Analitical Methods

Estimation of antiradical properties of antioxidants using DPPH– assay: A critical review and results

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Abstract

Applications of antioxidants are increasing due to their multiple roles in minimising harmful effects of oxidative stress. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is routinely practiced for the assessment of antiradical properties of different compounds. A detailed literature survey revealed use of different materials and methods for DPPH assay by different investigators resulted in variation in the values of reference standards and measured parameters of new antioxidants. In the present work a detailed kinetic study of antioxidants has been performed and comprehensive results in terms of effective concentration which scavenges 50% radical (EC50), antioxidant reducing power (ARP), stoichiometry and second order rate constant (k2) values have been reported with DPPH assay. Importance of selection of appropriate reference compounds and kinetic calculations are suggested. Few case studies of standard antioxidants have been discussed to emphasise the utilisation of appropriate methodology and reference compounds.

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1. Introduction

Assessments of antioxidant properties of natural compounds are very important because of their uses in medicine, food and cosmetics (Halliwell, 1997; Liu, 2003; Sánchez-Moreno, 2002). In living systems various metabolic processes and environmental stresses generate various reactive species. These are free radicals and mainly reactive oxygen species (ROS). Increased level of ROS can damage structure of biomolecules and modify their functions and lead to cellular dysfunction and even cell death. The cumulative effect of increased ROS can increase oxidative stress in systemic level and it is manifested in the form of a variety of health problems such as cancer, age related disease and cardiovascular diseases (Gruene, Shringarpure, Sitte, & Davies, 2001; Noguchi & Niki, 2000). Cellular ROS are regulated by interplay of complex antioxidant machineries in living systems. Nature has bestowed living systems with numerous antioxidant molecules. These natural antioxidants are known to minimise the adverse effects of free radicals in living system. Many of these naturally occurring antioxidants are now isolated, fully characterised, and available for various applications. The chemical structures of these pure compounds are very diverse in nature. Thus, it is expected that the correlation between structure and antioxidant properties will be a difficult preposition. Many of these natural antioxidants are actively considered as prophylactic and therapeutic agents for possible applications for radiation countermeasures (Weiss & Landauer, 2003), combating cancers and aged related disease (Blasko & Cordell, 1998).

DPPH assay is routinely practiced for assessment of free radical scavenging potential of an antioxidant molecule and considered as one of the standard and easy colorimetric methods for the evaluation of antioxidant properties of pure compounds. Though this radical has limited similarities with peroxyl radicals, this assay is in commonly used to measure the antioxidant content of wheat grain and bran, vegetables, conjugated linoleic acids, herbs, edible seed oils, and flours in different solvent systems including ethanol, aqueous acetone, methanol, aqueous alcohol, and benzene (Cheng, Moore, & Yu, 2006). DPPH is a stable radical in solution and appears purple colour absorbing at 515 nm in methanol. This assay is based on the principle that DPPH on accepting a hydrogen (H) atom from the scavenger molecule i.e. antioxidant, resulting into reduction of DPPH to DPPH2, the purple colour changes to yellow with concomitant decrease in absorbance at 515 nm. The colour change is monitored by spectrophotometrically and utilised for the determination of parameters for antioxidant properties. This method was conceptualised by Blois (1958) in which the first time H atom accepting ability of a stable free radical DPPH from cysteine molecule was demonstrated. After about three decades this assay has drawn attention for characterisation of antioxidant
properties. The original DPPH assay procedure has been adopted in different labs but with modifications for convenience. A detailed literature survey revealed that most of the studies are based on fixed reaction time ranging from 20–30 min instead of total reaction time that is actually required to attain steady state to complete this redox reaction (Molyneux, 2004). The DPPH assay thus appears simple in nature but due to its stable nitrogen radical and, many antioxidants might react with different kinetics or might not react at all. Further, due to reversibility of reaction between DPPH+ and antioxidant reduction of the radical will not be entirely due to the antioxidant as DPPH2 will convert into DPPH/C5 form due to reversibility. Therefore, reversibility of the reaction may lead to low reading of antioxidant capacity of many antioxidants. The implication of reversibility in this reaction was reported by Bondet et al. in the case isoegenol and it is equally emphasised by Huang et al. for underestimation of antioxidant capacity of other phenols bearing a similar structure type (Bondet, Brand-Williams, & Berset, 1997; Huang, Ou, & Prior, 2005). Because of this reason the antioxidant capacity of many antioxidants likely to be wrongly estimated.

Because of our interest in mechanistic studies with antioxidants based radioprotectors, we have made a survey of literature and observed various methodologies used for DPPH assay. For instance variation in concentrations of DPPH, sample volume and different units of measured parameters were considered. These practices have resulted in differences in IC50 values of even reference standards viz., ascorbic acid, butyrate hydroxyl toluene (BHT), gallic acid, butylated hydroxyl anisole (BHA) and trolox. For example, IC50 of ascorbic acid reported in the range from 11.85 to 629 μM. A detailed summary of these reported values are shown in Table 1. Similar variation in measured parameter exists for synthetic antioxidants BHT, the IC50 of this compound ranges from 25 to 393 μM. These reported values of reference standards are thus deceptive. Because of these variations with standard reference molecule, antiradical parameters of new molecules assessed by using DPPH assay are likely to be wrongly estimated.

Further, in DPPH assay the influence of the medium and possible role of depredonation of the phenolic group has also been outlined due to its important role (Foti, Daquino, & Geraci, 2004; Litwinienko & Ingold, 2003). DPPH assay is usually performed in methanol and to simulate in vivo predictability, DPPH assay is also reported in semi-aqueous media (methanol:buffer, 1:1(v/v)). The comparative studies of these two methods have not been attempted earlier.

In the present study we have focused on the two methods, (a) steady state reaction time and (b) fixed reaction time, to simultaneously estimate kinetics and effective concentration of DPPH scavenging by a number of antioxidants. The comparison of these two methods in this study revealed expected noticeable differences in addition; few case studies have been discussed to emphasize the use of suitable method. The findings from this study will be useful in the understanding and designing of new experiment to assess antiradical properties of chemically pure antioxidants or complex plant extracts with suitable reference standard.

2. Materials and methods

2.1. Chemicals

Sesamol, ferulic acid were purchased from Acros organics (Belgium) and melatonin, ascorbic acid, gallic acid, butyrate hydroxyl toluene (BHT), alpha-tocopherol, curcumin, alpha-lipoic acid, pentoxifylline, 1,1-diphenyl-2-picryl-hydrazil (DPPH) were purchased from Sigma chemicals, USA. Methanol (spectroscopic grade) was purchased from E. Merck (Germany).

2.2. Preparation of antioxidant solutions

Ascorbic acid, sesamol, alpha-tocopherol, curcumin, BHT, gallic acid, melatonin, alpha-lipoic acid, pentoxifylline, ferulic acid were dissolved in methanol for the preparation of stock solution (10 mM).

2.3. Estimation of antiradical properties

The antioxidant activities of different compounds were determined using DPPH free radical scavenging assay. A fresh solution of DPPH was prepared in methanol and the exact initial concentration of DPPH solution in methanol was calculated spectrophotometrically from a calibration curve (Eq. (1)).

\[
\text{ABS}_{515\text{nm}} = 10.500 \times [\text{DPPH}] - 1.4 \times 10^{-2}
\]  

(1)

The linear regression \( r^2 = 0.999 \) indicated the goodness of fitting. For each antioxidant tested, the kinetic measurements were carried using spectrophotometer model Cary Bio 100 (Varian, Australia). The temperature of the sample chamber was controlled by inbuilt peltier system. DPPH radical scavenging by H atom donating antioxidants in literature has been reported by using at least two methods (a) fixed reaction time and (b) steady state saturation method. We have carried out both the methods to compare their results.

2.3.1. Fixed reaction time

This is commonly practiced procedure in different labs. In this fixed reaction time, 1.5 ml of antioxidant solution was prepared in 50 mM phosphate buffer (pH 7.5) and mixed with 1.5 ml of 100 μM methanolic solution of DPPH+ (methanol:buffer,1:1(v/v)) and kept at room temperature for 30 min. The decrease in absorbance was monitored at 515 nm. DPPH radical scavenging capacity was calculated using following equation.

\[
\text{DPPH} \text{ scavenging effects} (\%) = ([\text{A}_0 - \text{A}_1]/\text{A}_0 \times 100)
\]  

(2)

\( A_0 \) and \( A_1 \) correspond to the absorbances at 515 nm of the radical (DPPH+) in the absence and presence of antioxidant respectively.

2.3.2. Steady state measurement

Steady state method of Brand-Williams, Cuvelier, and Berset (1995) was followed and the decrease in absorbance was monitored for different durations from 30 min to 6 h depending upon the antioxidant and its concentrations to obtain a steady state of decrease in absorbance of [DPPH]. The decrease in as a function of time is exponential in nature and plotted for different concentrations of antioxidant. The percentage of DPPH remaining at steady state was determined.

\[
\% \text{DPPH remaining} = (\text{A}_t/\text{A}_0) \times 100
\]  

(3)

where \( A_0 \) and \( A_t \) correspond to the absorbance at 515 nm of DPPH at initial and steady state respectively. \( A_t \) value was obtained at the steady state region where absorbance did not depict further observable decreases.

2.4. Calculation of rate constant and EC50

The DPPH radical scavenging reaction kinetics was followed as described earlier in methanol medium (Bartasiute, Westerink, Verpoote, & Niederlander, 2007; Bondet et al., 1997; Suja, Jayalekshmy, & Arumughan, 2004). The typical plots for fast, intermediate and slow kinetic categories are represented in Fig. 1a–c. Scavenging reaction between [DPPH] and the antioxidant [A] is represented by Eq. (4). The less reactive [A] can undergo...
interactions with another [DPPH] as well as [A] according to Eqs. (5) and (6). These secondary reactions may be of limited occurrence.

$$
\begin{align*}
\frac{1}{2} \text{DPPH} + \text{AH} & \rightarrow \text{DPPH} - \text{H} + \text{A} \\
\text{DPPH} + \text{A} & \rightarrow \text{DPPH} - \text{A} \\
\text{A} + \text{A} & \rightarrow \text{A} - \text{A}
\end{align*}
$$

The order of reaction kinetic of [DPPH] and [AH] is depended on the relative concentration of reactants. Since the concentration of [DPPH] is fixed at 60 μM and therefore with increasing concentration of antioxidant ([DPPH]< [AH]), the reaction follows pseudo first order kinetics as shown in Eq. (7)

$$
\ln[\text{DPPH}]_t = \ln[\text{DPPH}]_0 - k_{obs}t
$$

where, [DPPH]₀ is the concentration of radical at initial time zero and [DPPH]ₜ is the concentration of radical at time t, kobs is the pseudo first order rate constant. From Eq. (7) pseudo first order rate constant was calculated. This constant is linearly dependent on the concentration of antioxidants, and from the slope of this plot, second-order rate constants (k₂) were calculated to determine the radical scavenging capacity of the different compounds and the now equation 8 may rewritten as

$$
-d[\text{DPPH}]_t/dt = k_{obs}[\text{DPPH}^-]_t = -k_2[AH][\text{DPPH}^-]_t
$$

Thus in order to obtain the saturation time, the time required will be dependent on concentration and the type of antioxidant.

From Fig. 1 the percentage of [DPPH] remaining at steady state was determined and it was plotted against the molar ratio of antioxidant to DPPH in Fig. 2. The effective concentration (EC₅₀) value was determined for antioxidants. EC₅₀ is similar to LD₅₀ and/or IC₅₀ in biological measurements. This parameter is defined as the efficient concentration required to decrease the initial DPPH concentration by 50% (Brand-Williams et al., 1995; Lu & Foo, 2000). The value of EC₅₀ was expressed in terms of molar ratio of antioxidant to DPPH. A number of important antioxidant parameters viz. antioxidant reducing power (ARP), stoichiometry (number of mole of DPPH required to reduce 1 mol antioxidant) has been calculated from EC₅₀ values. ARP is inverse of EC₅₀ value, the larger the ARP the more efficient the antioxidant.

### Table 1

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>IC₅₀ Reported (μM)</th>
<th>IC₅₀ Value in μM</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic Acid</td>
<td>11.85</td>
<td>11.85</td>
<td>Sharma and Bhat (2009)</td>
</tr>
<tr>
<td></td>
<td>5.85 μg/ml</td>
<td>33.21</td>
<td>Kanimozhi and Prasad (2009)</td>
</tr>
<tr>
<td></td>
<td>91 μM</td>
<td>91</td>
<td>Mohamada et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>50 μg/ml</td>
<td>284</td>
<td>Charrer, Fonty, Gaillard-martinie, Ainouche, and Andant (2006)</td>
</tr>
<tr>
<td></td>
<td>62.4 μg/ml</td>
<td>354.3</td>
<td>Shurwaikar et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>110.77 μg/ml</td>
<td>629</td>
<td>Ricci et al. (2005)</td>
</tr>
<tr>
<td>BHT</td>
<td>19.4 μg/ml</td>
<td>88</td>
<td>Guangrong et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>21.30 μg/ml</td>
<td>97</td>
<td>Charrer et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>42.62 μg/ml</td>
<td>193</td>
<td>Koyong et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>86.6 μg/ml</td>
<td>393</td>
<td>Ricci et al. (2005)</td>
</tr>
<tr>
<td>Sesamol</td>
<td>48 μM</td>
<td>48</td>
<td>Erkan et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>23.23 μg/ml</td>
<td>168</td>
<td>Kanimozhi and Prasad (2009)</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>27.1 μg/ml</td>
<td>63</td>
<td>Gülcin (2006)</td>
</tr>
<tr>
<td></td>
<td>96.0 μg/ml</td>
<td>223</td>
<td>Mohamada et al. (2004)</td>
</tr>
</tbody>
</table>

### 2.5. Data analysis

All statistical analyses (calculation of EC₅₀, rate constant and other values were performed using Origin 7 software (Microcal origin, USA). The statistical significance were calculated from one way

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**Fig. 1.** The dependence of absorbance at 515 nm on time of incubation at a different concentration of antioxidant in the reaction mixture of DPPH (60 μM) in methanol medium. Representative kinetic scans of three different types of reaction categories are shown: (A) sesamol (fast), (B) Ferulic Acid (medium) and (C) Curcumin (slow). Three independent experiments were carried for each antioxidant.
**Fig. 2.** Calculation of EC50 value toward DPPH radical: A figure shows % decrease of DPPH with ratio of sesamol to DPPH. The data shown is average ± SD of three independent experiments.

ANOVA analysis and level was set to 0.05. Values of all parameters are expressed as mean ± SD of three independent measurements.

### 3. Results

#### 3.1. Kinetic behaviour and second order rate constant

The reaction of DPPH· radical with antioxidant is basically a kinetic driven process. Thus in the present study, first an attempt has been made to assess the nature of kinetic behaviour of disappearance of DPPH· radical with antioxidants viz., ascorbic acid, sesamol, alpha-tocopherol, curcumin, ferulic acid, gallic acid, BHT, melatonin, alpha-lipoic acid and pentoxifylline. Kinetic scans were performed for individual antioxidants till steady state saturation i.e. maximum decrease in DPPH· radical was attained. Fig. 1a shows the kinetic scan of sesamol in methanol, it is clearly observed that DPPH· radical scavenging by sesamol is fast and increases with concentration of sesamol. The time to reach steady state saturation appears to be <20 min. In a similar kinetic scan of ferulic acid (Fig. 1b), the time to reach the steady state was found to be between 2–3 h depending upon concentration. Though, at higher concentration the scavenging capacity was more but could not attain completion even up to 120 min. Similarly in the case of curcumin 80 min reaction time was not enough to complete the reaction at lower range (3–6 μM). Though, at slightly higher concentration (>10 μM) the time required achieving saturation was <30 min. Therefore, it is clear that the time duration of reaction to reach the steady state is different and antioxidants need to be categorised as slow, medium and fast kinetics. Therefore, in summary reaction time for scavenging of DPPH· radical with ascorbic acid, sesamol and alpha-tocopherol was <30 min whereas for ferulic acid, gallic acid and BHT it was between 30 min to 3 h. Therefore, these antioxidants can be classified under the categories of fast (<30 min), medium (30 min to 1 h) and slow (>1 h). The reaction time for BHT in the present as well as in the earlier study (Brand-Williams et al., 1995) was found to be around 6 h and can be considered as good standard for slow kinetics. Interestingly, in case of curcumin the scavenging reaction continued even up to 6 h at lower concentration and thus can be categorised under slow kinetics. This slow reaction could be possibly that total curcumin was not able to take part in the reaction and remained unused. Similar explanation was reported earlier with BHT (Brand-Williams et al., 1995). Antioxidants like melatonin, lipoic acid, and pentoxifylline have demonstrated poor reaction with DPPH even up to 2 mM (data not shown) and therefore values of various parameters EC50 and ARP could not be determined.

Further, from these kinetic scan second order reaction constant (k2) was calculated at 30 min (t = 0 to t = 30 min) for ascorbic acid, sesamol, alpha-tocopherol, curcumin, ferulic acid, gallic acid and BHT. The calculated values of k2 for gallic acid, ascorbic acid, curcumin, sesamol, alpha-tocopherol, BHT and ferulic acid are 4.0 (±0.4) × 10−5, 2.25 (±0.04) × 10−5, 2.1 (±0.12) × 10−5, 1.3 (±0.14) × 10−5, 0.85 (±0.2) × 10−5, 0.3 (±0.14) × 10−5 and 0.55 (±0.07) × 10−5 M−1 s−1 respectively. Dependence of the rate constant on sesamol concentration is shown in Fig. 3a. Our results are in agreement with reported values (Suja et al., 2004) and on arranging these values antiradical properties of these antioxidants can be arranged as gallic acid > ascorbic acid > curcumin > sesamol > alpha-tocopherol > ferulic acid > BHT. Gallic acid is about four times more efficient than sesamol; ascorbic acid and curcumin are two times more efficient than sesamol. Whereas sesamol is two and three times more efficient than ferulic acid and BHT respectively.

#### 3.2. Determination of effective concentration (EC50) and antiradical power (ARP)

EC50 value is defined as the amount of antioxidant necessary to decrease the absorbance of DPPH by 50% of the initial absorbance. Antiradical power (ARP) is used to define antioxidant action of an antioxidant and it is defined as reciprocal of EC50

\[ \text{ARP} = \frac{1}{\text{EC50}} \]

EC50 values for ascorbic acid and sesamol (fast reaction kinetic) were calculated as 0.17 and 0.22 whereas the corresponding ARP values were 5.9 and 4.5 respectively. The reported values of EC50 and ARP for ascorbic acid are 0.27 and 3.7 (Brand-Williams et al., 1995) and our values are found to be different from the reported values. Nenadis, Lazaridou, and Tsimidou (2007) reported EC50 and ARP values for ascorbic acid as 0.25 and 5, respectively and found to be similar to our results. In case of alpha-tocopherol (intermediate reaction kinetic), EC50 and ARP values were determined as 0.21 and 4.7 respectively. Under slow reaction kinetic category, EC50 value for gallic acid as determined from the steady state concentration of DPPH was around 0.07 which is similar to earlier reported value 0.08 (Brand-Williams et al., 1995) but differed from 0.11 (Nenadis et al., 2007). The EC50 for ferulic acid,
Comparison of antiradical parameters: The antioxidant concentration (EC₅₀) that caused 50% decrease in DPPH radical scavenging was determined as average ± SD of three independent experiments. (B) The dependence of the second order rate constant (kₙ) of DPPH radical scavenging on EC₅₀ of antioxidants. Stoichiometry values for ascorbic acid and sesamol were found to be 0.34 and 0.44 respectively. The reported stoichiometry and number of reduced DPPH radicals for ascorbic acid are 0.54 and 1.85 respectively and thus differs slightly from our values. Stoichiometry and number of reduced DPPH for sesamol was not reported earlier. In case of alpha-tocopherol the stoichiometry was 0.44 and number of reduced DPPH was 2.4 and these values were similar to the reported values of 0.5 and 2 respectively. Antioxidants curcumin, ferulic acid and BHT have reduced 3.7, 1.3 and 1.3 DPPH radical with stoichiometry of 0.3, 0.68 and 0.46 respectively. The reported value of reduced DPPH for ferulic acid was 1.2 and 1.3 DPPH radical scavenging. Therefore, after considering the EC₅₀ and ARP values the overall trend of antiradical action is reflected as gallic acid < curcumin < ascorbic acid < alpha-tocopherol < sesamol < BHT < ferulic acid. Lower value of EC₅₀ means higher antiradical power, therefore gallic acid is the most powerful and the ferulic acid is the least potent antioxidant. The complete details of the numerical values of these antiradical parameters are shown in Table 2.

3.3. Stoichiometry of reactions

One of the important parameters of antioxidant action is stoichiometry of reactants i.e. the amount of antioxidant required theoretically to reduce 100% of DPPH radicals. The stoichiometry value of antioxidant is determined by multiplying EC₅₀ value by stoichiometry of reactants i.e. the amount of antioxidant required. Therefore, after considering the EC₅₀ and ARP values the overall trend of antiradical action is reflected as gallic acid < curcumin < ascorbic acid < alpha-tocopherol < sesamol < BHT < ferulic acid. Lower value of EC₅₀ means higher antiradical power, therefore gallic acid is the most powerful and the ferulic acid is the least potent antioxidant. The complete details of the numerical values of these antiradical parameters are shown in Table 2.

3.4. Comparison of IC₅₀ of steady state (methanol medium) and fixed 30 min (semi-aqueous)

In the present study an attempt has been made to compare the IC₅₀ values obtained from the steady state kinetic and 30 min fixed reaction time (semi-aqueous medium) respectively. Different antioxidant concentrations (5 μM–2 mM) were taken in 1.5 ml 50 mM phosphate buffer and mixed with 1.5 ml of methanolic (100 μM) solution of DPPH and incubated for 30 min. Absorbance of this solution was measured at 515 nm. IC₅₀ for antioxidants in methanol viz., ascorbic acid, sesamol, alpha-tocopherol, curcumin, ferulic acid, gallic acid and BHT were 10.2, 13.5, 12.7, 9, 22, 4.2, and 18.8 μM respectively. Whereas, in semi aqueous medium, IC₅₀ for

![Fig. 3. (A) Plot of pseudo first order rate constant (kₙ) versus sesamol concentration. All measurements were carried out in methanol medium. Data are expressed as average ± SD of three independent experiments. (B) The dependence of the second order rate constant (kₙ) of DPPH radical scavenging on EC₅₀ of antioxidants.](Image 38x371 to 279x726)

### Table 2

Comparison of antiradical parameters: The antioxidant concentration (EC₅₀) that caused 50% decrease in DPPH estimated at steady state, antiradical power (1/EC₅₀), stoichiometry (EC₅₀ x 2), no of reduced DPPH (1/stoichiometry) and second order rate constant (kₙ) calculated from Eq. (8), at 30 min.

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>EC₅₀ (μ mole of antioxidant/μ mole of DPPH)</th>
<th>ARP</th>
<th>Stoichiometry</th>
<th>No of reduced DPPH</th>
<th>EC₅₀ (μM)</th>
<th>kₙ (second order rate constant) in 10⁻² μM⁻¹ sec⁻¹</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic Acid</td>
<td>0.07 ± 0.01</td>
<td>14.7 ± 0.72</td>
<td>0.14 ± 0.01</td>
<td>6.9 ± 0.35</td>
<td>4.2 ± 0.21</td>
<td>4 ± 0.4</td>
<td>0.99</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.17 ± 0.01</td>
<td>5.9 ± 0.01</td>
<td>0.34 ± 0.01</td>
<td>2.9 ± 0.01</td>
<td>10.2 ± 0.01</td>
<td>2.25 ± 0.07</td>
<td>0.91</td>
</tr>
<tr>
<td>Curcumin</td>
<td>0.13 ± 0.04</td>
<td>6.5 ± 1</td>
<td>0.3 ± 0.08</td>
<td>3.7 ± 0.08</td>
<td>8 ± 1</td>
<td>2 ± 0.12</td>
<td>0.94</td>
</tr>
<tr>
<td>Sesamol</td>
<td>0.22 ± 0.01</td>
<td>4.5 ± 0.14</td>
<td>0.44 ± 0.01</td>
<td>2.2 ± 0.07</td>
<td>13.5 ± 0.42</td>
<td>1.3 ± 0.14</td>
<td>0.97</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>0.21 ± 0.01</td>
<td>4.7 ± 0.22</td>
<td>0.44 ± 0.02</td>
<td>2.4 ± 0.11</td>
<td>12.7 ± 0.6</td>
<td>0.85 ± 0.2</td>
<td>0.98</td>
</tr>
<tr>
<td>Ferulic Acid</td>
<td>0.36 ± 0.04</td>
<td>2.75 ± 0.27</td>
<td>0.68 ± 0.07</td>
<td>1.3 ± 0.13</td>
<td>22 ± 2.12</td>
<td>0.55 ± 0.07</td>
<td>0.98</td>
</tr>
<tr>
<td>BHT</td>
<td>0.23 ± 0.12</td>
<td>3.43 ± 0.4</td>
<td>0.46 ± 0.24</td>
<td>1.3 ± 0.65</td>
<td>18.9 ± 7.21</td>
<td>0.3 ± 14</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Results are average of three independent experiments ± standard deviation.

Melatonin is weakly interacting with DPPH while pentoxifyline and γ-lipoic acid do not react.
antioxidants ascorbic acid, sesamol, alpha-tocopherol, curcumin, ferulic acid, gallic acid and BHT were 10.5, 13.5, 26, 18.5, 17.2, 2 and 36.1 μM respectively. On careful examination of the results in Table 3, it appears that antioxidant with fast and medium reaction kinetics with DPPH\(^*\) yields similar IC\(_{50}\) values, whereas for antioxidants under slow reaction kinetics, these values are different. For example, IC\(_{50}\) value of 18.8 μM for BHT obtained from the steady state kinetics is almost half of 36.1 μM when measured from the fixed 30 min incubation time. Similarly for alpha-tocopherol the value of 13 μM is half of 26 μM obtained from 30 min fixed reaction time. In case of curcumin different IC\(_{50}\) values of 8 and 18.5 μM were found for steady state and fixed time respectively. Interestingly for ferulic acid IC\(_{50}\) values in methanol and semi-aqueous medium were 22 and 17 μM respectively. Result suggests IC\(_{50}\) of ferulic acid and its derivatives were wrongly estimated in methanol in earlier study (Bandoniene et al., 2002; Brand-Williams et al., 1995). The detailed comparisons are presented in Table 3.

An attempt was also made to determine IC50 or EC50 values for melatonin, lipoic acid and pentoxifylline at higher concentration. As we have mentioned earlier that these antioxidants were poor scavengers of DPPH radicals in the concentration range (5–200 μM), we have chosen a concentration range from 200 to 2000 μM. Results suggest that alpha-lipoic acid did not scavenge DPPH radical even at higher concentrations. Melatonin was poor scavenger in the concentration range (200–2000 μM) with IC\(_{50}\) value of 1025 μM. Similarly, pentoxifylline (up to 2 mM) was found to be even poor scavenger than melatonin and scavenged only 20% free DPPH radical.

### 3.5. Chemical structure and DPPH\(^*\) antioxidant activity relationship

Chemical structure and spatial geometric configuration of molecules are important for its specific function. Interesting results were obtained in this study for example, ascorbic acid containing no phenolic hydroxyl group reacts with DPPH\(^*\) within a minutes. Sesamol contains only one hydroxyl group but reacts with two DPPH within 5–10 min and both comes under fast kinetic behaviour. It was earlier hypothesised that dioxy group of sesamol might be responsible for higher scavenging property (Suja et al., 2004). Our study confirms this hypothesis that sesamol with single hydroxyl group practically reduces two DPPH\(^*\) radicals. Alpha-tocopherol containing four hydroxyl groups reacts with DPPH in 5–30 min but EC\(_{50}\) values found to be similar to sesamol and follow intermediate reaction kinetic. In this study it was observed that gallic acid with the maximum number of hydroxyl groups (three) is the most powerful antioxidant but falls in the slow kinetic category. Though, it is not always the case that number of hydroxyl group is directly correlated with more antioxidative efficiency. Literature suggests that carnosic acid with two phenolic hydroxyl groups has a higher antioxidant power than rosmarinic acid with four phenolic hydroxyl groups (Erkan, Ayranci, & Ayranci, 2008). Gallic acid reduced around seven DPPH radicals which is somewhat unpredictable because gallic acid has only three phenolic hydroxyl groups. In case of curcumin which contains only two phenolic hydroxyl groups surprisingly curcumin has more antiradical efficiency than alpha-tocopherol with four hydroxyl groups. However, in some cases like ferulic acid one phenolic hydroxyl group reduces one DPPH radical. Alpha-lipoic acid, melatonin and pentoxifylline with no phenolic hydroxyl group reacted poorly with DPPH: Similar unexpected results based on the number of phenolic hydroxyl group per antioxidant molecule were reported in several studies (Brand-Williams et al., 1995; Erkan et al., 2008). It is therefore clear that the number of phenolic hydroxyl groups is not always the only factor determining the antioxidant activity of an antioxidant. Several studies suggests that structure–antioxidant activity for flavonoids and phenolic acids compounds depends on position of phenolic hydroxyl groups, presence of other functional groups in the whole molecule such as double bonds and their conjugation to hydroxyl groups and ketonic groups (Rice-Evans & Miller, 1996; Silva et al., 2002).

An attempt has been made to study the dependence of second order kinetics (k\(_2\)) on the effective concentration of antioxidant required to scavenge DPPH radical by 50% (EC\(_{50}\)). The k\(_2\) values as mentioned were correlated and a negative correlation was observed (R = −0.85) (Fig. 3b). Though, limited number of antioxidant compounds studied in this paper, dependence of effective concentration on second order kinetics (k\(_2\)) found to be good correlated. Many studies about the antiradical effectiveness of phenolic compounds are reported. The sequence as obtained could serve the purpose of comparison of effectiveness. But the sequence may greatly vary depending on the assay method and the way in which the results are analysed (Visioli & Galli, 1998).

## 4. Discussion

Natural antioxidants have demonstrated beneficial effects in maintenance of health, management of age related diseases, ameliorating the harmful effects of toxic agents both chemical and physical. Presence of antioxidant both, prior and after radiation exposure has shown its ability to reduce the harmful effects of radiation (Weiss & Landauer, 2003). Understanding of effects of natural (pure antioxidants) or synthetic antioxidants on the reduction of harmful effects of radiation in vitro and in vivo system is of particular interest of our research group (Chaudhury et al., 2006; Mishra, Bhardwaj, & Chaudhury, 2009).

Sesamol, curcumin, ferulic acid, are believed to be potential antioxidant based therapeutic agents. Antioxidants like gallic acid, BHT are used as food preservatives in industries and as reference standards in the evaluation of antioxidative properties of pure antioxidant compounds or plant extract of different nature (Erkan et al., 2008; Nenadis et al., 2007). On careful reviewing of literature, we have observed several anomalies in the reported antiproliferative properties of different pure antioxidants including the standard response compounds in many studies. The results of standard reference molecules were under estimated and used for depicting higher antioxidant properties in pure or plant. In order to emphasis this in the following sections we have discussed few examples as case studies.

### 4.1. First case study

Berberine is reported as a better antioxidant molecule in terms of ability of DPPH scavenging than ascorbic acid (Shirwalkar, Kuppusamy, & Punitha, 2006). The author incubated methanolic solution of DPPH (100 μM) with different concentrations of ascorbic acid and berberine for 20 min. The IC\(_{50}\) values of ascorbic acid...
and berberine were reported as 62.4 and 42.7 µg/ml respectively. The IC\textsubscript{50} values after converting to molarities as 354 µM and 126.94 respectively. Therefore the author in this paper summarised that berberine is better antioxidant than ascorbic acid. Our specific comments are: (i) it is not understood why concentration was expressed as weight by volume (µg/ml) when the molecular weights of these antioxidants are known? (ii) Reaction time for berberine was chosen randomly as 20 min and might not be sufficient for completion of the reaction. IC\textsubscript{50} value of 354 µM for ascorbic reported by Shirwaikar et al. (2006) was much higher than 10.2 µM (present study) and 11.85 µM of Sharma and Bhat (2009). Therefore, claiming that berberine is better antioxidant and overestimation of IC\textsubscript{50} value of ascorbic acid are likely to be erroneous and might be due to the wrong selection of reaction time.

4.2. Second case study

BHT is another standard reference compound used in DPPH assay. The IC\textsubscript{50} values of this molecule were reported in a wide range from 19.4 µM (Guangrong, Jiaxin, & Delhui, 2008) to 393 µM (Ricci et al., 2005). (1) In the present study the IC\textsubscript{50} was calculated as 18.9 µM (2) whereas Sharma and Bhat (2009) reported 60 µM using 30 min reaction time which is approximately three times higher than the value obtained by us. Interestingly use of different methods also resulted in the differences in estimated results. Recently in another study, scavenging properties of L-adrenaline, curcumin, caffeic acid and l-carnitine (Gülcin, 2006; Gülcin, 2009) were reported using DPPH assay and the reference standard were BHT and alpha-tocopherol. In that study 0.5 ml of DPPH (0.1 mM) in ethanol was mixed with 1.5 ml of different concentration of antioxidants. The effective concentration of DPPH used in this study was 25 µM which is not the recommended concentration to start the reaction (Sharma & Bhat, 2009). The reported IC\textsubscript{50} data was in different pattern viz. µg/ml, percentage and in µM. The representation of data in these different forms makes impossible to compare the data of different labs. The IC\textsubscript{50} values were reported by the author as 6.1 µg/ml for BHT and 10.3 µg/ml for alpha-tocopherol which after calculation in terms of molarities was found to as 27.68 and 23.91 µM respectively. These IC\textsubscript{50} values of BHT and alpha-tocopherol differ from our results.

After considering above cases in details we have noted few specific points: (i) in many cases concentration of antioxidants were taken higher than the concentration of DPPH; (ii) there was no limit of higher concentration selection in several studies, (iii) ambiguity in the selection of suitable reference standard molecule of which detailed study and results were reported, (iv) time of incubation not considered purposefully in many cases, which ranges from 10 min to 6 h and (v) many high throughput assays has been designed without considering the suitable reaction medium and time. It is practically difficult to include the reference of all related studies in a manuscript of this kind. However, we have observed in our studies that concentration less than 50 µM is suitable to provide practically all derived essential parameters irrespective of antioxidants used in this study. The calculation of rate constant (second order) was also difficult at higher concentration with practically no linearity in rate constant (k\textsubscript{obs}) was observed at antioxidant concentration higher than concentration of DPPH (60 µM). Similarly, rate of reaction was variable from first to second order even at concentration less than the concentration of DPPH in few cases (gallic acid and curcumin) when steady state time was considered. In many cases this variation remains unnoticed due to fixed time incubation study. Antioxidants like melatonin, alpha-lipoic acid, and pentoxifylline did not or poorly scavenged DPPH radical, though used in various in vivo studies with promising results. Therefore it is important to study the effective concentration range of these molecules before undertaking detailed in vivo studies(to be removed). DPPH assay is based on kinetic derived process that is, it may take one minute (ascorbic acid) to more than 180 min(curcumin) and therefore the assay should be performed in kinetic mode and estimation of antioxidant capacity of unknown antioxidant compounds must be counter-measured by using other antioxidant assays e.g. ABTS, FRAP, ORAC, etc.

A number of apparently similar and simple analytical methods for characterisation of antioxidants antiradical properties based on physicochemical properties are available in literature. These methods can be broadly classified under two sub heads viz., H atom donating ability (HAT) and electron transfer (ET) from antioxidants both resulting to neutralisation of free radicals. The majorities of HAT assays are kinetics based and involve a competitive reaction scheme in which antioxidant and substrate compete for radicals. ET based assays measure the capacity of an antioxidant in the reduction, which changes colour when reduced. ET assays include the ABTS/TEAC, CUPRAC, Folín–Ciocalteu and FRAP methods, each using different chromogenic redox reagents with different standard potentials (Apak et al., 2007). Thus, it is important to assess the reaction time before starting the detail study. Concentration of substrate (antioxidant) should be kept as minimum as possible. This will ultimately lead to good assessment of parameters related to antioxidant properties in these analytical methods also.

5. Conclusions

Our case studies and experimental results demonstrated that characterisation of antioxidant activities need to be systematically performed by DPPH assay. Our comparative results of steady state and fixed time (30 min) reconfirmed that fixed time incubation experiment underestimate radical scavenging activities for slow reacting molecules. Differences in the antiradical properties of antioxidant in methanolic and semiaquous media need to be understood for each antioxidant. This study is expected to benefit researchers working with new antioxidant and herbal extracts using DPPH assay.

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References

Apak, R., Güçlü, K., Demirata, B., Ozyürek, M., Celik, S. E., Bektașoglu, B., et al. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC Assay. Molecules, 12, 1496–1547.


