Original Article

Antioxidant properties of polyphenolic rich HPLC standardized extract of *Beta vulgaris* L. roots

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ABSTRACT:

OBJECTIVES: The present study was planned to evaluate the antioxidant effects of standardized *Beta vulgaris* L. or beetroot.
METHODS: The polyphenols present in lyophilized water extract of beetroot (BR) were quantified and standardized by HPLC. Deactivation of free radicals by BR was studied by *in vitro* biochemical assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide and superoxide radical scavenging activities. Nitrite content of BR was estimated. Moreover, inhibition of protein denaturation properties of BR was carried out.
RESULTS: HPLC chromatogram revealed presence of nine bioactive polyphenols in BR. Further, strong dose-dependent antioxidant properties were noted. IC_{50} (Inhibitory Concentration) of BR for DPPH, hydrogen peroxide and superoxide radical were 104.44μg/ml, 4.06μg/ml and 71.43μg/ml respectively. BR also showed enriched in nitrite content 3.359 mM/mg and it inhibited protein denaturation (IC_{50} 594μg/ml).
CONCLUSION: Beetroot was found to possess strong antioxidant property that may be due to presence of polyphenols.

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INTRODUCTION

Beta vulgaris L., also known as beetroot, is a leafy green vegetable cultivated throughout the world and is widely consumed. This vegetable is a well-known plant for its high nutritive and therapeutic value since ancient times [1-2]. Betalains (anthocyanins) and carotenoids are the major active constituents of beetroot and generally recommended as safe natural preservative [3-4]. Dietary polyphenols and nitrate has been demonstrated to have a range of beneficial vascular effects, including reducing blood pressure, inhibiting platelet aggregation, preserving or improving endothelial dysfunction, enhancing exercise performance [5-7].

It has been reported that beetroot is a nitrate rich vegetable [8]. Researchers have also shown, leaves of *Beta vulgaris* possess strong anti-oxidant properties [9-10].

But, till date there is very few information on its chemical and pharmacological actions.

Present study was aimed to standardize the water-soluble extract of beetroot and evaluate its anti-oxidant properties related to its pharmacological action.

MATERIALS AND METHODS

Chemicals:

Ultra-pure standards like, gallic acid, caffeic acid, chlorogenic acid, syringic acid, p-coumaric acid, sinapic acid, catechin, quercetin, coumarin, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) has been procured from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents like bovine serum albumin, phenazine methosulphate, nitroblue tetrazolium, nicotinamide adenine dinulcleotide, sodium pyrophosphate, N-1-naphthylethylenediamine dihydrochloride, sulfanilamide, phosphoric acid, sodium nitroprusside, hydrogen peroxide, methanol etc. were obtained from Sisco Research Laboratories Pvt. Ltd. (India). All reagents were of analytical grade.

Plant material and preparation:

Fresh roots of *Beta vulgaris* L. were obtained from a local market and identified by Botanical Survey of India, West Bengal [CNH/2016/Tech II/69/5]. Fresh roots were cut into small pieces, crushed with deionized water (1:2 w/v), filtered and lyophilized to powder. The powder obtained from the water extract of beetroot (BR) was standardized by HPLC and studied for antioxidant properties.

Drug standardization by HPLC:

HPLC analysis was done using instrument 1260 Infinity, Agilent Technologies, USA equipped with binary pump for solvent delivery, 20µl loop for injection and PDA (Photodiode Array) detector. Analyses were performed using reverse phase column, COSMOSIL, 5C18-MS-II (250mm x 4.6mm, 5µm). BR powder was dissolved in hydro-methanol (80:20) at a concentration of 1 mg/ml and filtered through 0.2 µm PDVF (Polyvinylidene fluoride) filter. Standard polyphenols like, gallic acid, caffeic acid, chlorogenic acid, syringic acid, p-coumaric acid, sinapic acid, catechin, quercetin and coumarin were prepared in hydro-methanol (80:20) at concentration 1 mg/ml. Polyphenols were eluted with some solvents such as 3% acetic acid (solvent A) and acetonitrile (solvent B) having following gradient: 0-5% B in 5 min, 5-15% B in 17 min, 15-20% B in 40 min, 20-50% B in 60 min, 50% B in 65 min, 50-0% B in 70 min. The solvent was delivered at a flow rate 0.8 ml/min. 20 µl of each standard and sample were injected for analysis and calibration. Detection was done at 320 nm and regression equation was derived from calibration procedures [11-12].

Determination of hydrogen peroxide scavenging activity:

Hydrogen Peroxide scavenging activity was determined with the method of Ruch *et al*. (1989) [13]. 2 ml of different concentrations of the extract were prepared and added to 0.6 ml of 40 mM of hydrogen peroxide (H_2O_2) solution in phosphate buffer (50 mM, pH 7.4). Solvent, without extract, mixed with H_2O_2 solution served as the control. The mixtures were incubated for 10 min at room temperature.

Then, the absorbance was measured at 230 nm. Percentage inhibition was calculated with the following formula:

Percentage hydrogen peroxide scavenging activity =

$$\left(1 - \frac{\text{Absorbance of Sample}}{\text{Absorbance of Control}}\right) \times 100$$

IC_{50} value was obtained from the graph plotted with radical scavenging activity against concentration.

Determination of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability:

DPPH scavenging activity was determined by the method of Sur *et al*. (2015) [14]. 1 ml of different concentrations of BR was added to 1 ml of DPPH solution (0.002% methanol solution). Solvent, without extract, mixed with DPPH solution served as the control. After 30 min incubation in dark, absorbance was measured at 517 nm. IC_{50} value was obtained from the graph plotted with radical scavenging activity against concentration.

Determination of superoxide radical scavenging activity:

Superoxide radical scavenging activity was determined with the method of Kakkar *et al*. (1984) [15]. 0.3 ml of BR was mixed with 1.2 ml of sodium pyrophosphate buffer (0.052 mM), 0.1 ml of phenazine methosulphate (186 µM), 0.3 ml of nitroblue tetrazolium (156 µM) and 1 ml water. Reaction was started with 0.2 ml of nicotinamide adenine dinulcleotide (780 µM) and stopped after 1 min by adding 1 ml of glacial acetic acid. The amount of chromogen formed was measured by recording color intensity at 560 nm. IC_{50} value was obtained from the graph plotted with radical scavenging activity against concentration.

Determination of nitrite content:

Nitrite content of the extract was determined by the method of Green *et al*. (1982) [16]. Sodium nitroprusside (5mM) in standard phosphate buffer saline (0.025M, pH 7.4) was incubated with different concentrations of BR at room temperature for 2.5 h. Griess reagent (1% sulfanilamide, 2% phosphoric acid, and 0.1% N-1-naphthylethylenediamine dihydrochloride) was then added and incubated for another 30 min. Absorbance was estimated at 546 nm. Nitrite content was estimated with sodium nitrite as the standard.

Determination of protein denaturation:

The protein denaturation was assessed following the method of Sur *et al*. (2003) [17]. In tubes, 0.05 ml of different concentrations of BR was added to 0.45 ml bovine serum albumin (5% aqueous solution). pH of the mixture was adjusted at 6.3 using 1 N HCl. The samples were incubated at 37°C for 20 min and then heated at 57°C for 3 min.

After cooling the samples, 2.5 ml of phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm. Control sample comprised of 0.05 ml distilled water instead of BR. Product control contained 0.45 ml distilled water instead of bovine serum albumin.

Statistical analysis:

Data were expressed as mean ± standard error of mean (SE). The data were analyzed statistically by a one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test for multiple comparisons, with computerized statistical package (SPSS version 20).

RESULTS

HPLC chromatogram:

Nine distinguished bioactive polyphenols were identified out of total twelve peaks of HPLC chromatogram (Fig. 1). The retention time, % relative area and absolute quantitative values are given in Table 1. The chromatogram revealed the amount of quercetin was highest in BR, followed by sinapic acid, p-coumaric acid, syringic acid, gallic acid, coumarin, caffeic acid, chlorogenic acid and catechin.
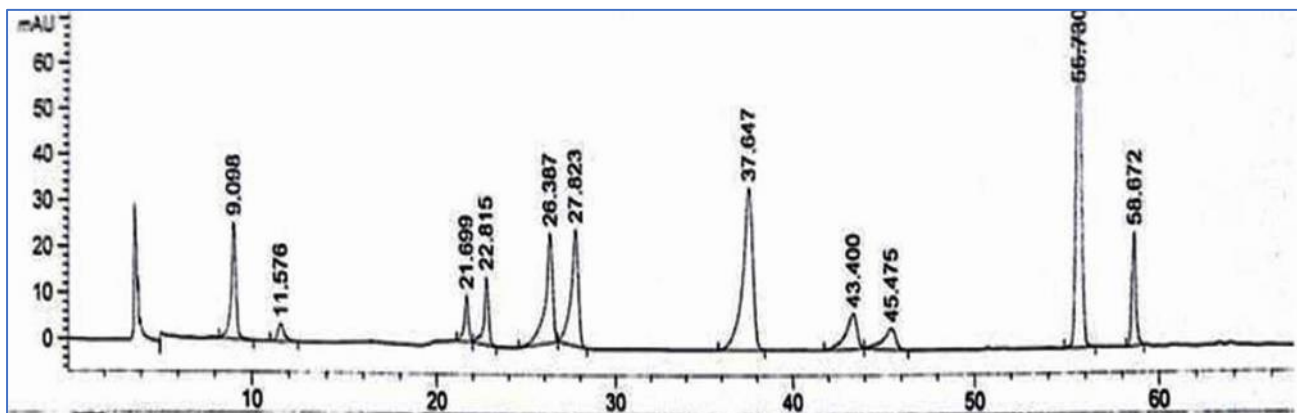

Figure 1: HPLC chromatogram of beet root extract (BR)

Table 1: Integration and quantification of polyphenols present in beetroot extract (BR) by HPLC

Peak No.	Retention time (min)	Relative area (%)	Absolute amount (µg/g of BR)	Peak Identification
1	8.31	2.40	-	Unknown
2	9.098	7.05	389.15±0.73	Gallic acid
3	11.576	1.43	28.62±0.42	Catechin
4	21.699	2.62	73.44±0.51	Chlologenic acid
5	22.815	4.27	104.20±0.73	Caffeic acid
6	26.387	11.17	495.06±0.48	Syringic acid
7	27.823	11.89	508.53±0.75	p-Coumaric acid
8	37.647	20.63	964.67±0.94	Sinapic acid
9	43.400	5.42	219.14±0.59	Coumarin
10	45.475	3.53	-	Unknown
11	55.730	23.61	1038.73±0.88	Quercetin
12	58.672	5.98	-	Unknown

Detection wavelength 320 nm; absolute value expressed as mean ± SE; N=3 in each test

Hydrogen peroxide scavenging activity:

Hydrogen peroxide scavenging activity of BR was found to significant as shown in Fig. 2. It was observed that the scavenging activity increased with the increase in the concentration of BR. The IC_{50} value of BR was only 4.06 µg/ml (Table 2).

DPPH free radical scavenging ability:

The scavenging abilities of DPPH free radical by BR was shown in Fig. 3. There was marked increase in the activity with the concentration of the extract. IC_{50} value of BR was 104.44 µg/ml (Table 2).
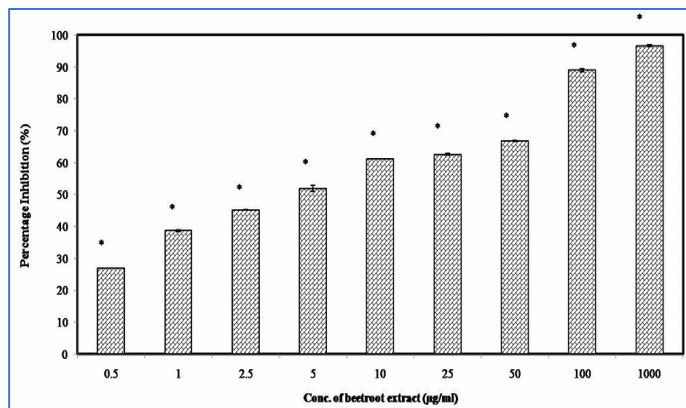

Figure 2: Hydrogen peroxide scavenging activity of beet root extract (BR)

Superoxide radical scavenging activity:

BR was found to inhibit superoxide radical in a dose-dependent manner (Fig. 4). The IC_{50} value of BR was noted 71.43 µg/ml (Table 2).

Nitrite content:

A dose-dependent increase in nitrite concentration was observed with beetroot extract as shown in Fig. 5. The nitrite content in BR was 3.359 mM/mg (Table 2).
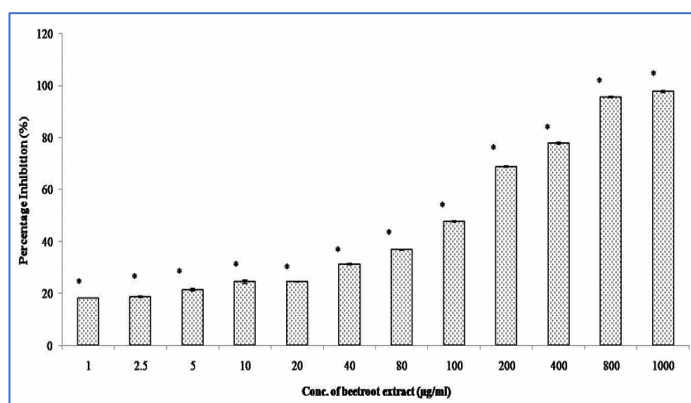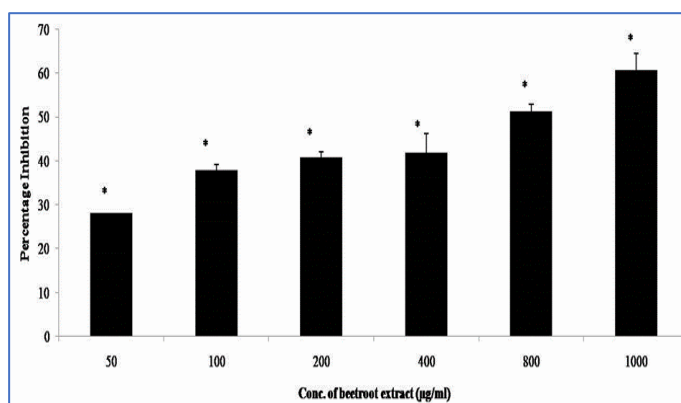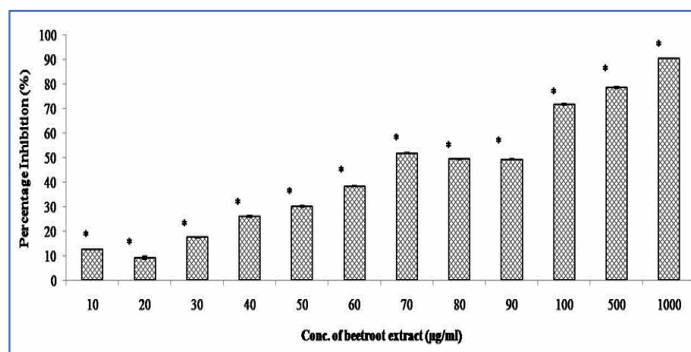
Protein denaturation:

A dose-dependent inhibition of protein denaturation was recorded with beetroot extract as shown in Fig. 6. The IC_{50} value was found to 594 µg/ml (Table 2).

Table 2: Effect of beetroot extract (BR) on antioxidant properties

Extract	50% Inhibitory concentration (µg/ml)				Nitrite content (nM/mg)
	H_2O_2 scavenging	DPPH scavenging	Superoxide radical scavenging	Protein denaturation	
Beetroot extract (BR)	4.06±0.016*	104.44±8.16*	71.43±5.39*	594±13.23*	3.359±0.45*

All data were expressed as mean±SE; N=6 in each test; Data were analyzed statistically by a one-way analysis of variance (ANOVA) and exhibited significant (*$p<0.001$).

Figure 3: DPPH scavenging activity of beet root extract (BR)

Figure 4: Superoxide scavenging activity of beet root extract (BR)
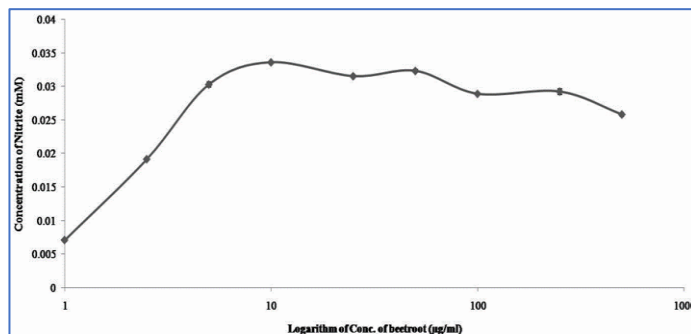
Figure 5: Distribution of nitrite content of beet root extract (BR)

Figure 6: Protein denature activity of beet root extract (BR)

DISCUSSION

Considerable scientific facts recommended that under situations of oxidative stress, reactive oxygen species (ROS) such as superoxide, hydroxyl, peroxyl radicals and reactive nitrogen species (RNS) such as nitric oxides and peroxynitrite are generated and the balance between anti-oxidation and oxidation is believed to play an important role in pathophysiology of human diseases like diabetes, cardiovascular diseases, neurodegenerative disorders, inflammation, arthritis and cancer [18]. Various enzymatic and non-enzymatic antioxidants present in the body help to scavenge these free radicals and reactive oxygen species [19]. Plants and foods are composed of an aggregation of several secondary metabolites, mainly polyphenols and thereby are rich source of natural antioxidants [20].

In the present study, HPLC analysis confirmed the presence of bioactive polyphenols such as quercetin, sinapic acid, p-coumaric acid, syringic acid, gallic acid, coumarin, caffeic acid, chlorogenic acid and catechin in beetroot extract.

The antioxidant properties of these polyphenols are mainly due to their redox properties, which allow them to act as either reducing agents or hydrogen donors or singlet oxygen quenchers [21].

Earlier it has been reported that leaves of beetroot have shown to possess significant antioxidant property [9-10]. Therefore, the present study was aimed to evaluate the antioxidant property of beetroot that is consumed as a vegetable in various parts of the world including India. It is established fact that hydrogen peroxide (H_2O_2) produced by different metabolic processes is converted into hydroxyl radicals, which damages cell membrane [22]. Beetroot extract showed significant scavenging of H_2O_2. Similarly, DPPH is a stable free radical, the nitrogen atom of which accepts hydrogen from antioxidants to produce diphenylpicryl hydrazine [23]. Thus, DPPH solution was used to evaluate the antioxidant property of BR extract. Present study reported BR extract showed 97.63% scavenging activity at 1000 µg/ml. Moreover, superoxide anion radical, the most harmful ROS, damages cell membrane by peroxidation of lipids [22] showed maximum superoxide anion radical scavenging activity 90.44% of BR at 1000 µg/ml.

Various leafy vegetables are known to be rich in nitrate and nitrite content. Dietary nitrate is essential in reducing blood pressure, inhibition of platelet aggregation and various other vascular effects [8]. Beetroot is known to be therapeutic source of dietary nitrate. Clinical trial observed beetroot juice was found to reduce blood pressure in hypertension patients [6]. Present study also revealed *Beta vulgaris* has enriched with nitrite molecules.

Protein denaturation means loss of biological properties of protein molecules. Denaturation of proteins is responsible for the cause of inflammation is conditions like rheumatoid arthritis, diabetes, cancer etc. [17]. Mechanism of denaturation is involved in alteration of electrostatic force, hydrogen, hydrophobic and disulphide bonds. Beetroot showed abilities to protect protein from denaturation indicating its relevant therapeutic properties.

From the above study, it may be concluded that beetroot extract has found to possess strong antioxidant properties and thus can be used as a natural antioxidant and may also be helpful in inflammation and other related disorders. Further investigations are necessary to corroborate the relationship of polyphenols present in BR with therapeutic applications.

ACKNOWLEDGEMENT

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