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Effects of beetroot (*Beta vulgaris*) supplements on selective enzymatic and non-enzymatic antioxidants in iron deficiency anemia

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Abstract: Iron deficiency anemia (IDA) is not only associated with iron deficiency but has shown strong association with the over production of free radicles and deficiency of antioxidant enzymes. The result of this imbalance is oxidative stress (OS) which is now considered as an important associated factor with various diseases. Treating IDA in most of cases with oral iron supplements results in more OS as iron is a transition metal. A more suitable alternate for iron supplementation is *Beta vulgaris* supplement, which being herbal in origin is far less associated with side effects. We studied effects of *beta vulgaris* supplements on enzymatic and non-enzymatic antioxidants in IDA patients. A significant increase in all study parameters were observed after treatment ($p<0.05$). When pre-supplemental values of super oxide dismutase (SOD) and reduced glutathione (GSH-PX) of IDA were compared with post-supplemental values, they were significantly low ($p<0.05$).A positive correlation was noted between the two antioxidants and hemoglobin (Hb) values suggesting a direct relationship between antioxidant status and Hb levels. Non enzymatic antioxidants included vitamin A,C and E. We also found a significant improvement ($p<0.05$) of these vitamins when compared with their initial values and the control group. Our study shows improvement of antioxidant status of anemic patients with 12 week supplementation of *Beta vulgaris.*

Keywords: *Beta vulgaris*, iron-deficiency anemia, oxidative stress, glutathione peroxidase, super oxide dismutase, non enzymatic antioxidants.

INTRODUCTION

Iron deficiency anemia (IDA) is considered to be a major public health issue which is globally effecting the health and wellbeing of people of all ages (Kulkarni *et al.*, 2022). According to the reports by World Health Organization (WHO) around 1.62 billion people are affected by IDA which is almost 24.8% of world population (WHO 2008). Though IDA is prevalent in developed countries also, but most of the burden occurs in developing countries such as Pakistan. According to a study by Usman *et al.*, 2019 about 65-78% of children and 39% of adolescents in Pakistan are suffering from IDA.

A negative iron balance that is particularly associated with either increase in iron requirements like during pregnancy or decrease supply as evident in malnutrition in children or increase loss of iron like in infections, are most frequently seen causes resulting in IDA (Sun *et al.*, 2021). IDA has been found to be associated with the risk of oxidative stress (OS), with a decrease in the antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) (Khalid *et al.*, 2019). Chemically, OS can be described as an oxidative imbalance due to decline in the functional redox activity of cells causing oxidative cell damage (Bathla & Arora 2022). This imbalance is now considered as an important contributing factor in various disease processes like alzheimer's, parkinsonism, cardiovascular (CVS) pathologies and various other inflammatory disorders. (Khalid & Ahmad 2012).

WHO suggests iron supplementation program for treatment and prevention of anemia ((Ebea *et al*. 2024). Conventionally, the daily recommended dose of oral iron is 100–200 mg iron/day in divided doses (Stoffel *et al*., 2020). Although the efficacy of oral iron supplements has been proved in literature, still IDA is prevalent particularly in developing and under develop countries ((Ebea *et al*. 2024). Studies have shown that oral iron supplements are associated with low compliance that is possibly due to side effects such as nausea, vomiting, colic, and diarrhea (Abd-El-Fattah *et al*., 2021). Use of herbal products seems to be a smart choice in any such scenario (Ali and Bilal 2023, Calixto, J.B., 2000).

The beetroot, *Beta vulgaris* belongs to the Chenopodiaceae family of plants. Its secondary metabolites betalains, betaine and nitrites are functionally the most key phytochemicals, providing health benefits. Betanin is the major betalain found in *Beta vulgaris (beetroot)* (Thiruvengadam *et al*., 2024). Chemically it is betanidin-5-O-beta-glucoside, having phenolic and cyclic

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amine groups and is responsible for the antioxidant activity (Babarykin *et al*., 2019).

Due to its nutritional values (table 1) and chemical composition (fig. 1) various health benefits of *Beta vulgaris* supplements have been identified and studied (Bangar *et al*., 2022). Beetroot has proven its role for the treatment of anemia by enhancing the oxygen carrying capacity of erythrocytes, lowering blood pressure due to its vasodilatory effect, preventing neural tube defects by increasing folate levels, etc. Studies have also proved red beet extracts provide some other various health benefits like good glycemic control by having hypoglycemic effect and providing potent antioxidant activity (Bangar *et al*., 2022).

Considering the risk factors and side effects which frequently accompany oral iron supplements and health benefits associated with natural herbal replacement, present study was designed to study different antioxidant parameters effected with the use of beetroot supplements in IDA patients.

MATERIALS AND METHODS

The study was carried out in public sector hospital and was approved from institutional review board. The study type is a Quasi experiment with a pre-test-post-test with control group design. Study was design for 12 weeks. Sample size was calculated on the basis of 5% significance level (two-tailed test), power 80%, standard deviation 10g/dl & a difference of Hemoglobin (Hb) concentration of 5g/dl. This comes out to be 46 patients in each group. Keeping a dropout rate of 20% and rounding it of, 60 patients in each group was required. Total 120 patients attending the outpatient department of medicine, who fulfilled the inclusion criteria were selected and enrolled in the study. Hb cut off value was 11gm/dl according to WHO criteria (WHO 2008). The nature of study was explained to the patients and written consent was obtained. Each follow-up visit was scheduled after 4 weeks. *Beta vulgaris* capsule were purchased and provided to the patients.

Each capsule contains 1500mg of dried beetroot in powder form. After all aseptic measures venous blood samples (10ml) were obtained from median cubital vein. Blood samples were collected in polystyrene standard tubes with ethylene diamine tetraacetic acid (EDTA) as anticoagulant. Serum was separated by centrifuging for about 10 minutes at 3000 rpm. Plasma was then aspirated off and erythrocytes were washed 3 times with 0.9% normal saline solution and centrifuging at 3000rpm after each wash. The hemolyzate was prepared and stored at 2oC for further analyses.

Inclusion criteria

Iron deficiency patients of either sex or ages, that ranging from 18 years to 70 years were recruited for anemic group, diagnoses based on hemoglobin level.

Exclusion criteria

Clinically significant renal and hepatic impairment, diagnosed diabetes mellitus or endocrine disorder, haemoglobinopathies and pregnancy.

Group-A: Control group .60 Non anemic patients
Group-B: Anemic group. 60 IDA patients treated with capsule *Beta vulgaris* 1500mg daily for 12 weeks.

A detailed history was taken and a complete clinical examination was performed at the time of enrollment. General physical examination included, height, weight, body mass index (BMI) and blood pressure. These physical parameters were assessed at each visit.

Hb estimation was done by Cynamethemoglobin Hb method (Drabkin's method) as mentioned by Stoltzfus and Dreyfuss (1988). SOD and GSH-PX activity were measured using commercial kits as described by Paglia and Valentine (1967). SOD and levels were expressed as U/g Hb.Vitamin A was estimated by the method of Bessey OA *et al*. 1946 and was expressed in µg/dl. Vitamin C in plasma was estimated by Natelson (1971) method and was expressed in mg/dl. The vitamin E was measured by the method Baker and Frank (1968) and was expressed in mg/dl. Commercially available kits were used for measurements all the vitamins. The data were analyzed with SPSS software version 22.

STATISTICAL ANALYSIS

The results were analyzed by applying One–way ANOVA and the Tukey test. Data were presented as mean±SD and $p<0.05$ was considered as level of significance.

RESULTS

A total of 143 patients attending OPD were screened for anemia. Out of these, 120 patients (60 patients in both groups) fulfilling the selection criteria were enrolled for study. IDA was diagnosed according to WHO criteria that is, Hb level less than 11g/dl. Study participants in control group had Hb levels greater than or equal to 11g/dl.IDA group was provided with the beetroot supplements for every 4 weeks at a time and were advised to come for a follow-up visit after every 4 week with a repeat advised blood test for refill of beetroot supplements for the next 4 weeks.

After a period of 12 weeks supplementation with beetroot, blood test showed improvements, not only in hematological parameters (results not shown here) but also in terms of oxidative stress. Our study results shows that both enzymatic and non-enzymatic antioxidants improved in every follow up visit (results not shown here). table 2 shows comparison of enzymatic antioxidant status namely SOD and GSH-PX of our study groups.

Table 1: Nutritional value of beetroot (Value per 100 grams (g)) (Bangar *et al*., 2022).

Calcium (Ca) (mg) 16	*Vitamins*	Energy (Kilo Joules, KJ) 180
Iron (Fe) (mg) 0.8	Vit C (as total ascorbic acid) (mg) 4.9	Water 87.6
Magnesium (Mg) (mg) 23	Vit B1 (Thiamine) (mg) 0.031	Protein 1.61
Phosphorus (P) (mg) 40	Vit B2 (Riboflavin) (mg) 0.04	Total Lipid 0.17
Potassium (K) (mg) 325	Vit B3 (Niacin) (mg) 0.334	Ash 1.08
Sodium (Na) (mg) 78	Vit B5 (Pantothenic acid) (mg) 0.155	Carbohydrate (by difference) 9.56
Zinc (Zn) (mg) 0.35	Vit B6 (Pyridoxine) (mg) 0.067	Sucrose 6.76
Copper (Cu) (mg) 0.075	Vit B9 (Folate) (total) (µg)	Fiber (Total) 2.8
Manganese (Mn) (mg) 0.329	Retinol (µg) 0	
Selenium (Se) (µg) 0.7	Beta carotene (µg) 20	
	Vit E (alpha tocopherol) (mg) 0.04	
	Vit K (µg) 0.2	

Table 2: Pre and post supplementation data for enzymatic antioxidants (Expressed as mean ±SD)

	Before supplement	After supplement	Control
SOD (U/gHb)	1716(±182)*	2114(±290)†	1865(±191)
GSH-PX(U/gHb)	44.78(±9.01)*	71.05(±15.12)†	52.41(±8.6)

*p value <0.05 (Significantly low values when compared with control group).
† p value <0.05 (Significantly greater values when compared with pre supplement values).

Table 3: SOD & GSH-PX values according to tertiles of Hb (expressed as Mean ±SD Range) of Anemic group before &after beetroot supplements.

		Hb >7<11 g/dl (Hb Before supplement)	Hb ≥11 <12 g/dl (Hb After supplement)	Hb ≥12 g/dl (Hb After supplement)
Hb g/dl	Mean± SD Range	9.3±1.1(7.3-10.7)	11.7±0.4(11.1-11.9)	12.8±0.2*(12-13.4)
SOD u/gHb	Mean± SDRange	1716(±182)*(729-1284)	2538±101(2015-3241)	3168±114 (2991-3324)
GSH-PX u/gHb	Mean± SDRange	44.78(±9.01)* (30.12-46.2)	60.14±1.8 (53.41-63.1)	68.68±1.6 (60.16-75.53)

*P value <0.05(significantly low when compared with values in highest tertile of Hb)

Table 4: Pre and post supplementation data for non enzymatic antioxidants (Expressed as mean ±SD)

	Before supplement	After supplement	Control
Vitamin C (mg/L)	0.68(±0.12)*	0.89(±0.16) †	0.71(±0.13)
Vitamin E(mg/L)	10±.3(±2.1)*	14.51(±2.4) †	12.01(±2.1)
Vitamin A(mg/L)	0.48(±0.16)*	0.78 (±0.11) †	0.61(±0.13)

* p value <0.05 (Significantly low values when compared with control group).
† p value <0.05 (Significantly greater values when compared with pre supplement values).

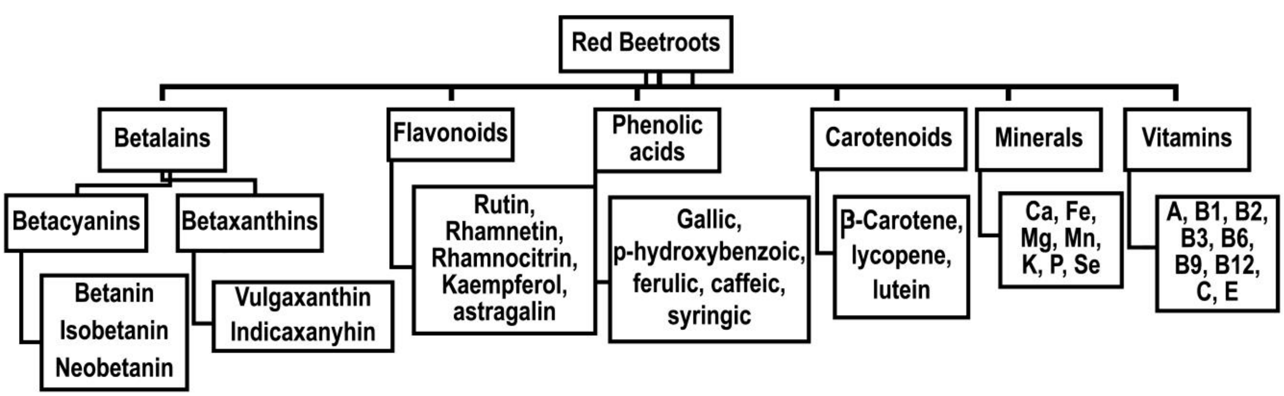

Fig. 1: Schematic presentation of the bioactive compounds present in the Beetroot

Our control group shows a better but non-significant ($p>0.05$) antioxidant level at the start of study when compared with IDA group. A significant improvement ($p<0.05$) of SOD and GSH-PX levels of IDA group were noted after completion of 12 weeks when compared with their baseline values and the control group. Table 3 shows the mean values of SOD and GSH-PX according to tertiles of Hb of anemic group before & after beetroot supplements. The upper tertile of Hb was directly associated with SOD and GSH-PX levels. Highest values of SOD and GSH-PX lies in the highest tertile of Hb ($P<0.05$) indicating a positive correlation of these two antioxidant enzymes with the improvement of Hb levels of our patients.

Table 4 shows non enzymatic antioxidants for the participants. Vitamin C levels were found to be non-significantly high ($p>0.05$) in control group when compared to the values at the beginning of study. Beetroot supplements shows significant improvement ($p<0.05$) when vitamin C levels were compared with the final value after 12 weeks and with the control group. Vitamin E and Vitamin A levels were significantly low ($p<0.05$) for IDA group when compared with control group. Significant improvement were noted for vitamin A and E when their final values after 12 week supplementation were compared with their baseline values and with the control group ($p<0.05$).

DISCUSSION

Vegetables are a known source of antioxidant nutrients and *Beta vulgaris* has been placed as one of most potent antioxidant vegetables (Thiruvengadam *et al*., 2024). They can be considered as an excellent source of energy and nutrients due to their moderate caloric value but high protein contents (Ceclu & Nistor 2020). Betaines are associated with remythylation of homocysteine. This remythylation reaction offers protective effects largely by regulating methionine metabolism by removing homocysteine. Abnormal levels of homocystein are associated with various disorders including tumors and cardiovascular diseases (Arumugam *et al*., 2021).

The beneficial role of antioxidant molecules has been associated with scavenging free radicals, hence preventing the oxidative damages on proteins, lipoproteins and DNA. Studies have mentioned that macromolecular oxidative damages leading to chronic cardiorespiratory diseases, various neurodegenerative disorders and even carcinogenic reactions may be benefited by the antioxidant compounds as in red beets. The results of studies on biochemical properties of *Beta vulgaris* have shown them to be rich in vitamin A, C, E and K along with different types of vitamin B (Punia *et al*., 2022). Although betalains, present in *Beta vulgaris* have not been much studied but still studies have identified their antioxidant activity and potential to clear off free radicals. Antioxidants can be endogenous in the form of various enzymatic compounds like SOD and GSH-PX or they may be exogenous that could be provided as vitamins in fruits, vegetables or by dietary supplements (Averill-Bates, 2023).

In present study, we studied the effect of *Beta vulgaris* supplements on two important antioxidant enzymes SOD and GSH-PX. We measured the OS by assessing the levels of these two enzymatic antioxidants before and after the completion of study in order to evaluate the efficacy of *Beta vulgaris* supplementation. We found that beetroot supplements managed to significantly increase the activity of GSH-PX & SOD hence protecting body cells against damages caused by OS. In a 6 weeks human study by Czlapka and Gut (2023), the effect of daily snacking of *Beta vulgaris* on OS and enzymatic and non-enzymatic antioxidants were studied. They found improvement in OS by an increase in antioxidants. Our results are in accordance to AL-Lamki (2017) and Kozłowska *et al*. (2020) who mentioned that antioxidant activity is related with betalain pigments and reported an improved activity of SOD and GSH-PX. Abd-El-Fattah *et al*. (2021) in his animal model study compared the effects of beetroot extract, beetroot powder and iron polymaltose complex in phenylhydrazine-induced anemic albino Wistar rats. Their study exhibited improvement in Hb and other RBC indices in addition to an increase in antioxidants (GSH and SOD).

Apart from enzymatic antioxidants, the non-enzymatic antioxidants also hold importance by protecting oxidative damages of polyunsaturated fatty acids through their role as hydrogen donor to the lipid peroxyl radicals. Alpha tocopherol (the primary constituent of vitamin E), Ascorbic acid (vitamin C), Beta-carotene (pro-vitamin A) are the utmost non enzymatic natural antioxidants (Didier *et al*., 2023). Various studies have reported *Beta vulgaris* to have a high content of both water and fat-soluble vitamins (Bangar *et al*., 2022).

The results of our studies are in accordance to the researchers who reported improvement in nonenzymatic antioxidants with the use of *Beta vulgaris* (Kozłowska *et al*., 2020, Farhan *et al*., 2024). Sukanya *et al*. (2023) also mentioned a high content of vit. C.

Vitamin E, being a fat soluble vitamin is the major free radical chain terminator in the lipophilic environment (Lu *et al*., 2024). Ascorbic acid is known to be very crucial in maintaining growth and wear and tear phenomenon of body tissues. It is also found to be involved in production of various hormones, neurotransmitters and immune system responses. Antioxidant property of vitamin C is due to its potential to donate electron and its antioxidant potential has been demonstrated in various studies suggesting ascorbic acid as a powerful water-soluble antioxidant vitamin in humans. It decreases the adverse effects of reactive oxygen species (ROS) causing damages to macromolecules such as proteins, lipids and DNA which have shown their relation in development of various types of tumors, cancer, neurodegenerative and CVS disorders. Also, Vitamin C being an antioxidant and reducing agent directly reacts with hydroxyl radicals, superoxide ions and various other lipid hydroperoxides. Moreover, it can also reinstate the antioxidant properties of vitamin E. Vitamin C has been proved to be essential for growth, wear and tear mechanism of tissues and overall human nutrition along with the production of different hormones immune responses and neuro-transmitters (Sun *et al*., 2023)

Among fat soluble vitamins, vitamin A was the first to be recognized. The term Retinoid is collectively used for its active forms. Among them, beta-carotene has a high antioxidant effect and has been found to have a significant role in decreasing redox reactions. This is very helpful in preventing harmful chain reactions resulting lipid peroxidation and DNA damages vitamin A and carotenoids have been found to be associated with various inflammatory and immunological disorders including CVS and respiratory systems (Didier *et al*., 2023).

CONCLUSION

Several studies involving both in vivo and in vitro designs have documented *Beta vulgaris* as an excellent nutritional source for iron and other trace metals and different vitamins particularly Vitamin C. Numerous studies have focused on identification of bioactive compounds present in *Beta vulgaris* and their potential health-beneficial biological properties. Among them hepatoprotective, cardioprotective antioxidant, antihypertensive, antimicrobial, anti-tumor, anti-diabetic, analgesic and anti-inflammatory properties are found to be associated with its bioactive compounds**.** Being a natural compound, researchers are focusing *Beta vulgaris* supplements to improve health and general wellbeing rather than to use synthetic compounds which are often associated with side effects. In present study we focused on the antioxidant potential of beetroot supplement by studying the changes in serum concentrations of selected enzymatic and non-enzymatic antioxidants in IDA patients. We found a positive association for our study parameters probably with betalain compounds of beetroot as suggested in previous studies. Yet further researches are strongly recommended and needed as most of them are done either on cell lines or have used animal models. We strongly recommend *in vivo* and *in vitro* experiments in order to identify and explore various health associated beneficial effects of *Beta vulgaris*. On the basis of the results of the present study, we may conclude that beetroot supplements used in IDA can no doubt improve antioxidant status of anemic patients.

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