Optimized and Comparative Antioxidant Assays and Its Applications in Herbal and Synthetic Drug Analysis as an Antioxidants

Shivraj Hariram Nile[1,2,*] C.N. Khobragade[1] and Se Won Park[2]

[1]*Biotechnology Research Laboratory, School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded-431606, Maharashtra, India;* [2]*Department of Molecular Biotechnology, College of Life & Environmental Sciences Konkuk University, Seoul- 143-701, South Korea*

Abstract: Drug development in the recent times often relies on use of natural and synthetic drugs that are promising candidates as therapeutic agents for prevention of diseases and disorders. They possess different chemical structures with wide range of therapeutic activities. Many natural and synthetic drugs act as antioxidant agents in various metabolic processes. Increasing epidemiological, clinical and experimental studies have shown that intake of antioxidants drugs provide protection against various disorders and diseases related to oxidative stress. The factors responsible for this oxidative stress are mainly free radicals, reactive nitrogen species (RNS) and reactive oxygen species (ROS). The antioxidant drugs act as free radical scavenging, reducing and metal chelating substances; Antioxidants also show inhibition of various metabolic enzymes and factors responsible for inflammation. The present paper reviews different *In vitro* assays for determination of antioxidant activities (Table **1**). The basic assays include DDPH assay, OH Scavenging assay, Reducing activity assay, TEAC assay, FCR assay, PRTC assay, ABTS assay, FRAP assay, ORAC assay, Ferric thiocynate assay, TRAP assay, Chemiluminescence assay, NBT assay, CUPRAC Assay.

Keywords: Herbal drugs, Synthetic drugs, Antioxidant, Assays.

INTRODUCTION

Natural and synthetic drugs are widely used as a source of therapeutic tools for the prevention or treatment of many diseases. These drugs are mainly polyphenols, chalcones (precursors of flavonoids), vitamins, carotenoids, and proteins in nature. Experimental and epidemiological studies have shown that many natural and synthetic drugs are involved in reduction of oxidative stress developed due to free radicals and act as antioxidants [1]. Antioxidant is a molecule having capacity of preventing or slowing the oxidation of molecules. Oxidation reaction transfers electrons from a substance to an oxidizing agent and produces free radicals leading to cell damage. Antioxidants terminate the chain reactions by removing free radical intermediates and inhibit other oxidation reactions by oxidizing themselves; for this reason, antioxidants are often considered as reducing agents [2]. Majority of complex life forms require oxygen for their existence; which is highly reactive molecule that damages living organisms by producing reactive oxygen species (ROS) [3]. However most of the cells contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids [4]. Antioxidants either remove or prevent these reactive species before they can damage vital cellular components of the cell or tissues [5]. Free radicals are the types of Reactive Oxygen Species (ROS) which includes all highly reactive oxygen containing molecules; these are mainly hydroxyl radicals, peroxy radicals, super oxide radicals, hypochlorite radicals, hydrogen peroxides, singlet oxygen, nitric oxygen radicals and various lipid peroxides [6, 7]. ROS are also capable of reacting with membrane lipids, proteins, nucleic acids, various metabolic enzymes and small molecules of living systems. The interaction of ROS and cellular components result in cellular damage and tissue injury prompting inflammation. These reactive oxygen species have been found to play an important role in the initiation and progression of various diseases such as cancer, gout, atherosclerosis, cardiovascular diseases, aging and respiratory diseases [8-12]. ROS also initiate a wide range of toxic oxidative reactions in body like initiation of lipid peroxidation, inhibition of metabolic enzymes, inhibition of membrane sodium potassium ATPase activity and oxidative modifications of proteins. All these toxic effects lead to development of inflammation [13], a fundamental protective response or a local response of living mammalian tissue injury. It is a body defense phenomenon in order to eliminate or limit the spread of injurious agent. Various components of inflammatory reaction can lead to various symptoms such as tissue injury, edema formation, and leukocyte infiltration [14]. Antioxidant activity could be measured simply by studying fat in a closed container with application of oxygen in reaction and measuring the rate of oxygen consumption during the reaction [15, 16]. The possible mechanism behind this action of antioxidants was explored when substance with antioxidative property is likely to be one that it gets readily oxidized by itself [17]. This paper explains the major *in-vitro* methods for determination of antioxidant assays for natural and synthetic drugs.

*Address correspondence to this author at the Biotechnology Research Laboratory, School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded- 431606, Maharashtra, India; Tel: +919823772087; Fax: +912462229245; E-mail: nileshivraj@gmail.com

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Table 1. Summary of Analytical Features of Some *in vitro* Scavenging Capacity Assays

Sr. No	Antioxidant Assays	Advantages	Disadvantages
1.	DPPH Radical Scavenging Assay	This technique is widely used as it is very easy, effective, and rapid way to study the herbal and synthetic drugs. No sample separation is needed. Potency of sample can be known.	This assay is easy but the DDPH reagent is very costly but comparatively cheap than other antioxidant reagent.
2.	ABTS Radical Scavenging Activity	This method basically determines antioxidant activity depending up on the concentration of antioxidant which might be biologically inactive. This assay is useful for measurement of antioxidant activity of mixed compounds, hence useful to distinguish additive and synergistic effects.	In this method the capability of the tested sample to react with $ABTS.^{+}$ rather than to inhibit the oxidative process. Less accurate, Time consuming, costly.
3.	ORAC Assay (Oxygen Radical Absorbance Capacity Assay)	ORAC Assay widely implies equally well for both antioxidants that exhibit distinct lag phase and those that have no lag phases. This assay has been broadly applied in food and dietary supplement industries as a method of choice to quantify the drugs.	This method has limitations for measurement of hydrophilic chain but ignores lipophilic antioxidants. In this method the Temperature control decreases reproducibility. This method requires costly instrument fluorometers, which may not be routinely available in laboratories.
4.	TEAC Assay: Total Antioxidant Capacity by Trolox Equivalent Antioxidant Capacity	TEAC assay is operationally simple and has been used in many research laboratories. It permits study over a wide pH range. Although frequently used at pH 7.4, the stability of ABTS radical at this pH has been reported to be problematic.	Least suitable to determine plasma antioxidant capacity because it relies on enzymatic activity. This technique has not been widely applied, which limits the possibility to compare results from different studies.
5.	NBT (Superoxide Radical Scavenging Activity) Assay	Accurate, less time consuming and easily performed with minimal requirements.	This method is not suitable for quantifying non enzymatic antioxidant.
6.	FRAP (Ferric Reducing Ability of Plasma) Assay	FRAP assay is simple, rapid, inexpensive, and robust and does not require specialized equipment. The FRAP assay can be performed using automated, semiautomatic, or manual methods	FRAP cannot detect species that act by radical quenching (H transfer), particularly SH group containing antioxidants like thiols, such as glutathione and proteins.
7.	TBA (Thiobarbituric Acid) Assay	TBA method is simple and does not need expensive instruments; however, both of the reactions need high temperature (60–100 °C) and long reaction time (30–60 min).	This method is handy and sensitive; the multiple reaction products make it complicated to quantitative detection of hydroxyl radical.
8.	FTC (Ferric Thiocyanate) Assay	Moderately accurate, less time consuming and easily performed with minimal requirements.	Iodometric hydroperoxide measurements have some limitations, such as poor sensitivity and selectivity, since it is possible the addition of some iodine across unsaturated bonds, oxidation of iodine by dissolved molecular oxygen and variations in reactivity of different peroxides.
9.	FCR (Folin-Ciocalteu Reagent) Assay	FCR Method is convenient, simple, and reproducible and often found excellent linear correlations between the “total phenolic profiles” and “the antioxidant activity”.	This method requires maximum time for standardization and lack of standardization of methods can lead to several orders of magnitude difference. If the standard used for calibration is highly reactive and gives a high absorbance, then the measured values of samples will be low.
10.	Peroxynitrite Scavenging Assay	It provides concurrent multisampling analysis with automated data storage, regression analyses, and calculation of oxidation inhibition rates. For screening crude extracts and typical assay results are presented.	peroxynitrite (ONOOH) is a powerful oxidizing agent that may cause depletion of thiol groups (-SH) and oxidation of many biomolecules causing damage similar to that observed when HO• is involved.

(Table 1) contd….

Sr. No	Antioxidant Assays	Advantages	Disadvantages
11.	Chemiluminescence Assay	The promising characteristic of this method is their productivity; commonly, one run takes a few minutes only; in addition, the assay can be easily automated and run in microwell plates.	In this method the detection needs special equipment that both set samples close to the detector and detects light at single-photon levels and, in addition, provides temperature control. The mechanism for chemical processes resulting in assay is not known in detail which may create problems with interpreting data obtained.
12.	Ferrous Ion Chelating Ability Assay	Simple and less time consuming, requires low cost chemicals but rarely used method for antioxidants activity analysis of herbal and synthetic drugs.	This technique has not been widely applied, which limits the possibility to compare results from different studies.
13.	HOCL Scavenging Assay	Simple and less time consuming, requires low cost chemicals but rarely used method for antioxidants.	Production of HOCl contributes to the mechanism of defense against foreign microorganisms; nevertheless HOCl may also attacks amines and sulphydryl groups of several biomolecules causing damage to the host cell structures.
14.	(CUPRAC) Cupric Ion Reducing Antioxidant Capacity	The measurement of aromatic hydroxylation with HPLC / electrochemical detection is more specific than the low-yield TBARS test, it requires sophisticated instrumentation. As a more convenient and less costly alternative.	It is difficult to measure the small lag times that may occur, and many substances in foods also absorb at 234 nm. Cu(II) alone does not induce the autoxidation of lipids. Instead, the reaction was initiated by antioxidants (e.g., tocopherols) present in the LDL.

DPPH RADICAL SCAVENGING ASSAY

This method is widely used for determination of free radical scavenging activity, it is based on the reduction of methanolic or ethanolic solution of colored free radical DPPH (α, α-Diphenyl-β-picrylhydrazyl) by reactive oxygen species or reactive nitrogen species which are responsible for oxidative stress in cellular tissues. It involves measurement of decrease in absorbance of DPPH at its absorption maxima of 515nm, which is directly proportional to the concentration of free radical scavengers added to DPPH solution. The activity is expressed as effective concentration [18]. In this method the drug was prepared by using ethanol. The scavenging activity of the drug was measured using the stable radical DPPH. The mixture was shaken vigorously and the absorbance was monitored at 515 nm after 45 min of incubation, when the reaction reached a steady state, α-Tocopherol or Qurecetin was used as reference compound. The inhibition percentage (%) of radical scavenging activity was calculated by using the formula [19].

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

ABTS RADICAL SCAVENGING ACTIVITY

ABTS method is based on interaction of antioxidant and $ABTS^+$ radical cation which gives a unique color showing maximal absorbance at 734, 645, and 815 nm. This assay is based on the principle that when 2, 2'-azinobis-(3- ethyl-benzothiazoline-6-sulphonic acid) {ABTS} is incubated with a peroxidase (such as metmyoglobin and H_2O_2, a relatively stable radical cation, $ABTS^+$, is formed. The formation of $ABTS^+$ on Interaction with Ferryl myoglobin produces a relatively stable blue-green color, measured at 645nm. Antioxidants in the samples suppress this color production to a degree that is proportional to their concentrations. In this equation, $HX\text{-}Fe^{III}$ = metmyoglobin, X – [Fe^{IV} = 0] = ferrylmyoglobin, ABTS = 2, 2' – azino-di-[3-ethylbensthiazoline sulphonate] [23, 24]. The 2, 2-azinobis-3-ethyl benzothiazoline-6-sulfonic acid diammonium salt (ABTS) was oxidized with potassium persulfate overnight and the working solution was then diluted with ethanol to an absorbance at 645 nm. A standard calibration curve was constructed for Trolox. An aliquot of each drug was mixed with ABTS radical cation working solution in cuvette and absorbance was read at 645 nm. The activity was expressed as the effective concentration of drug necessary to give a 50 % reduction in the original absorbance (EC_{50}) [20].

ORAC ASSAY: OXYGEN RADICAL ABSORBANCE CAPACITY ASSAY

The ORAC antioxidant method is utilized to test "Antioxidant power" of various natural and synthetic compounds. Basically in this method in the presence of antioxidant ROO^o abstracts a hydrogen atom from the antioxidant to form hydroperoxide (ROOH) and a stable antioxidant radical ($Ar0^o$); as a result, the damage to fluorescein induced by peroxyl radical is inhibited. The protective effect of an antioxidant is measured by assessing the area under the fluorescence decay curve (AUC) of the sample compared to that of the blank in which no antioxidant is present. The ROO^- scavenging activity was measured by monitoring the decay in fluorescence due to the oxidation fluorescein, known as Oxygen Radical Absorbance Capacity (ORAC) assay [21]. In this method ROO^- was generated by thermo decomposition of 2, 2'-azobis (2-amino-propane) dihydrochloride (AAPH). For this reaction an automated

plate reader with 96-well plates were used. Reaction mixtures in the sample wells contained, the following reagents dissolved in potassium phosphate buffer, pH 7.4, at the indicated final concentrations: Fluorescein was used as substrate, drug at various concentrations and AAPH. The scavenging effects are expressed as the relative trolox equivalent ORAC value, which was calculated by the following equation, where AUC represents the area under curve [22].

Relative ORAC value

$= [(AUC_{sample} - AUC_{blank}) / (AUC_{trolox} - AUC_{blank})] \times$ (mass of Trolox / mass of sample)

TEAC ASSAY: TOTAL ANTIOXIDANT CAPACITY BY TROLOX EQUIVALENT ANTIOXIDANT CAPACITY

This assay performed using Trolox (a water soluble analog of vitamin E) as standard to determine the Trolox Equivalent (TE). The antioxidants potential was calculated as total antioxidant capacity by Trolox equivalent antioxidant capacity which indicates that higher the oxygen radical absorbing capacity value, the greater the antioxidant power. The principle of this method is similar to ORAC assay [23].

The $ABTS^+$ radical cation was generated by mixing ABTS stock potassium persulfate. This mixture was kept at ambient temperature for 12-16 h until the reaction was complete and the absorbance was stable. The $ABTS^+$ solution was dilute with water to give an absorbance valve at 730 nm. The sample solution was mixed with the $ABTS^+$ solution. After 6 min of incubation at ambient temperature, the absorbance value of the mixture was measured at 730 nm and a well known antioxidant was used as a positive control. Triplicates were made for each test sample. The TEAC of the sample was expressed as trolox equivalent in millimoles per µg/ml or 1 mM (drugs) [24].

NBT (SUPEROXIDE RADICAL SCAVENGING ACTIVITY) ASSAY

This is an *In-vitro* method for measurement of superoxide radical scavenging activity by using NBT (Nitro blue tetrazolium)/ riboflavin reduction**.** This method is based on generation of super oxide radicals by auto oxidation of NBT/riboflavin in presence of light. NBT get reduced by super oxide radicals forming a colored complex formazon which can be measured at 570 nm. The capacity of drug as an antioxidant to inhibit the color formation to 50% is measured in terms of effective concentration (EC_{50}) [25, 26].

In this method Nitroblue tetrazolium chloride (NBT) salt were used in Na_2EDTA in buffer, hypoxanthine in KOH and the drug or compounds were added in 96-well microplates. The reaction was started by adding xanthine oxidase enzyme in buffer to the mixture. The reaction mixture was incubated at ambient temperature, and the absorbance at 570 nm was determined every 1 min up to 8 min using ELISA reader. Catechin and quercetin, well-known antioxidants, were used as positive controls. Triplicates were made for each test sample. The percent inhibitions, ratio was calculated according to the following formula [27].

$$\% \text{ inhibition} = \frac{[(\text{Rate of control reaction-rate of sample reaction})]}{[\text{Rate of control reaction})]} \times 100.$$

FRAP (FERRIC REDUCING ABILITY OF PLASMA) ASSAY

FRAP assay is a most widely used method for assessing the antioxidant activity. The reaction of antioxidant and FRAP reagent containing (TPTZ (2, 4, 6-tripyridyl-s-triazine) and $FeCl_3.6H_2O$ forms ferrous ion which increases the absorbance of the reaction complex. This increased absorbance is measured spectrophotometrically at 595 nm for determination of antioxidant activity. The stock solutions were prepared by using acetate buffer pH 3.6. The reagent TPTZ (2, 4, 6-tripyridyl-s-triazine) was prepared by using TPTZ solution in $Fecl_3.6H_2O$ solution. The fresh working solution was prepared by mixing acetate buffer, TPTZ solution and $Fecl_3.6H_2O$ solution and this mixture was called as FRAP solution. The working solution (FRAP) was warmed at 37 ^oC before use. Drugs were allowed to react with the FRAP solution for 30 min in dark condition. Absorbance of the colored product (ferrous tripyridyltriazine complex) was recorded at 595 nm. Trolox was used as standard and the standard curve was linear between 25 and 800 µM of Trolox. The results were expressed as µM TE/g fresh mass. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve [28].

TBA (THIOBARBITURIC ACID) ASSAY

This method also called as lipid peroxidation involves the measurement of lipid peroxidation. The method depends on isolation of microsomes from rat liver and induction of lipid peroxides by ferric ion leading to the formation of small amount of Malonaldehyde (MDA). TBA (Thiobarbituric acid) reacts with this formed MDA to form a pink colored complex, which can be measured spectrophotometrically at 532 nm and the results were expressed in terms of percent inhibition [29]. This assay was determined according to the method; Lecithin was sonicated in an ultrasonic cleaner by using phosphate buffer (pH 7.4) for 2 h at ice cold temperature. Sonicated solutions (liposome in phosphate buffer) were incubated with the test drug in the presence of $FeCl_3$ and ascorbic acid at 37 ^oC for1 h. Finally the reaction was terminated adding trichloroacetic acid and thiobarbituric acid (1% w/V), then the solution was heated at 100 ^oC. After 15 min, the color of the malonaldehyde (MDA)-TBA complex was measured at 532 nm spectrophotometrically. Quercetin or Acetylsalicylic acid was used as a positive control. Triplicates were made for each test sample. The inhibition ratio (%) was calculated by using following formula [30].

$$\% \text{ inhibition} = \frac{[(\text{Absorbance of control} - \text{Absorbance of test sample})]}{\text{Absorbance of control}} \times 100$$

FTC (FERRIC THIOCYANATE) ASSAY

Peroxides formed from oxidation of linoleic acid form a colored complex which is measured at 500 nm and the

intensity of this complex is proportional to amount of peroxides formed.The antioxidant capacity was determined by the ferric thiocyanate method (FTC) in the following manner. Drugs with different concentrations of TPC were mixed with phosphate buffer at pH 7 and linoleic acid in ethanol to obtain a final solution. The resulting solutions were incubated. Aliquots were removed at regular intervals, and $FeCl_2$/ammonium thiocyanate solution was added in order to allow any peroxides resulting from the oxidation of linoleic acid to react, forming a complex that can be detected spectrophotometrically at 500 nm. This step was repeated every 24 hr until the control (phosphate buffer plus linoleic acid) reached its maximum absorbance value. Therefore, high absorbance values indicated high levels of linoleic acid oxidation. Phosphate buffer was used as the reaction blank. The total antioxidant activity was expressed as the average of three independent determinations carried out in duplicate. The percentage inhibition of lipid peroxidation of linoleic acid was calculated by applying the following equation.

Inhibition of lipid peroxidation (%) = 100 – [(As/Ao) x 100]

Where, Ao is the absorbance of the control reaction (linoleic acid alone) while As is the sample drug or positive control antioxidant activity (1 mg/mL ascorbic acid). The inhibitory concentration 50 (IC_{50}) values were calculated from data obtained graphically, using a mathematical method based on the principle of the right angled triangle:

IE_{50} = D – [(A – 50 % max response) X] Y,

Where, A is the immediately higher response of 50% max response, B is the immediately lower response of 50% max response; D = log concentration corresponding to A response; C = log concentration corresponding to B response; X = D – C; and Y = A – B [31, 32].

FCR (FOLIN-CIOCALTEU REAGENT) ASSAY

The exact chemical nature of the Folin-Ciocalteu reagent is not known, but it is accepted that it contains phosphomolybdic/phosphotungstic acid complexes. The chemistry behind the FCR assay counts on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue complexes that can be monitored spectrophotometrically at 750–765 nm. The FCR assay was carried out by using Folin-Ciocalteu Reagent (FCR). The assay was carried out using drug in methanol was mixed with FCR previously diluted with double distilled water, saturated sodium carbonate (Na_2CO_3) and distilled water. The mixture was incubated at room temperature for 25 min and centrifuged at 2000 g for 10 min. The supernatant were collected and absorbance was recorded at 725 nm using a spectrophotometer. The data were expressed as mg of Q4'G equivalents on dry weight basis; where gallic acid used as reference standard [33].

PEROXYNITRITE SCAVENGING ASSAY

This method is used to measure peroxy radical scavenging activity. The peroxynitrite is a culprit in many reactions along with its chemical precursors, superoxide and nitric oxide. The antioxidant activity is measured by monitoring the $ONOO^-$ induced oxidation of dihydrorhodamine 123 (DHR 123) to rhodamine 123 on a microplate fluorescence spectrophotometer at 485 nm. According to this method $ONOO^-$ was synthesized as described before. Prior to each study, the concentration of the stock peroxynitrite solution was determined spectrophotometrically in NaOH ($\varepsilon_{302\ nm}$=1670 M^{-1} cm^{-1}). A stock solution of DHR 123 in dimethyl formamide was purged with nitrogen and stored at – 20 ^oC. The working solution of DHR 123 diluted from the stock solution was placed on ice in the dark immediately before the determinations. The buffer (sodium chloride, sodium phosphate (pH=7.4) and (potassium chloride) was purged with nitrogen and placed on ice before use. At the beginning of the experiments, DTPA was added to the buffer. Reaction mixtures in the sample wells contained, in a final volume of reagents at the indicated final concentrations: DHR 123, drug at various concentrations and $ONOO^-$. In a parallel set of experiments the assay were performed in the presence of $NaHCO_3$ [34, 35].

CHEMILUMINESCENCE ASSAY

Samples Preparation

A stock solution of quercetin or Acetylsalicylic acid was prepared using dimethyl sulfoxide (DMSO).This solution was further diluted to 5 times in DMSO to obtain different concentrations. The natural and synthetic drugs were diluted in phosphate buffer, pH 7.4, to obtain final concentrations of defined volume. DMSO was also used to prepare both stock solutions of natural and synthetic drugs.

Assay

Sample of each test compound or DMSO as control for quercetin or Acetylsalicylic acid and phosphate buffer pH 7.4 as control for drug, were mixed with phosphate buffer and luminol solution was added to yield a final concentration. H_2O_2 was then added to final concentration of reaction mixture. The reaction was started by adding HRP at a final concentration of known volume, The chemiluminescence was measured for 10 minute at 25 ^oC with an Autolumat LB953 apparatus (EG & g Berthold, Githersburg, MD). Based on the measurement of the area under the time course of the chemiluminescence curve for quercetin and drugs the estimation of the relative inhibitory activity of each drug was tested at different concentrations, The inhibition ratio (%) of each sample was calculated as;

Inhibition (%) = 100X AUC_1 / AUC_0

Where AUC_0 and AUC_1 represent the area under the curve observed for the control and in the presence of the sample solution, respectively [36, 37].

FERROUS ION–CHELATING ABILITY ASSAY

The ferrous ion–chelating potential of the test drugs was evaluated by this method. In this method the test drugs in methanol were added to $FeCl_2$. The reaction was initiated by adding $FeCl_2$. Further ferrozine was added. The mixture was shaken vigorously and kept at ambient temperature for 10 min. Absorbance of the solution was then measured at 562 nm; Quercetin or Acetylsalicylic acid was used as a positive

control. Three replicates were made for each drug sample. The percent of inhibition of ferrozine–Fe^{2+} complex formation was calculated according to the following equation [38].

% inhibition = [(absorbance of control – absorbance of sample reaction)/absorbance of control] x 100

HOCL SCAVENGING ASSAY

In the analytical methods for *in vitro* determination of HOCl scavengers, this oxidant is obtained from the enzymatic system myeloperoxidase/H_2O_2/Cl^- or by acidifying commercial sodium hypochlorite to pH 6.2 with sulphuric acid. The former approach can be applied if the sample species do not interfere with HOCl generation (e.g. inhibition of myeloperoxidase activity or direct reaction with H_2O_2. In the second, the determination of the concentration of HOCl solution must be performed daily. The HOCl scavenging activity was measured by monitoring the HOCl-induced oxidation of luminol. HOCl was prepared immediately before use by adjusting the pH of a 1% (v/v) solution of NaOCl to diluted sulphuric acid. The concentration of HOCl was further determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 M^{-1} cm^{-1} [39].

OH RADICAL SCAVENGING ACTIVITY

OH radical were generated by using the Ferric ion (Fe^{3+})/ascorbic acid reaction system. The detection of OH radicals was carried out by measuring the amount of formaldehyde generated from the oxidation of dimethyl sulfoxide. The reaction mixture contained EDTA, Fe^{3+}, DMSO in phosphate buffer, pH 7.4, individual drug solution and Ascorbic acid in phosphate buffer was added finally to initiate the reaction, trichloroacetic acid was used to terminate the reaction. The contents were observed spectrophotomertically at 412 nm for the detection of formaldehyde. Mannitol or Acetylsalicylic acid (1mM) was used as a reference compound for comparative study [40].

(CUPRAC) CUPRIC ION REDUCING ANTIOXIDANT CAPACITY

This is a novel hydroxyl radical scavenging antioxidant activity assay for water-soluble antioxidants. Reactive oxygen species (ROS) may attack biological macromolecules giving rise to oxidative stress-originated diseases. Since OH is very short-lived, secondary products resulting from OH attack to various probes are measured. In this method aminobenzoate, 2,4- and 3,5-dimethoxybenzoate probes were used for detecting hydroxyl radicals generated from an equivalent mixture of $FeII^+$ EDTA with hydrogen peroxide. The produced hydroxyl radicals attacked both the probe and the water soluble antioxidants in 37 ^oC incubated solutions for 2 h. The CUPRAC absorbance of the ethylacetate extract due to the reduction of Cu (II)-neocuproine reagent by the hydroxylated probe decreased in the presence of OH scavengers, the difference being proportional to the scavenging ability of the tested compound. A rate constant for the reaction of the scavenger with hydroxyl radical can be deduced from the inhibition of color formation. The second-order rate constants of the scavengers were determined with competition kinetics by means of a linear plot A0/A as a function of C *scavenger* / C *probe* where A0 and A are the CUPRAC absorbance of the system in the absence and presence of scavenger, respectively and C is the molar concentration of relevant species [41, 42].

CONCLUSION

In this review, numerous antioxidant assays were reported; they differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions, and expression of results. When selecting the most appropriate antioxidant assay method, a researcher should consider the proper and easily rebuttable analysis conditions, substrate and concentration of antioxidants should simulate real biological systems as much as possible for herbal and synthetic analysis. Antioxidant activity occurs by different mechanisms which means employing a method depending on one mechanism may not reflect the true antioxidant capacity. The total antioxidant capacity value should include assays applicable to both lipophilic and hydrophilic antioxidants and regards the similarity and differences of both hydrogen atom transfer (HAT) reaction and electron transfer (ET) reaction-based methods have to be designed to comprehensively evaluate the antioxidant capacity of a sample. The main aim of this paper is to describe and give comparable evaluation for a high throughout assay that can be used to monitor oxidation reduction progress of herbal and synthetic drugs. These assays will be a valuable tool for identifying better antioxidants for herbal and synthetic drugs analysis.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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PATIENT CONSENT

Declared none.

ABBREVIATIONS

ADP = Adenosine diphophate

AMP = Adenosine monophophate

APS = Ammonium per sulphate

ATP = Adenosine triphophate

BSA = Bovine serum albumin

CDCl3 = Deuteriochloroform

CI = Chemical Ionization

DPPH = Diphenyl picrylhydrazyl

DTT = Dithiothreitol

EC = Enzyme commission

ED = Effective dose

EDTA = Ethylene diamine tetra acetic acid

EDTA = Ethylene Diamine Tetra-Acetic Acid

FRAP = Ferric reducing antioxidant power

GMP = Guanisine monophophate

HAP = Hydroxyapetite

HMP = 2-hydroxy-6-methylpurine

IC_{50} = Concentration of the inhibitor that is required for 50% inhibition *in vitro*

LPO = Lipid Peroxidase

M = Molar (mol/L)

NBT = Nitro blue tetrazolium

OD = Optical Density

ROS = Reactive Oxygen Species

SDS = Sodium dodecyl sulfate

TBS = Tris buffer saline

TCA = Trichloroacetic acid

TEMED = N, N, N', N' tetramethyl ethylenediamine

WST = Water soluble tetrazolium salt

XO = Xanthine Oxidase

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