MEASUREMENT OF ANTIOXIDANT ACTIVITY OF PLANT EXTRACTS

SARITA SRIVASTAVA

DEPARTMENT OF BOTANY, C.M.P. DEGREE COLLEGE, UNIVERSITY OF ALLAHABAD, PRAYAGRAJ 211002

Abstract: Among the SET methods, the most used are 2,2-di-phenyl-1-picrylhydrazyl (DPPH radical scavenging capacity assay), ferric reducing (FRAP) assay, Trolox equivalent antioxidant capacity (TEAC or ABTS) assay, copper reduction (CUPRAC) assay and reducing power assay (RP). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay is the most used antioxidant assay for plant extract. In this assay, a molecule or antioxidant with weak A-H bonding will react with a stable free radical DPPH• (2,2-diphenyl-1-picrylhydrazyl, λ_{max}=517 nm) causing discoloration of the molecule.

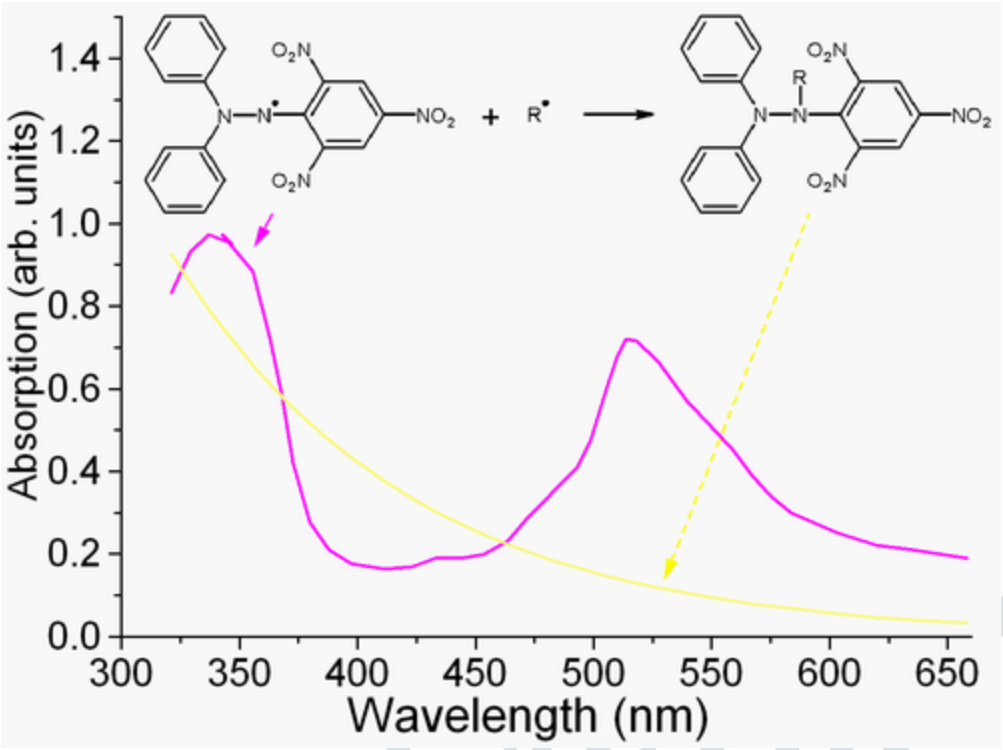
Keywords: DPPH, FRAP, TEAC, ANTIOXIDANT, FREE RADICAL, ABTS.

DPPH is a common abbreviation for the organic chemical compound **2,2-diphenyl-1-picrylhydrazyl**. It is a dark-colored crystalline powder composed of stable free radical molecules. DPPH has two major applications, both in laboratory research: one is a monitor of chemical reactions involving radicals, most notably it is a common antioxidant assay,[1] and another is a standard of the position and intensity of electron paramagnetic resonance signals.

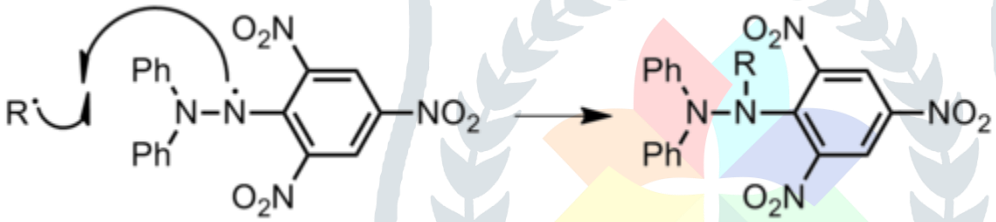
Properties and applications

DPPH has several crystalline forms which differ by the lattice symmetry and melting point. The commercial powder is a mixture of phases which melts at ~130 °C. DPPH-I (m.p. 106 °C) is orthorhombic DPPH-II (m.p. 137 °C) is amorphous and DPPH-III (m.p. 128–129 °C) is triclinic.[2]

DPPH is a well-known radical and a trap ("scavenger") for other radicals. Therefore, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. Because of a strong absorption band centered at about 520 nm, the DPPH radical has a deep violet color in solution, and it becomes colorless or pale yellow when neutralized. This property allows visual monitoring of the reaction, and the number of initial radicals can be counted from the change in the optical absorption at 520 nm or in the EPR signal of the DPPH.[3]



Because DPPH is an efficient radical trap, it is also a strong inhibitor of radical-mediated polymerization.[4]



Inhibition of polymer chain, R, by DPPH.

As a stable and well-characterized solid radical source, DPPH is the traditional and perhaps the most popular standard of the position (g-marker) and intensity of electron paramagnetic resonance (EPR) signals – the number of radicals for a freshly prepared sample can be determined by weighing and the EPR splitting factor for DPPH is calibrated at g = 2.0036. DPPH signal is convenient that it is normally concentrated in a single line, whose intensity increases linearly with the square root of microwave power in the wider power range. The dilute nature of the DPPH radicals (one unpaired spin per 41 atoms) results in a relatively small linewidth (1.5–4.7 G). The linewidth may however increase if solvent molecules remain in the crystal and if measurements are performed with a high-frequency EPR setup (~200 GHz), where the slight g-anisotropy of DPPH becomes detectable.[5][6]

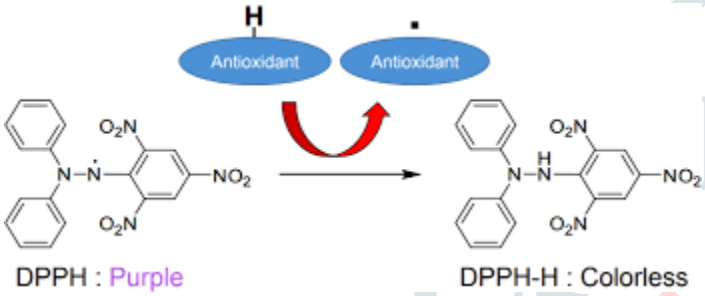
Whereas DPPH is normally a paramagnetic solid, it transforms into an antiferromagnetic state upon cooling to very low temperatures of the order 0.3 K. This phenomenon was first reported by Alexander Prokhorov in 1963.[7][8][9][10]

Antioxidant Assays 1. DPPH assay (2, 2-diphenyl-1-picrylhydrazyl) The radical scavenging activity of different extracts was determined by using DPPH assay according to Chang et al. (2001). The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517nm. Ascorbic acid (10mg/ml DMSO) was used as reference. Principle 1, 1 Diphenyl 2- Picryl Hydrazyl is a stable (in powder form) free radical with red color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (HA) can be written as,

(DPPH) + (H-A) DPPH-H + (A)

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. Reagent preparation 0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol. Working procedure Different volumes (2 - 20µl) of plant extracts were made up to 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 min. After 20 min, the absorbance of the mixture was read at 517 nm. 3ml of DPPH was taken as control. The % radical scavenging activity of the plant extracts was calculated using the following formula, Where RSA is the Radical Scavenging Activity; Abs control is the absorbance of DPPH radical + ethanol; Abs sample is the absorbance of DPPH radical + plant extract. 2 Phosphomolybdenum assay the antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto (1999). Principle This method is based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by sodium sulfide. The obtained phosphomolybdenum blue complex is oxidized by the addition of nitrite and this causes a reduction in intensity of the blue colour. Reagent preparation Reagent was prepared by adding 0.588ml of sulphuric acid, 0.049g ammonium molybdate and 0.036g sodium phosphate. The final volume was made up to 10ml with Dis. H2O. The working procedure of 10mg of plant extract was dissolved in 1ml of DMSO. 100µl from the prepared sample was taken and 1ml of reagent solution was added to it and incubated in a boiling water bath at 95°C for 90 min. After 90 min, the absorbance of the solution was read at 695 nm. Ascorbic acid (10mg/ml DMSO) was used as standard. The Phosphomolybdenum reduction potential (PRP) of the studied extracts were reported in percentage. 3 Super oxide anion radical scavenging activity Superoxide dismutase (SOD) is a metalloenzyme that catalyze the dismutation of superoxide radical into hydrogen peroxide (H2O2) and molecular oxygen (O2) and consequently provide an important defense mechanism against superoxide radical toxicity (Nishikimi et al., 1972). Principle The principle involved in this assay is the conversion of Nitroblue Tetrazolium (NBT) into NBT diformazan via superoxide radical. SOD utilizes the highly water-soluble tetrazolium salt and that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O2 is linearly related to the xanthine oxidase (XO) activity and is inhibited by SOD. Reagent preparation 156µl NBT was prepared in 10ml of 100mM phosphate buffer (pH 8). 468µM NADH solution was prepared in 10ml of 100mM phosphate buffer (pH 8). 60µM PMS in 10ml of 10mM PO4 buffer pH 8. 10mg extract in 0.1ml DMSO+ 0.9 ml PO4 buffer. Working procedure 1ml of NBT solution, 1ml of NADH solution, 0.1ml of plant extract (10mg in 0.1ml DMSO and 0.9ml PO4 buffer) and 0.1ml of PMS solution were added together and incubated at 25°C for 5 min. After 5 min the absorbance was read at 560 nm. 4 Hydroxyl radical scavenging activity Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to the cell (Halliwell and Gutteridge, 1981). Principle HRS assay is used to find the scavenging activity of free hydroxyl radicals (which damage the body cells) like hydrogen peroxide in the presence of different concentrations of plant sample. The model used is ascorbic acid-iron-EDTA model of hydroxyl radical generating system. This is a totally aqueous system in which ascorbic acid, iron and EDTA conspire with each other to generate hydroxyl radicals. Reagent preparation Iron-EDTA solution was prepared by mixing 0.13% ferrous ammonium sulphate and 0.26% EDTA. 0.018% EDTA was prepared by dissolving 0.018g EDTA in 100ml dist. H2O. 0.22% ascorbic acid was prepared by dissolving 0.22g ascorbic acid in 100ml dist. H2O. 17.5% TCA was prepared by dissolving 17.5g TCA in 100ml of dist.H2O. Nash reagent was prepared by adding 7.5g ammonium acetate, 0.5ml of glacial acetic acid and 0.2ml of acetone to 100ml dist.H2O. Working procedure Various concentrations of extract (250, 500, 750, 1000µg) were taken and 1ml of iron EDTA solution, 0.5ml of EDTA solution, 1ml of DMSO and 0.5ml of ascorbic acid were added to it. The mixture was incubated in a boiling water bath at 80 to 90°C for 15 min. After incubation, 1ml of ice-cold TCA and 3ml of Nash reagent were added and the reaction

mixture was incubated at room temperature for 15 min. The absorbance was read at 412 nm. The % hydroxyl radical scavenging activity is calculated by the following formula, Where HRSA is the Hydroxyl Radical Scavenging Activity, Abs control is the absorbance of control and Abs sample is the absorbance of the extract. 5 Metal chelating activity Principle Ferrozine can quantitatively chealate with Fe2+ and form a red coloured complex. This reaction is limited to the presence of other chelating agents and results in a decrease of the red colour of the ferrozine-Fe2+ complex. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas et al., 2000). The antioxidants present in plant extract form a coordinate complex with the metal ions (chelating activity) and inhibit the transfer of electrons. Thus, oxidation reaction is arrested, and no free radicals are produced. Reagent preparation 2mM ferrous chloride was prepared by dissolving 2.5mg of FeCl2 in 10ml of dist.H2O. 5mM ferrozine was prepared by dissolving 24mg in 10ml of dist.H2O. Working procedure 100µl of plant extract (10mg in 1ml DMSO) was added to 50µl of 2mM ferrous chloride and 200µl of 5mM ferrozine solution. The solution was mixed thoroughly and incubated in the dark at room temperature for 10 min. The absorbance was read at 562 nm.



Mechanism

CONCLUSION:

Conventional methods for the measurement of antioxidant activity are still needed and specific methodological protocols are complex and require a long testing time. One of the important selection parameters of the antioxidant test is the working pH. There are tests operating in acidic (FRAP), neutral (CUPRAC) or alkaline (Folin–Ciocalteu) conditions.

Additionally, the applicability of the antioxidant test to both hydrophilic and lipophilic antioxidants is an important factor. While the ABTS and CUPRAC tests can measure both hydrophilic, and lipophilic antioxidants, some methods only measure hydrophilic antioxidants (FRAP and Folin–Ciocalteu), and others only apply to hydrophobic systems (DPPH).

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