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Research Article

A 96 WELL-MICROTITER PLATE ABTS BASED ASSAY FOR ESTIMATION OF ANTIOXIDANT ACTIVITY IN GREEN LEAFY VEGETABLES

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ABSTRACT

A 96 well-microtiter plate-ABTS based decolorization assay was developed for the screening of antioxidant activity of various extracts such as food extracts, plasma antioxidants and synthetic antioxidants. Oxidation of 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) with potassium persulfate generated cation radical $ABTS^{\bullet+}$and reduction followed in the presence of hydrogen-donating antioxidants. Optimization was carried out for different parameters like concentration, incubation time, volume of the reagent and sample, reagent preparation, pH using the buffer. 96 well-microtiter plate-ABTS based method denotes very good intra- and inter-assay analysis. IC_{50} values of standards and methanolic extracts of selected green leafy vegetables showed per cent coefficient of variation (CV) 4.1% and 5% for inter- and intra-day assay results respectively and was a significant achievement for the sample volume maintained at 5µl. The inter-day and intra-day accuracy were proved by the low values relative standard deviations (RSD) did not exceed 4.5%, indicating high reproducibility and precision of the method. This 96 well-microtiter plate-ABTS based decolorization assay method has advantages over the techniques like ferryl myoglobin/ABTS assay, such as no involvement of an intermediary radical, suppression of rapidly reacting antioxidants, low volume of sample, reaction at desired pH, and analysis of huge number of sample in a single run.

Keywords: ABTS, antioxidant activity, leafy vegetable, microtiter plate.

INTRODUCTION

Protective role of antioxidants to prevent chronic illnesses such as heart disease, stroke, cancer, Alzheimer's disease, rheumatoid arthritis, and cataracts (Halvorsen *et al*., 2002; Manian *et al*., 2008) is well evident from the

literature (Gey, 1990; Rodríguez and Costa, 2006). Free radicals like super oxide, hydroxyl radical, hydrogen peroxide, and transition metals (Hosseinimehr *et al*., 2007; Raghuveer and Tandon, 2009) are part of aerobic life generated at low level during physiological functions (Halliwell, 1991). Unstable free radicals cause damage to proteins, DNA, lipids in cells and resulting imbalance between the generation of reactive oxygen species (ROS) and the antioxidant enzymes (Manian *et al*., 2008; Aurelia and Gheorghe, 2011) and results in the pathogenesis of various diseases such as cancer, diabetes, aging, oxidative stress and metabolic syndrome. Natural antioxidants from leafy vegetables and fruits (Halvorsen *et al*., 2006) due to their photochemical constituents (Miller *et al*., 1993; Miller and Rice-Evans, 1996) and synthetic compounds like butylhydroxyanisole, butylhydroxytoluene, gallates (Litescu *et al*., 2011) prevent the free radical damage by its protective role such as reacting with them, chelating catalytic metals and by playing as oxygen scavengers. In the intervening time, ingestion of several synthetic antioxidants such as butylhydroxytoluene and butylhydroxyanisole has been reported toxic to man (Lobo *et al*., 2010) and natural antioxidant from plants and their parts showed free-radical scavenging properties of plant origins could have great significance as therapeutic agents in ageing process and free radical-mediated diseases.

Dietary plants are rich in various forms of antioxidants like carotenoids, phenolic compounds, benzoic acid derivatives, flavonoids, proanthocyanidins, stilbenes, coumarins, lignans, and lignins (Lindsay and Astley, 2002) and total antioxidant potential depends on the synergic and redox interaction among the different molecules present in food (Ramadan *et al*., 2003; Ramadan and Moersel, 2007; Anna *et al*., 2011). Total antioxidant capacity of various plants sources (Pellegrini *et al*., 2003) can be analyzed by several analytical methods (Giardi *et al*., 2010) and spectrometric techniques (Thaipong *et al*., 2006) rely on the reaction of a radical, radical cation or complex with hydrogen atom donating an antioxidant molecule.

Two types of antioxidant inhibition assays are namely, the extent of the scavenging by hydrogen- or electron-donation of a pre-formed free radical, as well as assays involving the presence of antioxidant system during the generation of the radical. Decolorization assays measure total antioxidant activity of pure and mixed compounds and ABTS radical cation decolorization assay has attractiveness for its cleanness. ABTS can be oxidized by potassium persulphate (Pellegrini *et al.*, 2003; Thaipong *et al.*, 2006) generates ABTS cation radical (ABTS•+) and absorbance diminution at 743 nm can be monitored in the presence of standard chosen vegetable extracts (Pellegrini *et al*., 2003) as antioxidants. An improved technique for the generation of ABTS involves the direct production of the blue/green ABTS chromophore through the reaction between ABTS and potassium persulfate (Miller and Rice Evans, 1997). A microplate adaptation of ABTS method described previously by Wong *et al*., (2004) with inter-assay coefficient of variation (CV) was 14.1% for plasma constitutes with irrespective of pH.

The objective of the present study was to develop an efficient 96 well microplate based ABTS antioxidant activity method at desired pH for the estimation of large number of samples from broad range of antioxidant compounds originating from food, plasma, and plant extracts.

MATERIALS AND METHODS

Reagents

Experiments were performed using Biotek Microplate reader (Powerwave XS) capable of 96 well plate reading with temperature control. 96 well U bottom Greiner polystyrene microplate was used for analysis. ABTS 7mM and 2.45mM potassium peroxodisulphate were mixed and dissolved in double distilled water. The solution was then diluted with distilled water in a 1:9 v/v ratio. The solution was incubated for 12 h in the dark and the reagent could be usable for 15 days if stored in the dark at 4°C. Oxidation of the ABTS starts without delay, but the absorbance was not maximal and stable until more than 6 h had elapsed. Under the conditions used here for the preparation of the $ABTS^{\bullet+}$, about 60% of the ABTS present was oxidized to the radical cation form. The absorbance spectrum of $ABTS^{\bullet+}$ at different concentrations reveals the maximum absorbance at 734nm (Re *et al*., 1999). The calculated extinction coefficient of $ABTS^{\bullet+}$ at 734 in water was $1.48X\ 10^4\ mol^{-1}\ lcm^{-1}$.

Methanol extracts

Five different plant species used for the study were authenticated by Forest Research Institute, Dehradun (India) and Botanical Survey of India, Dehradun (India). Each collected plant material was air-dried in darkness at ambient temperature (30°C), homogenized in a laboratory blender, prepared plant material (10g) was transferred to flasks with 200 ml of methanol solvent and kept on magnetic stirrer at room temperature for about 24h. Then infusions were filtered through Whatman No.1 filter paper and supernatants were evaporated to dryness. The solvent was completely removed by rotary evaporator and obtained greenish gummy exudates and crude extract was stored at 4°C in refrigerator and used for antioxidant activity.

For the analysis of phenolic compounds and food extracts, $ABTS^{\bullet+}$ solution was diluted with respective mixture of solvent and desired pH buffer (1:1) and to an absorbance of 0.70 ± 0.1 at 734 nm and maintained at respective temperature (Somanjana *et al*., 2017). Generally the stability of radical cation and antioxidant activity depend partially upon pH of the medium (radical stable at pH 4.5). Use of 50mM glycine–HCl buffer, pH 4.5 or acetate buffer, pH 4.5 is preferred for fruit extracts. A 190 µl volume of reagent was pipetted into a microtiter well with succeeding addition of 10 µl of sample/standard (gallic acid, ascorbic acid). Absorbance was measured at λ=735 nm. A reagent blank reading was taken (A_0) and after addition of sample, the absorbance (A_6) reading was taken after 6 min after initial mixing. For calculating the antioxidant activity, values before the start of decrease of the absorbance (A_0–A_6) and the last measurement value was used. Values were calculated accordance to formula: $A = A0 - A6$.

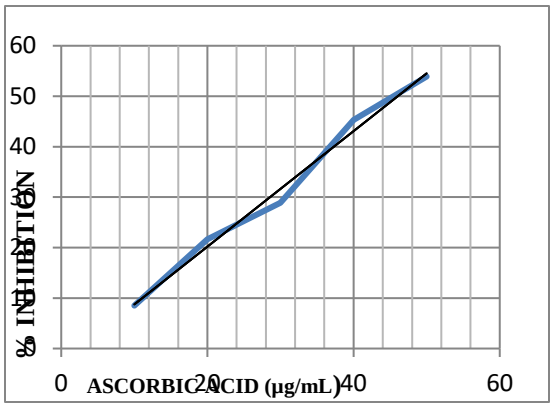
Statistical analysis

All the experiments were carried out three times, each concentration five readings. Results were expressed as mean values with standard deviations (mean±SD).

RESULTS AND DISCUSSION

Microplate assays are commonly used in immunological, microbiological, food, molecular biology, research analysis, because of low sample, low concentration and availability of source (Nakayama, 1998; Somanjana *et al*., 2017) and antioxidant assay on microplate could be advantage for screening large samples. L-ascorbic acid of 10-50 µg/ml and decolorization of the $ABTS^{\bullet+}$reduction of

the radical cation as the percentage inhibition of absorbance at 734 nm is used to measure the antioxidant activity of the range of flavonoids, carotenoids, phenolics, and some plasma antioxidants (Walid, 2012). The principle behind the ABTS reactions is to achieve optimization. ABTS method described earlier showed volume 200 µl (Gupta *et al*., 2009; Somanjana *et al*., 2017). Illustrated method was linear over the range of 10-50 µg/ml for L-ascorbic acid) and 8-40 µg/ml for gallic acid (Fig.1 and Table 1). Calibration curves of five points were organized on dissimilar days and the results obtained were used to calculate the equation of the line by using least squares regression method. The linearity of calibration graphs and adherence to Beer's law were validated by the good value of the correlation coefficient. IC_{50} values of natural antioxidants were very powerful over synthetic antioxidants (Table 2). Ascorbic acid was good antioxidant over gallic acid and BHT and similar observation was reported earlier (Somanjana *et al*., 2017).



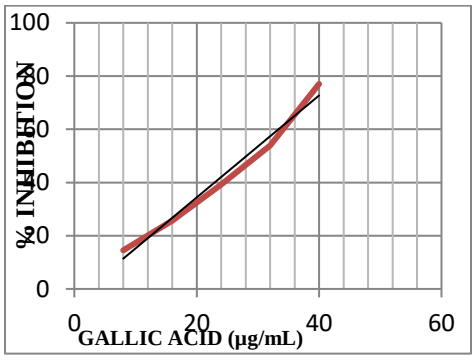


Fig.1. ABTS free radical scavenging activity of A. Ascorbic acid B. Gallic acid.

Table 1. Assay of ascorbic and gallic acid antioxidant activity.

Parameters	**Ascorbic acid**	**Gallic acid**
Conc (µg/ml)	10-50	5-40
CC	0.98	0.98
Slope	1.144	1.916
Intercept	-2.7	-3.95
%RSD	1.91	1.82
SE	0.02	0.0365
S_a	0.0104	0.00214
S_b	0.034	0.00811
$S_{y/x}$	0.00246	0.003

S_a= sd of intercept; S_b= sd slope; $S_{y/x}$, sd of residuals. n=10; cc=correlation coefficient SE=standard error

Table 2. IC_{50} values of methanol extracts

Standard/methanolic extracts	**IC_{50}Value (µg/ml)**
BHT	27
Gallic acid	35
L-Ascorbic acid	46
Menthaspicata	250.8
Coriandrumsativum	270.5
Trigonellafoenum-graecum	382.5
Spinaciaoleracea	412.8
Raphanussativus	430.5

The results demonstrate that for standards, the reaction with $ABTS^{\bullet+}$ was complete by 1-3 min and for leafy extracts showed an auxiliary little inhibitory effect up to 4 min reaction (Fig. 2). Earlier reports showed that these were completed in 5 min (Somanjana *et al*., 2017), in other case incubation time was optimized to 10 min after initial mixing for obtaining precise value (Anna *et al*., 2011).The chance of solvent effect on the assay could be minimized by assigning blank value to the sample value. The standards L-ascorbic acid and gallic acid showed 50% inhibition at below 50 and 40 µg/ml concentrations respectively (Table 2). The inter-day and intra-day precisions were examined by analysis of L-ascorbic acid 5 µg/ml and 10 µg/ml concentrations and gallic acid 8 µg/ml and 16 µg/ml concentrations each, three times a day for three consecutive days (Table 3).

Table 3. Evaluation of accuracy.

Intra-day assay results

Standard/Sample	IC_{50}*	%CV
L-Ascorbic acid	46 ± 0.98	2.13
Gallic acid	35 ± 0.51	1.45
Menthaspicata	250.8 ± 11.2	4.4
Raphanussativus	430.5 ± 12.9	2.99

Inter-day assay results

Standard/Sample	IC_{50}*	%CV
L-Ascorbic acid	46 ± 0.74	1.6
Gallic acid	35 ± 0.95	2.7
Menthaspicata	250.8 ± 9.4	3.7
Raphanussativus	430.5 ± 15.9	3.69

* n= 10, mean ± SD

The inter-day and intra-day accuracy were proved by the low values relative standard deviations (RSD) for the results did not exceed 4.5%, proving the high reproducibility of the results and the precision of the method**.** This good level of precision was suitable for quality control analysis of antioxidant activity of plant extracts. The factors that may affect the % RSD or CV values was the storage of extract, preparation of experiment, use of micropipette, time incubation, temperature, reagent preparation, age and storage conditions. The optimum maintenance of proper conditions of above mentioned factors could give high precision values. The prescribed 96-well microtiter plate ABTS based method denotes very good intra, inter assay analysis with a

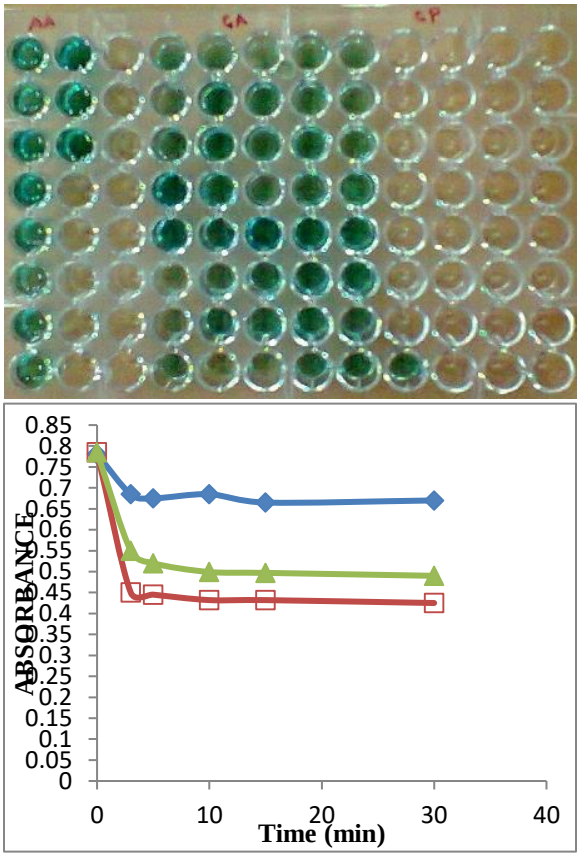


Fig. 2 A. 96-well microtiter plate with ABTS. B. Effect of time on ABTS radical cation decolorization assay,L-Ascorbic acid (Blue), BHT (Green), Gallic acid (Red).

value of coefficient of variation (CV) 4.1, 5 % when the sample volume maintained at 5µl which was a low value compared to other existing methods (Anna *et al.*, 2011; Somanjana *et al*., 2017). Earlier methods have limitations

such as, not validated, lacked stability, pH optimization, incubation time and so on (Roberta *et al*., 1999). Low volume of sample size leads to increase in coefficient variation, decreases correlation coefficient values.

CONCLUSION

Present study showed that 96-well microtiter plate ABTS based assay for estimation of antioxidant activity has advantages over the techniques like ferryl myoglobin/ABTS assay, such as no involvement of an intermediary radical, suppression of rapidly reacting antioxidants, low volume of sample, reaction at desired pH, and analysis of huge number of sample in a single run. This method can be used for extracts of different solvents with respective blank. The main advantages of this assay are reducing labor, time, cost of assay, sample volume.

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CONFLICT OF INTERESTS

We declare that we have no conflict of interest.

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