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Comparison of Antioxidant Activity of *Cichorium intybus* by Using Different Reported Antioxidant Assays

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Present work reports *in vitro* antioxidant and radical scavenging capacity of different organic and inorganic fractions of *Cichorium intybus* which are effective for the curing of liver disorders, gallstones and infection of the urinary tract. These are the traditionally used herbs against degenerative diseases so it is an important task to evaluate their antioxidant activity. Extraction of herb was carried out in aqueous as well as in different organic solvents. Antioxidant activity and radical scavenging potential of the seven fractions were studied by using different antioxidant assays such as ABTS {2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid)}, FRAP (ferric reducing antioxidant power), TPC (total phenolic contents), DMPD (N,N-dimethyl-*p*-phenylene diamine) and DPPH (diphenyl-1-picrylhydrazyl) radical scavenging assay. Trolox equivalent antioxidant activity (TEAC) of the aqueous and organic fraction of these herbs was determined by calculating the percentage inhibition of the coloured radical solution after reaction with sample and standard antioxidants by comparing with the standard curve formed by trolox as standard antioxidant. Ferric reducing antioxidant power and TPC decolourization assays, *C. intybus* showed a wide range of antioxidant activity. Using total phenolic content assay the amount of total phenolics components for different fractions of *C. intybus* ranged from 0.465-4.807 μM and ferric reducing antioxidant power assay TEAC values ranged from 1.013-6.133 μM which showed the presence of many antioxidant components in composition.

Key Words: *Cichorium intybus*, 2,2'-Azino-*bis*(3-ethylbenzothiazoline-6-sulpohonic acid), Trolox equivalent antioxidant capacity.

INTRODUCTION

The free radical chain reactions are usually produced in the mitochondrial respiratory system[1]. Free radicals or oxidative injury now appears the fundamental mechanism underlying a number of human neurologic and other disorders[2]. For instance, in diabetes, increased oxidative stress which co-exists with reduction in the antioxidant status has been postulated[3]. It is generally assumed that frequent consumption of plants derived phytochemicals from vegetables, fruit, tea and herbs may contribute to shift the balance toward an adequate antioxidant status. This interest in natural antioxidant, especially of plant origin, has greatly increased in recent years[4].

Genus comes from the ancient Arabic word Chikourych, which was the name for one species of salad vegetable, for chicory[5]. Common names: (chicory; succory; blue sailor; coffee weed and witloof[5]. The leaf chicory (*Cichorium intybus*), a Mediterranean plant, is widely grown in Europe, Western Asia, Egypt and North America.

Beginning with the ancient Egyptians, Greeks and Romans, chicory leaves were used as salad greens. Chichory is still popular today in many European countries[6]. Herbalists have used chicory for rheumatic and liver complaints[7]. Chicory is used medicinally for gout, hemorrhoids, as a digestive aid, diuretic, laxative, tonic and mild sedative. Additionally, chicory is regarded as a cooling, alternative herb in Ayurvedic medicine[8].

Chicory, the dried root of *Cichorium intybus* Linne (Compositae), is used as a folk remedy treatment for gallstones, hepatitis, jaundice, inflammations, warts, tumors and cancer[9] and as a tonic and an additive that enhances bitterness flavour, colour and form of coffee[10]. The antihepatotoxic effects of the root callus extracts were found to protect against carbon tetra-chloride induced hepatocellular damage[11], its water soluble extract improved lipid metabolism[12], chicory was found to have potential colon tumor inhibitory properties[13] and its methanol extract was reported to have antiinflammatory activity[14]. It contains the bitter sesquiterpenoid lactones, lactucin and intybin[15] as well as cichoriin (coumarin glucoside).

Their is no comprehensive study on the radical scavenging and antioxidant capacity has been undertaken as yet. The objective of the present study is to evaluate radical scavenging and antioxidant potential of different organic and aqueous

extracts of *C. intybus* by using trolox equivalent antioxidant capacity (TEAC) assay, ABTS decolourization essay, FRAP assay, TPC assay, DMPD free radical scavenging assay and DPPH assay.

EXPERIMENTAL

Cichorium intybus was purchased from a local market, Paparr Mandi, Lahore, in January 2010 and identified by Muhammad Ajaib (Taxonomist), Department of Botany, Government College University, Lahore.

Extraction of antioxidant components: Socked 10 of finely ground herb in 100 % methanol (4 × 200 mL) at room temperature with mild shaking (2 × 24 h). The extract was filtered out and the residue was extracted again to ensure complete extraction. From 100 % of the filtrate, methanol was evaporated under reduced pressure to obtain a crude residue. The residue was re-suspended in distill water (200 mL). The aqueous solution was successively partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol (4 × 25 mL for each extraction). The non-aqueous layer was separated and stored at 4 ºC until used for further analysis.

Chemicals and standards: Standard antioxidant such as trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxlic acid), N,N-dimethyl-*p*-phenylene diamine (DMPD) ICN biomedical I, *n*-hexane, acetone, methanol, acetic acid, ethyl acetate, ethyl alcohol, ferric chloride, sodium chloride, sodium acetate, dichloromethane, potassium per sulfate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ferric chloride all of them are E. Merk, deionized water, (ABTS) 2,2-azino-*bis*-3-ethylbenzothiazoline-6-sulphonic acid, I-diammonium salt, Aldrich chemical Co., TPTZ (2,4,6-tripyridal-s-triazine), hydrochloric acid. Follin-Ciocalteau's reagent and potassium persulfate (di-potassium peroxdisulfate) were purchased from Fluka (UK). HPLC grade ethanol was purchased from Rathburn Chemicals Ltd. (Walkerburn, Peebleshire, Scotland). Spectrophotometric measurements were made on UV-1700 PharmaSpec. UV-Visible spectrophotometer, Shimadzu, Japan equipped with temperature control device. All the solutions were made in triplicate and experiments were performed three times. The results obtained were averaged.

$ABTS^{\bullet+}$ radical cation decolourization assay: (In buffer and methanolic media): "ABTS" radical scavenging assay depends upon the scavenging activity of ABTS radical cation generated from a reaction between ABTS and 3.49 mL of (10 mM) potassium persulfate and making the total volume to 14.28 mL by adding 0.780 mL of deionized water in it. The antioxidant components reduce bluish green ABTS radical to colourless native ABTS depending upon their nature and quantity on a time-dependant scale. Trolox is generally used as a standard antioxidant for comparison purposes. For the determination of trolox equivalent antioxidant capacity of the extracts, $ABTS^{\bullet+}$ decolourization assay was followed[16]. ABTS radical cation was produced by a reaction between ABTS and potassium persulfate (7.00 and 2.45 mM final concentrations, respectively) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use and intense bluish green coloured stable radical cation ($ABTS^{+}$) was generated. To study the antioxidant activity of standard antioxidant and indigenous medicinal herb, the ABTS stock solution was diluted with PBS buffer (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 ºC. Then 3.49 mL of diluted $ABTS^{\bullet+}$ solution (A_{734} nm = 0.70 + 0.020) was transferred into the cuvette and noted down the absorbance as A_o. Then added 10 µL of sample solution, the absorbance reading was taken as 25 ºC exactly 1 min after initial mixing and upto 6 min. Appropriate blank were run in each case. 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 was calculated by the following formula.

$$\text{Inhibition (\%) (at734 nm)} = \left(1 - \frac{I_f}{I_o}\right) \times 100$$

where I_o = absorbance of radical cation solution before addition of sample and I_f = absorbance after addition of the sample. $ABTS^{\bullet+}$ radical cation also prepared by using methanol. The resultant data was plotted between concentration of antioxidants and that of trolox for the standard reference curve.

Total phenolic contents assay (TPC): Total phenolic contents of the extracts were determined by an reported method[17]. Stock solution of gallic acid was made by dissolving 0.500 g gallic acid in 10 mL of C_2H_5OH in a 100 mL conical flask and diluted it to volume with double distilled water. Sodium carbonate solution was prepared by dissolving 200 g of anhydrous sodium carbonate in 800 mL of distilled water. After boiling and subsequent cooling of the solution, a few crystals of sodium carbonate were added. The solution was stand for 24 h, filtered and volume was raised to 1 L with distilled water. To prepare a calibration curve, 0, 1, 2, 3, 5 and 10 mL of stock solution of phenol were added into 100 mL conical flask separately and then diluted to volume with distilled water. The final solutions contained concentrations of 0, 50, 100, 150, 250 and 500 mg/L gallic acid, the effective range of assay. From each calibration solution and sample or blank, 40 µL were pipetted into separate cuvettes and to each 3.16 mL of D. water was added. Folin-Ciocalteu's reagent (200 µL) was added and mixed well. After 8 min, 600 µL of Na_2CO_3 solution was mixed thoroughly in the solution. The solution was allowed to stand at 20 ºC for 2 h and absorbance of each solution was noted at 765 nm against the blank. A concentration *versus* absorbance linear plot was thus obtained. Alternately, they can be left at 40 ºC for 0.5 h before reading the absorbance and noted the absorbance at 765 nm. Create a calibration curve with standard and determine the level in sample.

Ferric ion reducing antioxidant power (FRAP) assay: The ferric ion reducing capacity of plant extract was measured according to the reported method[18]. Freshly prepared FRAP solution contained 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM hydrochloric acid solution and 2.5 mL of 20 mM ferric chloride solution. The mixture was incubated at 37 ºC throughout the reaction period. 3 mL of FRAP reagent was mixed with 100 µL of sample and 300 µL of distilled water. Absorbance readings were taken at 593 nm after every minute for 6 min. Results were compared with standard curve of $FeSO_4$.

DMPD/$FeCl_3$ decolourization assay: DMPD stock (100 mM) solution was prepared by dissolving 209 mg of DMPD in 100 mL of deionized water. The coloured radical cation ($DMPD^{\bullet+}$) was obtained by taking 1 mL of this stock solution to a 100 mL of measuring flask and adding into it 0.2 mL of 0.05 M ferric chloride solution, then diluting the solution to 100 mL by adding 0.1 M acetate buffer (pH 5.25). One milliliter of this solution was directly placed in 1 mL quartz cell and its absorbance was measured at 517.5 nm. An optical density of 0.900 + 0.100 unit of absorbance was obtained and it represents the uninhibited signal. Then 2.90 mL of diluted $DMPD^{\bullet+}$ solution (A_{517} nm = 0.900 + 0.100) was transferred in a cuvette and noted down the absorbance as A_o. Then added 10 µL of sample solution, the absorbance reading was noted at 30 ºC exactly 1 min after initial mixing and up to 6 min. Appropriate blank were run in each case. All determinations were carried out at least three times in succession and in triplicate at each separate concentration level of the standards and sample. The percentage inhibition of radical cation at 517 nm was calculated and plotted as a function of concentration of antioxidant and trolox for the standard reference data.

$$\text{Inhibition (\%) of } A_{505} = \left(1 - \frac{A_f}{A_o}\right) \times 100$$

A_o = Absorbance of uninhibited radical cation. A_f = absobance measured after 4-6 min after the addition of antioxidant samples.

Chemical reaction:

$$DMPD_{(uncoloured)} + \text{oxidant } (Fe^{3+}) + H \rightarrow DMPD^{\bullet+}{}_{(purple)}$$

$$DMPD^{\bullet+}{}_{(purple)} + AOH \rightarrow DMPD^{+}{}_{(uncoloured)} + AO$$

(Antioxidant)

2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH) assay: 2,2-Diphenyl-1-picrylhydrazyl free radical scavenging potential was found by using a previously reported method[19]. DPPH is one of a few stable and commercially available organic nitrogen radicals and has a UV-vis absorption maximum at 515 nm. Upon reaction, solution colour fades; the reaction progress is monitored by a spectrophotometer.

Briefly, DPPH solution (3 mL, 25 mg/L) in methanol was mixed with appropriate volumes of neat or diluted sample solutions (0.1 mL). The reaction progress of the mixture was monitored at 517 nm over a time period of 0.5 h until the absorbance becomes stable. Upon appropriate reduction, the purple colour of the solution changed to yellow diphenylpicrylhydrazine. The percentage of the DPPH remaining ($DPPH_{rem}$ %) was calculated as

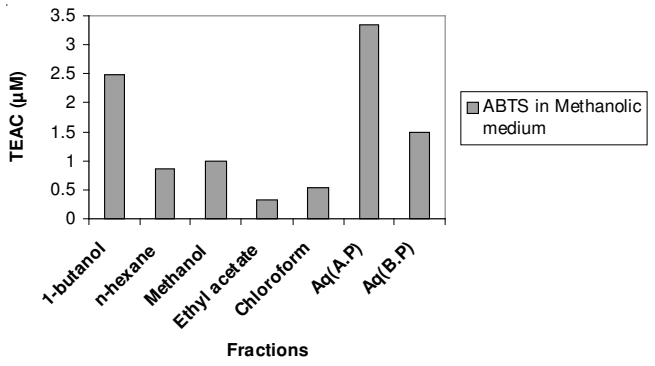
$$DPPH_{rem}\ (\%) = 100 \times \frac{[DPPH]_{t=t}}{[DPPH]_{t=0}}$$

where $[DPPH]_{t=0}$ = concentrat ion of DPPH radical before reaction with antioxidant samples and while DPPHrem is proportional to the antioxidant concentration 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 517 nm as a function of time (min) was plotted for each fraction of the samples. EC_{50} value, which is the concentration of a substance that reduces the amount of DPPH radical to half of the original concentration under experimental conditions, was also determined for each fraction.

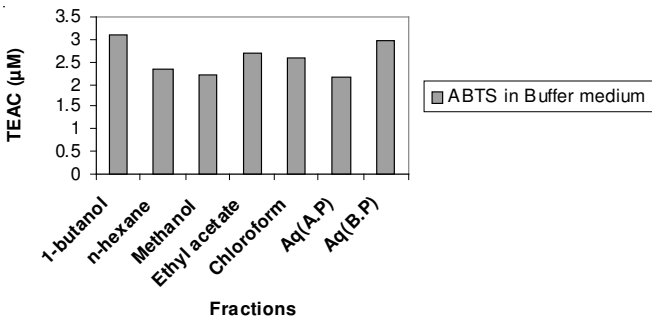
RESULTS AND DISCUSSION

$ABTS^{\bullet+}$ decolourization assay: The ABTS decolourization assay was applied to evaluate *in vitro* radical scavenging potential of different fractions of *C. intybus*. Trolox equivalent antioxidant capacity (TEAC) values were obtained by comparing the percentage inhibition values of fractions samples with the standard trolox curve. Column graphs are plotted for the TEAC values of each fraction of the sample (Fig. 1). Two media were used for the generation of ABTS free radical, one was methanloic and other was buffer medium, but methanolic medium shows more percentage inhibition. Trolox equivalent antioxidant capacity values ranged from 0.325-3.329 µM of trolox equivalents in methanolic medium. Among different fractions aq. extract (after partitioning) shows maximum TEAC values than other six fractions. In methanolic medium maximum TEAC value (3.329) is given by aq. extract (after partitioning), which indicates that it has maximum and ethyl acetate shows minimum radical scavenging activity. Decreasing order of radical scavenging activity of different fractions in methanolic medium is given as aq. extract (after partitioning) > 1-butanol > aq. extract (before partitioning) > methanol > *n*-hexane > chloroform > ethyl acetate. While in buffer medium, decreasing order of TEAC values as 1-butanol > aq. extract (before partitioning) > ethyl acetate > chloroform > *n*-hexane > methanol > aq. extract (after partitioning). As shown in (Fig. 1A-C).

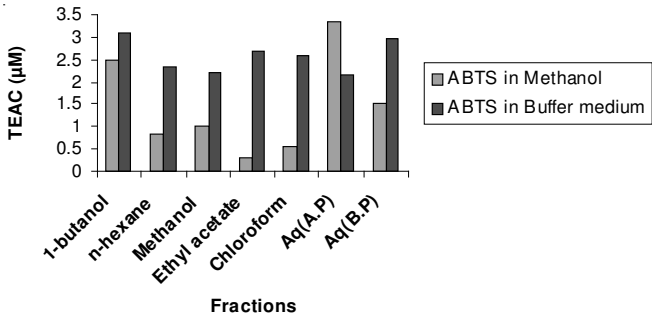
Total phenolic contents: Follin-Ciocalteu's reagent (FC reagent) is usually used in the laboratories for the determi-



(A)



(B)



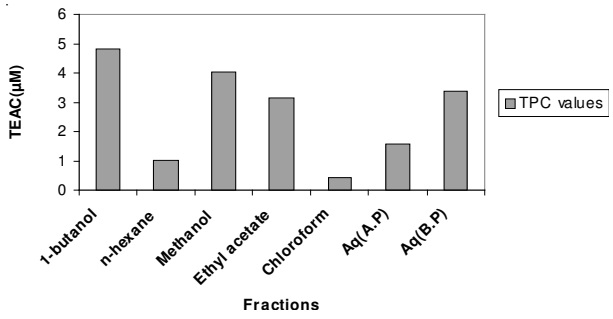
(C)

Fig. 1. Trolox equivalent antioxidant capacity values of extracts of *Cichorium intybus* by ABTS assay (A) in methanolic medium (B) in buffer medium, (C) comparison between methanolic and buffer medium

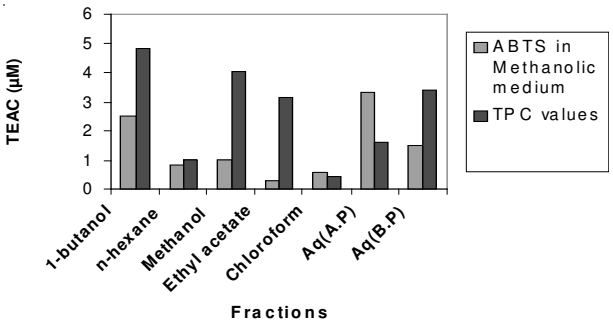
nation of phenolic components in plants/herbal extracts and other fractions. Phenolic compounds react FCR only under basic conditions at pH 10. Hydroxyl moieties of phenolic compounds have the ability to reduce yellow coloured FC reagent to blue colour. The change in the colour is monitored spectrophotometrically at 765 nm. Total phenolic content values ranged from 0.465-4.807 µM of GAE/100 g of dry weight (Fig. 2). Employing TPC assay, decreasing order of antioxidant activity of different fractions of *C. intybus* was found to be 1-butanol > methanol > aq. extract (before partitioning) > ethyl acetate > aq. extract (after partitioning) > *n*-hexane > chloroform.

Ferric reducing antioxidant power (FRAP): The FRAP assay is employed to assess antioxidant power by knowing the ability of the sample to reduce ferric to ferrous ion at low pH. The FRAP assay as developed by Benzie and Strain which involves a single electron reduction of the $Fe(TPTZ)_2(III)$ complex (pale yellow) to the $Fe(TPTZ)_2(II)$ complex (blue) by sample antioxidants at acidic pH. Any antioxidant species with lower reduction potential than that of Fe(III)TPTZ salt (0.7 V) can reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ contributing to Ferric reducing antioxidant power value[20]. This reduction is progressed spectrophotometrically at 593 nm. Appearance of intense blue colouration show the presence of reducing components in the sample. The original method of Benzie and Strain uses a 4 min interval but we noted that the reaction/colour change is in progress even after 4 min interval. Absorbance readings, therefore, were taken at a 6 min interval after addition of sample to TPTZ reagent allowing the reaction to reach a steady state. Ferric reducing antioxidant power values for different fraction ranged from 1.013-6.133 µM, which shows the maximum TEAC values as compared to other antioxidant assays mentioned in this artical (Fig. 3). Decreasing order of TEAC values of *C. intybus* as aq. extract (before partitioning) > ethyl acetate > methanol > aq. extract (after partitioning) > 1-butanol > *n*-hexane > chloroform.

N,N-Dimethyl-*p*-phenylene diamine/$FeCl_3$ decolourization assay: DMPD stock solution was prepared by dissolving 209 mg of DMPD in 100 mL of deionized water. The $DMPD^{\bullet+}$ was obtained by taking 1 mL of this stock solution to a 100 mL of measuring flask and adding into it 0.2 mL of 0.05 M $FeCl_3$ solution, then diluting the solution to 100 mL by adding 0.1 M acetate buffer of pH 5.25. One mL of this



(A)



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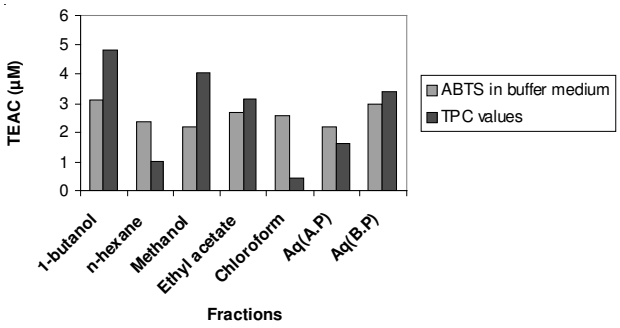
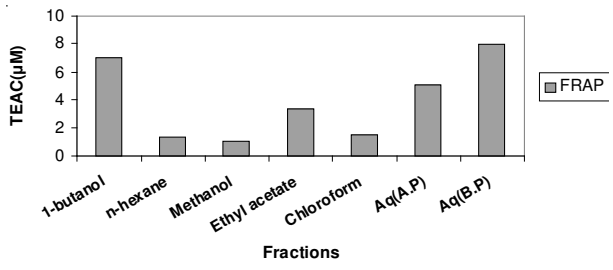
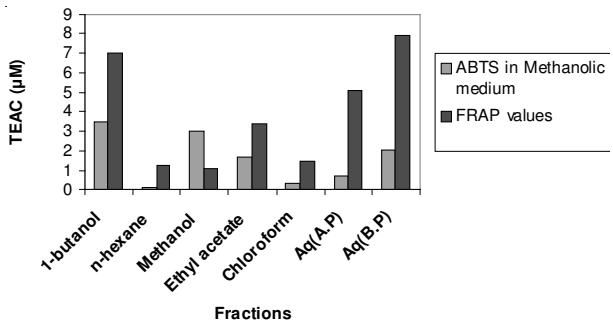


(C)

Fig. 2. (A) Trolox equivalent antioxidant capacity values of *C. intybus* by using TPC assay (B) comparison between TEAC values of ABTS in methanilc medium and TPC values (C) comparison between TEAC values of ABTS in buffer medium and TPC values



(A)



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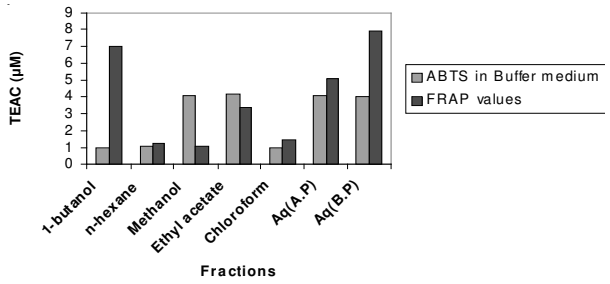

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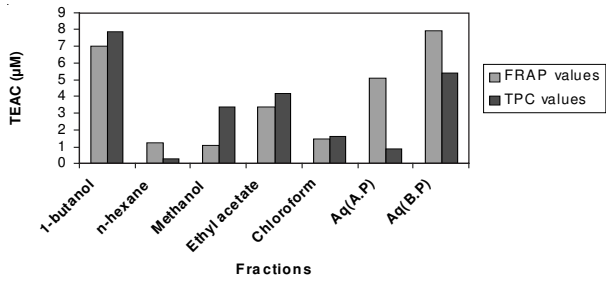
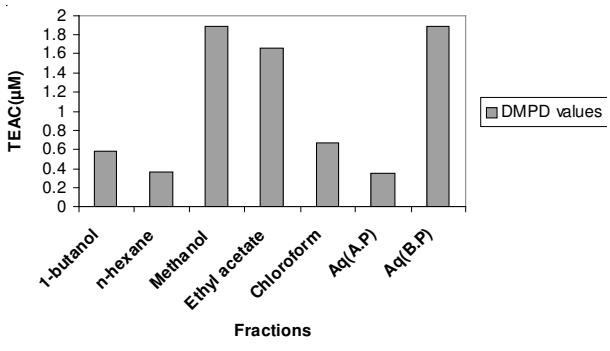


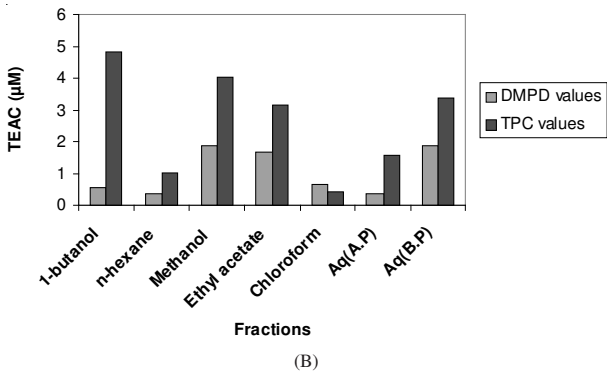
(D)

Fig. 3. (A) Trolox equivalent antioxidant capacity values of the fractions of *N. hindostana* by FRAP assay (B) comparison between TEAC values of ABTS in methanolic medium and FRAP values (C) comparison between TEAC values of ABTS in buffer medium and FRAP values (D) comparison between FRAP values and TPC values

solution was directly placed in 1 mL quartz cell and its absorbance was measured at 517.5 nm. Then 2.90 mL of diluted $DMPD^{\bullet+}$ solution (A_{517} nm = 0.900 + 0.100) was transferred in a cuvette and noted down the absorbance as A_o. Then added 10 µL of sample solution, the absorbance reading was noted at 30 ºC exactly 1 min after initial mixing and up to 6 min. Appropriate blank were run in each case. N,N-Dimethyl-*p*-phenylene diamine values ranged from 0.355-1.890 µM, which shows the minimum TEAC values as compared to other antioxidant assays mentioned in this artical (Fig. 4). Decreasing



(A)



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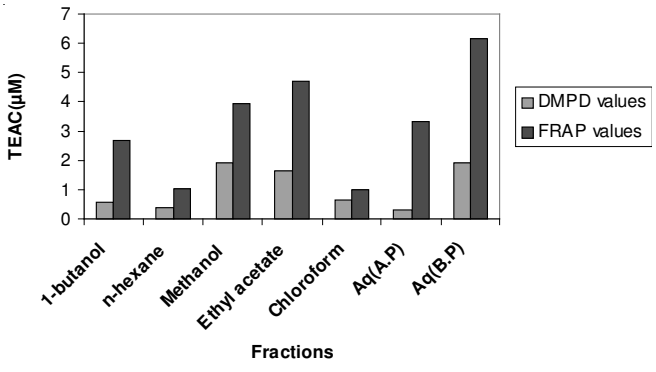

(C)

Fig. 4. (A) Trolox equivalent antioxidant capacity values of the fractions of *C. intybus* by DMPD assay (B) comparison between TEAC values of DMPD and TPC (C) comparison between TEAC values of DMPD and FRAP

order of TEAC values of *C. intybus* as aq. extract (before partitioning) = methanol > ethyl acetate > chloroform > 1-butanol > *n*-hexane > aq. extract (after partitioning).

2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH): DPPH is a preformed stable radical used to measure radical scavenging activity of antioxidant samples and has a UV visible absorption maximum at 515 nm. Upon reaction the solution colour fades. The progress of the reaction is monitored by a spectrophotometer. 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay was performed by taking parameters, the absorbance of antioxidant and the time for completion of the reaction of antioxidants, into consideration. Kinetic curves obtained by plotting absorbance against time showed that all the fractions of *C. intybus* contained minimum range of radical scavenging activity (Fig. 5). It is clear from the figure that all the curves are less steeper, showing slow reaction of antioxidant components with DPPH radical.

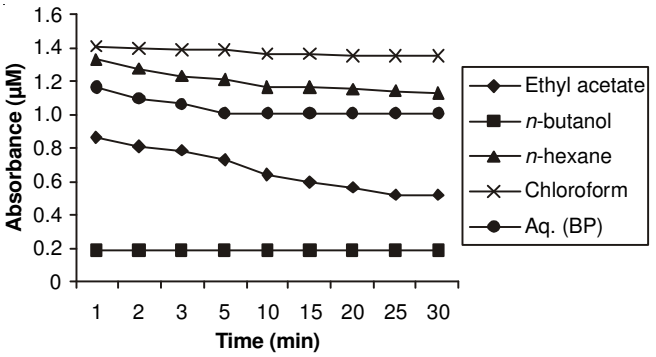

Fig. 5. Absorbance values of different fractions of *C. intybus* by DPPH assay

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