ANALYTICAL SCIENCES FEBRUARY 2014, VOL. 30

Quantification of Antioxidants by Using Chlorpromazine Hydrochloride: Application of the Method to Food and Medicinal Plant Samples

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Chlorpromazine hydrochloride (CPH) (3-(2-chloro-phenothiazine-10-yl)-propyl] dimethylamine hydrochloride) has been the subject of a large number of studies employing a broad spectrum of oxidants, and chosen to examine the course of electron transfer reactions. We report on a method to determine the antioxidant activity of some food and medicinal plants using the oxidation of CPH by chromium(VI) to form a stable CPH radical in the 1:1 orthophosphoric acid–ethyl alcohol (OPA-EtOH) medium. The pink color of the control solution was measured at λ_{max} of 530 nm. Nine standard antioxidants have been studied by this method, along with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The EC_{50}, TEC_{50}, antioxidant efficacy and the stoichiometric values for antioxidants have been evaluated. The radical scavenging activity expressed as EC_{50} ranged from 9.2 µg/mL in *Camellia sinensis* to 448.18 µg/mL in *Cuminum cyminum*. The application of a simple and versatile antioxidant capacity assay for dietary polyphenols and medicinal plant extracts, which are commonly used in Ayurveda opens its relevance in the field of antioxidant analysis.

Keywords Antioxidants, chlorpromazine hydrochloride, chromium(VI), spectrophotometry, free radical

(Received August 1, 2013; Accepted December 6, 2013; Published February 10, 2014)

Introduction

Free radicals are unstable species because they have unpaired electrons and seek stability through electron pairing with biological molecules.[1] Oxidative stress is caused by an imbalance between the effects of pro-oxidants such as reactive oxygen species and defense mechanisms such as antioxidants in tissues, with the balance being shifted in favor of the pro-oxidants.[2] Antioxidants can reduce oxidative damage caused to cells and biomolecules, defend cancer causing agents, and lower the risk of cardiovascular disease, diabetes, and dementia including Alzheimer's disease.[3,4] Free radicals generally play a role in the pathogenesis of chronic degenerative diseases including cancer, autoimmune, inflammatory, cardiovascular, neurodegenerative diseases and aging. Owing to these facts, synthetic and natural compounds with potential antioxidant activity are receiving increased attention in biological research, medicine and pharmacy.[5]

Ascorbic acid is one of the most important water-soluble vitamins present in the human diet, since it helps the body functions such as forming connective tissue, bone, teeth, blood vessel walls, and assists the body in the assimilation of iron and amino acids, present naturally in a wide range of natural food comprised of fruits and vegetables.[6] As an antioxidant, vitamin E acts at cell membranes and prevents the propagation of free-radical reactions.[7] Gallic acid seems to have anti-fungal and anti-viral properties, acts as an antioxidant, helps to protect our cells against oxidative damage and exhibits cytotoxicity against cancer cells, without damaging healthy cells. Synthetic phenolic antioxidants, such as butylated hydroxy anisole (BHA) or tertiary butylhydroquinine (TBHQ) posses good antioxidant capacity although those have been questioned due to possible side effects for human health.[8] The main structural feature responsible for the antioxidative and free-radical scavenging activity of phenolic derivatives is the phenolic OH-group from which hydrogen atoms can be donated to the free radicals, thus stopping the propagation chain during the oxidation process.

Based on the chemical reactions involved, antioxidant capacity assays can be divided into two categories: hydrogen atom transfer reaction (HAT) based assays such as the oxygen radical absorbance capacity (ORAC) assay and the total radical trapping parameter (TRAP) methods and single-electron transfer (SET) reaction based assays such as 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), trolox equivalent antioxidant capacities (TEAC),[9] cupric reducing antioxidant capacity (CUPRAC),[10] 2,2-diphenyl-1-picrylhydrazyl (DPPH)[11] and ferric reducing and antioxidant power (FRAP).[12] These methods require special equipment and technical skills for the analysis. It may also be time consuming because they depend on the oxidation of a substrate that is influenced by the temperature, pressure *etc*. We also very often experience in the antioxidant capacity assays a shortage of reagents. Because of some limitations in using DPPH as a reagent, it becomes very important and essential to develop new, sensitive and simple methods for quantifying antioxidants.

A method has been developed to determine the antioxidant activity of some food and medicinal plants by using the chromium(VI) oxidized chlorpromazine hydrochloride (CPH)

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radical of which the odd electron gives a strong absorption maximum at 530 nm. CPH is a drug used in psychopharmacology which forms a colored radical cation through partial oxidation; it is fairly stable with major implications in biological systems. It has been the subject of a large number of studies employing a broad spectrum of oxidants and chosen to examine the course of electron-transfer reactions. Ce(IV), Br_2, Fe(III), and Co(III) have all been shown to oxidize CPH *via* a one-electron pathway and to produce a paramagnetic cation characterized by an intense visible absorption band (λ_{max} 523 nm) and a complex ESR spectrum.[13] CPH radical may appear in both aqueous and non aqueous medium.[14] The main advantages of this method are the fact that the pink color turns to colorless upon the addition of an antioxidant, and the use of metal for the oxidation instead of enzymes. This method is easy to perform, reproducible and comparable with other method, such as DPPH. Currently, there is a growing interest toward natural antioxidants since plant products are part of the vegetarian diet and a number of them exhibit medicinal properties. Several Indian plants are also being used in Ayurvedic and Siddha medicines and had been documented in Ancient Indian literature.[14-17] Epidemiological and *in vitro* studies on medicinal plants and vegetables strongly supported this idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems.[18]

Experimental

Reagents and extract preparation

A 5-mM CPH ($C_{17}H_{19}ClN_2S$; molar mass, 355.33 g/mol; Sigma-Aldrich, St. Louis, MO) solution was prepared in ethyl alcohol in a 10-mL standard flask, and protected from light by being wrapped in photosensitive paper. A 1-mM potassium dichromate (Merck, Germany) solution was prepared by dissolving 15 mg of potassium dichromate in distilled water. Further dilution was suitably made. All of the standard antioxidant, DPPH and other chemicals were purchased from Sigma Aldrich. Double-distilled ethanol and water were used throughout the experiment. An orthophosphoric acid (Qualigens Fine Chemicals, Mumbai, India)–ethyl alcohol (OPA + EtOH; 1:1) mixture was prepared. Different concentrations of antioxidant solutions as per the requirement for the assay were prepared. Depending on the solubility, different solvents, such as cold water, hot water and ethanol were selected.

Fruits, vegetables and the plant samples were collected from local markets of Mysore, and were identified by Department of Botany, University of Mysore, India. They were peeled, dried for 2 h in an oven at 80°C, weighed and then homogenized by using a blender after adding a sufficient quantity of deionized water. The solution was refluxed for 30 min, then cooled and the homogenate was centrifuged. The supernatant (juice fraction) was recovered and used directly for the assay after suitable dilution. Aqueous extracts were prepared just before the start of the experiments so as to prevent any undesired degradation reaction, and then assayed at least in triplicate; the results were averaged. All infusions were analyzed as fresh as possible for reliability of the results.[19]

Instrument

A Jasco Model UVIDEC-610 ultraviolet-visible (UV-Vis) spectrophotometer with 1.0-cm matched cells was used for all absorbance measurements. A CM 101 cyclo-mixer and REMI centrifuge (Bombay, India) was used to homogenize and centrifuge the extracts. The ESR spectrum was recorded using a Varian V-4546 aqueous flat sample cell at 298K.

DPPH method

DPPH radical scavenging activity was determined according to a method described earlier.[20] The test samples (10 – 100 µL) were mixed with 0.8 mL of Tris–HCl buffer (pH 7.4) to which 1 mL of DPPH (500 µM in ethanol) was added. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517 nm in a UV-Vis spectrophotometer. The radical scavenging activity was measured as a decrease in the absorbance of DPPH. The radical scavenging potential was expressed as the EC_{50} value, which represents the sample concentration at which 50% of the DPPH radicals are scavenged.

CPH radical scavenging activity

To a final volume of 2.5 mL the reaction mixture containing 4×10^{-4} mol L^{-1} CPH, a 2.0-mL 1:1 OPA + EtOH mixture, a 2×10^{-5} mol L^{-1} Cr(VI) solution, 100 µL of various concentrations of antioxidants and 100 µL ethyl alcohol were added. After 10 min, the change in the absorbance of the colored solutions with reference to the control was recorded at 530 nm. The linearity was ascertained by plotting the % CPH activity *versus* the concentration of the antioxidant. The change in the absorbance produced by reduced CPH was used to evaluate the ability of test compounds to act as free-radical scavengers. Thus, it can be measured directly by the quantification of antioxidants. The activity was expressed as percentage CPH scavenging relative to the control which does not contain an antioxidant using the following equation:

$$\text{CPH scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100,$$

where A_{control} and A_{sample} are the absorbances of the control and the antioxidant, respectively.

Parameters measured

The EC_{50} value expresses the amount of antioxidant. The lower is EC_{50} the higher does the antioxidant power becomes. TEC_{50} can be calculated using a graph of the absorbances of the EC_{50} concentration of an antioxidant against the time. The kinetic behavior of an antioxidant compound can be classified as follows: TEC_{50} < 5 min rapid; 5 – 30 min intermediate; and > 30 min slow. The antioxidant stoichiometric can be calculated by using the following equation:[21]

$$n = \frac{A_0 - A_f}{\varepsilon c_0 l},$$

where A_0 is the absorbance of the radical at t = 0; A_f absorbance of the radical at the steady state; ε the molar extinction coefficient for CPH radical; c_0 the initial concentration of phenolic compound and l the optical path length of cuvette (cm). Hence, a higher 'n' value implies a good antioxidant.

The antiradical efficiency (AE) can be calculated using the formula.[22]

Results and Discussion

Optimum conditions for free-radical formation

The optimum conditions for the proposed procedure were established by performing systematic investigations. Rapid formation of the free radical with the maximum stability and

sensitivity at 530 nm was achieved by varying one parameter at a time and fixing the others to be constant for a series of solutions. The optimum conditions for reasonable measurements were 0.2 mL of 20-mM CPH, and 2.0 mL of a 1:1 orthophosphoric acid–alcohol mixture, with 0.1 mL of a 0.5-mM Cr(VI) solution at room temperature. The absorbance of the pink-colored free radical was monitored from 0 to 50 min in an interval of 1 min (Fig. 1), allowing a constant reading from 10 to 30 min. The solution was thus found to be stable for about 30 min. Ten minutes incubation time was selected for further studies.

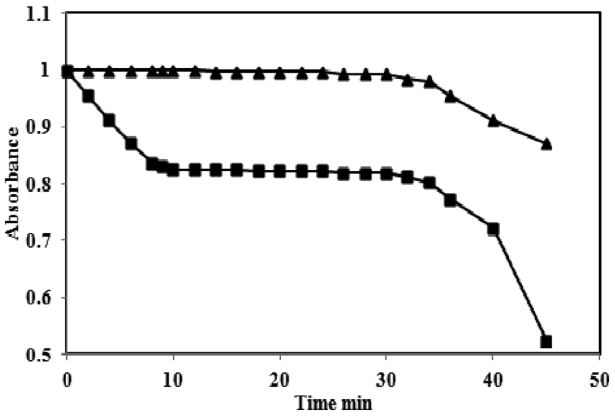

Fig. 1 Stability of the colored product. 4×10^{-4} mol L^{-1} CPH, 2.0 mL 1:1 OPA + EtOH mixture, 2×10^{-5} mol L^{-1} Cr(VI) solution and 200 µL ethyl alcohol or control (▲) and 1 µM ascorbic acid (■).

Spectral characteristics

The absorption spectrum of the optimized solution was scanned on a spectrophotometer in the wavelength region 450 – 590 nm against the distilled water and the maximum absorption was found to be 530 nm. The spectra (Fig. 2a) were recorded by using various concentrations of ascorbic acid (6.24 – 25 µM).

Using the above optimized conditions of the oxidation reaction of CPH with Cr(VI) in a 1:1 OPA + EtOH mixture, the electron spin resonance spectra was recorded at 298K. The spectrometer settings were as follows: microwave frequency, 9.0 GHz; microwave power, 10 dB; center field, 3350 G; receiver gain, 125. The modulation frequency was 100 kHz; modulation

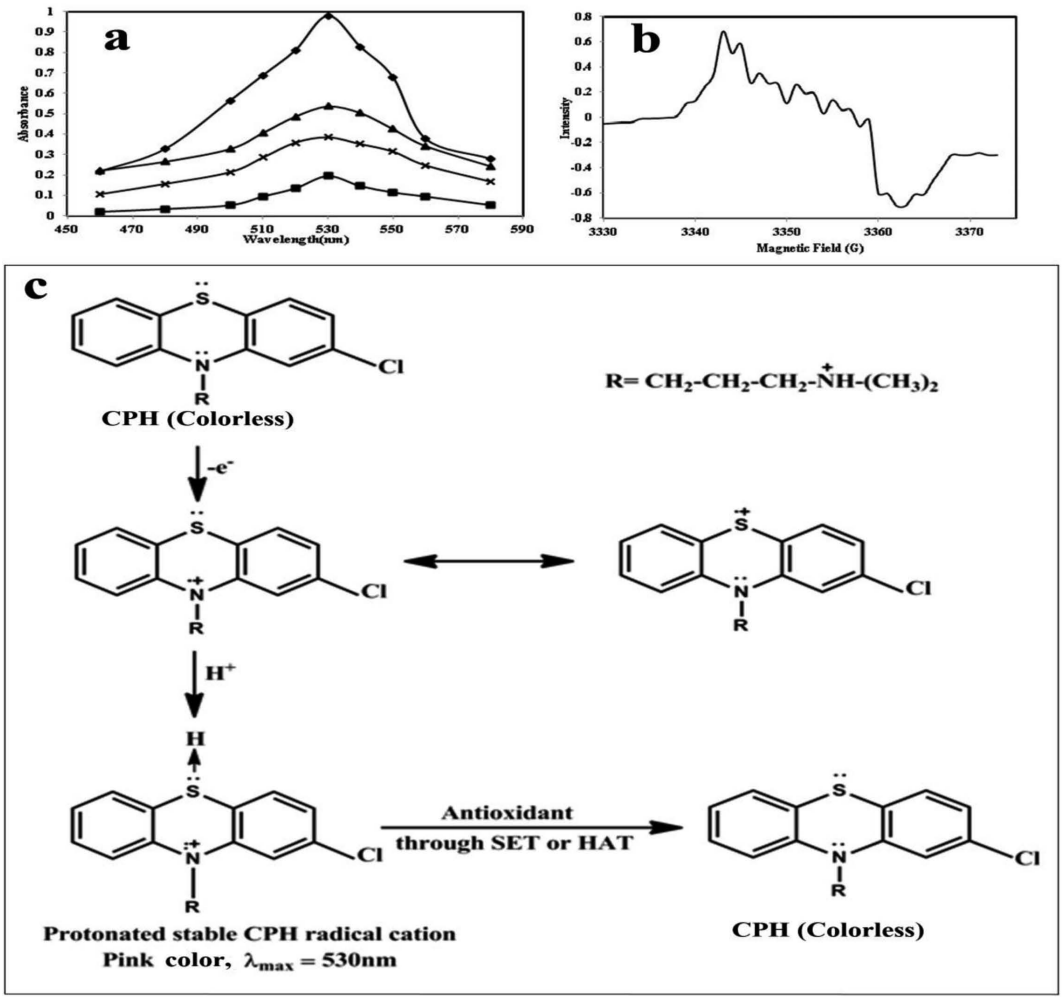

Fig. 2 a. Absorption spectra of 4×10^{-4} mol L^{-1} CPH, 2.0 mL 1:1 OPA + EtOH mixture, 2×10^{-5} mol L^{-1} Cr(VI) solution, and 200 µL ethyl alcohol and various concentrations of ascorbic acid. (■) Control, (▲) 6.24 µM, (×) 12.5 µM, (◆) 25 µM. b. ESR spectrum of the solution of 4×10^{-4} mol L^{-1} CPH, 2.0 mL 1:1 OPA + EtOH mixture, 2×10^{-5} mol L^{-1} Cr (VI) solution. c. Proposed reaction mechanism for the formation of CPH free radical and the final colorless product.

Table 1 EC_{50} and TEC_{50} values

Antioxidant	R^2		[a]EC_{50}/μM		TEC_{50}/min		Stoichiometry		Antioxidant efficacy ($\times 10^{-3}$)	
	CPH	DPPH	CPH	DPPH	CPH	DPPH	CPH	DPPH	CPH	DPPH
Trolox	0.994	0.989	13.5 ± 0.03	13.98 ± 0.01	4	6	1.85	1.8	18.5	12.0
Ascorbicacid	0.994	0.981	7.8 ± 0.01	5.15 ± 0.01	3	1	3.2	2.8	43.0	19.42
TBHQ	0.983	0.978	1.38 ± 0.02	1.69 ± 0.04	41	58	4.5	3.7	17.7	10.2
Gallic acid	0.973	0.988	6.65 ± 0.02	5.33 ± 0.03	6	49	2.3	2.9	25.0	38.0
Quercetin	0.984	0.965	6.3 ± 0.02	5.8 ± 0.03	7	56	4	4.3	22.7	31.0
BHA	0.987	0.982	2.8 ± 0.02	1.12 ± 0.02	10	4	1.1	2.8	36.0	22.3
Rutin	0.967	0.969	6.09 ± 0.02	4.96 ± 0.02	40	55	17.3	5.04	4.11	3.7
Eugenol	0.973	0.989	1.21 ± 0.01	1.19 ± 0.01	50	55	1.6	1.8	16.5	15.0
Catechin	0.977	0.984	8.9 ± 0.03	7.34 ± 0.02	105	110	4.4	5.2	1.07	1.23

a. EC_{50} values are represented as mean ± SD (n = 5).

amplitude 5 G; time constant 81.92 ms and sweep time 5.243 s. Figure 2b shows the ESR spectrum of the reaction system after 30 s from the preparation. The asymmetric and broad spectrum was thought to be due to the CPH radical because of the similarity to the reported ESR spectrum for the electrochemically oxidized CPH in 0.1 M KCl.[23,24] The ESR spectrum could be explained as arising from a splitting pattern due to the interaction with two equivalent hydrogens in the methylene group and the ring nitrogen giving rise to a five-line spectrum. An interaction with the two more methylene will split each of these lines into another three lines. Due to the large number of lines and the relatively low spin density of the unpaired electron at the hydrogens most distant from the ring nitrogen, such a predicted hyperfine pattern will not be resolved. A possible interaction with the nitrogen of the side chain will further complicate the hyperfine pattern.

Extraction efficiency with different solvents

The extraction efficiency for methanol–water, ethanol–water and water to extract from plants and food samples was investigated, and the results were 0.3, 0.45 and 0.6%, respectively. The antioxidant activity was maximum when water was used as an extraction solvent for the *Citrus limon* extract. When this was repeated for other samples, similar results were obtained. Hence, water was selected as the extracting solvent.

Analytical appraisals of the method

In order to test the applicability of the recommended method, the absorbance of a series of solutions, containing various amounts of antioxidants, were recorded at 530 nm. The system obeyed Beer's law for all of the antioxidants tested. The concentration range was 25 – 6.25 μM for ascorbic acid and 25 – 3.125 μM for trolox; the calibration graph exhibited a straight line with a negative slope. The molar absorptivity of trolox, ascorbic acid, TBHQ and rutin was in the order of 10^4 L mol^{-1} cm^{-1}, while the others in the order of 10^3 L mol^{-1} cm^{-1}.

To assess the precision of the method, determinations were carried out for some of the antioxidants with different EC_{50} under the optimum conditions. The results revealed that the RSD values were 0.22, 0.13, 0.30 and 0.71 for trolox, ascorbic acid, gallic acid and BHA, respectively. The low RSD values indicate the high precision with respect to the proposed method. In order to estimate the accuracy of the method known concentration of the antioxidants were added to a determined amount of antioxidants. It was then calculated by using the concentration of antioxidants recovered in relation to that added; the recoveries were 99.2, 98.8, 99.1, and 99.4% for trolox, ascorbic acid, gallic acid, and BHA, respectively. From the values it can be concluded that the proposed method is sufficiently accurate.

Reaction sequence and stoichiometry

Cr(VI) oxidizes CPH to a stable, pink-colored CPH cation radical[24] in an acidic medium that absorbs at a λ_{max} of 530 nm. A similar oxidative product was found to be stable in 1:1 orthophosphoric acid–alcohol media. The mechanism for the reaction may be suggested as in Fig. 2c. The free radical is released by the oxidation of CPH. Under the reaction conditions, the examined CPH loses one electron upon oxidation in the presence of Cr(VI), forming an electrophilic intermediate species, a CPH radical cation. The cationic nature of the species was confirmed by passing through a Dowex-50 cationic exchange resin. When an antioxidant is added to the radical, it decolorizes the radical by reducing it. The antioxidant activity with an excess amount of the CPH radical in order to exhaust the H-donating capacity of antioxidants was tested.

Antioxidant activity of compounds according to the CPH radical scavenging method

The absorbance decreases as the result of a color change from pink to colorless as the radical is scavenged by antioxidants through the donation of hydrogen to form the stable reduced CPH (Fig. 1). For most of the antioxidants tested, a fast decay was observed, followed by a slower step until equilibrium was reached. The antioxidant stoichiometry is related to the structure of the antioxidant (Table 1). The number of hydroxyl groups available and the reaction products are capable of further reacting with the CPH radical and influence the stoichiometry by increasing it.

A possible explanation for the difference in the antioxidant efficiencies found in this study may be an implication of the emulsion.[25] However, small oil droplets and the lipophilic nature of antioxidants would improve their efficiencies in oil-in-water emulsions. On the other hand, a high surface-to-volume ratio, where the surface is nearly the whole phase and poor solubility of the lipophilic antioxidant in the aqueous phase may be the reason for obtaining contradictory results.[26]

Within a group, further distinction in terms of antioxidant activity could be made on the basis of the number of hydroxyl and methoxy groups. The addition of *o*-methoxy groups increased the antioxidant activity due to improved stabilization of the phenoxyl radical.[27] However, the addition of an

Table 2 EC_{50} values for some food and medicinal samples

Sample		R^2		EC_{50} µg/mL^{-1}		*t*-test[b]	F-test[c]
Common name	Botanical name	CPH	DPPH	CPH[a]	DPPH[a]		
Tea decoction	*Camellia sinensis*	0.997	0.994	9.2 ± 0.9	8.3 ± 0.7	2.24	1.65
Clove	*Eugenia caryophyllus*	0.992	0.989	11.2 ± 1.8	10.1 ± 0.8	1.37	5.06
Ginger	*Zingiber officinale*	0.993	0.998	70.4 ± 2.4	72.5 ± 1.4	1.96	2.94
Pepper	*Piper nigrum*	0.984	0.988	134.7 ± 2.9	131.3 ± 1.7	2.39	2.91
Cumin	*Cuminum cyminum*	0.989	0.991	448.18 ± 2.7	450.7 ± 2.4	2.09	1.27
Lemon	*Citrus limon*	0.995	0.987	15.4 ± 1.3	13.8 ± 0.9	2.7	2.1
Tulsi	*Ocimum tenuiflorum*	0.994	0.994	35.5 ± 2.5	38.5 ± 1.7	2.68	2.16
Neem	*Azadirachta indica*	0.992	0.998	45.9 ± 1.4	47.6 ± 1.2	2.71	1.36
Allspice	*Pimenta dioica*	0.984	0.981	60.67 ± 2.4	62.9 ± 1.2	2.1	4.0
Guduchi (amrutha balli)	*Tinospora cordifolia*	0.983	0.989	127.6 ± 2.5	130.4 ± 1.3	2.5	3.7

a. Mean ± standard deviation (n = 5). b. Tabulated t-value for four degrees of freedom at P (0.95) is 2.78. c. Tabulated F-value for (4,4) degrees of freedom at P (0.95) is 6.39.

o-hydroxyl group decreased the antioxidant activity.[28]

The more rapidly does the absorbance decrease, the more potent is the antioxidant activity of the compound in terms of the hydrogen donating ability.[29] Euginol, (+)-catechin, BHA, ascorbic acid, TBHQ, gallic acid, rutin, quercetin and trolox resulted in such a very rapid decrease in the absorbance. The percentage inhibition increased with ortho substitution of monophenols that had a methoxy group, which acts as electron donor. Substitution with a methoxy group was more effective than with a hydroxyl group.

The EC_{50} values are extremely diverse, and were in the range of 1.21×10^{-6} M for eugenol to 13.5×10^{-6} M for trolox in the CPH radical scavenging method. Among the tested antioxidants, (+) catechin reacted slowly, while ascorbic acid, gallic acid, trolox and quercetin needed less time to react. The antioxidant efficacy comprises two aspects such as the electron-donating capacity and the rate of the reaction in order to easily characterize the behavior of the substance as an antioxidant. We can see that the compounds considered as less reactive show lower AE value. This characteristic is of great importance in biological systems since free-radicals have very short half-lives.[30]

The EC_{50} values are comparable for most of the antioxidants in both CPH and DPPH methods. The small differences in the values for some antioxidants may be due to the different pH values of the two methods. DPPH can only be dissolved in organic media (especially in alcoholic media), not in aqueous media, which is an important limitation when interpreting the role of hydrophilic antioxidants.[31] The absorbance of DPPH radical at 517 nm after reacting with an antioxidant is decreased by light,[32] oxygen, and the type of solvent.[33] It was concluded that the antioxidant capacity decreases above a certain limit of water content of the solvent, since a part of the DPPH coagulates, and it is not easily accessible to the reaction with antioxidants.[34] It was also reported that the reaction of DPPH with eugenol was reversible.[35] This would result in falsely low readings for the antioxidant capacity of samples containing eugenol and other phenols bearing a similar structure.

Applications

Herbs and vegetables contain free-radical scavengers, like polyphenols, flavonoids and phenolic compounds. In the present work, we evaluated the free-radical scavenger activity of aqueous extracts of some of them. The results showed that the method can be applied to test the antioxidant activity of plant extracts. The concentration (µg/mL) of food that was required to scavenge 50% of radicals, and their time requirements were calculated. Among the ten extracts tested for *in vitro* antioxidant activity using the CPH and DPPH method, the crude aqueous extracts of *Camellia sinensis, Eugenia caryophyllus* showed antioxidant activity, with EC_{50} values of 9.2 ± 0.9, 11.2 ± 1.8 µg/mL, respectively. *Cuminum cyminum* showed weak antioxidant activity, with EC_{50} values of 448.1 ± 2.7 µg/mL. Similarly common Indian medicines like *Ocimum tenuiflorum*, *Azadirachta indica*, *Pimenta dioica* showed good antioxidant activity. The chemical constituents present in the extract, like tannins, reducing sugars and proteins present in the extract may be responsible for such activity. The phytochemical tests indicated the presence of alkaloids, glycosides, tannins, and flavonoids in the crude extract. Several such compounds are known to possess potent antioxidant activity. Some of these constituents have already been isolated from this plant. Hence, the observed antioxidant activity may be due to the presence of any of these constituents. The results were statistically evaluated in terms of the Student's *t*-test and the variance ratio F-test. The values calculated were found to be less than the tabulated values at the 95% confidence level, indicating no significant differences in the accuracy and precision of the recommended method and the reference method (Table 2).

Conclusions

The antioxidant activity and the nutritional values of vitamins, minerals, *etc*., aid in interpretating the clinical results obtained as various food products tested in biological models for chronic diseases. It is sensible to anticipate that high-antioxidant foods have greater potential to reduce free radicals in the body. As a consequence, it becomes important to know the antioxidant content of foods in addition to knowing basic nutritional information.

The incubation time for the method is 10 min, the color of reaction mixture turns to a clear solution after becomig reduced by antioxidant and the radical is stable for about 30 min. This method proves to be advantageous in serving for studying antioxidant activities. The antioxidant activity of various foods can be determined easily and rapidly using the new CPH method. The tendency in antioxidant activity obtained by using the CPH method is comparable to tendencies found using other methods mentioned in the literature.

Acknowledgements

One of the authors, N. A., thanks Department of Science and Technology (DST), New Delhi for financial support, University of Mysore for providing research facilities and Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology (IIT), Madras for spectral data analysis.

Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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