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Assays for Natural Antioxidant

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ABSTRACT

Cellular damage or oxidative injury arising from free radicals or reactive oxygen species (ROS) now appears the fundamental mechanism underlying a number of disorders. Free radicals are generated through normal metabolism of drugs, environmental chemicals and other xenobiotics as well as endogenous chemicals, especially stress hormones (adrenalin and noradrenalin). Antioxidants are an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. Plant extracts and their constituents as a natural source of antioxidants have been extensively reviewed. In addition to extracts, numerous naturally occurring compounds are useful as antioxidant, ranging from alpha tocopherol and β carotene to plant antioxidants such phenolic compounds. The objective of the investigation performed was to determine the antioxidant properties of herbs that are commonly available and to indicate which of them can become a new source of natural antioxidants for food, cosmetic and pharmaceutical industries.

***Keywords**: Assay, Plants, Free Radicals, Antioxidants, Scavenging*

INTRODUCTION

Oxygen, an element indispensable for life, can, under certain circumstances, adversely affect the human body. Oxidation is essential in many living organisms for the production of energy to fuel biological processes. However, the uncontrolled production of oxygen derived free radicals is involved in the onset of many diseases such as atherosclerosis, rheumatoid arthritis, and cancer as well as in degenerative processes associated with ageing. [1] There have been strong evidences indicating that free radicals cause oxidative damage to lipids, proteins, and nucleic acids.

Most of the potentially harmful effects of oxygen are due to the formation of reactive oxygen species (ROS). The uncontrolled production of ROS and the unbalanced mechanism of antioxidant protection result in the onset of many diseases and accelerate ageing. In addition, under pathological conditions or oxidative stress, ROS are overproduced and result in peroxidation of membrane lipids, leading to the accumulation of lipid peroxides; however, they are removed by antioxidant defence mechanisms.

Antioxidants are considered as possible protective agents, reducing oxidative damage from ROS in the human body and retarding the progress of many chronic diseases, as well as lipid peroxidation. Therefore, there is a growing interest in substances that exhibit antioxidant properties, which are supplied to humans and animals as food components or as specific pharmaceuticals. Antioxidants may be defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. [2]

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation. These compounds may be synthesized in the body or obtained from the diet. [3] The different antioxidants are present at a wide range of concentrations in body fluids and tissues, with some such as glutathione or ubiquinone mostly present within cells, while others such as uric acid are more evenly distributed. Some antioxidants are only found in a few organisms and these compounds can be important in pathogens and can be virulence factors. [4]

Plants have developed an array of defence strategies (antioxidant system) to cope up with oxidative stress. The anti-oxidative system includes both enzymatic and non-enzymatic systems.

The non enzymatic system includes ascorbic acid (vitamin C); ά-tocopherol, carotenes etc. and enzymatic system include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) and polyphenol oxidase (PPO) etc. The function of this antioxidant system is to scavenge the toxic radicals produced during oxidative stress and thus help the plants to survive through such conditions.

MECHANISM OF ACTION OF ANTIOXIDANTS

The redox properties of antioxidants play an important role in absorbing and neutralising free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. [5] In doing so, the antioxidants themselves become oxidised. This urges the constant need of antioxidants to replenish them. The mechanism of antioxidants work has two functions; the first function is that they act as the giver of the hydrogen atom, which is a main function. Antioxidants, which have such main functions, are referred to as primary antioxidants. They can provide hydrogen atoms at a faster rate to the lipid radical (R*, ROO*) or change it to a more stable form. It is a chain breaking step. The second function is a secondary one, which is a preventive step. It reduces the rate of auto-oxidation with a variety of mechanisms beyond the auto-oxidation mechanism of chain termination by radical conversion of lipids to form more stable [6] i.e., by scavenging initiating radicals, such antioxidants can thwart an oxidation chain from ever setting in motion. The effectiveness of an antioxidant in the body depends on which free radical is involved, how and where it is generated, and where the target of damage is present.

SCREENING METHODS OF ANTIOXIDANT ACTIVITY

This review intends to be comprehensive to cover the reported assays which have few influence and applications. Choosing an adequate assay is critical to investigate the antioxidant activity of foods and biological samples. The chemistry behind all the above mentioned assays has been reviewed here emphasising the need of discovery of a convenient method for the quick quantitation of antioxidants.

1. **(1,1-Diphenyl-2-picryl-hydrazyl) radical scavenging assay (DPPH)** [7]
   DPPH (1,1-Diphenyl-2-picryl-hydrazyl) is a stable free radical with red colour. On scavenging, these free radicals turn to yellow. In this assay, 1.2 ml of test sample is added to 0.1 ml of 1 M Tris-HCl buffer (pH 7.9) and mixed with 1.2 ml of 5 mM DPPH in methanol. The reaction mixture is then kept in dark at room temperature for 30 min. The absorbance of the resulting solution is measured at 517 nm. Phenolic organic acids can be used as standard (e.g. Gallic acid). The decrease of the absorbance at 517 nm is calculated as the percentage of inhibition by the following equation,
   % Inhibition = $[(A_0 – A_1)/A_0] \times 100$
   A_0 - absorbance of control & A_1 - absorbance of the tested sample
   It is expressed as Standard phenolic acid equivalent and defined as mg of Gallic acid equivalents per 1 g of sample.
2. **(2,2'-azino-bis3-ethylbenzthiazoline-6-sulfonic) radical cation decolourisation assay (ABTS)** [7]
   ABTS is based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce a radical cation. The ABTS reagent is prepared by mixing 5 ml of 14 mM ABTS with 5 ml of 4.9 mM potassium persulphate ($K_2S_2O_8$) and the mixture is kept in dark at room temperature for 16 h. The reagent absorbance is then adjusted to 0.700 ± 0.02 at 734nm with distilled water and used for the assay purposes. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, its reduction in the presence of hydrogen-donating antioxidants is measured spectrophotometrically at 734nm. Decolourisation assays measure the total antioxidant capacity in both lipophilic and hydrophilic substances. The effects of oxidant concentration and inhibition duration, of the radical cation's absorption are taken into account when the antioxidant activity is determined. Trolox is used as a positive control. The activity is expressed in terms of Trolox-equivalent antioxidant capacity for the extract or substance (TEAC/mg).
3. **Ferric reducing antioxidant power assay (FRAP)** [8]
   In this the compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants. FRAP reagent is prepared by mixing the reagents such as; 0.1 M acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and 20 mM ferric chloride in the ratio 10:1:1 (v/v/v). 10 ml of testing sample is mixed with 300 ml of FRAP reagent, incubated at room temperature for 15 min and the absorbance is read at 593nm. The assay involves $FeSO_4.7H_2O$ as a standard reference and with different concentrations of which the standard curve was plotted. The FRAP values are expressed in mole Fe^{2+}/mg dry weight of the test sample.
4. **Reducing power Assay (RPA)** [8]
   In this assay, 1.0 ml extract is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) is mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM) and absorbance is measured at 700 nm. Ascorbic acid, Butylated hydroxyanisole (BHA) can be used as positive control.
5. **Lipid peroxidation inhibitory activity (Anti-lipid peroxidation assay) (LPIA)** [9]
   The reaction is then inhibited by TCA-TBA solution. 2ml of 10% TCA-0.67% TBA made in 50% acetic acid is added to 1 ml of the reaction mixture and boiled for 1 hour at 100°C, followed by centrifugation for 5 min at 10,000 rpm. The supernatant is observed for absorbance at 535 nm against blank. Induced vitamin E is used as a standard. Reaction mixture without test sample and $FeSO_4$ is used as control. Inhibitory activity was expressed as EC50 value, which is sample concentration at which 50 % of lipid peroxidation was inhibited.
6. **Hydrogen peroxide scavenging activity assay (HPSA)** [10]
   In this method, 1.0 ml of 0.1 mM H_2O_2 and 1.0 ml of various concentrations of test sample are mixed together. 2 drops of 3% ammonium molybdate, 10 ml of 2 M sulphuric acid and 7.0 ml of 1.8 M potassium iodide are added to the reaction mixture. The mixed solution is then titrated with 5.09 mM NaS_2O_3. Appearance of yellow colour is marked as the end point of the reaction. The reaction mixture without test sample is used as control. Percentage of scavenging of hydrogen peroxide is calculated as follows
   % Inhibition = $(V_0 - V_1) / V_0 \times 100$
   V_0 - volume of NaS_2O_3 solution used to the titrate control & V_1 - volume of NaS_2O_3 solution used in titrate the test mixture.
7. **Hydroxyl radical scavenging activity assay (HRSA)** [10]
   The Fenton Reaction is used in this method, 60 µl of 1.0 mM $FeCl_2$, 90 µl of 1mM 1,10- phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2 and 1.5 ml of test solution with various concentrations are mixed together. H_2O_2 is added to the reaction mixture in order to initiate the reaction and the mixture is kept for incubation at room temperature for 5 min. After incubation the absorbance of mixture is read at 560 nm using a spectrophotometer and the hydroxyl radicals scavenging activity is calculated.
8. **Superoxide anion scavenging activity assay** [11]
   The superoxide anion radicals are produced in 2 ml of phosphate buffer (100 mM, pH 7.4) with 78µM β-nicotinamide adenine dinucleotide (NADH), 50µM nitro blue tetrazoliumchloride (NBT) and test samples at different concentrations. The reaction mixture is kept for incubation at room temperature for 5 min. It is then added with 5-methylphenazinium methosulphate (PMS) (10 µM) to initiate the reaction and incubated for 5 min at room temperature. The colour reaction between superoxide anion radical and NBT is read at 560 nm. Gallic

acid is used as a positive control agent for comparative analysis. The reaction mixture without test sample is used as control and without PMS is used as blank. The scavenging activity is calculated as follows,

% Scavenging activity = [(Absc – Abss)/Absc] × 100

9. **Oxygen radical absorbance capacity (ORAC) assay** [12]
   The capacity of a compound to scavenge peroxyl radicals, generated by spontaneous decomposition of 2, 2'-azobis,2- amidinopropane dihydrochloride (AAPH), was estimated in terms of standard equivalents, using the ORAC assay. The reaction mixture (4.0 ml) consist of 0.5 ml extract in phosphate buffer (75 mM, pH 7.2) and 3.0 ml of fluorescein solution both are mixed and pre-incubated for 10 min at 37°C. Then, 0.5 ml of 2, 2'-azo-bis, 2- amidinopropane (AAPH) dihydrochloride solution is added and immediately the loss of fluorescence (FL) is observed at 1 min intervals for 35 min. The final results are calculated using the differences of areas under the FL decay curves between the blank and a sample and are expressed as micromole Trolox equivalents (TE) per gram (μmol TE g-1).
10. **Ferric Thiocyanate method (FTC)** [13]
    The extracts (4.0mL) would be dissolved in ethanol (4.0mL) and mixed with 4.1 mL 2.5% linolic acid (in ethanol), 8.0 mL phosphate buffer (0.02M, pH 7.0) and 3.9 mL distilled water will be added to make up a volume to 20 mL. BHT will be taken as positive control and a solution without extracts will be taken as negative control. The mixture will be incubated at 40-45ºC for 30º min. Ethyl alcohol (75%) 9.7 mL and 0.1 mL ammonium thiocyanate (30%) will be added to 0.1 mL of the mixture. The readings are taken at an interval of 24 h for one week.
11. **Thiobarbituric acid Method (TBA)** [13]
    To 1.0 ml of the FTC sample used above will be added 2.0 mL of TCA and 2.0 mL of TBA 0.8%(w/v) TBA in 1.1%(w/v) sodium dodecyl sulphate solution and this mixture will be placed in a boiling water bath at 100ºC for 10 min. After cooling it will be centrifuged at 3000rpm f or 20 min. and absorbance of the supernatant would be measured at 532 nm using UV -visible spectrophotometer.

The interest in natural antioxidants has grown in the recent years with the awareness of several deadliest diseases. The result of which, is the development of several assays seen above for the measurement of total antioxidant capacity of a biological sample or any food. Indeed, these assays are being utilised in medical field as well as food industries. Due to the complexity of the composition of foods or other biological sample, studying each antioxidant individually is costly and inefficient. Therefore, it is very appealing to researchers to have a convenient method for the quick quantitation of antioxidants.

There is a long list of antioxidant plant of which some have been discussed in Table 1.

TABLE 1: ANTIOXIDANT POTENTIAL IN PLANTS

Sr. No.	Plant	Part	Method to determine Antioxidant potential	Reference
1.	*Aegle marmelos*	Fruit Pulp	DPPH, FRAP, ABTS, NOS, SRSA	[14]
2.	*Aerva lanata*	Leaves	DPPH, SRSA, HRSA	[15]
3.	*Amorphophallus campanulatus*	Tubers	DPPH, NOS, FRAP	[16]
4.	*Amorphophallus paeonifolius*	Tubers	DPPH, FRAP, ABTS	[17]
5.	*Asparagus racemosus*	Root	DPPH	[18]
6.	*Azadirachta indica*	Flower & Seed Oil	DPPH	[19]
7.	*Bacopa monnieri*	Aerial Parts	CAT, SOD	[20]
8.	*Bletilla striata*	Roots	DPPH, FRAP	[21]
9.	*Bridelia retusa*	Stem Bark	SOD, CAT	[22]
10.	*Calocedrus formosana*	Leaf, Bark, Heartwood	DPPH, NBT	[23]
11.	*Camellia sinensis*	Leaves	FRAP, DPPH	[24]
12.	*Canavalia ensiformis*	Seeds	DPPH, FRAP	[25]
13.	*Capparis spinosa)*	Leaves	DPPH ABTS	[26]
14.	*Cassia tora*	Leaves	DPPH, SOD, NOS	[27]
15.	*Centaurea calcitrapa*	Aerial Parts	DPPH	[28]
16.	*Centella asiatica*	Leaves	FRAP, DPPH	[29]
17.	*Chlorella marina*	Microalga	DRSA, FRAP	[30]
18.	*Cinnamomum verum*	Leaf	ABTS, DPPH, MCA, HRSA, FRAP	[31]
19.	*Citrus limon*	Fruits	DPPH, FRAP	[32]
20.	*Citrus aurantium*	Peel	DPPH, FRAP	[33]
21.	*Clinacanthus nutans*	Leaves	DPPH, FRAP	[34]
22.	*Costus pictus*	Leaves & Rhizomes	DPPH, TBARS, FRAP, SRSA	[35]
23.	*Croton caudatum*	Leaves	DPPH, FRAP, HRSA, TCM	[36]
24.	*Cucumis callosus*	Seeds	DPPH, HPRSA	[37]
25.	*Dendrophthoe falcate*	Leaves	DPPH	[38]
26.	*Eugenia jambolana*	Fruits	DPPH	[39]
27.	*Ficus glomerate*	Stem Bark	NBT	[40]
28.	*Ficus racemosa*	Leaves	DPPH, FRAP, PRA	[41]
29.	*Garcinia indica*	Fruits	DPPH	[42]
30.	*Hibiscus sabdariffa*	Leaves	ABTS, DPPH, FRAP	[43]

31.	*Ichnocarpus frutescens*	Whole Plant	TBARS, GSH, CAT, SOD	[44]
32.	*Juglans nigra*	Fruits	DPPH, NOS	[45]
33.	*Leucas plukenetii*	Whole Plant	TBARS, LPA	[46]
34.	*Limonia acidissima*	Fruit Pulp	ABTS, DPPH, FRAP	[43]
35.	*Machilus odoratissima*	Bark	DPPH	[47]
36.	*Malus domestica*	Fruits	DPPH	[48]
37.	*Medicago sativa*	Roots	DPPH, ABTS, ICA	[49]
38.	*Melia azedarach*	Leaves	DPPH	[50]
39.	*Mentha arvensis*	Leaf	DPPH, ABTS, FRAP	[51]
40.	*Mentha piperita*	Leaves	DPPH	[52]
41.	*Momordica charantia*	Fruits	DPPH, HPRSA	[53]
42.	*Morinda citrifolia*	Fruits	Peroxides Value, DPPH	[54]
43.	*Moringa oleifera*	Leaves	TBARS, CAT, LPO SOD	[55]
44.	*Olea europaea*	Fruits & Leaves	TRAP	[56]
45.	*Parkia biglobosa*	Fruits	TAC, DPPH, FRAP	[57]
46.	*Phoenix dactylifera*	Fruits	TBARS, DPPH	[58]
47.	*Phylanthus emblica*	Fruits & Seeds	Peroxides Value	[59]
48.	*Piper cubeba & Piper nigrum*	Fruits	DPPH,	[60]
49.	*Pistacia lentiscus*	Fruits	DPPH	[61]
50.	*Plumbago zeylanica*	Root	FTC, TBA, DPPH	[62]
51.	*Pongamia pinnata*	Leaves	DPPH, ABTS, NOS, SRSA	[63]
52.	*Portulaca oleracea*	Whole Plant	DPPH, FRAP, NOS	[64]
53.	*Pouzolzia zeylanica*	Whole Plant	DPPH, ABTS, HRSA, FRAP	[65]
54.	*Raphanus raphanistrum*	Aerial Parts	DPPH	[66]
55.	*Rumex vesicarius*	Leaves	ABTS, DPPH, FRAP	[43]
56.	*Sargassum wightii.*	Seaweeds	FTIR Analysis	[67]
57.	*Silybum marianum*	Seeds	DPPH	[68]
58.	*Smilax campestris*	Rhizomes	TRAP	[69]
59.	*Solanum pseudocapsicum*	Leaf	DPPH, ABTS, NBT	[70]
60.	*Syzygium cumini*	Fruit Pulp	ABTS, DPPH, FRAP	[43]
61.	*Tephrosia spinosa*	Aerial Parts	LPA, SOD, CAT, GSH	[71]
62.	*Tetrapleura tetraptera*	Fruits	TAC, DPPH, FRAP	[57]
63.	*Thymus vulgaris*	Whole Plant	PRA, DPPH	[72]
64.	*Toddalia asiatica*	Roots	DPPH	[73]
65.	*Torilis leptophylla*	Whole Plant	DPPH, SRSA, PMA, HRSA, HPSA, ABTS, FRAP	[74]
66.	*Trachyspermum ammi*	Seeds	TBARS, SOD, GSH	[75]
67.	*Vitex trifoliate*	Roots	SRSA, HRSA, LPA, DPPH, TBARS	[76]
68.	*Vitis Vinifera*	Leaves	TBARS, GSH	[77]
69.	*Withania somnifera*	Roots	DPPH, FRAP, HPRSA, NOS, CUPRAC	[78]
70.	*Zingiber officinale*	Essential Oil & Oleoresin	ABTS	[79]

CONCLUSION

Free radicals have been implicated in the etiology of large number of major diseases. They can adversely alter many crucial biological molecules leading to loss of form and function. Such undesirable changes in the body can lead to diseased conditions. Antioxidants can protect against the damage induced by free radicals acting at various levels. Dietary and other components of plants form major sources of antioxidants. The relation between free radicals, antioxidants and functioning of various organs and organ systems is highly complex and the discovery of 'redox signalling' is a milestone in this crucial relationship. Recent research around various strategies to protect crucial tissues and organs against oxidative damage induced by the free radicals. Many novel approaches are made and significant findings have come to light in the last few years. Coordinated research involving biomedical scientists, nutritionists and physicians can make significant difference to human health in the coming decades. Research on free radicals and antioxidants involving these is one such effort in the right direction.

ABBREVIATION

ABTS: 2,2'-azobis-3-ethylbenzthiazoline-6-sulphonic acid
CAT: Catalase activity (CAT)
CUPRAC: Cupric Reducing Antioxidant Capacity
DPPH: 1,1-diphenyl-2-picrylhydrazyl
DRSA: De-oxyribose radical scavenging activity
FRAP: Ferric Reducing/Antioxidant Power
FTC: Ferric thiocyanate (FTC)
GSH: Reduced Glutathione

HPRSA: Hydrogen peroxide radical scavenging activity
HRSA: Hydroxyl radical scavenging activity
ICA: Iron Chelating Activity
LPA: Lipid peroxidation inhibition activity (TBARS)
NBT: Nitroblue tetrazolium chloride (NBT) assay,
NOS: Nitric oxide scavenging
ORAC: Oxygen Radical Absorbance Capacity
PMA: Phosphomolybdate assay
SOD: Superoxide dismutase activity
SRSA: Superoxide radical scavenging activity
TAC: Total Antioxidant Capacity (TAC) Assay
TBA: Thiobarbituric acid (TBA)
TCM: Thiocyanate Method

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