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Modified 2,2'-Azinobis(3-Ethylbenzo Thiazoline)-6-Sulphonic Acid Radical Cation Decolorization Assay for Antioxidant Activity of Human Plasma and Extracts of Traditional Medicinal Plants

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Abstract

2,2'-Azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical cation decolorization assay has been applied under different pH conditions for the determination of trolox equivalent antioxidant capacity (TEAC) of biological and food samples. Due to innate nature of ABTS radical cation, it is unstable at physiological pH. Further, its sensitivity is also lost at acidic pH. No single method has yet claimed to cover both drawbacks of the ABTS procedure. The present investigation was aimed at developing an improved ABTS decolorization method for the measurement of antioxidant activity of human plasma and herbal extracts with greater stability and sufficient sensitivity. A relatively stable ABTS radical cation was generated through a reaction between ABTS and potassium persulfate in the presence of sodium acetate buffer at pH 6.5 (20 mM). The electron or hydrogen atom transfer from antioxidative components of plasma or herbal extracts reduced the radical cation. Standard antioxidants like trolox, butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT) and ascorbic acid demonstrated stable end-point readings showing a promising aspect of determination of total antioxidant activity (TAA) of all the other samples with reference to these indicators. The modified assay proved to be highly reproducible. TEAC values for plasma samples ranged between 1.410–2.025 mM with mean percent relative standard deviation (% RSD) 1.64 and between 1.72–1.98 with mean % RSD 1.27 for older and modified ABTS assays, respectively. The efficacy of the assay was ascertained further by determining antioxidant activity of organic and aqueous extracts of *Cuscuta reflexa* and *Terminalia arjuna.*

Keywords: ABTS radical cation, antioxidant activity, TEAC, plasma, potassium persulfate.

1. Introduction

A number of decolorization assays are being employed for the measurement of total antioxidant activity of body fluids, food, herbal extracts, and pure compounds.[1–17] Amongst these assays, ABTS (2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid; (2*Z*,2'*Z*)-2,2'-(hydrazine-1,2-diylidene)bis(3-ethyl-2,3-dihydrobenzo[*d*]thiazole-6-sulfonic acid)) radical cation decolorization assay as developed by Miller *et al.*[1] have gained popularity for its simplicity. ABTS, a phenothiazine drug, when reacting with a suitable oxidizing agent, like potassium persulfate, forms ABTS radical monocation that has been reported to be stable in pH 6.5–3.5 range. On either side of this pH there is an accelerated degradation of ABTS into its metabolites. At physiological pH of 7.4, Cano *et al.*[18] and Erel[19] have expressed their reservations about the stability of ABTS radical cation. In order to determine the antioxidant activity of both plasma antioxidants and citrus fruits at pH 7.4, Re *et al.* have developed an improved ABTS assay.[20] Since the stability of ABTS radical cation and the antioxidant activity depend partially upon pH of the radical cation medium, the reproducibility and accuracy may not be expected for biological and abiological samples when using ABTS assay at pH 7.4. Recently, Ozgen *et al*. reported that ABTS radical cation

was more stable at pH 4.5 and could be used for the measurement of antioxidant activity of small fruits that have lower pH values.[21] In case of plasma samples, the sensitivity of ABTS assay at pH 4.5, however, was markedly affected. In the present study, the assay was modified to obtain a more stable radical cation with comparable sensitivity for total antioxidant capacity (TAC), for both herbs and plasma samples. The results obtained were subsequently compared with ABTS assay performed at pH 7.4.

Oxygen, although essential for existence of life, is paradoxically involved in the generation of reactive oxygen species (ROS) in living organisms. The over-production of ROS has reportedly been implicated in more than 100 degenerative diseases including cardiovascular, diabetes and cancer.[22–25] Since phytochemicals like flavonoids, terpenes and other related compounds are thought to be active against ROS generation, exploration of new natural sources of such compounds may be of great interest for the scientists. In the sub-continental region, traditional medicinal plants have extensively been used for the control and cure of various degenerative diseases. These herbs are either used as dietary supplement or directly as a treatment of several diseases. *Cuscuta reflexa* and different parts of *Terminalia arjuna* have been used as folk medicine for various diseases. The bark, stem and leaves of *T. arjuna* have been used for the treatment of various cardiovascular diseases in the sub-continental region. *In vivo* and *in vitro* studies have shown that *T. arjuna* is cardioprotective,[26–29] gastroprotective,[30–31] antigenotoxic[32] and antimutagenic herb.[33–34] It has been reported that tannins, triterpenoid, saponins (arjunic acid, arjunolic acid, arjungenin, arjunglycosides), flavonoids (arjunone, arjunolone, luteolin), gallic acid, ellagic acid, oligomeric proanthocyanidins (OPCs), phytosterols, calcium, magnesium, zinc, and copper are the major constituents of *T. arjuna*.[35–36]

Cuscuta reflexa is used as alterative, anthelmintic, carminative and purgative agent by the traditional medicine practitioners. The plant is employed in treatment of urinating problems, jaundice, muscle pain and chronic coughs. Antibacterial, antiviral, antisteroidogenic and psychopharmacological activity of *C. reflexa* have also been reported.[37–38]

Until now, no comprehensive study has been undertaken for evaluation of *in vitro* antioxidant potential of *T. arjuna* and *C. reflexa*. Efforts have been undertaken in the present study to evaluate antioxidant activity of these herbs though an improved ABTS assay. The antioxidant activity observed through our study was compared to those determined by other contemporary assays, in order to correlate it with their anti-disease activity.

2. Materials and Methods

All the chemicals used were of the highest purity grade available. Trolox (Hoffman-La Roche) (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Aldrich Chemical Co., Gillingham, Dorset, UK) was used as an antioxidant standard and its solution was prepared in phosphate buffered saline at pH 7.4. Fresh working standards were prepared daily on dilution with either methanol or double distilled water. ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) diammonium salt, potassium persulfate (dipotassium peroxodisulfate, $K_2S_2O_8$), ascorbic acid, reduced glutathione, BHT (butylated hydroxytoluene; 2,6-di-*tert*-butyl-4-methylphenol), BHA (butylated hydroxyaniline; 2-*tert*-butyl-4-methoxyphenol), gallic acid (3,4,5-trihydroxybenzoic acid), uric acid (2,6,8-trihydroxypurine), 2,2'-methylenebis(6-*tert*-butyl-4-methylphenol) and kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one) were obtained from Fluka (UK) and HPLC grade ethanol from Rathburn Chemicals Ltd. (Walkerburn, Peebleshire, Scotland). Experiments were performed on UV-1700 Pharma-Spec UV-Visible Spectrophotometer, Shimadzu, Japan equipped with CPS controller. All the experiments were performed three times and results obtained were averaged. Data obtained, except where specifically mentioned, is mean ±SD (n = 3).

2. 1. Sample Preparations

Solutions of standard antioxidants were prepared in either methanol or double distilled water, which depended upon the solubility of the antioxidant.

The blood samples were taken from five apparently healthy volunteer men (average age 30 years), with informed consent. For plasma, the blood samples, pre-mixed with sodium citrate in clinical concentrations, were centrifuged at 5000 rpm and 4 °C for 30 min. Plasma was separated and stored in refrigerator at 4 °C. All samples were analyzed within three days. To check and compare reproducibility of the modified method with the older ABTS versions, % RSD and mean % RSD values were calculated. Stem (TAS), bark (TAB) and leaves (TAL) samples of *T. arjuna* and *C. reflexa* (CR) were obtained in dried form from a local market in Lahore, Pakistan. A finely ground amount (TAS 50 g, TAB 35 g, TAL 10 g and CR 50 g) of these medicinal herbs was soaked in methanol in 1:20 (w/v) in cork-fitted flasks separately for 24 h at 30 °C and 240 rpm. The following day, extract obtained was filtered and stored at 4 °C, while the residue was re-soaked in methanol in the same proportion at 240 rpm and 30 °C for 24 h. The filtrate obtained on the second day was mixed with the filtrate from the first day. The methanolic filtrate was concentrated at 30 °C using rotary evaporator. The solid concentrate obtained was weighed and suspended in appropriate volume of distilled water (Table 1). Subsequently, fractions of aqueous extract were further extracted in equal volumes of organic solvents (25 × 3 mL) of varying polarity according to the scheme shown in figure 1.

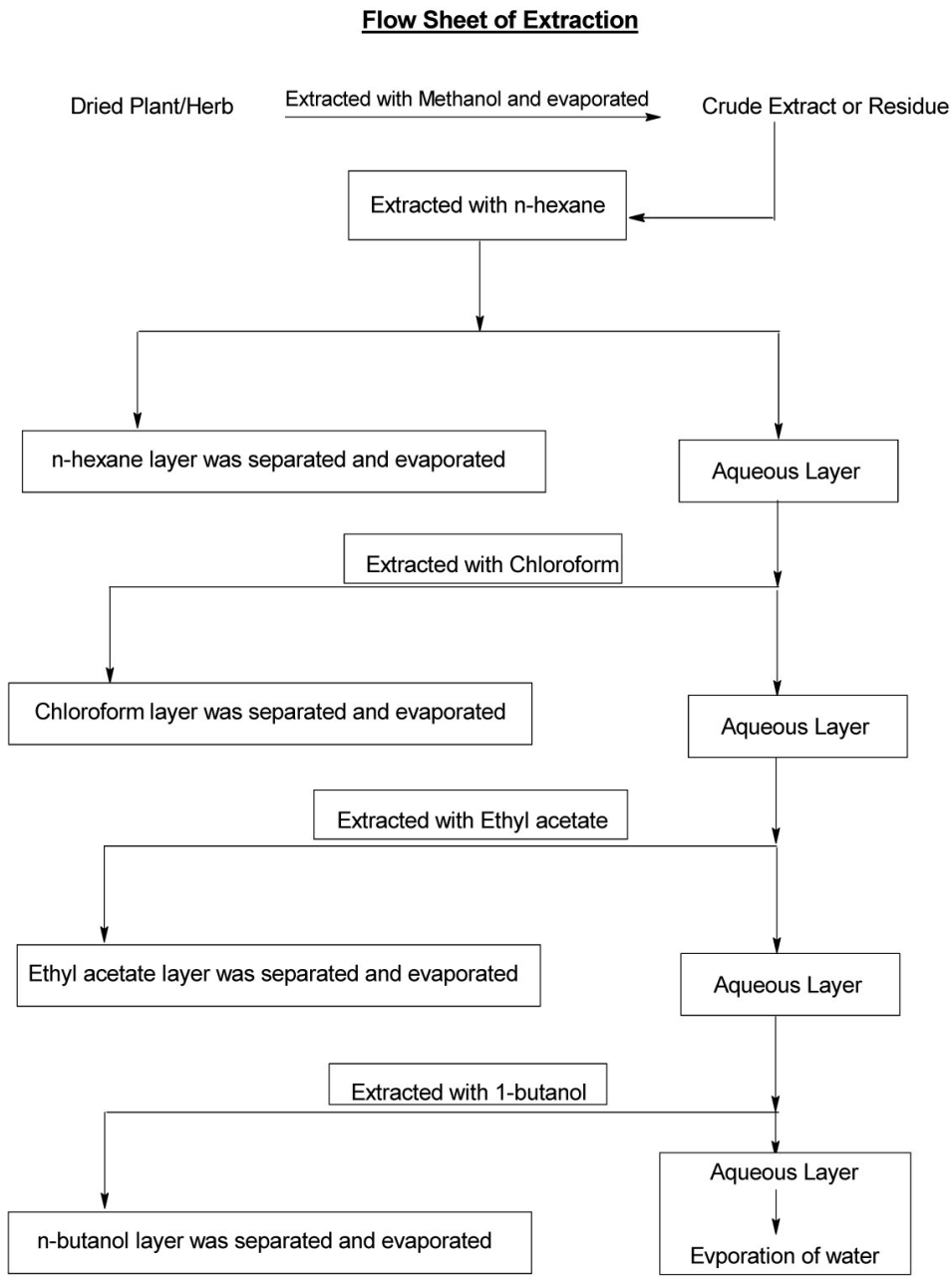

Figure 1. Flow sheet diagram showing extraction of antioxidant components of *T. arjuna* and *C. reflexa* in different solvents

Table 1. Mass of residue obtained and stock solutions formed for different fractions of *T. arjuna* and *C. reflexa*

Fraction	Plant/herb Sample							
	TAS (g)	**Stock Solution (mg/ml)**	**TAL (g)**	**Stock Solution (mg/ml)**	**TAB (g)**	**Stock Solution (mg/ml)**	**CR (g)**	**Stock Solution (mg/ml)**
Methanol	0.100	20.00	0.038	7.74	0.055	11.18	0.100	20.00
n-Hexane	0.190	19.00	0.050	3.33	0.030	3.00	0.580	58.00
Chloroform	0.110	11.00	0.250	25.00	0.940	94.00	1.420	94.66
EtOAc	0.100	9.09	0.030	3.00	0.190	7.60	0.950	95.00
1-Butanol	1.170	39.00	0.810	67.50	0.280	23.33	1.670	167.00
Aq. (AP)	4.980	14.20	1.040	2.45	5.430	27.15	2.840	10.88
Aq. (BP)	8.080	23.00	1.871	4.40	8.614	43.07	10.881	26.26

The residue obtained at each extraction step was weighed and dissolved in appropriate volume of respective solvent to obtain stock solution to be used in antioxidant assays (Table 1).

2. 2. ABTS$^{\bullet+}$ Decolorization Assay Protocol

ABTS$^{\bullet+}$ (ABTS radical cation) assay protocol, as developed by Re *et al.*,[20] was followed for the analysis described in this article. ABTS was dissolved in double distilled water to a 7 mM concentration. ABTS^{+} was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand, in the dark, at room temperature for 12–16 h before use. For study of antioxidant activity of standard antioxidant and plasma samples, the ABTS stock solution was diluted with PBS buffer (pH 7.4) to an absorbance of 0.70 (±0.02) at 745 nm respectively, and equilibrated at 30 °C. For herbal extracts, dilutions were made in the respective organic solvents with which they had been extracted from aqueous solutions. After addition of 10 μL of neat or diluted stock solution (as necessary) to 2.99 mL of diluted ABTS^{+} solution (A = 0.700 ± 0.020), the absorbance was measured at 30 °C, within exactly 1 min intervals for 8 min. Solvent blanks were run in each assay for accurate readings. All determinations were carried out at least three times in succession and in triplicate at each separate concentration level of the standards. The percentage inhibition of absorbance at 734 nm (I_{734}) was calculated by the following formula.

$$I_{734} = \left(1 - \frac{A_f}{A_o}\right) \times 100 \qquad (1)$$

Where A_o is the absorbance of radical cation solution before addition of sample/standard antioxidants and A_f is the absorbance after addition of the sample/standard antioxidants. The resultant data was plotted between concentration of antioxidants and that of trolox for the standard reference curve.

2. 3. Modified ABTS$^{\bullet+}$ Decolorization Assay Protocol

ABTS was dissolved in double distilled water to a 7 mM concentration. ABTS$^{\bullet+}$ was produced by reacting ABTS stock solution (in 20 mM sodium acetate buffer, pH 6.5) with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the study of antioxidant activity of standard antioxidants and plasma samples, the ABTS stock solution was diluted with 20 mM sodium acetate buffer (pH 6.5) to absorption value of 0.70 ± 0.02 at 734 nm and equilibrated at 30 °C. After addition of 10 μL of neat or diluted stock solution to 2.99 mL of diluted ABTS$^{\bullet+}$ solution (A_{734nm} = 0.700 ± 0.020), the absorbance reading was taken after intervals of exactly 1 min, for 8 min. Appropriate solvent blanks were run in each assay for accurate readings. In case of plasma samples, the radical cation solution, after mixing with plasma samples were incubated at room temperature for 30 min. All determinations were carried out at least three times, at each separate concentration of the standards and subsequent samples. The percentage inhibition of absorbance at 734 nm (I_{734}) was calculated as follows.

$$I_{734} = \left(1 - \frac{A_f}{A_o}\right) \times 100 \qquad (2)$$

Where A_o is the absorbance of radical cation solution before addition of sample/standard antioxidants while A_f is the absorbance after addition of the sample/standard antioxidants.

2. 4. 2,2'-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay (DPPH Assay)

DPPH solution (3 mL, 25 mg/L) in methanol was mixed with appropriate volumes of neat or diluted sample solutions. The reaction progress of the mixture was monitored at 515 nm over a time period until T_{EC50} was obtained. Upon appropriate reduction, the color of the solution faded. The percentage of the DPPH remaining was calculated as

$$\%DPPH_{rem} = \frac{[DPPH]_{T=t}}{[DPPH]_{T=0}} \times 100 \qquad (3)$$

Where $[DPPH]_{T=0}$ is the concentration of DPPH radical before reaction with antioxidant samples and $[DPPH]_{T=t}$ is the concentration of DPPH radical after reaction with antioxidant sample at time t. A kinetic curve showing the scavenging of DPPH radical in terms of decrease in absorbance at 593 nm as a function of time (min) was plotted for each fraction of the samples. The concentration that causes a decrease in the initial DPPH concentration by 50% (EC_{50}), and the time needed to reach the steady state with EC_{50} concentration (T_{EC50}) were measured from the equation 3.

2. 5. Total Phenolic Content Assay

Stock solution of gallic acid was made by dissolving 0.5 g gallic acid in 10 mL of ethanol in a 100 mL volumetric flask and diluting to volume with doubly distilled water. Sodium carbonate solution was prepared by dissolving 200 g of anhydrous sodium carbonate in 800 mL of double distilled water. After boiling and subsequent cooling of the solution, a few crystals of sodium carbonate were added. The solution was let to stand for 24 h, filtered and volume was increased to 1 L with double distilled wa-

ter. To prepare a calibration curve, 0, 1, 2, 3, 5 and 10 mL of phenol stock solution were added into 100 mL volumetric flask separately and then diluted to volume with double distilled water. The resultant solutions contained concentrations of 0, 50, 100, 150, 250 and 500 mg/L gallic acid.

From each calibration solution and sample or blank, 40 μL were pipetted into separate cuvettes, and to each 3.16 mL of double distilled water was added. Folin–Ciocalteu reagent (200 μL) was added and mixed well. After 8 min, 600 μL of sodium carbonate solution was mixed thoroughly in the solution. The solution was allowed to stand at 40 °C for 30 min and absorbance of each solution was determined at 765 nm against the blank (without phenolic solution). A concentration versus absorbance linear plot was thus obtained. The concentration of total phenolic compounds of each fraction of TAS, TAL, TAB and CR (as milligram of gallic acid equivalent (GAE)), as determined by using the following equation was obtained from the standard gallic acid plot.

$$\text{Absorbance} = 0.0557 \times [\text{gallic acid (mg/L)}]$$

2. 6. Total Antioxidant Activity

Total antioxidant activity of aqueous and organic extracts of both plants was determined according to the method employed by Mitsuda *et al.*[39] The solution, which contained 100 L each of neat or diluted plant extract of both plants in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). Each solution was then incubated at 37 °C in sealed bottles in dark. The solution without added extract was used as a blank, while the solutions containing 100 μL (50 μg/20 μL) of trolox were used as positive control. At intervals of 24 h during incubation, 0.1 mL of each solution was transferred to a beaker containing 3.7 mL of ethanol. After addition of 0.1 mL each of $FeCl_2$ (20 mM in 3.5% HCl) and thiocyanate solution (30%) to ethanolic sample, the solution was stirred for 1 min. The absorbance values of the solutions measured at 500 nm were taken as lipid peroxidation values.

3. Results and Discussion

3. 1. Modification of $ABTS^{\bullet+}$ Decolorization Assay

The basic principle underlying the $ABTS^{\bullet+}$ decolorization assay is that $ABTS^{\bullet+}$, on reaction with $K_2S_2O_8$, forms a greenish blue radical cation. Standard and sample antioxidants that are able to transfer an electron to ABTS radical cation scavenge the color of the solution proportionate to their amount. The extent of scavenging depends both upon the concentration of antioxidant and time duration for the reaction under analysis. Standard ABTS assay (pH 7.4) used 734 nm as the absorption maximum. First derivative UV-Visible spectrum of the ABTS radical cation in sodium acetate buffer (20 mM, pH 6.5) is shown in Figure 2. It is quite clear from the figure that the change in pH of the medium did not disturb the position of absorption maximum as indicated by zero crossing at 734 nm.

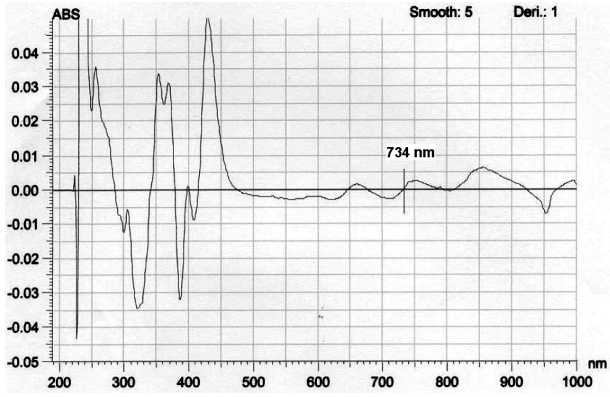

Figure 2. First derivative spectrum of ABTS radical cation in sodium acetate buffer (20 mM, pH 6.5)

ABTS decolorization assay has most widely been used at pH 7.4 (PBS, 5 mM). The effectiveness of the assay at pH 7.4 has been questioned due to the vulnerability of ABTS radical cation to degradation at higher pH values.[18–19] Ozgen *et al.*[21] has recommended that the antioxidant activity of the fruits, which occur naturally at low pH, should have been evaluated by ABTS radical cation stabilized at pH 4.5. Using pH 4.5, stability of ABTS radical cation was appreciably improved. However, its sensitivity to biological samples was lost. The need was, therefore, felt to modify the assay for determination of antioxidant activity of both biological and abiological samples at a single pH with greater accuracy.

The stability and sensitivity of ABTS radical cation was checked at different pH values using standard antioxidants, herbal and plasma samples. Figure 3 shows stability of ABTS radical cation at different pH.

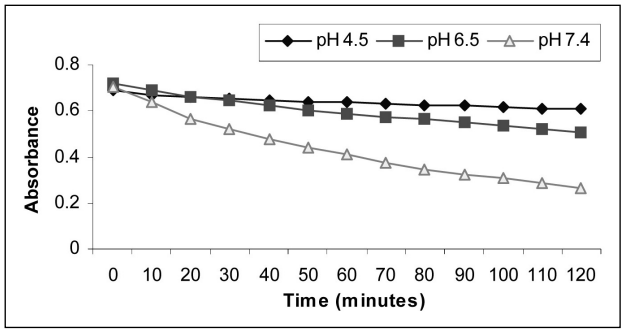

Figure 3. Absorbance over time of ABTS radical cation standardized to an initial A_{734} of 0.7 at pH 4.5, 6.5 and 7.4

It is apparent from the figures that increase in the p-H of radical cation solution resulted in decrease of stability of the cation, with the lowest stability being observed at pH 7.4. Although maximum stability of ABTS radical cation was observed at pH 4.5, the sensitivity of assay with regards to plasma samples was greatly affected. The

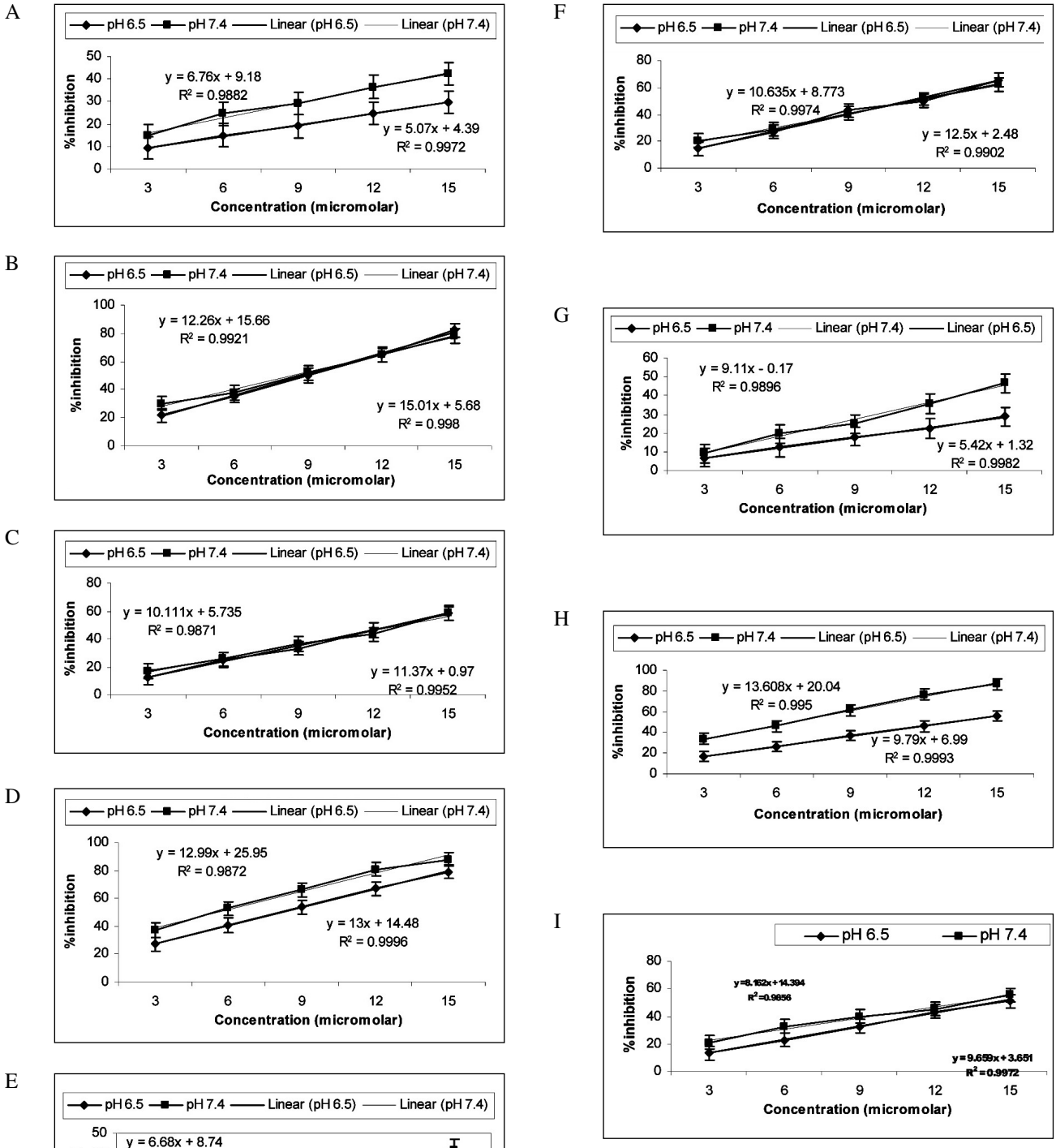

Figure 4. Effect of concentration of antioxidants on the inhibition of the $ABTS^+$ in sodium acetate buffer (20 mM, pH 6.5) and PBS (5 mM, pH 7.4) (A) ascorbic acid, (B) BHT, (C) trolox, (D) reduced glutathione, (E) uric acid, (F) BHA, (G) gallic acid, (H) kaempferol, (I) 2,2'-methylenebis(6-*tert*-butyl-4-methylphenol)

maximum stability and sensitivity of ABTS radical cation, with respect to both plant and plasma samples, were observed at pH 6.5 (20 mM sodium acetate buffer).

3. 2. Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The method was used to measure the antioxidant activity of both lipophilic and hydrophilic standard antioxidants and plasma samples by measuring the extent of scavenging of ABTS radical cation at 734 nm on addition of the sample solution for 8 min for standard antioxidants and plant extracts, and 30 min for plasma samples. Dose-response curves were obtained by plotting the percent inhibition of the original absorbance value (fixed at 0.7 ± 0.02 absorbance units) for a range of concentrations of different standard antioxidant compounds. The kinetics of reaction showed that the reaction with standard antioxidant was completed in 0.5–2 min range, by reaching a stable end-point. TEAC values of the sample solutions were obtained by comparing the percent inhibition with standard curve of trolox.

A comparison of the dose-response curves of different standard antioxidants by the modified and the old version of $ABTS^{\bullet+}$ decolorization assays is shown in Figure 4. The values of coefficient of determination (R^2) obtained for both assays demonstrate that there was a high degree of correlation between the concentrations of a given antioxidant compound and % inhibition of the radical cation. A general trend of relative decrease in the antioxidant activity (in terms of % inhibition of the radical cation) of the standard phenols using the modified assay may be attributed to the greater stability of the radical at pH 6.5 value. This also, in turn, minimized the effect of pH, which was reported to cause about 5–20% increase in the observed antioxidant value of standard phenols due to instability of ABTS radical cation at higher pH values (pH 7.4).[21]

The TEAC assay which was originally described by Miller *et al.*[1] and later modified by Re *et al.*[20] is based upon scavenging of ABTS radical cation on addition of sample antioxidants. The extent of scavenging of radical anion, which is detected spectrophotometrically at 734 nm, can therefore be related to the amount of antioxidants present in the sample. TEAC value can be determined for all the compounds/samples, which are able to scavenge $ABTS^{\bullet+}$ radical cation by comparing their scavenging activity with that of trolox, a water-soluble analogue of vitamin E. The modified assay was found to be highly reproducible. TEAC values for plasma sample ranged between 1.410–2.025 mM and 1.72–1.98 mM for older version of ABTS assay and the modified ABTS assay, respectively (Table 2). The mean % RSD was found to be 1.64 and 1.27 for older and modified versions of $ABTS^{\bullet+}$ assay, respectively.

TEAC values for different fractions of *C. reflexa* and stem, bark and leaves of *T. arjuna* were also determined using both assays. Comparative analyses of TEAC values of different fractions of the two plants employing two assays are shown in Figure 5.

TEAC values obtained by using the modified $ABTS^{\bullet+}$ assay were comparable with those found by the older version of ABTS assays. A general trend of slightly decreased antioxidant activity with the modified assay may be attributed to the increased stability of ABTS radical cation at pH 6.5. The results showed greater precision with the modified assay showing its superiority over the older versions.

3. 3. Total Phenolic Content (TPC) Assay

Phenolic compounds have been reported to be very powerful antioxidants due to the presence of hydroxy groups in their structures.[40] For TPC, a previously reported assay was adopted.[41] Amongst the three parts of *T. arjuna*, high phenolic contents were found in butanol, methanol, aq. (AP) and aq. (BP) fractions of TAB and butanol, methanol, aq. (AP) and ethyl acetate fractions of TAS. Chloroform, ethyl acetate and butanol fractions of CR also showed high content of phenolic compounds (Figure 6). High TPC values may be attributed to the previously reported tannins, triterpenoid, saponins, flavonoids, gallic acid, ellagic acid, OPCs, and phytosterols that are found in *T. arjuna*.[35–36] These results are also depictive of a correlation between the polyphenolic content and the antioxidant activity of the respective fraction, which is in agreement with the already reported results regarding other plants.[42–43]

Table 2. TEAC values (mM) of human plasma using old version of ABTS assay and the modified ABTS assay

Sr. No.	Plasma	TEAC using Old version of $ABTS/K_2S_2O_8$ Assay (pH 7.4) (mM)*	%RSD	Mean %RSD	TEAC using Modified $ABTS/K_2S_2O_8$ Assay (pH 6.5) (mM)*	%RSD	Mean %RSD
1.	Subject 1	2.025	1.72		1.901	1.41	
2.	Subject 2	1.740	1.54		1.902	1.24	
3.	Subject 3	1.499	1.21	1.64	1.750	1.25	1.27
4.	Subject 4	1.990	1.81		1.980	1.12	
5.	Subject 5	1.410	1.91		1.720	1.35	

* The data shown is mean ±SD (n = 5)

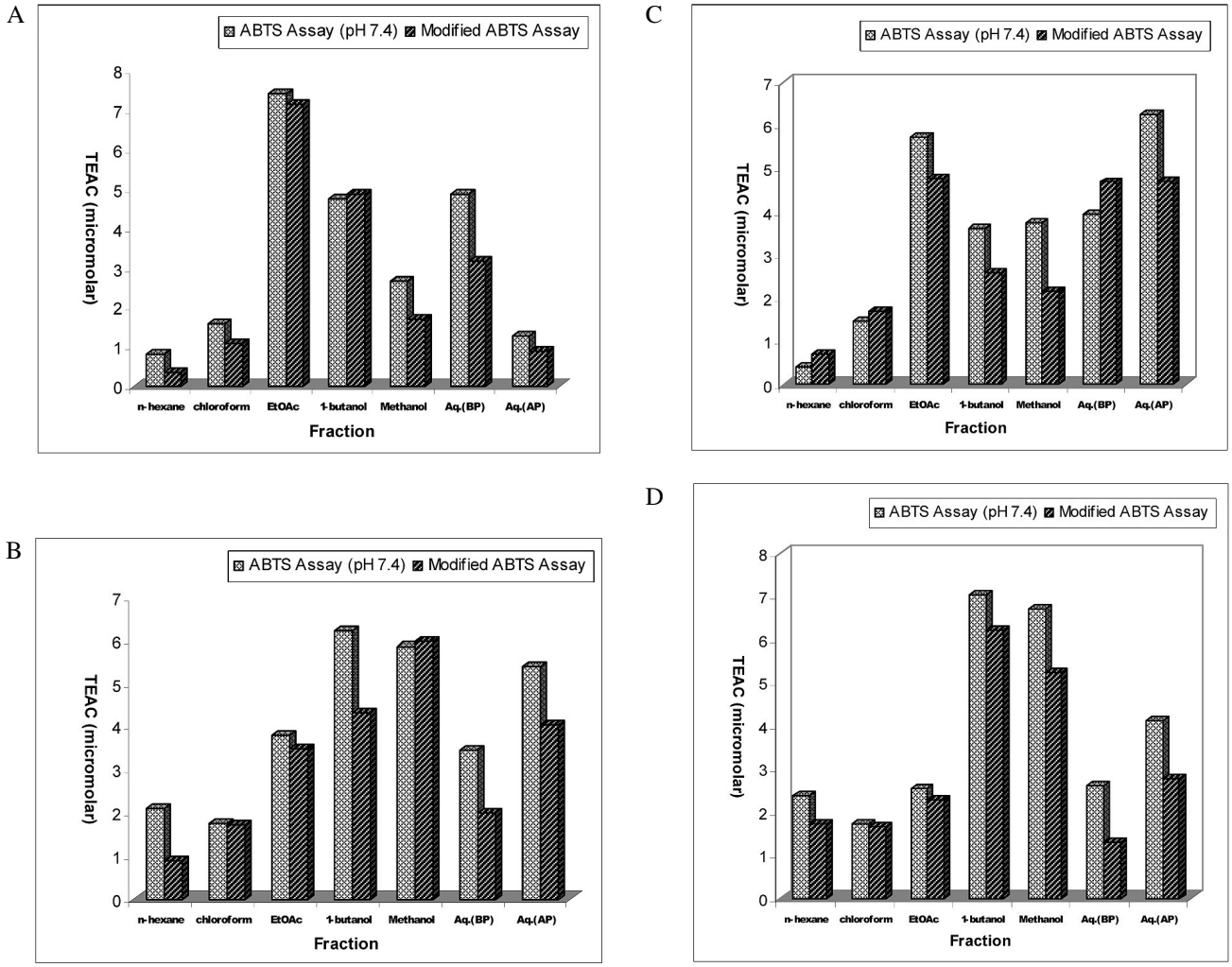

Figure 5: A comparative analysis of TEAC values (mM) of different fractions of (A) TAS (B) TAL (C) TAB and (D) CR, using ABTS assay (pH 7.4) and the modified assay.

n-hexane, chloroform (TAS & TAL) and n-hexane and methanol (CR) fractions were used neat; chloroform, aq. (BP) (CR), EtOAc, 1-butanol, methanol, aq. (BP), aq. (AP) (TAL) and EtOAc, aq. (BP) (TAS) fractions were 10 times diluted while all the other fractions were 20 times diluted for both the ABTS assay (pH 7.4) and the modified ABTS assay. In the case of TAB n-butanol, methanol, aq. (AP), aq. (BP) fractions were 20 times diluted; EtOAc was 10 times diluted and n-hexane fractions were used neat for both assays. Chloroform fraction of TAB was used as neat for the modified assay while it was 10 times diluted for ABTS assay (pH 7.4).

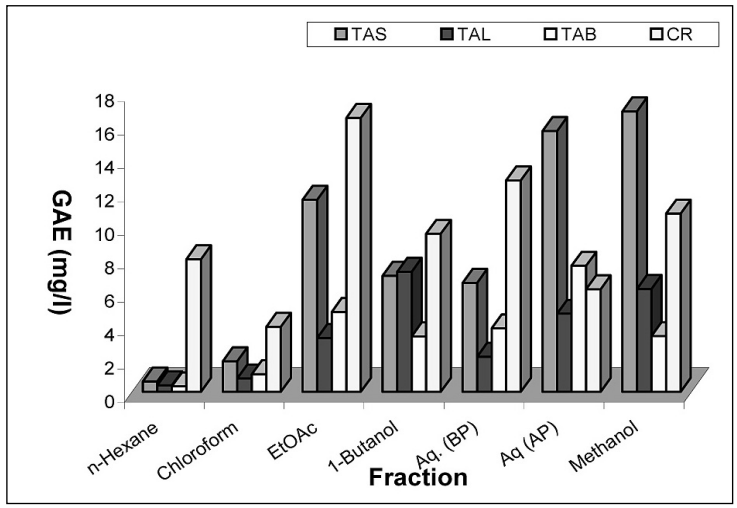

Figure 6. Comparison of TPC analysis of different fractions of stem, leaves and bark of *T. arjuna* and *C. Reflexa* (butanol fraction of TAS, butanol, aq. (BP), aq. (AP) and methanol fractions of TAB; EtOAc and butanol of CR; and chloroform fraction of CR are 100, 20 and 10 times diluted, respectively)

Table 3. EC_{50} and T_{EC50} values of different fractions of *C. reflexa* and stem, bark and leaves of *T. arjuna*

Fraction	Plant/herb Sample							
	TAS		TAB		TAL		CR	
	EC_{50} µg of dried mass/mL of DPPH	T_{EC50} (min)	EC_{50} µg of dried mass/mL of DPPH	T_{EC50} (min)	EC_{50} µg of dried mass/mL of DPPH	T_{EC50} (min)	EC_{50} µg of dried mass/mL of DPPH	T_{EC50} (min)
Methanol	5.5	3	3.1	5	8.6	40	665.9	12
n-Hexane	Nil*	Nil*	416.6	46	18.156	11	966.6	75
Chloroform	61.1	20	1044.4	38	416.6	20	1577.5	3
EtOAc	2.5	20	12.7	25	50.0	65	158.3	4
1-Butanol	3.6	55	8.6	42	11.25	45	278.3	8
Aq. (AP)	5.9	10	6.7	34	2.0	75	362.7	30
Aq. (BP)	19.7	45	18.4	15	7.3	37	739.4	12

* *n*-hexane fraction of TAS did not show any radical scavenging activity

3. 4. DPPH Free Radical Scavenging Activity

DPPH is a commercially available stable free radical, which has been widely used for estimating scavenging activity of pure antioxidants and extracts of herbs.[44–45] Violet-colored DPPH radical, after accepting an electron or hydrogen atom from the antioxidant compounds, is converted into a colorless or somewhat yellow diamagnetic DPPH molecule.[46] The free radical scavenging activity of aqueous and organic extracts of TAS, TAB, TAL and CR were investigated. A plot between % DPPH remaining and the time after addition of neat or diluted stock solution of TAB is shown in Figure 7. The graph shows a sharp fall in the absorbance of DPPH in the first 5 min after addition of extract from bark of *T. arjuna*, and then becomes more moderate and gradual for the next 35 min. This behavior indicated the presence of both slow-reacting and fast-reacting antioxidants in the samples. The radical scavenging activity was found to be in the decreasing order of methanol > 1-butanol > aq. (AP) > EtOAc > aq. (BP) > *n*-hexane > chloroform for TAB. Kinetic graphs for the rest of the fractions of *T. arjuna* and *C. reflexa* were obtained and EC_{50} and T_{EC50} values for each extract were determined (Table 3). The data showed methanol, 1-butanol, EtOAc, aq. (AP) and aq. (BP) as the best extracting solvents for all three parts of *T. arjuna.* The radical scavenging activity of *C. reflexa* was found to be comparatively low. Ethyl acetate, 1-butanol and aq. (AP) showed relatively good radical scavenging activity.

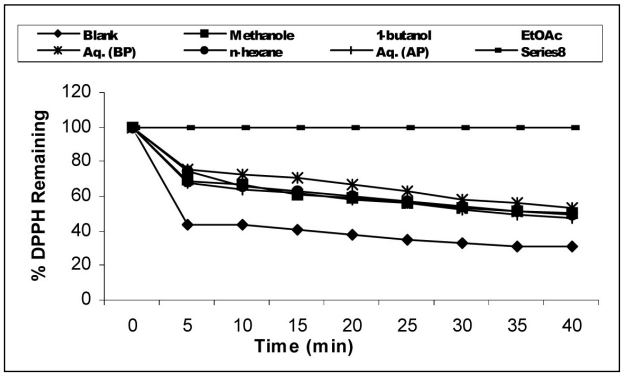

Figure 7. DPPH free radical scavenging activity of bark of *T. arjuna*. Data are mean ±SD (n = 3)

3. 5. Total Antioxidant Capacity Assay

Thiocyanate method was employed to determine total antioxidant activity of the extracts of *C. reflexa* and *T. arjuna*. During linoleic acid oxidation, peroxides are formed with the ability to oxidize Fe^{2+} to Fe^{3+}. Iron(III) ion on reaction with thiocyanate ions (SCN^-) forms a complex which can be measured spectrophotometrically at 500 nm. The added standard or sample solutions compete to inhibit or slow down oxidation of linoleic acid and thus formation of peroxide values. The low peroxidation values indicate high antioxidant power of the sample and vice versa. Antioxidant activity of aqueous and organic fractions of both plants was determined as a function of time, with standard antioxidant trolox being utilized as a positive control. The results showed that all the fractions of both plants have strong antioxidant activity (Figure 8). The antioxidant components of the samples were potent to the extent that even after incubation of 192 h, there was observed a slight increase in the peroxidation value.

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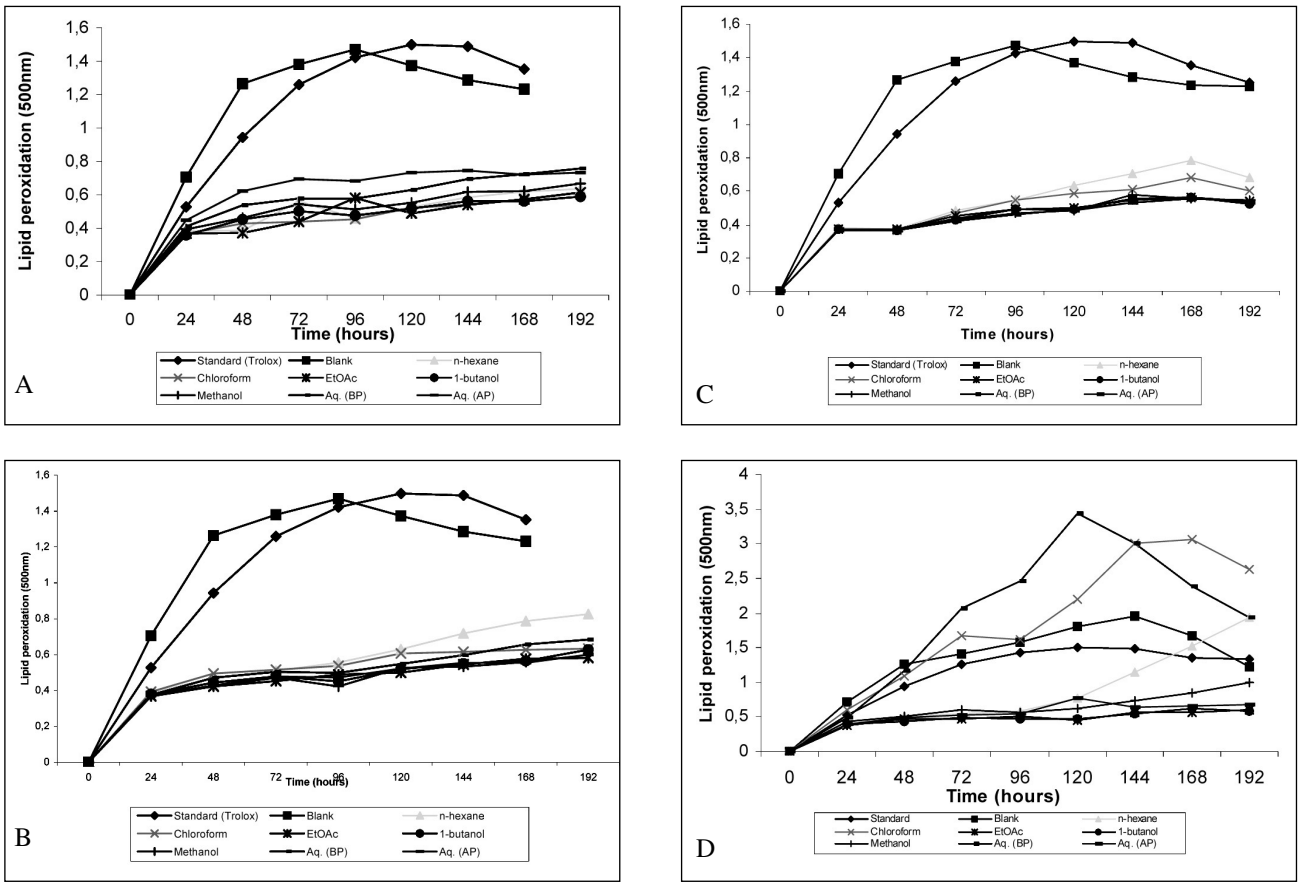

Figure 8. Antioxidant activity (in terms of peroxidation value) of different fraction of (A) CR, (B) TAS, (C) TAB and (D) TAL in the linoleic acid system.

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Povzetek

Radikal kation 2,2'-azinobis(3-etil benzotiozolin)-6-sulfonske kisline (ABTS) je bil pod različnimi pH pogoji uporabljen kot test z razbarvanjem za določitev troloks ekvivalentne antioksidativne kapacitete (TEAC) bioloških in prehrambenih vzorcev. Zaradi lastne nestabilnosti ABTS radikal kationa je nestabilen pri fiziološkem pH. Občutljivost the metode se izgubi tudi pri kislem pH. Nobena posamezna metoda še ni odpravila obeh slabosti ABTS metode. S sedanjo raziskavo smo želeli razviti izboljšano ABTS metodo z razbarvanjem za meritev antioksidativne aktivnosti človške plazme in rastlinskih ekstraktov, ki bi bila manj občutljiva na pH in hkrati dovolj občutljiva. Z reakcijo med ABTS in kalijevim persulfatom v prisotnosti natrijevega acetatnega pufra pri pH 6.5 (20 mM) je nastal relativno stabilen ABTS radikal kation. Prehod elektrona ali vodikovega atoma z antioksidativne komponente v plazmi ali rastlinskem ekstraktu reducira radikal kation. Običajni antioksidanti, kot so troloks, butil hidroksianizol (BHA), butil hidroksitoluen (BHT) in askorbinska kislina kažejo stabilne končne vrednosti pri določanju celotne antioksidativne aktivnosti (TTA) za vse vzorce glede na referenčne indikatorje. Modificirana metoda se je izkazala za visoko ponovljivo. TEAC vrednosti za vzorce plazme so bile med 1.410–2.025 mM s srednjim odstotnim relativnim standardnim odklonom (% RSD) 1.64 za prvotno ABTS metodo in 1.72–1.98 s srednjim % RSD 1.27 za modificirano ABTS metodo. Učinkovitost metode smo dodatno dokazali z določitvijo antioksidativne aktivnosti organskih in vodnih ekstraktov *Cuscuta reflexa* in *Terminalia arjuna.*