts of wormwood (*Artemisia absinthium* L) on DPPH radicals.

As can been seen, the investigated extracts showed significant differences ($p < 0.05$) in AA at concentrations lower than 1.0 mg ml$^{-1}$. It is evident that the interaction of a potential antioxidant with DPPH depends on the type and concentration of the investigated extract. The following order of AA has been established: ethyl acetate > methanol > *n*-butanol > chloroform > petroleum ether > remaining water extracts. Also, the investigation showed that the AA increased with increasing the concentration of all extracts. When the concentrations of methanol extracts increased from 0.060 to 2.0 mg ml$^{-1}$, the antiradical effect on DPPH radicals increased from 12.50 to 100%, while in the presence of *n*-butanol extract it increased from 4.08 to 96.06%. However, at a concentration of 2.0 mg ml$^{-1}$, the AA of chloroform and petroleum ether extracts were 84.82 and 78.26%, respectively. The remaining water extract of wormwood possesses the lowest AA (at concentration of 2.0 mg ml$^{-1}$, AA = 16.67%), which indicated that the important antioxidative compounds are present in very small amounts, or they are not present at all. These compounds were probably extracted by some other of the solvents used in the successive extractions.

The activity of the ethyl acetate extract is the highest. The very high AA = 71.61% was detected in the presence of 0.060 mg ml$^{-1}$ (Fig 2(b)). Concentration of 0.5 mg ml$^{-1}$ reduces all DPPH radical molecules, ie the ESR signal vanishes, indicating an excellent AA on DPPH radicals (100%). Obviously, there were more antioxidant components present in this extracts which could act rapidly with DPPH radicals and reduce almost all DPPH radical molecules. This observation could indicate that AA is due to a variety of components including both hydrophilic and lipophilic compounds in the herb.

Natural phytochemicals such as phenolic compounds found in numerous herbs, commonly involve an aromatic ring as part of the molecular structure, with one or more hydroxyl groups. They can act as antioxidants as their extensive conjugated $\pi$-electron systems allow ready donation of electrons or hydrogen atoms from the hydroxyl moieties to free radicals.[29] The less reactive aroxyl radicals were obtained during this reaction, which stabilizes their structure by electron delocalization (forming aryl radicals).[34] The antiradical efficiencies based on this mechanism are the typical for different phenolic acids (chlorogenic acid, syringic acid) and flavonoids (possessing two or three hydroxyl groups on the carbon in the B or C ring of the molecules), whose presence has been proved in wormwood. Monophenols (coumaric acid, vanillin and vanillic acid), probably possess poor efficiency, which may be explained by the presence of an electron withdrawing group (CHO, COOH).[35] The reduction potentials of the hydroxyl groups of polyphenols may change in different solvent systems, depending on their state of protonation/deprotonation.[36] Another mechanism proposed for AA on DPPH radicals is 'scavenging' activity (one DPPH$^{\bullet}$ molecule forms complex with one aryl radical).

The antioxidative effect of the different extracts of wormwood was investigated by the ability of the extract to scavenge hydroxyl radicals. This is very important because of the fact that hydroxyl radicals were mentioned as the major active oxygen species causing lipid oxidation.[37] To test the reactions of hydroxyl radicals with extracts of wormwood, the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\bullet}OH$) was used as a source of hydroxyl radicals. Using the spin trap, such as DMPO, it is possible to convert reactive hydroxyl radicals to stable nitroxide radicals (DMPO-OH adducts) with spectral hyperfine splittings that reflect the nature and structure of these radicals. The relative intensity of free radical formation can be determined because the ESR spectroscopy signal is directly related to the concentration of spin adducts. The height of the peaks in the spectra is proportional to the number of radical adduct molecules in the accumulating system. However, it is important to recognize that the ESR spectra of a particular spin adduct have unique characteristics that are dependent on the specific spin trap used and the free radical trapped, serving thus as sensitive and specific markers of the presence of a particular free radicals species.

As shown in Fig 4(a), the reaction of $Fe^{2+}$ with $H_2O_2$ in the presence of spin trapping agent DMPO generated a 1:2:2:1 quartet of lines with hyperfine coupling parameters ($a_N = a_H = 14.9$ G). The intensity of ESR signal, corresponding to the concentration of formed free radicals, was decreased in the presence of 0.060 mg ml$^{-1}$ of ethyl acetate extract of wormwood (Fig 4(b)).

The intensity of the ESR signal decreased after addition of all the investigated extracts (Fig 5). The

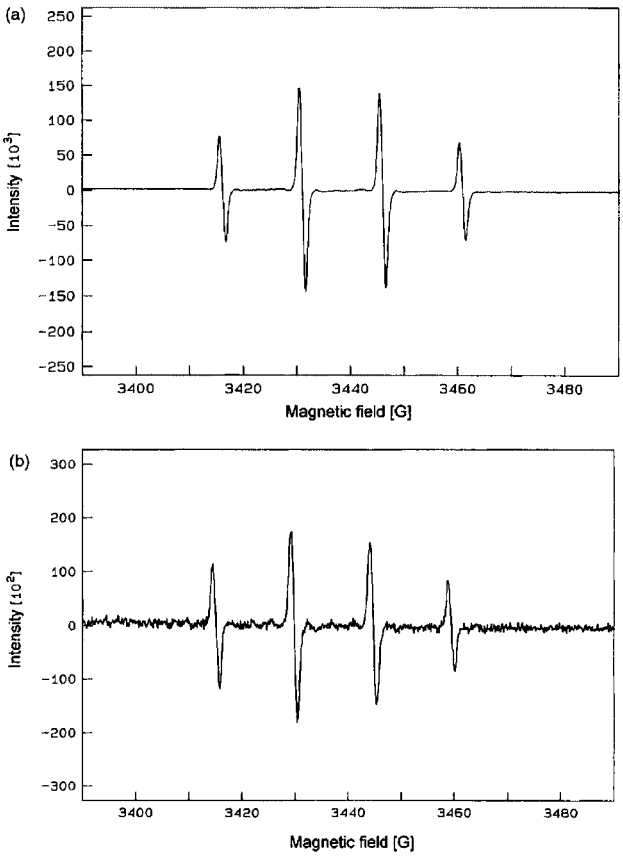


**Figure 4.** ESR spectra of DMPO-OH spin adduct recorded 5 min after mixing of (a) 0.2 ml 0.3 M DMPO, 0.2 ml 10 mM $H_2O_2$, 0.2 ml 10 mM $Fe^{2+}$ (blank); (b) same as blank but with 0.060 mg ml$^{-1}$ of ethyl acetate extract of wormwood.

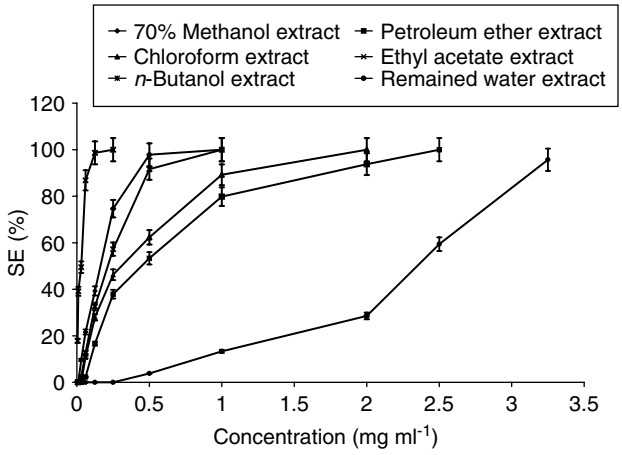


**Figure 5.** Scavenging effect of different concentrations of methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol and remaining water extracts of wormwood (*Artemisia absinthium* L) on DMPO-OH spin adduct.

scavenging effect (SE) of the extract was defined as:

$$SE(\%) = 100 \cdot (h_o - h_x)/h_o$$

where $h_o$ is the height of the second peak in the ESR spectrum of DMPO-OH spin adduct of the blank and $h_x$ is the height of the second peak in the ESR spectrum of DMPO-OH spin adduct of the probe. Values for

a given concentration are not significantly different at the level of 0.05.

The following order of antioxidative activity has been established: ethyl acetate > methanol > *n*-butanol > chloroform > petroleum ether > remaining water extract. Also, the investigation showed that the antioxidative activity increased with increasing the concentration of all extracts ($p < 0.05$).

The concentration of 0.060 mg ml$^{-1}$ of ethyl acetate is more effective in scavenging hydroxyl radicals (SE = 86.85%) in comparison with the DPPH antiradical activity AA = 71.61% (Fig 3).

Hence, at the concentration of 0.250 mg ml$^{-1}$, ethyl acetate extract inhibits completely the formation of hydroxyl radical. At this concentration, methanol extract produced SE = 74.65% and *n*-butanol extract SE = 57.24%. The lowest scavenging effects were observed for the petroleum ether (SE = 16.64%) and for chloroform (SE = 27.95%) extracts.

The remaining water extract exhibited a high SE (95.68%) only at a high concentration (3.250 mg ml$^{-1}$). Generally, the high concentrations of all investigated extracts produced a high scavenging effect.

The mechanism of scavenging activity of phenolic compounds is not clear but they may act as scavengers of hydroxyl radical by acting as chain-breaking antioxidants, as hydrogen donors with the formation of less reactive flavonoid (aroxyl-ArO) radical ($ArOH + {}^{\bullet}OH \rightarrow ArO^{\bullet} + HOH$). The initially formed aroxyl radical may also be an efficient hydroxyl radical scavenger ($ArO^{\bullet} + {}^{\bullet}OH \rightarrow HO{-}Ar(=O)$) and stable products of quinonic structure are obtained.[38,39] Numerous studies offer evidence that polyphenols act as electron donors in aqueous media, which is in accordance with their one-electron reduction potentials. The net result of this mechanism is also stable aroxyl radicals.

There are no differences in the order of SE and AA of the investigated extracts. All of investigated extracts are more effective on the DMPO-OH scavenging than in the DPPH test. A regression curve was constructed for the values obtained by each test (DPPH, OH). As can be seen by the coefficient of regression obtained ($r^2 = 0.987$, ie $r^2 = 0.9831$), the two tests showed high linear correlation, as examplified for methanol and ethyl acetate extracts (Fig 6). The significant difference between DPPH and hydroxyl radical assays at lower concentrations of methanol and ethyl acetate extracts can be explained by the fact that some natural compounds are good iron chelators. It was suggested that flavonoid compounds and phenolic acids with *o*-dihydroxyl groups might be exerting their protective effects through chelation of metal ions during the Fenton reaction, or by the altering iron redox chemistry.[40,41]

A complex system of synergy effects and different symbiosis between certain substances (phenolic acids, flavonoids, tannins, terpenes, and many others) is also very important for antioxidative activity.[42]

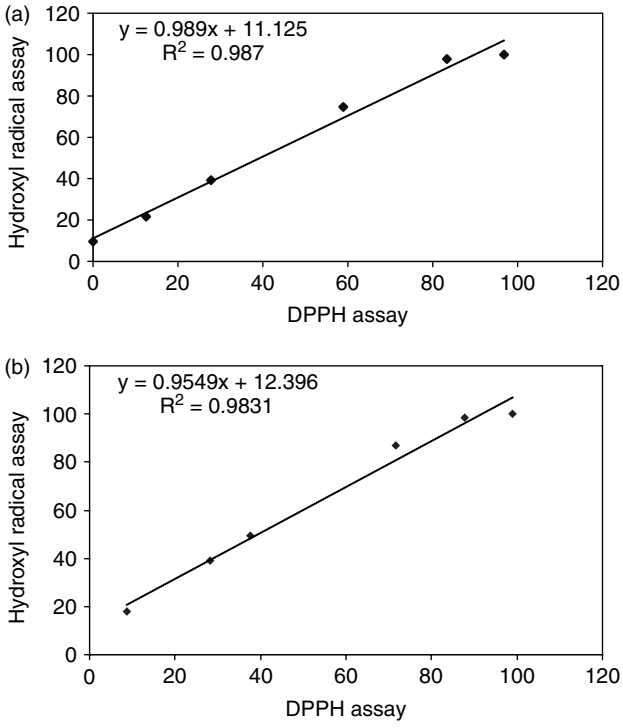


**Figure 6.** Correlation between DPPH and hydroxyl radical assays for 70% methanol (a) and ethyl acetate (b) extracts.

The scavenging properties of antioxidant compounds (flavonoid and phenolic acids) are often associated with their ability to form stable radicals. For example, quercetin and chlorogenic acid (the main antioxidative compounds in wormwood) can scavenge radicals effectively and usually give rise to semiquinone free radicals. In alkaline solution, these free radicals are stable enough to be simply examined by ESR spectroscopy.[43] Under our experimental conditions quercetin displayed the highest radical intensity in alkaline medium (Fig 7(a,b)), possibly due to the partial structure of hydroquinone in the B ring. The eight peaks observed in the ESR spectrum and hyperfine coupling constants ($a_H{}^{2'} = 1.5$G $a_H{}^{5'} = 0.7$G and $a_H{}^{6'} = 2.7$G) are typical of quercetin *o*-semiquinone radicals (Fig 7(a)).

Jovanovic *et al*[44] suggested that, in the flavonoids which have a 2,4-dihydroxy-acetophenone (rutin, quercetin)-like A ring and a catechol-like B ring, the antioxidant active moiety is clearly the B ring because this ring possesses a lower reduction potential.

The analysis of ESR spectra presented in Fig 7(b) showed that the line pattern and splitting constants ($a_H{}^6 = 5.03$ G, $a_H{}^5 = 2.42$ G and $a_H{}^2 = 1.25$ G) were typical of *o*-semiquinone anion radicals of chlorogenic acid, as secondary type of radicals (Fig 7(b)). These radicals are insufficiently reactive and they can disappear by several mechanisms.[45]

## CONCLUSION

In conclusion, this study indicates that the extracts obtained from whole plant of wormwood have

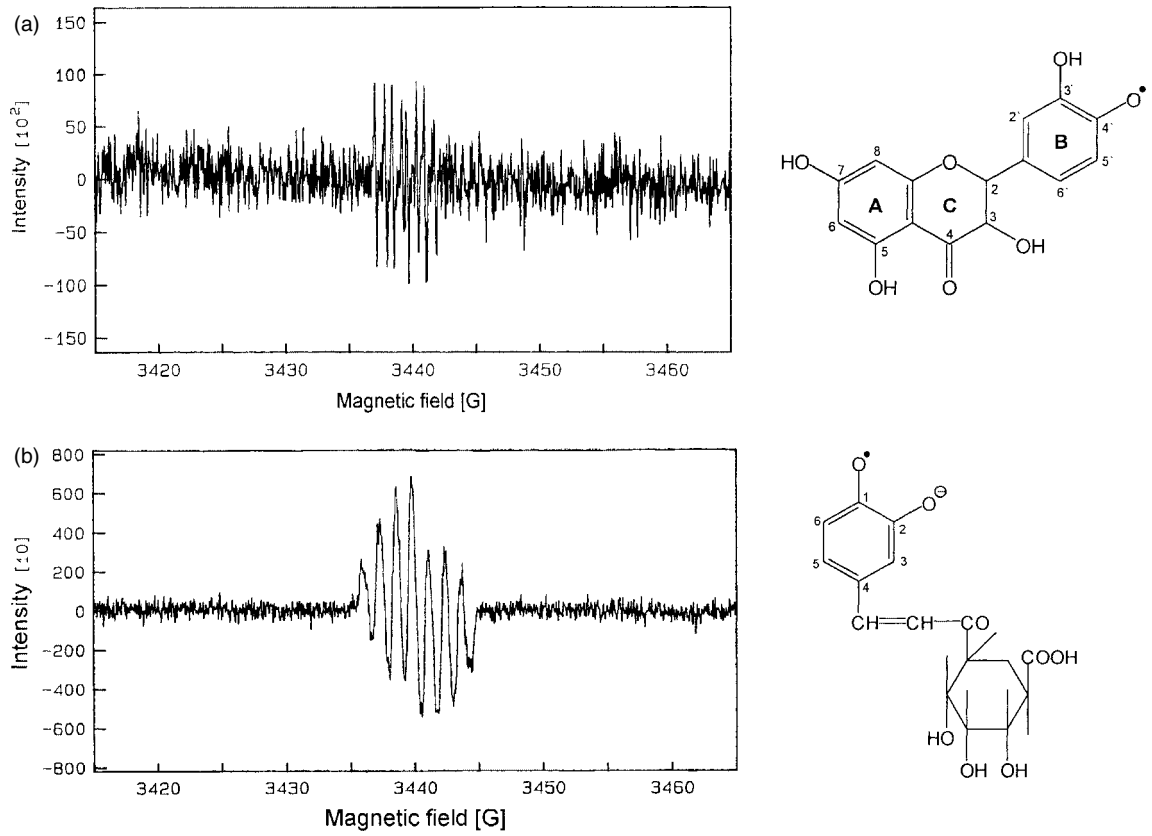


**Figure 7.** ESR spectra of quercetin (a) and chlorogenic acid (b).

significant free radical scavenging activity on stable DPPH and high reactive hydroxyl radical, the extent of which depends on the type of the solvent used for the extraction. Herb extracts contain polyphenols with different chemical behaviours which can exhibit their antioxidative activity by simultaneous hydrogen atom donation to free radicals, electron transfer which is strongly solvent-dependent, and by metal chelating.

More work should be done to characterize individual phenolic compounds of the extracts of wormwood in order to assign certain antioxidant effects to individual compounds of the resulting extracts. In future studies it will be desirable to employ experimental conditions that can more specifically reflect the various gastric/intestinal microenvironments, which are pertinent to the uptake of nutritient and plant secondary metabolites in the human system.

## ACKNOWLEDGEMENT

This research is part of the project 'Biologically Active Constituents of Wild Growing Plants as Natural Sources for Pharmacy, Cosmetics and Foodstuff Industry' (Project no 1862), which is financially supported by the Ministry of Science, Technologies and Development of the Republic of Serbia.

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