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In vitro study on the use of natural iron chelation sources incorporated to the diet for control of iron overload condition

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Abstract
The effect of incorporation of dietary iron chelators to the diet for the control of complications of iron overload was investigated this *in vitro* study. An array of 15 test meals was designed based on four dietary sources, red rice, turmeric, fresh milk and black tea against the standard meal. The designed diets were subjects to *in vitro* digestion and effect on the total phenolic content, potential iron intake and antioxidant activities in the bio-accessible fraction was investigated against the standard meal. The results indicated that test meal that included turmeric, red rice and black tea to be most effective diet from the dietary formulations under study. The study concluded that the total phenolic content, potential iron intake, iron chelation ability and antioxidant activities significantly (p value<0.05) change in the bio-accessible fraction of the diets added with dietary iron chelation sources in a manner that favors control of iron overload.

Keywords: Antioxidant activity, dietary intervention, iron chelation, iron overload, natural iron chelators

1. Introduction
Iron overload is considered as a rising health problem in many industrialized countries [1]. Basically iron overload is classified as primary or genetic iron overload and secondary or acquired iron overload [2]. In primary iron overload condition genetic mutations in HEF gene is found to be the main causative agents while certain non HEF related causes such as hepcidin also is found to be causing genetic hemochromatosis [3]. In secondary iron overload conditions as in pathophysiological conditions such as thalassemia and sickle cell anemia, ineffective erythropoiesis that downgrade action of hepcidin and transfusional iron are found as leading causes of iron overload [4]. Certain risk factors are found to enhance the iron overload condition genetic or otherwise. Heavy consumption of alcohols [5], iron fortified foods and red meat[1] are identified as some of the risk factors. Since the human body is not equipped to control the excess iron in the body, excess iron stored in the body causes damage to vital tissues and organs due to both primary and secondary iron overload. Some of the adverse conditions are cardiac complications, liver complications, endocrine complications such as diabetes mellitus and bone complications such as osteopenia and osteoporosis. Iron overload related complications is the major cause of death in thalassemia patients [6]. When excess iron is found in the body, they tend to produce ROS (Reactive Oxygen species) via Fenton reactions [7]. This result in increase of the oxidative stress in the body and depletion of the antioxidant stores in the cells. Thus, left untreated iron overload state is a fetal condition and means of controlling iron overload condition should receive the same attention as that of other public health conditions such as obesity and diabetes.

At present in pathophysiological conditions related to iron overload such as thalassemia, iron chelation therapy is used as an effective means of controlling iron overload condition. The high cost of the iron chelating drugs causes a great economic burden to the families and nations with higher prevalence of thalassemia and other iron overload related complications. In Sri Lanka, where there is an intermediate prevalence of thalassemia, 5% of the national health budget is spent on the disease management of thalassemia [8]. More than half of the total cost of treatment is on the medications taken by the thalassemia patients and close to 90% of the cost of medications are on iron chelation drugs [9]. In many developing countries, comprehensive iron chelation therapies are not practiced because of the high cost [10]. In addition to the economic burden, there are risk of noncompliance and adverse side effects [11] due to the prolong use of the synthetic iron chelating drugs. It is found that 5% reduction of the treatment cost of thalassemia can be achieved by 25% reduction of the iron chelating drug, deferoxamine [12].

The use of natural iron chelators is proposed as a cheaper and safe therapeutic alternative for the iron overload and related complications [13]. The natural iron chelators are group of compounds found in natural sources that can form complexes with iron. These natural iron chelators should be able to remove free, reactive excess iron in the body, reducing the iron related Fenton reactions in the serum, compete with iron binding proteins such as transferrin and ferritin and reduce deposition of excess iron in vital organs [13]. Plant polyphenols resembling the action of iron chelating drugs are named as 'phytochelators' and they are proposed as one of the most feasible alternative for synthetic iron chelating drugs [14]. Polyphenol compounds such as tannins, phytates, flavonoids, curcumin etc. were found to exhibit iron chelation properties in *in vitro* and *in vivo* studies [15]. Due to toxicological aspects of the polyphenolic extracts, unavailability of less invasive mode of delivery to the body and pressure from pharmaceutical companies the clinical use of phytochelators is not practiced.

It is possible to use dietary constituents that have a strong iron binding ability to suppress the Fenton reactions and other unfavorable effects of iron overload condition [16]. Food sources such as rice bran, turmeric, fresh milk, wheat grass, grapes, berries, pomegranate, green tea and black tea that are rich in phytochelators are considered as good dietary sources for control of iron overload condition [16]. Yet the prospect of a dietary intervention for control of complications of iron overload condition through incorporation of dietary sources of natural iron chelators is not investigated. Therefore the aim of this study is to investigate the potential dietary intervention for control of iron overload condition through incorporation of food sources rich in natural iron chelators to the diet through an *in vitro* assay. Here in the study effect to bioactivity of parameters; iron bio-accessibility, iron chelation ability and antioxidant potential with the incorporation of natural iron chelators to the diet was investigated in favors of control of iron overload. Thus, the findings of the study can be used to identify whether there is a favorable effect for control of iron overload through a dietary intervention to extend the study for further *in vitro* and *in vivo* testing before clinical application.

2. Materials and methods

2.1 Preparation of test meals

The four dietary sources rich in natural iron chelators namely, turmeric, red rice, fresh milk and black tea were selected for the analysis. The standard meal plan per day was prepared without including any of known dietary sources rich in natural iron chelators. Then 15 test meals were formulated by incorporating four dietary sources to the standard diet plan singly and in combinations of two, three and four. The list of constituents of the meals with the individual component weights is shown in table 1. All the food items were purchased from local markets and supermarkets in Malabe area, Colombo, Sri Lanka and meal plans were cooked following general household recipes in Sri Lanka. All the components of the test meals, one meal at a time, was combined and homogenized in the food blender (PHILIPS, Model no- HR2001) to a creamy consistency. After blending each test meal were stored at -1°C in separate containers until further analysis (all the diets were subjected to *in vitro* digestion within one week of preparation).

2.2 *In vitro* gastro-intestinal digestion

The meal plans that were homogenized to stimulate mastication were subjected to stimulated gastro intestinal digestion according to method published in previous study [17] and as modified in previous study [18]. In brief, firstly the homogenized samples were completely thawed by keeping them at room temperature and then 10g of the homogenized test meals were weighted using analytical balance (KERN ABJ-NM/ABS-N, Model – ABS 220-2). Then the samples were mixed with 50ml of 0.9% NaCl and 4.0ml of pepsin (Sigma Aldrich, St. Lousis, MO, USA) solution (40mg/ml in 0.1M HCl). The pH of the mixture was adjusted to 2.0 with an addition of 8 ml of 0.1M HCl. After that the mixture was incubated for 1 h in Labtech shaking water bath (DAIHAN LABTECH, Korea) at 37°C and 100rpm.

For the intestinal phase with dialysis, the dialysis tubing cellulose membrane (SIGMA-ALDRICH, USA, average flat width- 33 mm, MWCO- 12,000 Da) was cut into 15.0 cm segments, both outer and inner surfaces were rinsed with 0.9% NaCl solution and one end was sealed with clips. After that the prepared dialysis bags were filled with 5.5 ml 0.5M $NaHCO_3$ and the other end of the dialysis bag were sealed with clips without leaving any air bubbles inside. Then the sealed dialysis bags were immersed into each gastric digested sample immediately after digestion. Then the samples were incubated for 45 min in the shaking water bath at 37°C and 100 rpm. After this step the pH was brought to 6.5 with addition of $NaHCO_3$ to reflect the transition from gastric phase to intestinal phase. The pancreatin- bile mixture was prepared by dissolving 2mg/ml pancreatin (Sigma Aldrich, St. Lousis, MO, USA) and 12mg/ml bile extract (Sigma Aldrich, St. Lousis, MO, USA) in 0.1M $NaHCO_3$. Then 18 ml of the prepared pancreatin-bile mixture were added to each digesta and were further incubated in shaking water bath for 2 h at 37°C and 100 rpm. At the end of the incubation period, the dialysis bags were removed from the beakers and were carefully rinsed with water and dried using a paper towel. After that the content of the each dialysis bag was transferred quantitatively into measuring cylinders and diluted a final volume of 14 ml with 0.9% NaCl. Finally diluted dialyzed fractions were filtered through Whatman filter paper and were stored at −18°C until analysis.

2.3 The total phenolic content

The total phenolic content of the dialyzed fraction was analyzed using Folin- Ciocalteu method as published in previous work [19] and modified in another work [2]. Firstly 0.5ml of the test sample and 0.1 ml of 0.5N Folin- Ciocalteu (FC) reagent (MP Biomedicals LLC, France) were mixed in a glass test tubes and were incubated at dark in room temperature for 15 min. Then 7.5% sodium carbonate (HiMedia Laboratories pvt. Ltd., India) was added to the mixture and was incubated again at dark in room temperature for 2 h. The absorbance was measured at 760nm using UV/VIS (Ultra Violet/ Visible) spectrometer (Thermo Scientific GENESYS 10S Series). The standard curve of gallic acid (Duksan pure chemicals, Korea) was prepared at the concentration range 0-250 ppm and the total phenolic content was expressed as µg gallic acid equivalent (GAE) per gram of diet.

2.4 Iron bio-accessibility

The iron content of the dialyzed fraction of the test meals was estimated following the previously published procedure [21] using Atomic Absorption Spectrometric method after subjecting to wet ashing. Concisely, 0.5ml of the test sample was introduced to 50ml beaker and then to that 5ml of 65% nitric acid was introduced. After that the samples were

incubated in water bath at 95°C for 2 h. Then 2.5 ml of 65% HNO_3 was added and were incubated in water bath at 95°C until a clear solution appears. Once a clear solution appeared, the samples were removed from the water bath, cooled and was diluted 1:1 using deionized water. The diluted samples were filtered through syringe filters (Thermo Scientific, PVDF- 0.45µm) and were stored until analysis. The iron content of the ashed samples was analyzed using Flame Atomic Absorption spectrometer (Thermo Scientific iCE 3500) under the operating conditions; wave length- 248.3 nm, flame- Air/ Acetylene oxidant, band pass - 0.2 nm. The iron standard curve was prepared at the concentration range 1-5 ppm using ferrous ammonium sulphate (Research-lab Fine Chem Industries, India). A blank solution contained 0.5ml of deionized water instead of test sample.

2.5 Ferrous chelation ability

The iron chelation ability of the dialyzed fraction of the test meals were investigated through the ferrozine assay following the previously published procedure [22]. Firstly 1 ml of the test sample was introduced to the glass test tube and then to that 1 ml of 0.1mM of ferrous sulphate and 0.25mM of extra-pure (Min. Assay 97%) sodium 4-[3-pyridin-2-yl-5-(4-sulfophenyl)-1,2,4-triazin-6-yl]benzenesulfonate (Ferrozine) (Research-lab Fine Chem Industries, India) were introduced and shaken well. The mixture was allowed to stand in room temperature for 10 minutes. Then the absorbance of the samples and the control were measured at 562 nm against the blank (deionized water) using the UV/VIS spectrometer. The control contained 1 ml of deionized water, 1 ml of 0.1 mM $FeSO_4$ and 1 ml of 0.25 mM of ferrozine. The ferrous chelation ability was expressed as a percentage using the Eq. 1.

Equation 1- Ferrous chelation ability

Chelating activity% = ((Absorbance of control – Absorbance of sample)/ Absorbance of control) X 100

2.6 Free radical scavenging ability

Free radical scavenging ability of the dialyzed fractions were performed using the DPPH assay according to the previously published work[23] with a slight modification as proposed in another work[24]. Briefly, 0.5 ml the test samples were added to the clean, dry test tubes and to that 1 ml of methanolic 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma Aldrich, St. Lousis, MO, USA) was added and shaken well for 10 seconds. After that the samples were left at dark for 45 minutes and the absorbance were measured at 517 nm using UV/VIS spectrometer against the blank (methanol). The 2mM and 1mM ascorbic acid (VWR International, Belgium) were used as positive controls. The free radical scavenging ability was calculated using the formula Eq. 2.

Equation 2- Free radical scavenging ability

Free radical scavenging activity% = ((Absorbance of control – Absorbance of sample)/ Absorbance of control) X 100

The control sample contained 0.5ml of methanol and 1 ml of 0.1mM DPPH.

2.7 Total antioxidant capacity

The total antioxidant capacity of the dialyzed fraction of the test meals was analyzed according to the previously published method[25]. The tubes containing 0.3 ml of the test sample, 3 ml of the reagent solution (28 mM of sodium phosphate (Research Lab Fine Chem Industries, India) and 4mM ammonium molybdate (Alpha Chem Laboratories, Ambala) in 0.6M sulphuric acid) were incubated at 95°C for 90 min in water bath. Then the mixture was cooled to room temperature and the absorbance of the each sample was measured at 695nm using UV/VIS spectrometer against the blank (deionized water). The gallic acid standard curve was prepared in the range 50-200 ppm and the total antioxidant capacity of the test samples were expressed as µg gallic acid equivalent (GAE) per gram of diet.

2.8 Multivariate analysis of variance

MANOVA (Multivariate analysis of variance) was performed using SAS version 9.0 (SAS Insitute Inc, USA) to identify the most effective test meal for control of iron overload condition under *in vitro* analysis. The categorical variable, 15 test meals were considered as the independent variable and five variables under analysis (iron bio-accessibility, ferrous chelation ability, free radical scavenging ability, total antioxidant capacity and total phenolic content) as the dependent variables. The MANOVA was performed to test the hypothesis at 95% confidence level.

H_0 = There is no contrast in variables between the standard meal and the test meal

H_1= There is a contrast in variables between the standard meal and the test meal

In the diet plans with a significant contrast the Wilks' Lambda value was taken as the measure of the effectiveness. The meal plan with lowest Wilks' Lambda value was taken as the most effective diet plan as per the interpretation of Wilks' Lambda value provided in previously studies [26].

2.9 Statistical analysis

All data from the study were presented as mean ±SD of two replicates and the difference between the standard and the test meals were analyzed in duplicates using two sample t-test under 95% confidence interval. Pearson correlation co-efficient analysis was used to test the relationship between total phenolic and the iron bio-accessibility and antioxidant activities. All the analyses were conducted using MINITAB 17 and MS Excel 2013.

Table 1: The constitutes and component weights of test meals

	Sample ID	Bread/ g	Dhal curry/ g	Chicken curry / g	Green beans / g	Pol sambol/ g	Vegetable salad/ g	chili paste/g	Banana/g	Fried white rice / g	White rice / g	Red rice /g	yellow fried rice /g	Yellow red rice /g	Yellow white rice /g	Fresh milk /ml	Milk tea /ml	Black tea /ml
Standard meal	D0	100	86	233	56	56	56	15	116	220	220							
Single chelators	D1	100	86	233	56	56	56	15	116	220	220					375		
	D2	100	86	233	56	56	56	15	116	220	220							375
	D3	100	86	233	56	56	56	15	116			440						
	D4	100	86	233	56	56	56	15	116						440			
Combination of two chelators	D5	100	86	233	56	56	56	15	116	220	220						375	
	D6	100	86	233	56	56	56	15	116					440				
	D7	100	86	233	56	56	56	15	116				220		220	375		
	D8	100	86	233	56	56	56	15	116				220		220			375
	D9	100	86	233	56	56	56	15	116			440						375
	D10	100	86	233	56	56	56	15	116			440				375		

Combination of three chelators	D11	100	86	233	56	56	56	15	116				220		220		375	
	D12	100	86	233	56	56	56	15	116			440					375	
	D13	100	86	233	56	56	56	15	116					440		375		
	D14	100	86	233	56	56	56	15	116					440				375
Combination of four chelators	D15	100	86	233	56	56	56	15	116					440			375	

3. Results and discussion

Many dietary sources were found to exhibit characters in favor of control of iron overload condition [16]. Prior to device of a dietary intervention it is necessary to identify whether the bio-accessible fraction of the dietary sources exhibit the ability to control iron overload condition and the effect of other components of the diet on the action of those dietary sources. Decrease or increase of *in vitro* potential uptake (dialyzed fraction) of iron compared to the control and iron chelation ability, free radical scavenging ability, total antioxidant capacity and total phenolic content of the dialyzed fractions are shown in table 2.

Table 2: Percentage decrease/increase of the potential iron intake, total phenolic content, iron chelation ability, free radical scavenging ability and total antioxidant capacity of the dialyzed fractions of the test meals

No	Dietary source incorporated	The total phenolic content (µg GAE/g)[a]	Percentage Increase/ (decrease) of the potential iron intake	Percentage of iron chelation ability	Free radical scavenging%	The total antioxidant capacity (µg GAE/ g)[a]
C1	Standard meal	60.71 ±4.70	-	5.92 ±0.34	18.33 ±0.35	26.43 ±1.19
P15	Turmeric +red rice +fresh milk+ black tea	175.06 ±5.72*	37.77%	15.48±0.67*	52.94 ±1.23*	33.81 ±0.38
P14	Turmeric +red rice + black tea	238.76 ±10.54*	(39.88%)*	91.45 ±0.22*	76.16 ±1.25*	29.77 ±0.16*
P13	Turmeric+ red rice + fresh Milk	140.95 ±4.46*	(18.78%)	15.09 ±0.34*	70.92 ±0.33*	25.77 ±0.78
P12	Red rice + fresh milk + black tea	219.60 ±15.70*	(56.69%)*	0.47 ±0.00	77.89 ±2.02*	26.56 ±0.52*
P11	Turmeric + fresh milk +black tea	145.22 ±10.84	(69.69%)*	92.58 ±0.89*	24.92 ±0.12*	234.88 ±4.73*
P10	Red rice + fresh milk	63.46 ±1.69	(62.84%)*	1.34 ±0.11*	61.96 ±0.82*	19.21 ±1.41
P9	Red rice + black tea	44.17 ±3.78	(40.92%)*	3.32 ±0.22	73.53 ±4.19*	18.37 ±1.69*
P8	Turmeric + black tea	100.40 ±2.65	(14.40%)*	84.99 ±0.89*	55.87 ±0.74*	125.72 ±5.30*
P7	Fresh milk + turmeric	38.98 ±0.84	(57.49%)*	4.49 ±0.11	55.99 ±2.96*	56.83 ±0.53*
P6	Red rice + turmeric	44.91 ±2.62*	(28.04%)	85.31 ±0.22*	43.31 ±0.80*	89.56 ±6.61*
P5	Fresh milk + black tea	1.85 ±0.16	(60.57%)*	(3.00 ±0.45)*	12.02 ±0.20*	16.78 ±1.09*
P4	Turmeric	103.66 ±5.42	(37.24%)	94.47 ±0.45*	53.78 ±1.16*	296.64 ±14.05*
P3	Red rice	53.24±0.63	ND	6.95 ±0.45	27.16 ±0.74*	83.48 ±6.08*
P2	Black tea	112.19 ±3.49	(47.42%)*	80.33 ±0.11*	15.48±1.61	18.55 ±0.88*
P1	Fresh milk	(43.71 ±0.75)*	(63.04%)*	90.13 ±0.34*	12.16±1.48	19.38 ±1.61*

Values are presented as mean ±SD; n=2, [a] µg gallic acid equivalent per gram of diet (wet basis), 'ND' means not detectable, negative values indicated within brackets are disregarded as experimental errors expect in the column for effect on potential iron intake and the * indicated the values that showed a significant difference (p value<0.05) against the standard meal for values in one column.

In the investigations for alternative and novel iron chelating agents, natural or otherwise, the effect of these chelators on the dietary intake of iron is mostly overlooked. But as iron overload results in increase in the gastro-intestinal absorption of iron[4] it is essential to investigate on the effect of incorporation of natural iron chelators to the diet on the potential dietary intake of iron. In the analysis it was assumed that undigested test meals contained same amount of iron as that of the standard meal as the increase of iron content due to addition of dietary iron chelating sources is negligible. The results showed at 95% confidence level that potential iron intake had decreased within a range of 14.40% to 69.69% compared to that of the standard meal. But no particular pattern was observed in the decrease of the potential iron intake with the incorporation of the food sources rich in iron chelators. The decrease of potential intake of iron (63.04%) from the diet that contained fresh milk was more than that of the other test meals that contain only one source of chelators. Calcium in milk is found to compete with iron and inhibit the release of iron from the food systems in the lumen of the small intestine [27]. Lactoferrin in milk and in synovium fluid was found to decrease the potential iron intake in adults [28].

The test meal containing the turmeric, black tea and fresh milk showed the highest decrease (69.69%) in potential iron intake. Turmeric, when added to the diet alone, showed no significant decrease of the iron bioavailability against the control diet. Curcumin, active group in turmeric, was found to have iron chelation ability by binding with Fe^{2+} and Fe^{3+} in the body fluids such as serum [29]. But very little evidences exist on the effect of curcumin on iron in food matrix. In this study, it was observed that curcumin when present alone, had an insignificant effect towards the decrease of iron in the food matrix but once it was complemented with other sources rich in iron chelators it had an enhanced effect on the decrease of potential iron intake. This enhanced effect on decrease of iron bioavailability in both turmeric and black tea could be attributed to presence of lactoferrin in milk. Availability of polyphenols decreases considerably in the process of digestion[18]. But lactoferrin has a protective effect towards polyphenols[30]. Thus, in turn in the presence of lactoferrin, polyphenols in turmeric and black tea reduces the iron bioavailability in higher extend than when present alone.

The mode of action of drugs used for control of iron overload is iron chelation. The test meals 7, 9, 10 and 12 that showed a decrease in iron chelation ability compared to that of the standard meal contained red rice in the diet. Phytic acid (inositol hexakisphos phate /IP6) extracted from the rice bran was reported to be the most potent iron chelator in the nature [31, 15]. The findings of the current study depicted that even though phytic acid extract showed strong iron chelation ability, in the presence of other constituents of red rice and food items in the meal its chelation ability was hindered. The processing of grains such as steaming, soaking and heating destroy or eliminate phytic acid [32] and phytic acid is a fiber associated compound [33]. So fibers found in red rice could limit the bioavailability of phytic acid.

The highest chelation ability was observed in the diet containing only turmeric. The high chelation ability of the turmeric could be direct result of its major active compound, curcumin. Both *in vivo* and *in vitro* studies have reported

curcumin to be a strong iron chelator with high affinity to both Fe^{2+} and Fe^{3+} [29, 34]. The chelation ability seemed to be higher when the sources rich in iron chelators (except red rice) were incorporated to the diet separately not in combinations. This hindrance on the chelation ability could be due to the fiber in red rice or due to some polyphenol found in red rice whose action is enhanced with the protective effect of lactoferrin in fresh milk. But further investigations are needed to identify the mode of action and functional group responsible for hindrance of the chelation action by red rice. Most of the commercial iron chelation drugs shows a higher affinity to Fe^{3+} because such agents are more specific in their metal chelation. But as chelatable iron pool contain both Fe (II) and Fe (III) [35] here the affinity to Fe^{2+} was investigated using ferrozine assay. The reduction Fe^{2+} control the free radical mediated damages to the cells by supressing Fenton reactions as well.

The chelatable iron loosely bind with molecules such as albumin, citrate and acetate in a manner than they retain the ability to form ROS through Fenton reactions and Haber-weiss reaction [35] and the synthesis of ROS can be accelerated by redox recycling of ferrous and ferric irons[36]. Thereby in iron overload condition free radical mediated damages are inevitable and considerably deplete the antioxidant potential in the body [37]. Provision antioxidants along with iron chelating agents can mobilize iron from soft or hard tissues and assist in removal of excess iron from the body [38]. The antioxidative potential of digesta can be measured using various assays. Here in this study free radical scavenging ability and the total antioxidant assay were used for investigation of total antioxidant effect.

The free radical scavenging ability of the digesta was investigated using the DPPH radical scavenging assay. The test meals containing combination of fresh milk, red rice and black tea showed highest percentage of inhibition of 77.89% closely followed by diet containing combination of black tea, turmeric and red rice 76.16% In test meals that contain black tea, fresh milk and combination of black tea and fresh milk in each showed less %inhibition than the standard meal. Lactoferrin in milk and black tea in comparison to green tea has very little antioxidant activity [39].

Measuring the total antioxidant capacity of sample is useful in determining which antioxidant activity has the most contribution toward the total antioxidant activity. The test meal 4(296.64 µg GAE/l), 11 (234.88 µg GAE/l) and 8 (125.72 µg GAE/l) showed high antioxidant capacity compared to test meals. These three diet plans showed high iron chelation ability where it was reported that iron chelation ability of test meal 4, 11 and 8 to be 94.47%, 92.58% and 84.99% respectively. This clearly indicated that iron chelation ability to be the most prominent antioxidant activity in the bio-accessible fraction.

The total phenolic content was considered as a crude measure of the amount of the natural chelators remained after digestion. The bio-accessible polyphenol amount was found to be spread within a wide range of 1.85 to 236.76 µg GAE per gram of diet. The potential iron intake, iron chelation ability and total antioxidant activity showed no significant (p value<0.05) correlation with total phenolic content. Thus, the action of these three parameter is a combined function of many components in the diet and matrix effect and cannot be only attributed to polyphenols even though most of the natural iron chelators are polyphenols. Previous study [40] also reported that the iron chelation ability of natural chelators present in food systems is a function of many known and unknown components in the food system and the matrix effect (Eg- pH and redox potential) [16, 40]. But the total phenolic content and the free radical scavenging ability of the diets showed a strong correlation (r^2 – 0.58, p value<0.05) at 5% level of significance. But according to the linear regression analysis (figure 1) the total phenolic content can only predict the free radical scavenging ability up to 31.3% ($R^2_{adj.}$ – 31.3%). This indicated that the free radical scavenging ability of the diet was a function of the total phenolic content but other components in the diet too contribute towards the free radical scavenging ability. The finding of similar studies also indicated that free radical scavenging ability of natural chelator was due to phenolic compounds present in the chelators [40, 22].

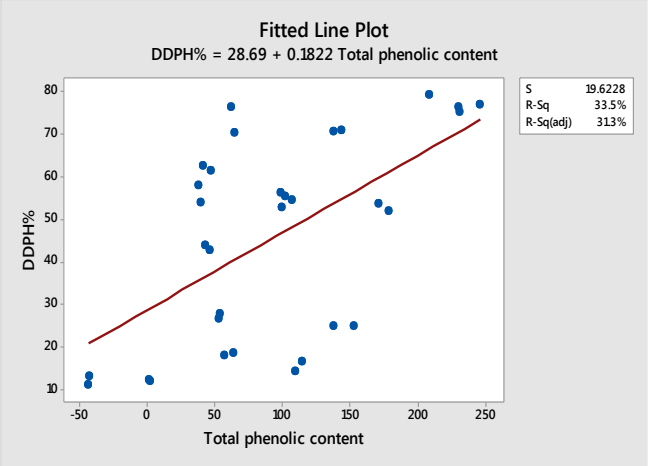

Fig 1: Correlation analysis between the total phenolic content and free radical scavenging ability

The free radical scavenging ability (DPPH%) of the bio-accessible fraction of the diets showed a significant (p value<0.05) positive linear correlation to the total phenolic content of the bio-accessible fraction of the diet.

The MANOVA was used to identify the most effective test meal for control of the iron overload condition based on the five parameters tested. The test meal 14 (Wilks' lambda value-0.0001637) that contained turmeric, red rice and black tea was found to be the most effective in control of iron overload condition under the six parameters investigated. When considering about the individual parameters for the test meal 14 it showed a high iron chelation ability (91.45%), free radical scavenging ability (76.16%) and the total phenolic content (238.76 µg GAE per gram of diet). From the findings of the study it could be seen that dietary formulations displaying high phenolic content, high iron chelation ability and high free radical scavenging power in the bio-accessible fraction has the most effect towards the control of iron overload condition. That was observed for the diet containing turmeric, red rice and black tea out of the four food sources rich in iron chelators investigated under the *in vitro* conditions in the present study.

4. Conclusion

It can be concluded that the bio-accessible fraction of the test meals display characters that are favorable for control of the complications of iron overload. Thus, it is possible to use dietary sources rich in natural chelating compounds in the diet to control the complications of the iron overload. From the four dietary sources under study it was concluded that the

most effective dietary intervention represents the meals containing high amount of turmeric, red rice and black tea. Based on the findings of the present study, it is possible to establish dietary guidelines to control iron overload condition and use it as a supplementary or alternative treatment for expensive iron chelation therapy. Further studies are needed to affirm the finding of the present study ideally with the use of cell-lines and experimental animal models.

5. Declaration of interest
None

6. Author contribution
All authors contributed equally in the study

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