

# Determination of Free Radical Scavenging Activity of Plant Extracts Through DPPH Assay: An EPR and UV–Vis Study

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**Abstract** Several methods have been developed to assess the radical scavenging activity. Among them, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) spectrophotometric method is one of the most widely applied and is appreciated for its reliability. In this study, a comparison of two spectroscopic methods (electron paramagnetic resonance (EPR) and ultraviolet–visible (UV–Vis) spectroscopy) was performed analysing the spectroscopic features of DPPH in mixed ethanol/water solution and the free radical scavenging properties of myrtle leaves extracts and citrus juices. When DPPH was dissolved in mixed solvents, EPR enabled to identify the aggregation phenomena that occur when high amounts of water were employed. On the contrary, UV–Vis revealed only small differences in the absorption maximum among solutions with increasing water contents, without detecting aggregation.  $EC_{50}$  values of myrtle leaf extracts and citrus juices calculated from UV–Vis data were lower than those calculated with EPR. In myrtle extracts, the DPPH depletion measured by UV–Vis

was not concentration dependent, revealing the interference in the analysis of the decomposition products of the antioxidants, which absorb at 517 nm. EPR spectroscopy was proven to be most reliable with all types of matrix since it is not dependent on the chemical composition of the extract.

**Keywords** EPR spectroscopy · UV–Vis spectroscopy · Antioxidants · DPPH · Myrtle · Citrus juice

## Introduction

Antioxidants have received much attention in modern society for the beneficial effects that they exert on human health. It is well established that antioxidant molecules can prevent cardiovascular and neurodegenerative diseases by scavenging free radicals, mainly reactive oxygen species, produced during cell metabolism (Zafra-Stone et al. 2007). The antioxidant application fields are manifold ranging from the cosmetic to the food industry. Synthetic antioxidants, like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are frequently used as additives in food processing to prevent deterioration due to oxidation processes. However, the safety concerns about the use of such additives have led to an increasing interest in natural antioxidants coming from the secondary plant metabolism.

A variety of methods have been developed to assess antioxidant activity (AA), but the complexity of substrates like food or biological matrixes prevents from having a reliable method for antioxidant activity quantification (Huang et al. 2005).

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH<sup>•</sup>) method is widely applied for AA assay and is thought to be one of the most reliable especially for fruits, vegetable

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juices, and plant extracts (Magalhães et al. 2008; Moon and Shibamoto 2009; Sanchez-Moreno 2002). This method is based on the ability of the antioxidant to scavenge DPPH, reducing its original concentration and turning the colour of the solution from purple to yellow (Brand-Williams et al. 1995). For the DPPH assay, several protocols have been reported, differing for the concentration of the DPPH solution ranging from 22.5 to 250 mM, for the solvents or mixtures of solvents used to dissolve DPPH or to prepare the extracts and for the reaction time. Sharma and Bhat (2009) showed a strong influence of the reaction medium on the  $EC_{50}$  values. Similarly, Stasko et al. (2007) showed that in reaction mediums with high water content, aggregation phenomena of the DPPH molecules can occur thus reducing the availability of DPPH in the reaction with the antioxidants.

The spectrophotometric assay, due to its simplicity, is the most extensively used method to determine the depletion of DPPH in the presence of an antioxidant. In recent years, electron paramagnetic resonance (EPR) spectroscopy has been applied to evaluate the antioxidant properties of wines, teas, fruits, juices (Dos Santos et al. 2009; Polovka 2006; Polovka et al. 2003; Tzika et al. 2008) as well as to evaluate the stability of  $\gamma$ -irradiated foods (Polovka et al. 2006) and to test the radical scavenging ability of processed fruits (Oszmianski et al. 2008) and plant extracts (Maisuthisakul et al. 2007).

This study was designed to perform a direct comparative analysis of the outcomes obtained with ultraviolet–visible (UV–Vis) and EPR spectroscopies. The comparison was carried out analysing the spectroscopic features of DPPH in ethanol- or methanol–water solutions and the radical scavenging activity of different foodstuffs. In this work, myrtle leaves and fruits of some citrus species were selected as plant material. Myrtle (*Myrtus communis* L.) is an aromatic plant whose leaves (or berries) are used for the production of the Sardinian typical liqueur and are an interesting source of antioxidant compounds with medicinal properties (Tuberoso et al. 2010). Citrus fruit is one of the most important antioxidant sources, is widely appreciated for its nutritional value and for the presence of bioactive compounds, such as, ascorbic acid, polyphenols, and flavonoids (Ladaniya 2008).

## Materials and Methods

### Chemicals

All reagents and solvents were of analytical grade, unless otherwise specified and used without further purification. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Alfa Aesar (London, UK), methanol and

ethanol (95%), Folin-Ciocalteu reagent, sodium carbonate, and gallic acid from Aldrich (Milan, Italy) while ethanol absolute from Carlo Erba (Rodano (MI), Italy).

### Free Radical Scavenging Activity of Plant Material

#### *Plant material*

The leaves of six myrtle cultivar namely: ‘Daniela’, ‘Angela’, ‘Barbara’, ‘Grazia’, ‘Maria Antonietta’ and ‘Giovanna’, grown in an experimental orchard of the University of Sassari, Faculty of Agriculture located in Oristano (Sardinia, Italy) were used in this study. Leaves were harvested at the beginning of June, selected to remove damaged ones, washed, immediately frozen and stored at  $-80^{\circ}\text{C}$  until extraction.

Grapefruit, cv Star Ruby, blood oranges, cv Moro and Sanguinello, lemons cv Verna and mandarins cv Fortune were grown in an experimental orchard (Central Western Sardinia, Italy), receiving standard horticultural practices. Fruits were harvested when commercially mature and washed.

#### *Extraction Procedure*

Two grams of frozen myrtle leaves were ground and mixed with 50 mL of 95% ethanol. The mixture was stored in the dark at  $4^{\circ}\text{C}$  for 4 h and was shaken to ensure complete extraction. Then, it was filtered (Whatman 113), and the extract was stored in the dark at  $4^{\circ}\text{C}$  for a maximum of 3 days until analysis.

For the juice extraction, the citrus were squeezed with a small laboratory hand reamer, filtered, and immediately analysed. All extracts were performed in triplicate.

#### *Determination of Free-Radical Scavenging Capacity*

The free radical scavenging activity of myrtle and citrus extracts was determined by UV–Vis and EPR spectroscopies. Each extract, without further modification, was analysed with EPR to ensure the absence of free radical species, which could interfere with EPR measurements.

The extract solutions were prepared through dilution with ethanol 95%. A fixed volume of plant extract solution (4.75 mL) at different dilutions was mixed with 250  $\mu\text{L}$  of DPPH 1 mM in ethanol 95% and stored in the dark at room temperature for 30 min. At the end of the reaction period, the same solution was used to measure EPR and UV–Vis spectra. Concentrations of myrtle extracts were referred to the original stock solution with a concentration of 40 mg/mL.

The free radical scavenging activity of the plant extracts was expressed as  $EC_{50}$ , that is, the concentration of extract

required to reduce the initial concentration of DPPH by 50%. This value was estimated by plotting the percentage of inhibition over sample concentration.

For EPR measurements, the percentage of inhibition, was calculated as follows: percent inhibition =  $100 \times (I_0 - I_i)/I_0$ , where  $I_0$  is the intensity of the EPR spectrum of the control solution (mixture without plant extract), while  $I_i$  is the intensity of the EPR spectrum of the sample.

For UV–Vis measurements, the formula was corrected according to the colour of the solution when 100% of the initial DPPH was converted into non-radical species by the antioxidants present in the plant extract, as demonstrated by the EPR spectra. In this case, the percentage of inhibition was calculated as follows: percent inhibition =  $100 \times (A_0 - A_S)/(A_0 - A_i)$ , where  $A_0$  is the absorbance at 517 nm of the DPPH solution without antioxidant,  $A_S$  is the absorbance of the sample and  $A_i$  is the absorbance of the solution when 100% of DPPH is reduced.

### DPPH Features in Mixed Solvents

In this set of experiments the results obtained with EPR and UV-Vis spectroscopies were compared. In order to evaluate the reliability of UV-Vis results even in the presence of DPPH aggregated forms due to increasing water contents in the reaction mixtures, three DPPH concentrations and different water/ethanol and water/methanol ratios were tested.

### Solutions

Three stock DPPH solutions  $4.0 \times 10^{-4}$  M,  $8.0 \times 10^{-4}$  M, and  $8.5 \times 10^{-3}$  M were prepared in absolute ethanol. Stock solutions were stored in the dark at 4 °C, and the analyses were performed within 3 days. An aliquot of each DPPH stock solution was added to ten different reaction mixtures containing increasing water contents. DPPH spectra were also recorded using buffered ethanol as solvent. In this case, the final composition of the reaction mixture was: 40% (v/v) of AcOH/AcONa buffer pH 5.5, ethanol 60% (v/v) and DPPH  $8.5 \times 10^{-4}$  M.

Similar experiments were carried out using methanol as solvent. In this case, only one DPPH concentration ( $8.5 \times 10^{-4}$  M in methanol) was tested. A test with buffered methanol as solvent, prepared as described above, was also performed.

### EPR Measurements

EPR measurements were carried out at room temperature using a Bruker EMX spectrometer operating at the X-band (9.4 GHz) using a quartz flat cell. EPR instrument

setting were: center field, 3460 G; sweep width, 100 G; modulation amplitude, 1 G; microwave power, 20 mW (10 dB); receiver gain,  $5 \times 10^5$ . For each sample, at least two spectra were recorded. For the quantification of the DPPH concentration present in each sample, double integration is required since the spectra were measured as second derivative of the absorption. EPR spectra showed that the line width was not dependent by DPPH concentration being similar in all samples. For this reason, instead of doubly integrating, the height of the central line was taken as a measure of the intensity of the signals. The central line was used only when a pure quintet spectrum was present, that is, when all DPPH was in solution. This was possible for all the measurements because in the ethanol 95% solutions, DPPH was always in solution, and no aggregation phenomena took place.

### UV–Vis Measurements

UV–Vis readings were carried out with a spectrophotometer Perkin-Elmer Lambda 35. The spectrophotometric DPPH assay measures the absorbance of the DPPH antioxidant solution at 517 nm; however, a spectrum in the range between 400 and 700 nm was recorded. Percentages of inhibition were calculated using the absorbance at 517 nm as generally reported in the literature for the determination of DPPH radical.

### Determination of Total Polyphenol Content

For polyphenol analysis, myrtle and citrus extracts were prepared as described above and measured with a Cary 1E spectrophotometer (Varian, Palo Alto, CA, USA). Total polyphenols were determined by the Folin–Ciocalteu colorimetric method (Di Stefano and Cravero 1991) using gallic acid as standard. Results were expressed as milligrams/100 mL of gallic acid equivalents.

### Statistical Analysis

One-way analysis of variance was carried out with the MINITAB software using a unifactorial complete randomized block design. Mean comparisons were calculated by Fisher's least significant differences test at  $P \leq 0.05$ .

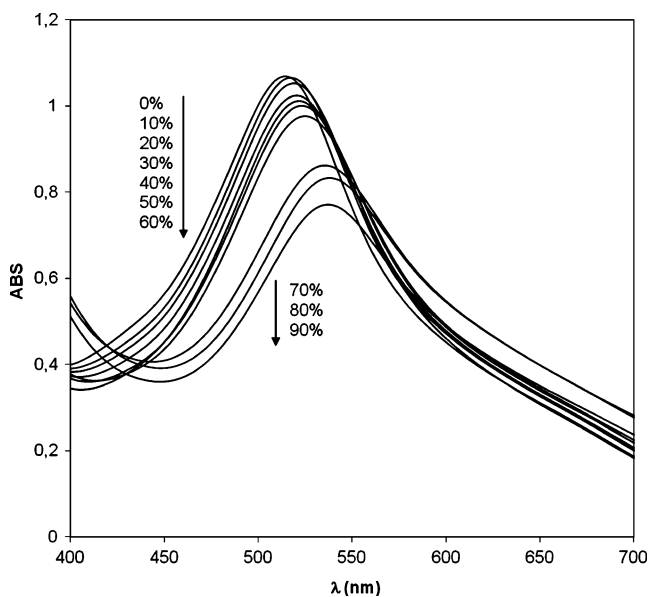
## Results and Discussion

### DPPH in Mixed Solvents

In agreement with previous studies (Stasko et al. 2007), DPPH aggregation processes, due to high water ratios and/

or high DPPH concentrations, can be clearly detected with EPR measurements. By contrast, such occurrences cannot be observed when the analyses were performed by UV–Vis spectrophotometric method. In this case, the absorption maxima of DPPH solutions containing different ratios of ethanol/water were dependent on the mixture composition: increasing the amount of water the absorption maximum increased its wavelength (Fig. 1). Similarly, the extinction coefficient was strictly related to the ethanol/water ratio in DPPH solution, thus preventing from comparing the results from the solutions with different percentages of water.

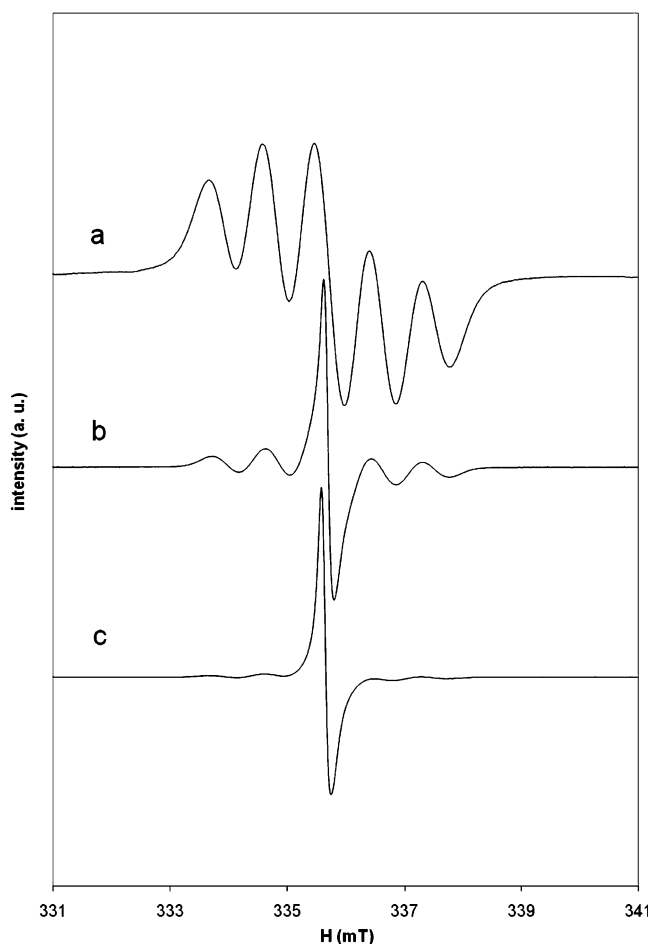
The EPR spectrum of DPPH aggregated form is characterized by the presence of an unusually narrow line with disappearance of the hyperfine splitting. The EPR spectra of DPPH  $8.5 \times 10^{-5}$  M in mixed ethanol water solutions showed aggregation in mixtures containing 70%, 80%, and 90% (v/v) of water. In these solutions, despite the presence of water, where DPPH is completely insoluble, no precipitation was observed. With a DPPH concentration of  $8 \times 10^{-4}$  M, DPPH molecules begin to aggregate with 30% (v/v) of water. In these conditions, the quintet spectrum was still present, but the singlet began to be evident. The singlet spectrum cannot be due to other radicals since no antioxidants, able to react with DPPH forming new paramagnetic species, were present in the solution. With increasing water content, the quintet spectrum decreased its intensity; the two forms overlapped



**Fig. 1** Electronic absorption spectra measured on solutions containing increasing water/ethanol ratios (from 0% to 90%) having the same concentration of DPPH ( $8.5 \times 10^{-5}$  M). In the last three spectra (from 70% to 90% of water), aggregation phenomena take place as evidenced by EPR spectra

up to 55–60% (v/v) of water (Fig. 2b, c), then only the singlet spectrum remained. Contrary to what was observed with low DPPH concentration, precipitation was evident a few minutes after the preparation of the solutions. With the high concentration of DPPH, precipitation of solid DPPH occurs but the intense colour of the DPPH solutions usually prevents the detection of these precipitates. In these conditions, precipitation was evident only when UV–Vis spectra and not single wavelength measurements were recorded.

The relationship between aggregation and DPPH concentration was clear, analyzing EPR spectra recorded with an intermediate concentration (DPPH,  $4 \times 10^{-4}$  M). In this case, the onset of aggregation occurred with 50% of water in the reaction mixture. Even in this situation, although a spectrum was registered, UV–Vis did not allow to identify aggregation. With high water contents, that is, 55% and 60% (v/v), precipitation was starting to take place. The results obtained in methanol/water mixtures



**Fig. 2** EPR spectra of  $8 \times 10^{-4}$  M DPPH in ethanol/water mixtures. **a** 100% ethanol, **b** 45% ethanol (55% H<sub>2</sub>O), and **c** 40% ethanol (60% H<sub>2</sub>O). In the last spectrum, aggregated DPPH was present but without precipitation

were similar to those described for solutions containing ethanol and water. EPR spectra of these solutions showed that the onset of aggregation occurred with 60% (v/v) of water in the reaction mixture with a DPPH concentration of  $8.5 \times 10^{-5}$  M, as demonstrated by the increase of the intensity of the central line in the five-line signal.

The chemical behaviour of DPPH was also studied in buffered methanol and ethanol solutions. Both EPR and UV–Vis measurements revealed some differences between buffered methanol and pure methanol. As expected, the absorbance of a solution of DPPH  $8.5 \times 10^{-5}$  M in buffered methanol (with 40% (v/v) of water) was lower than that measured in pure methanol. Furthermore, a wavelength shift of the absorption maximum was observed. The comparison of the buffered methanol solution and the mixture containing the same amount of water revealed a higher absorption in buffered than in methanol/water solution. The same was not found by Sharma and Bhat (2009) who observed no significant differences in the absorption at 517 nm between methanol and buffered methanol with DPPH concentrations lower than 100  $\mu$ M. With higher DPPH concentrations the absorbance measured in buffered methanol was slightly higher than in pure methanol (Sharma and Bhat 2009).

EPR spectra confirmed what was observed in spectrophotometric measurements. Even in this case, the intensity of the five-line signal was higher in buffered methanol than in methanol 60% (v/v), while the ethanolic solutions showed an opposite trend. The comparison of the UV–Vis spectra of methanol 60% (v/v) and buffered methanol (AcOH/AcONa pH 5.5, 60% (v/v) of methanol) demonstrated that the latter showed an absorption maximum higher than that observed in non-buffered solution. These data confirm the influence of pH of the reaction mixture on the stability of DPPH (Ozcelik et al. 2003). However, the optical properties of buffered solutions containing DPPH also depend on the type of solvent used (Ozcelik et al. 2003). This could explain the different behaviours of DPPH solutions observed in our study when ethanol 60% (v/v) and buffered ethanol were used as solvents. In fact, contrary to that observed for methanol, the reaction mixture containing ethanol 60% (v/v) had higher absorbance than the buffered solution with the same amount of ethanol.

#### Radical Scavenging Activity of Myrtle Extracts

In the determination of radical scavenging activity of myrtle extracts, no aggregation phenomena occurred since solutions with low amounts of water were used (ethanol 95%). In these cases, the EPR spectra were characterized by the presence of five lines, and no narrow single line was observed.

The antiradical activity of myrtle extracts was dependent on the cultivar (Table 1). Anyway, the numerical values were not compared with those of known antioxidants, such as BHT and BHA, because the aim of the present work was to compare the outcomes of the two spectroscopic techniques.

Leaves of the cultivar ‘Angela’ showed the highest DPPH scavenging activity, as indicated by the lowest  $EC_{50}^{EPR}$  value, whereas the lowest DPPH depletion was found in the cultivar ‘Maria Antonietta’ revealing a low antioxidant “power” of the extract. The other leaf extracts namely: ‘Grazia’, ‘Giovanna’, ‘Barbara’ and ‘Daniela’ showed very similar scavenging activities ranging from 30.36 to 36.80  $\mu$ g/mL. The different antioxidant activities among myrtle cultivars could be related to the relative concentration of antioxidant compounds such as galloyl glucosides, ellagitannins, galloyl-quinic acids and flavonol glycosides (Romani et al. 2004; 1999; Tuberoso et al. 2010).

Despite the use of the same solution, the two spectroscopic techniques gave contrasting outcomes. The  $EC_{50}$  values obtained with EPR significantly differed from those achieved from UV–Vis spectroscopy for all myrtle cultivars except for “Barbara” leaves.  $EC_{50}$  values determined with EPR were always lower than those attained by UV–Vis, with differences between the two methods ranging from 6.84 to 28.9  $\mu$ g/mL. The differences in the  $EC_{50}$  values obtained with UV–Vis and EPR increase in the order: ‘Barbara’, ‘Grazia’, ‘Maria Antonietta’, ‘Angela’ and ‘Giovanna’. Such results prevent to find a clear relationship between data obtained with EPR and UV–Vis applicable to different extracts. A possible explanation of this behaviour relies on the lack of corrections of the results due to the oxidation products of the antioxidants. In spectrophotometric assay, a correction of the results should be taken

**Table 1** Antioxidant activity of the extract of myrtle cultivars as determined by EPR and spectrophotometric (UV–Vis) method

Myrtle cultivar	Spectrometric method	
	EPR $EC_{50}$ ( $\mu$ g/mL)	UV–Vis $EC_{50}$ ( $\mu$ g/mL)
Barbara	34.24 $\pm$ 1.3a	41.08 $\pm$ 6.9a
Giovanna	32.15 $\pm$ 2.3b	61.05 $\pm$ 9.8a
Maria Antonietta	42.46 $\pm$ 5.6b	60.73 $\pm$ 5.0a
Grazia	30.36 $\pm$ 5.3b	44.62 $\pm$ 6.8a
Angela	28.50 $\pm$ 8.4b	46.10 $\pm$ 3.1a
Daniela	36.80 $\pm$ 1.6	

Means in row followed by different letters differ significantly for Fisher’s LSD,  $P \leq 0.05$

LSD least significant difference



into account if the plant extracts or the antioxidants are coloured and absorbed at 517 nm. In this study, no correction for the colour of the solution was applied since all the extracts examined were colourless and did not show a significant absorbance at 517 nm even when the highest analysed concentration was used, that is, a dilution of 1:100 of the ethanolic extract. On the contrary the decomposition products of both DPPH and the antioxidants have significant absorptions at the same wavelength, therefore results were corrected considering the absorbance of a solution where all the DPPH radical form is reduced. In order to detect the complete reduction of the radical DPPH, EPR spectra were recorded. By contrast in EPR spectroscopy, no correction was made since it identifies only the DPPH radical, and none of the compounds present in the extract is a paramagnetic species.

In UV–Vis measurements, the differences in absorbance values measured at 517 nm among solutions with increasing amounts of extract were very low for all myrtle cultivars, revealing no proportionality between the extract concentration and absorbance. Increasing the amount of added extract, the colour changed from dark purple, in mixtures without antioxidant, to dark brown, as the extract, added in large amounts, depleted all the DPPH present in the solution. The very small differences in the absorption spectra prevented to obtain reliable results in the case of the cultivar ‘Daniela’ even if the solutions used for EPR and UV–Vis assays were the same. For this reason, the results of the spectrophotometric assay were not reported in Table 1 for this cultivar. In the other cases, the differences between the samples were small but  $EC_{50}^{UV-Vis}$  values could be obtained.

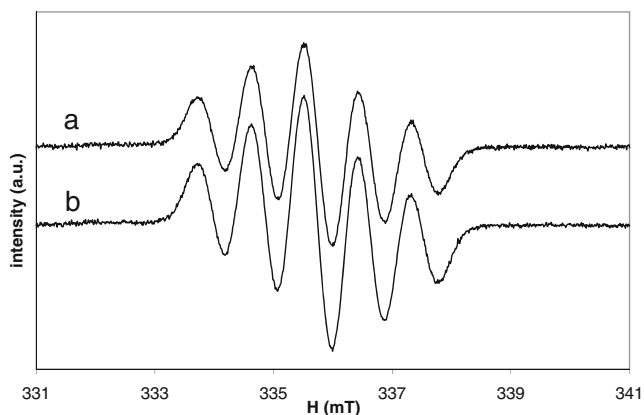
Contrary to what was observed for UV–Vis measurements, the decrease in DPPH signal intensities measured by EPR was concentration dependent for all the extract, and

significant differences were observed among solutions with increasing amounts of extract.

#### Radical Scavenging Activity of Citrus Juices

Citrus juices analysed in our study had quite high antioxidant activity. Blood oranges antioxidant activity accounts for  $EC_{50}^{EPR}$  values of 135.09 and 128.61  $\mu\text{L}/\text{mg}$  DPPH, for ‘Moro’ and ‘Sanguinello’ juice, respectively, while the other  $EC_{50}^{EPR}$  juices analysed were 228.95, 183.96, and 221.25  $\mu\text{L}/\text{mg}$  DPPH, respectively for ‘Fortune’ mandarin, ‘Verna’ lemon and ‘Star Ruby’ grapefruit. Our results confirm other reports which assign to blood oranges the highest AA (1.03–7.05 mM trolox equivalents) compared to other citrus juices (Rapisarda et al. 1999).

Results attained from EPR and UV–Vis spectroscopies showed lower differences than those obtained for myrtle extracts (Table 2). In the juice of ‘Fortune’ mandarins and in ‘Sanguinello’ oranges, no significant differences were found between the two methods. Instead, for the other citrus cultivar, the outcome obtained with the two methods differed significantly: with  $EC_{50}^{EPR}$  values higher than  $EC_{50}^{UV-Vis}$ . The DPPH depletion was dose dependent in both methods of analysis. This is probably related to the different compounds and oxidation products involved in antioxidant reaction which absorb at the wavelength considered. Furthermore, measuring the  $EC_{50}^{UV-Vis}$  no correction of the results was applied for the colour of the antioxidant solution. Even when working with coloured samples like ‘Moro’ and ‘Sanguinello’, the final solution was colourless since the high antioxidant activity of such juices allow to dilute the sample up to lose their characteristic red colour. In general, all citrus juices analysed in our study had quite high antioxidant activity.



**Fig. 3** EPR spectra of  $8.5 \times 10^{-4}$  M DPPH in methanol/water mixtures. **a** 60% methanol and **b** buffered methanol

**Table 2** Antioxidant activity of juice of ‘Fortune’ mandarins, ‘Verna’ lemons, ‘Sanguinello’ and ‘Moro’ oranges, and ‘Star Ruby’ grapefruit as determined by EPR and spectrophotometric (UV–Vis) method

Citrus cultivar	Spectrometric method	
	EPR $EC_{50}$ ( $\mu\text{L}/\text{mg}$ DPPH)	UVVis $EC_{50}$ ( $\mu\text{L}/\text{mg}$ DPPH)
Fortune	228.95 $\pm$ 7.45a	227.06 $\pm$ 0.22a
Verna	183.96 $\pm$ 4.49a	155.84 $\pm$ 2.03b
Sanguinello	128.61 $\pm$ 9.06a	130.36 $\pm$ 10.56a
Moro	135.09 $\pm$ 4.21a	121.85 $\pm$ 3.43b
Star Ruby	221.25 $\pm$ 3.19a	152.52 $\pm$ 10.40b

Means in row followed by different letters differ significantly for Fisher’s LSD,  $P \leq 0.05$

LSD least significant difference

The small differences found between EPR and UV–Vis results using citrus juices let us suppose that other compounds may be involved in the antioxidant activity, with respect to myrtle extracts. This was further supported by the fact that, in our study, a low correlation between AA and juice polyphenol content was found. Gardner et al. (2000) considered ascorbic acid as the major antioxidant in orange juices, accounting for more than 65% of their total antioxidant activity. This might be the possible explanation of the low differences between EPR and UV–Vis results. As a matter of fact, dehydroascorbate, which is the primary oxidation product of ascorbate, does not absorb at the wavelength considered in this work (Smirnoff 1996) thus ruling out any possibility of interference of oxidation product in the spectrophotometric assay.

### Polyphenol Content

Myrtle extracts showed remarkable differences in polyphenol content among cultivars, with values ranging from 264.14 to 162.25 mg/100 mL for ‘Giovanna’ and ‘Grazia’, respectively (Table 3). Significant differences were observed for citrus juice polyphenol content as well.

Lemon juice had the lowest phenolic content, with 47.93 mg/100 mL, while high phenolic concentration was found in Sanguinello oranges and Fortune mandarins, averaging 86.97 and 84.30 mg/100 mL, respectively (Table 4).

Total antioxidant activity was not related with total phenol content both in citrus juices and myrtle extracts.

### Conclusions

Our data clearly show that there could be differences in the EC<sub>50</sub> values obtained with the two applicable spectroscopic methods for the determination of the radical scavenging activity of DPPH, EPR, and UV–Vis. Since the samples

**Table 3** Total polyphenol content in myrtle leaves extract

Myrtle cultivar	Polyphenol concentration (mg/100 mL)
Barbara	183.58±2.7d
Giovanna	264.14±3.5a
Maria Antonietta	178.98±0.7d
Grazia	162.25±2.1e
Angela	234.74±2.2a
Daniela	191.72±3.9c

Means in column followed by different letters differ significantly for Fisher’s LSD,  $P \leq 0.05$

LSD least significant difference

**Table 4** Total polyphenol content in citrus juices

Citrus cultivar	Polyphenol concentration (mg/100 mL)
Star Ruby	63.04±5.19c
Moro	68.82±1.48b
Sanguinello	86.97±1.08a
Fortune	84.30±1.30a
Verna	47.93±2.47d

Means in column followed by different letters differ significantly for Fisher’s LSD,  $P \leq 0.05$

LSD least significant difference

used in the two assays were the same, these differences were certainly not due to the solutions used but to the method itself. Since DPPH is an intensely coloured radical, both EPR and UV–Vis spectroscopies could be used for its quantification in a sample. However, when comparing the two techniques, EPR is more sensitive and reliable than absorption spectroscopy because it is not influenced by the nature of the antioxidants or of their decomposition products. The absence of factors, like decomposition products or other radicals produced during the reaction, influencing the measurements, makes the EPR spectroscopy a reliable tool in a wide range of conditions. The only other possible limitation in using this technique is due to aggregation phenomena but since these are easily recognized this does not constitute a serious problem. According to the present results, the estimation of radical scavenging capacity performed by EPR is more reliable than UV–Vis measurements even in the case of clear or not coloured samples. In fact, the spectrophotometric methods are very sensitive to the colour of the solutions since they are able to detect all the chemical species absorbing at 517 nm. Based on these considerations, the spectrophotometric method could be considered as the method of choice in the case of clear and not coloured samples, but aggregation phenomena that occur in the presence of solutions with high water contents can prevent its use for the determination of radical scavenging activity.

This observation is particularly important because it means that the spectrophotometric DPPH assay cannot be considered of general use, but preliminary measurements are needed in order to determine if, in that particular case, it can be used or not. Therefore, parallel measurements performed with EPR and UV–Vis highlight different aspects of the same chemical reaction.

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