

Simplified Methods for Microtiter Based Analysis of *In Vitro* Antioxidant Activity

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Abstract

Context: The research in exploring the antioxidant activity of pharmaceuticals has recently been increased considerably and to determine such property a variety of testing methods are available. However, the major drawback of these conventional tests is their large reaction volumes varying from 2 to 6 ml that in turn demand high quantity of reagents and biological resources. **Aims:** Thus, this work was focused for optimization of routinely used five antioxidant experiments such as superoxide radical ($O_2^{\cdot-}$) inhibition, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) quenching, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) scavenging, chelating ability of ferrous ion and reducing power by means of 96-well plates in minimal reaction volume ranging from 60 to 200 μ l. **Subjects and Methods:** To authenticate the processes some renowned standards such as ascorbic acid, butylated hydroxyanisole, Trolox as well as ethylenediaminetetraacetic acid were used, and their calibration curves were prepared. **Results:** Analysis depicted similar activities of all references as reported by traditional protocols suggesting validation of standardized procedures. **Conclusion:** Thus, the recommended systems clearly improve original one in a number of ways. First, the methods provide concurrent multi-sample investigation with automatic data storage. Second, the approach is time-saving with the use of a multichannel pipette. Third, assays are inexpensive as the use of chemicals is reduced by 10 times. Fourth, the investigating pure compounds or extracts are required in low quantity. Fifth, except $O_2^{\cdot-}$ quenching assay, all the methods are applicable to lipophilic and aqueous components both.

Key words: 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical, chelating ability, 2, 2-diphenyl-1-picrylhydrazyl radical, reducing power, scatter plots, superoxide radical, 96-well plates

INTRODUCTION

Antioxidant activity is a broadly used term to characterize substances with the ability of scavenging or neutralizing free radicals.^[1] These radicals can be produced inside human body by both exogenous and endogenous sources such as inflammation, immune cell activation, excessive exercise, mental stress, infection, ischemia, cancer, ageing, water pollution, alcohol, cigarette smoke, heavy metals, industrial solvents, certain drugs, radiation, and cooking.^[2] The presence of these reactive components in excess amount can generate a phenomenon known as oxidative stress, a deleterious process that can alter the structure of proteins, lipids, lipoproteins, and DNA.^[3] Consequently, a number of disorders may propagate that can lead to degenerative, cardiovascular, renal, neurological, liver, and autoimmune diseases.^[4] In this backdrop, antioxidant components externally supplied

through diets are required to neutralize excess radicals, protect cells against toxic effects and prevent ailments. Nowadays, several synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) are routinely used in foods and medicines.^[5] However, carcinogenic nature, strict legislation on the use of synthetic food additives and consumer preferences have shifted the attention of industrialists from synthetic to natural antioxidants.^[6] Thus, there has been

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a worldwide trend toward the use of natural substances as therapeutic antioxidants to reduce risk factors of human from deadly diseases.^[7]

To date, a great number of *in vitro* methodologies have been established to measure the efficacy of natural antioxidants either as pure compound or extract. However, there are several impediments that limited the applicability of these techniques and handling samples with different concentrations.^[8] For instance, an enormous volume of reaction mixture ranging from 3 to 6 ml is required for conducting conventional superoxide radical (O_2^-) scavenging activity and reducing power assay. As a consequence, not only reagents are utilized in high quantity but the biological materials are also needed in significant amount. This becomes of major concern in the case of pure components or complex samples that have been isolated in low extent. Besides, both these techniques offer robust and time-consuming processes demanding their modification in a simplified way. While, methods of 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) as well as 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical quenching activity are operationally simple and have been used in many research laboratories; however high price of these chemicals is the foremost and unavoidable limitation from the routine analysis.^[9]

Therefore, the development of new, easy to perform, rapid and inexpensive methods are an urgent call to determine radical scavenging potentiality of pharmaceuticals, nutraceuticals, and foods. In this context, microplate technology can be considered as an effective step toward more sensitive and fast measurement of spectral absorption values.^[10] Eventually, some antioxidant experiments such as DPPH, ABTS, and reducing power tests have been modified by adopting 96 as well as 48-well plates in a reaction volume of 220-1500 μ l.^[10-14] However, to the best of our knowledge, no previous study has been performed to simplify O_2^- inhibition and chelating ability of ferrous ion processes. In this backdrop, here we describe rapid, small-scale, high-throughput microplate-based methodologies for those aforementioned antioxidant assay using 60-200 μ l reaction mixture. The modified approach is more precise, sample-saving, rapid, and eco-friendly as well which would be more suitable for substituting the traditional laborious techniques. Overall, the proposed assays are easy to execute for manual screening of antioxidant capacity of a large number of samples in a day.

SUBJECTS AND METHODS

Chemicals

Sodium dihydrogen phosphate, disodium hydrogen phosphate, L-methionine, nitroblue tetrazolium (NBT), riboflavin, ferrous chloride, ferrozine, trichloroacetic acid (TCA), potassium ferricyanide, ferric chloride, DPPH, ABTS,

sodium persulfate, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), BHA, and Trolox were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All chemicals were of analytical reagent grade.

Instrument

In this study, Bio-Rad iMark™ Microplate Reader (USA) was used for absorbance reading and spectra recording. The machine is comprised an eight-channel, vertical path length photometer that measures the absorbance of contents in 96-well microtitration plate at specific wavelengths such as 415, 450, 490, 595, 655, and 750 nm. Before every read, the plate can be shaken at low, medium, or high speed for 0-999 s to mix the reaction mixture.

Superoxide radical scavenging assay

Original method

Each 3 ml reaction mixture sequentially contained 750 μ l sodium phosphate buffer (50 mM, pH 7.8), 390 μ l methionine (13 mM), 300 μ l EDTA (100 nM), 1300 μ l of standards or antioxidant compounds, 250 μ l NBT (75 mM), and 6 μ l riboflavin (2 mM). The reaction was started by turning on a fluorescent lamp (15 W), and illumination was run for 10 min. The production of violet color was monitored by measuring absorbance at 560 nm. Tubes with identical reaction mixture were kept in the dark and served as blanks.^[15]

Proposed method

Methionine solution was prepared by dissolving 0.074 g in 5 ml water (2 nM). 0.004 g EDTA was dissolved in 10 ml water to make 0.1 nM solution. NBT and riboflavin were prepared daily by dissolving 0.004 g in 5 ml (0.1 nM) and 100 ml (1 nM) water, respectively. In 200 μ l reaction mixture, x μ l of sample was added followed by serial inclusion of water (83-x μ l), 50 μ l buffer, 26 μ l methionine, 20 μ l EDTA, 17 μ l NBT, and 4 μ l riboflavin. The plate was shaken for 10 seconds in medium speed by microplate reader instrument, and initial absorbance was measured at 595 nm. Further, the 96-well plates were placed under 15 W lights and incubated for 10 min at room temperature to initiate the reaction. Finally, change in absorbance was recorded at the same wavelength, and initial absorbance was deducted to nullify background color. Ascorbic acid at different concentrations (10-150 μ g/ml) was considered as a reference in this technique.

The degree of scavenging was calculated by the following equation:

$$\text{Scavenging effect (\%)} = \left\{ \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right\} \times 100$$

DPPH radical scavenging assay

Original method

About 5 ml of standard or antioxidant compound solution was mixed with 5 ml of 0.008% DPPH prepared in 50% ethanol. The reaction was incubated for 30 min at room temperature in the dark. Decolorization of DPPH solution was measured at 528 nm.^[16]

Proposed method

About 0.004 g DPPH reagent was dissolved in 100 ml methanol or 4:1 aqueous methanol solution depending on nature of investigating component. In 96-well plates, x μ l sample and 200-x μ l DPPH solution were added. The plate was incubated for 30 min at room temperature in the dark, and finally, absorbance was recorded at 595 nm wavelength. To authenticate the process, three synthetic antioxidants including ascorbic acid, BHA, and Trolox at different concentrations ranging from 1 to 50 μ g/ml were used.

The degree of scavenging was calculated by the following equation:

$$\text{Scavenging effect (\%)} = \{(\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control}\} \times 100$$

ABTS radical scavenging assay

Original method

At first, ABTS was dissolved in water to prepare 7 mM concentrated solutions. Further, ABTS radical cation (ABTS⁺) was generated by reacting the stock solution with 2.45 mM of potassium persulfate followed by incubation in the dark at room temperature before use. After 12-16 h, the ABTS⁺ solution was diluted with ethanol or PBS (pH 7.4) to an absorbance of 0.7 at 734 nm. 1 ml of diluted ABTS⁺ solution was mixed with 10 ml of standards or antioxidant compounds and absorbance was measured against the appropriate solvent blank at 734 nm.^[17]

Proposed method

ABTS radicals were prepared freshly in each time according to the original method. After 12-16 h, ABTS⁺ solution was diluted to an absorbance of 0.7 ± 0.02 at 750 nm. In 200 μ l reaction mixture, x μ l standard was added followed by addition of 200-x μ l reagent solution. The plate was shaken for 10 seconds at medium speed, and absorbance was measured at 750 nm following 5 min incubation in the dark. Herein, three references such as ascorbic acid, BHA, and Trolox were used at different concentrations (1, 3, 5, and 10 μ g/ml).

The degree of scavenging was calculated by the following equation:

$$\text{Scavenging effect (\%)} = \{(\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control}\} \times 100$$

Chelating ability of ferrous ion

Original method

About 1850 μ l of standards or antioxidant compounds were incubated with 50 μ l Fe²⁺ (20 μ M) (ammonium ferrous sulfate) in 5% ammonium acetate, pH 6.9. Reaction was initiated by addition of 100 μ l of ferrozine (100 μ M) and after 10 min incubation absorbance was read at 562 nm.^[18]

Proposed method

About 10 mg ferrous chloride was dissolved in 40 ml water to prepare 3 nM solutions. While 0.012 g ferrozine was dissolved in 5 ml water (0.12 nM). In 200 μ l reaction mixture, x μ l sample was added to 96-well plates along with 5 μ l of ferrous chloride and mixed well. Then, 10 μ l ferrozine was included followed by addition of 185-x μ l of water or methanol depending on investigating component. The system was incubated at room temperature for 10 min, and absorbance was measured at 595 nm. EDTA at concentrations of 5, 10, 15, and 20 μ g/ml was considered as a reference.

The degree of scavenging was calculated by the following equation:

$$\text{Scavenging effect (\%)} = \{(\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control}\} \times 100$$

Reducing power

Original method

About 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of K₃[Fe(CN)₆] (1% w/v) were added to 1 ml of sample or antioxidant compounds dissolved in distilled water. The resulting mixture was incubated at 50°C for 20 min, followed by addition of 2.5 ml of TCA (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect an upper layer of solution (2.5 ml), mixed with distilled water (2.5 ml) and 0.5 ml of FeCl₃ (0.1%, w/v). The absorbance was then measured at 700 nm against blank sample.^[19]

Proposed method

Reagents were prepared as per the original method. In 96-well plates, 10 μ l of sample solution, 25 μ l of buffer and 25 μ l of K₃[Fe(CN)₆] were added sequentially. The mixture was incubated for 20 min at room temperature, and reaction was stopped by adding 25 μ l of TCA solution. Further,

85 μl of water and 8.5 μl of FeCl_3 were added to each well. The contents were mixed, incubated for another 15 min at room temperature and absorbance was measured at 750 nm. Ascorbic acid, BHA, and Trolox at concentrations of 10, 30, 50, and 70 $\mu\text{g/ml}$ were considered as standards in this technique.

Data analysis

All data were expressed as mean \pm standard deviation of three independent experiments. The sample concentrations providing 0.5 of absorbance or 50% of antioxidant activity were calculated from graphs of antioxidant activity percentages and regarded as EC_{50} value. Linear regression and correlation analyses were performed using Microsoft Excel[®] (USA).

RESULTS AND DISCUSSION

Superoxide radical scavenging assay

Superoxide anion ($\text{O}_2^{\cdot-}$) is generated by one-electron reduction of oxygen and acts as the precursor of most reactive oxygen species. Dismutation of this primary radical can produce hydrogen peroxide that in turn may be partially reduced to one of the strongest oxidants in nature, hydroxyl radical. In addition, the anion may react with other radicals such as nitric acid resulting a more powerful oxidant, peroxy nitrite.^[20] These harmful components can initiate cellular damage that may lead to different pathophysiological conditions.^[3] Therefore, evaluation of superoxide anion scavenging is extremely important for determining antioxidant activities. To assess such potentiality of pharmaceuticals, Martinez *et al.* proposed an *in vitro* method based on the generation of $\text{O}_2^{\cdot-}$ by auto-oxidation of riboflavin in the presence of light. The radical in turn reduced NBT to a blue colored formazan that can be measured photometrically. However, decrease in absorbance indicated consumption of $\text{O}_2^{\cdot-}$ by antioxidants present in resulting mixture.^[15]

This original method has been revised herein by adopting 96-well plates to reduce the reaction volume from 3000 to 200 μl at minimal expense of samples. In addition, less concentration of all reacting reagents was used as compared to the traditional one. Besides, the maximum absorption wavelength (λ_{max}) was changed from 560 nm to 595 nm due to the limitation of the automated analyzer. The proposed technique was standardized by using ascorbic acid as a reference which showed excellent radical scavenging activity [Figure 1]. At the concentration of 10, 50, and 100 $\mu\text{g/ml}$, the reference was able to inhibit $11.751 \pm 2.732\%$, $25.547 \pm 1.838\%$, and $41.217 \pm 0.285\%$ radicals, respectively, which reached to the level of $59.6 \pm 3.085\%$ at the dose of 150 $\mu\text{g/ml}$.

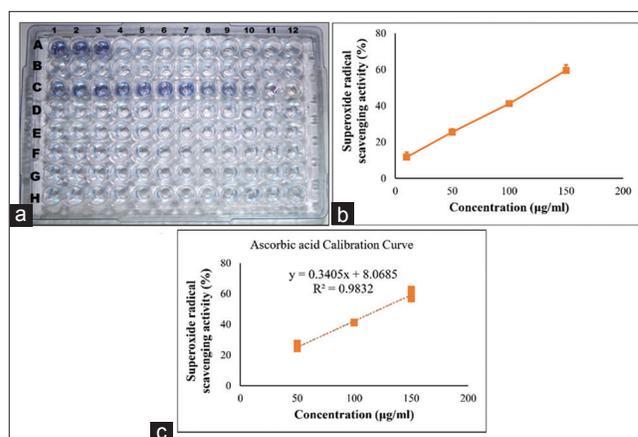


Figure 1: Superoxide radical scavenging activity (a) Photograph of 96-well plates presenting dose-dependent radical quenching potentiality of ascorbic acid with respect to control (Lane A1-3: Blank, B1-3: 10 $\mu\text{g/ml}$, B4-6: 50 $\mu\text{g/ml}$, B7-9: 100 $\mu\text{g/ml}$, B10-12: 150 $\mu\text{g/ml}$), (b) graphical presentation of the effect of ascorbic acid on radicals in terms of percent inhibition, (c) scatter plot of the standard showing Pearson's correlation coefficient (R^2). Experiments were triplicated, and values are presented as mean \pm standard deviation

DPPH radical scavenging assay

Use of DPPH to analysis antioxidant potentiality dates back to 1950s, when Brand-Williams *et al.* published the first technique to quantify scavenging property of components using a spectrophotometer.^[21] Presently, this commercially available stable nitrogen-centered free radical is the most extensively used reagent to determine radical scavenging activity of nutraceuticals. The violet colored radical possesses a characteristic absorption and readily accepts electron from antioxidant compounds. Consequently, it is reduced to yellow colored diphenylpicrylhydrazine that can be measured through colorimeter.^[22] However, a large volume of freshly prepared DPPH solution (1-5 ml) is required each time to conduct the conventional method. To overcome the limitation, protocol has been later modified by adopting microwell-based technique to make the assay simpler as well as faster.^[23] So far, few researchers have validated and described the assay by applying 96-well plates although reaction mixture was in the range of 220-300 μl .^[12]

For further simplification, miniaturization of volume and reduction in expenditure of radicals, the original method has been improved herein. In this context, the stock solution was prepared with the low concentration of DPPH and reaction system was standardized to only 200 μl signifying less use of the costly reagent. Besides, λ_{max} was changed from 528 nm to 595 nm due to limitation of the automated analyzer. To authenticate the technique, three standards were used, and results have been summarized in Table 1. Ascorbic acid exhibited a dose-dependent response by inhibiting $30.183 \pm 4.255\%$, $65.463 \pm 1.766\%$, $89.506 \pm 1.138\%$, and

Table 1: Antioxidant activity of standards

Antioxidant assays	Ascorbic acid	BHA	Trolox	EDTA
Superoxide radical scavenging assay	123.147±15.628	NT	NT	NT
DPPH radical scavenging assay	7.695±0.023	7.447±0.024	9.613±0.022	NT
ABTS radical scavenging assay	3.18±0.001	3.654±0.017	8.618±0.226	NT
Chelating ability of ferrous ion	NT	NT	NT	11.814±0.585
Reducing power	18.739±0.007	32.215±6.907	36.095±0.001	NT

The results are presented in EC₅₀ values (µg/ml) corresponding to effective concentration at which 50% radicals are scavenged or absorbance is 0.5. Experiments were triplicated, and values are presented as mean±SD. SD: Standard deviation, NT: Not tested, DPPH: 2, 2-diphenyl-1-picrylhydrazyl, ABTS: 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), BHA: Butylated hydroxyanisole, EDTA: Ethylenediaminetetraacetic acid, EC₅₀: Effective concentration 50%

91.716 ± 0.189% of radicals at the level of 5, 10, 25, and 50 µg/ml, respectively. While BHA showed 36.832 ± 0.576%, 68.829 ± 3.029%, 86.314 ± 0.383%, and 91.144 ± 0.197% scavenging ability of DPPH[•] at those above-mentioned concentrations. However, Trolox presented lower potentiality by quenching 31.134 ± 1.267%, 52.457 ± 3.168%, 92.617 ± 0.198%, and 95.654 ± 0.31% radicals at the level of 5, 10, 25, and 50 µg/ml, respectively [Figure 2]. Nevertheless, our findings were found to be in agreement with the previous reports.^[24-26]

ABTS radical scavenging assay

Original ABTS^{•+} scavenging assay was proposed by Wolfenden and Wilson where the radical cation was generated by activation of metmyoglobin with hydrogen peroxide in the presence of ABTS.^[27] Later the method was modified by direct production of blue-green ABTS^{•+} chromophore through reaction between ABTS salt and a strong oxidizing agent such as potassium persulfate or potassium permanganate.^[17] However, the blue colored ABTS^{•+} is converted back to its colorless neutral form in the presence of hydrogen-donating antioxidant compounds measured by decrease of its characteristic absorption spectrum.^[28] As a consequence, the assay becomes operationally simple, and nowadays it has been routinely used in many research laboratories to determine radical scavenging activity of components. However, with sophistication and advancement in instrumental techniques, the protocol has experienced several modifications, while basic principle remained unique. Recently, microplate adaptation of ABTS radical quenching method has been described by some investigators in a reaction volume of not <255 µl.^[10]

In an aim to reduce the quantity of reaction mixture and use of expensive reagent, the assay was revised herein. The system was optimized in resulting solution of only 200 µl, and three references were considered for validation [Figure 3]. Analysis showed that all standards possessed strong radical scavenging capacity that incremented in a dose-dependent manner. At the concentration of 1, 3, 5, and 10 µg/ml, radical scavenging abilities of ascorbic acid were 15.16 ± 0.647%, 47.07 ± 3.38%, 79.15 ± 5.586%, and 89.82 ± 2.387%,

respectively, depicting strong antioxidant potential. Whereas, BHA presented 15.37 ± 7.246%, 39.97 ± 1.923% of quenching ability at the levels of 1 and 3 µg/ml that gradually elevated to 69.02 ± 7.333% and 91.42 ± 1.931% at the dose of 5 and 10 µg/ml. Conversely, inhibition activities of Trolox were 8.21 ± 7.451%, 19.34 ± 8.542%, 29.43 ± 7.597%, and 58.81 ± 3.661% at those concentrations ranges demonstrating moderate activity. Thus, it can be said that ascorbic acid and BHA presented extremely powerful potential while Trolox exhibited lower effects [Table 1]. However, properties of Trolox were detected to be in accordance with the previously published reports.^[26,29]

Chelating ability of ferrous ion

Some transition metals including Fe²⁺, Cu²⁺, and Pb²⁺ as well as Co²⁺ can trigger oxidative stress and magnify cellular damage. Among them, Fe²⁺ has ability to react with H₂O₂ and produces highly reactive hydroxyl radical via Fenton's reaction. Thus, antioxidants with metal chelating ability may be of beneficial use in the treatment of several pathophysiological disorders.^[30] To determine such activity, Dinis *et al.* proposed a method where ferrozine competitively reacts with Fe²⁺ forming red complexes. When other chelators are present in reaction mixture, this complex formation is disrupted, and red color of solution decreases. Thus reduction in absorbance signifies Fe²⁺ trapping potentiality of investigating component.^[18] The assay is a quite popular technique for determination of antioxidant property of samples, though the method has not being modified so far for higher sample throughput and better reproducibility.

In this background, this study was conducted in an effort to improve the traditional method. As shown in Figure 4, EDTA, the reference, revealed exceptional dose-dependent chelating pattern at the tested concentrations. The chelating activity of 18.18 ± 3.04% and 38.82 ± 0.961% was achieved at the concentration of 5 and 10 µg/ml of EDTA, respectively. While it increased steadily to 65.99 ± 2.333% and 89.23 ± 0.209% at the level of 15 and 20 µg/ml of standard. In a recent study, Chai *et al.* reported ferrous ion chelating potentiality of EDTA following classical method which was found to be in agreement with our data.^[31]

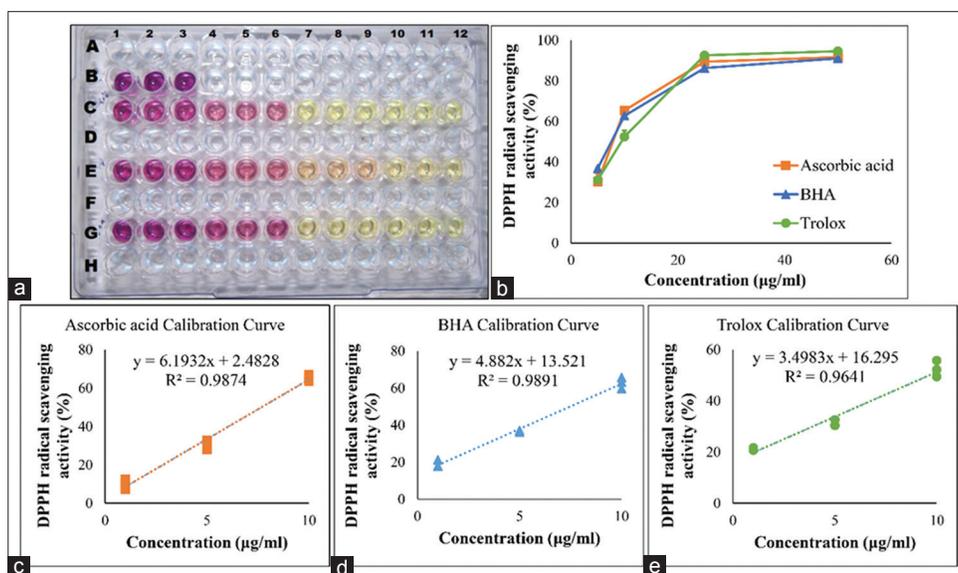


Figure 2: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (a) Photograph of 96-well plates where Lane B represents negative control; Lane C, E, and G depicts DPPH quenching potentialities of ascorbic acid, butylated hydroxyanisole (BHA), and Trolox, respectively. The standards were investigated at different concentrations such as 5, 10, 25, and 50 µg/ml in triplicates, (b) graphical presentation of the effect of three references on radicals in terms of percent inhibition. Scatter plot of ascorbic acid (c), BHA (d) and Trolox (e) was prepared to calculate Pearson's correlation coefficient (R^2). Experiments were triplicated, and values are presented as mean \pm standard deviation

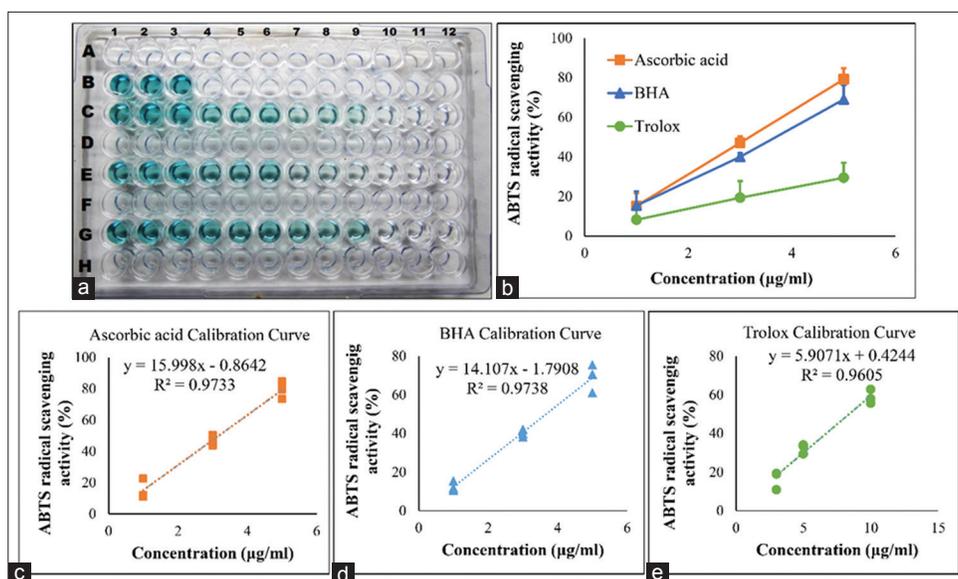


Figure 3: 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity (a) Photograph of 96-well plates where Lane B represents negative control; Lane C, E, and G depicts ABTS⁺ quenching potentialities of ascorbic acid, butylated hydroxyanisole (BHA), and Trolox, respectively. The standards were investigated at variable concentrations of 1, 3, 5, and 10 µg/ml in triplicates, (b) graphical presentation of the effect of three references on radicals in terms of percent inhibition. Scatter plot of ascorbic acid (c), BHA (d), and Trolox (e) was prepared to calculate Pearson's correlation coefficient (R^2). Experiments were triplicated, and values are presented as mean \pm standard deviation

Reducing power

It is well-known that free radicals are molecules, atoms or ions that contain unpaired electrons; as a result, they are highly reactive in nature. Antioxidants are substances that are stable enough to donate their own electrons to stabilize radicals and inhibit further damages.^[5] Thus, determination

of reducing the power of a substance may serve as a significant indication for its antioxidant potentiality. In that view, Oyaizu described a process based on reduction of Fe^{3+} to Fe^{2+} depending on the activity of investigating compounds which in turn generates $KFe [Fe(CN)_6]$ (Prussian blue) appropriate for spectrophotometric measurement.^[19,32] Thus increase in absorbance denotes an increment in reducing

power as well as antioxidant effects. However, originally the assay was designed for plasma, but after realizing its applicability, it is now used in an enormous number of matrixes.^[4] Subsequently, the methodology has been modified using 48 well microtitration plates in a reaction mixture of 1500 μl .^[13]

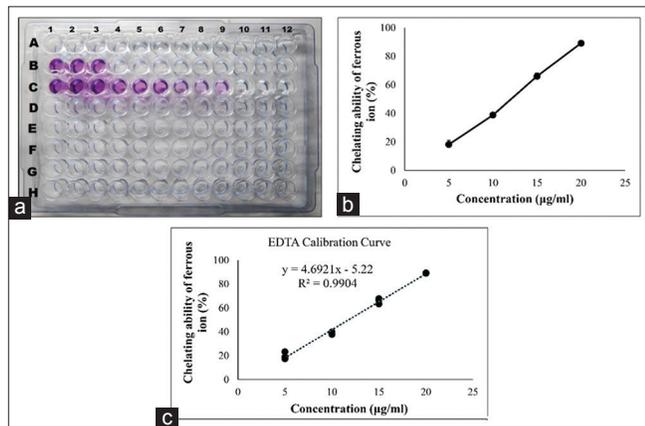


Figure 4: Chelating ability of ferrous ion (a) Photograph of 96-well plates presenting dose-dependent activity of ethylenediaminetetraacetic acid (EDTA) in respect to negative control (Lane B1-3: Blank, C1-3: 5 $\mu\text{g/ml}$, B4-6: 10 $\mu\text{g/ml}$, B7-9: 15 $\mu\text{g/ml}$, and B10-12: 20 $\mu\text{g/ml}$), (b) graphical presentation of the effect of EDTA in terms of percent chelation, (c) scatter plot of the standard showing Pearson's correlation coefficient (R^2). Experiments were triplicated, and values are presented as mean \pm standard deviation

To perform the test in 96-well plates, the assay needs to be re-designed. In this context, the protocol has been modified herein and to fulfill requirement resultant solution was deducted to only 60 μl . Moreover, the centrifugation step as described in original technique has been removed to simplify the method. Besides, λ_{max} was changed from 700 to 750 nm due to limitation of automated analyzer. To authenticate the revised technique, three standards were used, and their activities have been presented in Figure 5. All the references were capable of reducing Fe^{3+} to Fe^{2+} in a linear dose-dependent manner. Ascorbic acid exhibited a dose-dependent response by increasing absorbance 0.218 ± 0.011 , 0.909 ± 0.025 , 1.2 ± 0.0618 , and 1.2 ± 0.017 at the level of 10, 30, 50, and 70 $\mu\text{g/ml}$, respectively. While BHA showed 0.145 ± 0.015 , 0.526 ± 0.074 , 0.731 ± 0.018 , and 0.922 ± 0.014 reducing power at those above-mentioned concentrations. However, Trolox presented lower potentiality by increasing absorbance of 0.07 ± 0.011 , 0.445 ± 0.041 , 0.698 ± 0.08 , and 0.933 ± 0.003 at the level of 10, 30, 50, and 70 $\mu\text{g/ml}$, respectively. Thus, it can be observed that ascorbic acid possesses the strongest reducing effect followed by BHA and Trolox [Table 1].

CONCLUSION

The work presents improved version of routinely used antioxidant assays based on microplate reader technique. Major advantage of the proposed methods is a reduction in reaction volume ranging from 200 μl for DPPH and

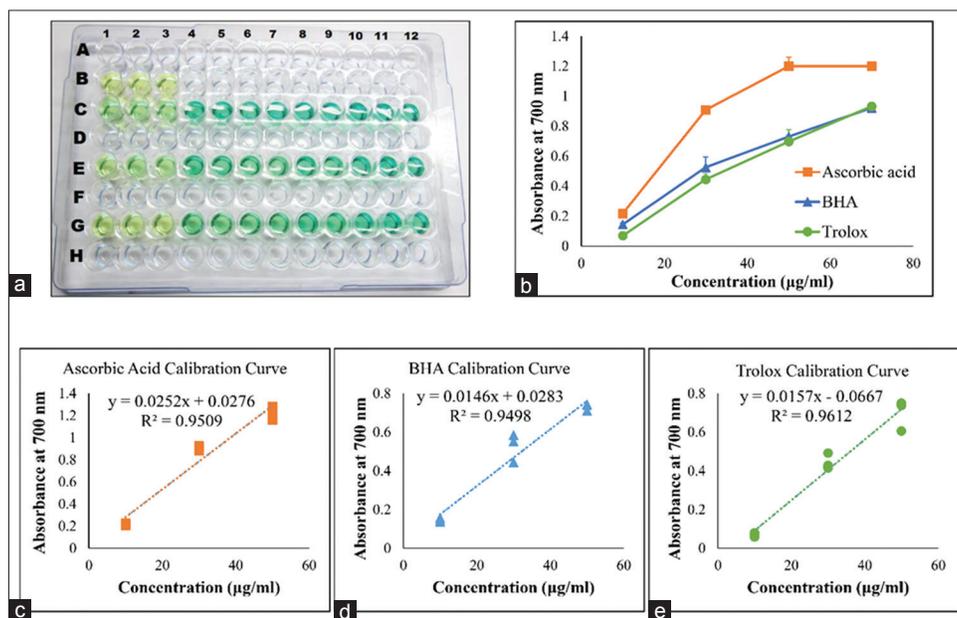


Figure 5: Reducing power (a) Photograph of 96-well plates where Lane B represents negative control; Lane C, E, and G depicts reducing power activity of ascorbic acid, butylated hydroxyanisole (BHA), and Trolox, respectively. The standards were investigated at variable concentrations of 10, 30, 50, and 70 $\mu\text{g/ml}$ in triplicates, (b) Graphical presentation of the reducing ability of three references in terms of increase in absorbance. Scatter plot of ascorbic acid (c), BHA (d) and Trolox (e) was prepared to determine Pearson's correlation coefficient (R^2). Experiments were triplicated, and values are presented as mean \pm standard deviation

ABTS radical scavenging protocols to 60 μ l in the case of reducing power method. In addition, this study provides the first time attempt for optimization of $O_2^{\cdot-}$ quenching as well as chelating ability of ferrous ion assays in 96-well plates at per our knowledge. The modified techniques may be considered as economically inexpensive, time-saving and most importantly useful for analysis of a large number of samples in a day.

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