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Iron-Chelating Property of Phytic Acid in Thalassaemia: An *In vitro* Study

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AB, SC and TKC designed the study. Authors AB and DC performed the statistical analysis. Authors AB and SC wrote the protocol. Authors AB and DC wrote the first draft of the manuscript. Authors AB and TKC managed the analyses of the study. Authors AB and TKC managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The aim of this study is to report an oral iron chelator phytic acid (PA), on blood samples from different types of thalassaemic patients of various age groups and physical conditions.
Study Design: The *In vitro* iron chelating effect was evaluated by ferritin assay using ELISA.
Methodology: Blood from the iron-overloaded 30 Thalassaemic patients of different age groups, body weights and heights were collected and the serum was separated. Patients without any history of blood transfusion and chelation therapy were taken as control group. The ELISA based ferritin assay was performed with standard phytic acid (40% water solution, Fluka), using Desferrioxamine (DFO) as control (0.5g Deferoxamine mesylate USP, Novartis, USA). The serum ferritin levels were recorded in two different conditions (treated with DFO and PA) at different time intervals (10, 30 and 60 min) by measuring absorbance at 450 nm.
Results: The Kolmogorov-Smirnov test of patient samples in two different conditions showed that the ferritin concentration, treated by DFO and PA, was significantly decrease

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in PA treated group compared to DFO, when used in equivalent concentrations, at intervals of 10 min (*P*=0.760),30 min (*P*=0.537) and 60 min (*P*=0.055).
Conclusion: The common iron chelators DFO or Deferiprone used as monotherapy may lead to transient and incomplete removal of iron, while PA showed a more complete and sustained removal of ferritin due to specific chemical binding at a wider pH range. However, further trial is required to establish its maintenance dose, comparative efficacy and mechanism of action.

Keywords: Iron chelator; phytic acid; ferritin; thalassaemia; desferrioxamine.

1. INTRODUCTION

Thalassaemia is one of the most common genetic disorders worldwide. About one-tenth of Indian patients of thalassaemia are found in West Bengal (Eastern India) of which 11-13% are carriers [1]. In most of the cases blood transfusion is the only management for their survival, which causes 'iron accumulation', one of the leading causes of clinical deterioration and even deaths. The excess iron is deposited in the form of ferritin, which needs to be removed from the patient's body by iron-binding drugs called as *chelators* or *iron chelators*. Ferritin is a soluble globular protein complex consisting of 24 protein subunits and is the main intracellular non-toxic iron storage protein of our body. It can be detectable in patient's serum when present in higher concentration, due to transfusional iron-overload. This form of iron may cause oxidative tissue damage and increased iron uptake into several vital organs.

With the introduction of iron chelators desferrioxamine (DFO) in the 1970s, death from iron overload has diminished dramatically [2,3]. Cohorts of patients born between the 1960s and 1990s showed progressively improved survival rate, mainly as a result of decreased cardiac toxicity. Successful iron chelation is therefore essential for the optimal management of these patients [2,3,4,5].

Although DFO has been the major iron-chelating treatment of transfusional iron overload patients, compliance is a major hindrance in achieving optimal therapeutic results. The iron chelators when administered parentally may cause local irritation and swelling, after sub-cutaneous infusion. The oral iron chelation with deferiprone (L-1: Kelfer) since 1987, is found to be useful but showed poor efficacy when used alone, as compared to oral DFO, used as routine therapy. Moreover, it can cause agranulocytosis, neutropenia and zinc deficiency in diabetic thalassaemic patients [6], while toxicity involving joints has been reported frequently in Indian patients [7]. However, a new oral chelator deferasirox (Asunra) introduced in 2007, showed several complications in transfusion-nondependent thalassaemics, including nausea, vomiting, abdominal pain, diarrhea, skin rashes in patients with iron overload problem from dietary intake [8]. Ferritin-iron may be released during boiling of the food material that contain phytic acid as well as during *in vitro* gastric digestion, as *In vitro* ferritin-iron is efficiently released from the ferritin molecule during cooking and at gastric pH [9].

Phytic acid (PA), a naturally occurring phytochemical has a wide range of pharmacological properties including antioxidative, antiinflammatory, anticancer and iron-chelation. Thus, the aim of this study is to introduce a nontoxic, cost effective, oral iron chelator of natural origin that can easily mobilize as iron-chelator complex, with a property of hexidentate binding of iron ions. The present study was conducted to evaluate the clinical efficacy of the herbal compound PA as new oral iron chelator, alternative to the available drugs [10]. Earlier reports showed that the PA may decrease the plasma cholesterol and triglycerides [11], as well the

bioavailability and therefore, increases toxicity of heavy metals like cadmium and lead [12,13]. Moreover, PA may reduce the *In vitro* cell proliferation in different cell lines, including erythroleukaemia and human mammary cancer cells [14], with *In vitro* iron chelating property [15].

2. MATERIALS AND METHODS

2.1 Selection and Description of Participants

Patients with HPLC-screened documented beta thalassaemia and HbE-beta thalassaemia genotypes were included in this primary analysis. Total 30 patients of age group of more than one year with steady-state Hb values (greater than 5.5 gm/dl, unrelated to transfusion) were enrolled. Both male and female patients included in this study were in the age group of 4 to 34 years (average age is 19 years). Patients without any history of (H/O) blood transfusion and chelation therapy were taken as control group; whereas the patients with H/O blood transfusion were taken as group I and without any H/O of blood transfusion as group II (Table 1). All groups (control, group I and II) were treated with DFO and PA in different concentrations, separately. The ethical permission was taken from all the patients recruited for sampling.

2.2 Technical Methods used

The chelation effect of PA (~40% in water solution, Fluka, USA) was investigated by ELISA ferritin chelation method (Accu Bind, ELISA Micro wells, Monobind Inc. Lake Fprest, CA92630, USA.) using plasma from the Thalassaemic patient's, and DFO (Novartis, 0.5g deferoxamine mesylate USP) as control. The serum was separated from the whole blood of thirty Thalassaemic patients and the pretreated serum (without addition of DFO and PA) ferritin level was recorded from each patients sample. According to Accu Bind ELISA Ferritin Assay (human kit), approximate serum ferritin reference ranged for 16-220 ng/ml for normal adult males, and 10-124ng/ml for female. Patients of all the groups were iron overloaded, according to serum ferritin reference ranges, and their ferritin values are presented in Table 2.

Serum with ferritin level of 461-2813ng/ml was mixed with DFO (0.0012gm/ml) and PA (0.003 ml/ml) separately, and incubated for 10, 30 and 60 min. The serum ferritin level was then recorded, using Accu Bind ELISA Ferritin Assay kit (human kit), by measuring the OD at 450 nm to calculate the concentration of ferritin.

2.2.1 ELISA ferritin assay (accu bind)

About 25µl of patient samples and controls were taken in each micro well and added with 100µl of ferritin biotin reagent. The micro plate was then gently swirled for 20-30 sec, and incubated for 30 min at room temperature. After incubation, the contents of the micro wells were discarded and washed 3 times with 300µl of wash buffer. Then 100µl of ferritin enzyme conjugate was added into each well and incubated for 30 min at room temperature.

The contents of each of the micro-wells were then discarded and washed 3 times with 300µl of wash buffer, and added with 100µl of working substrate solution and kept for 15 minutes at room temperature. Finally, 50µl of stop solution was added to each well and mixed gently for 15-20 sec. the optical density was read at 450 nm in a Elisa reader to calculate the results.

Table 1. Profile of patients

Patient Sl. No.	Types of Thalassaemia	Group	Sex	Age (year)	Pretreated ferritin values (ng/ml)
P1	Beta	Control	Male	19	637
P2	Beta	Control	Male	16	708
P3	Beta	Control	Female	34	1637
P4	E-Beta	Control	Male	5	628
P5	E-Beta	Control	Male	9	828
P6	Beta	Group I	Male	9	655
P7	Beta	Group I	Male	4	700
P8	Beta	Group I	Female	10	525
P9	Beta	Group I	Male	5	795
P10	E-Beta	Group I	Male	11	850
P11	E-Beta	Group I	Male	14	500
P12	Beta	Group I	Male	6.6	515
P13	Beta	Group I	Male	9.6	2500
P14	E-Beta	Group I	Male	12.6	756
P15	Beta	Group I	Male	4	625
P16	Beta	Group I	Male	5	551
P17	Beta	Group I	Female	5	1125
P18	E-Beta	Group I	Male	10	721
P19	E-Beta	Group I	Male	11	1778
P20	E-Beta	Group I	Female	3	1352
P21	Beta	Group II	Male	14	501
P22	Beta	Group II	Female	6.6	461
P23	E-Beta	Group II	Female	19.6	810
P24	Beta	Group II	Male	8	972
P25	E-Beta	Group II	Male	11	576
P26	E-Beta	Group II	Male	18	1467
P27	E-Beta	Group II	Male	11	2813
P28	E-Beta	Group II	Female	6	740
P29	E-Beta	Group II	Female	27	1005
P30	E-Beta	Group II	Female	8	990

2.2 Statistical Analysis

Quantitative data were presented as difference between per-treatment value and treatment after PA and DFO. Results from spectroscopic data were compared among the three time intervals (10, 30 and 60 min). The statistical significance of two different conditions and time intervals were analyzed by utilizing K-S test. The decreasing *P*-value <0.055 (for 60 minutes) of PA was considered statistically significant, compared to the DFO control.

3. RESULTS AND DISCUSSION

We observed the decrease of serum ferritin level, recorded by measuring OD at 450 nm, in different time intervals (10, 30 and 60 min). The results of Kolmogorov-Smirnov test of the patient sample in two different conditions revealed that the mean number of ferritin chelation by PA is significantly higher than that of DFO (Fig. 1), when added in equivalent concentrations and studied up to 10, 30 and 60 min of reaction (Table 2).
Kolmogorov-Smirnoff (KS) test was performed by taking the difference of the pretreated

value. It can be concluded from the p-value of each log normal distribution of data that chelation by DFO and PA have different effects in patients. The result of time-wise *P*-values in two different conditions treated with DFO and PA was compared. Lower *P*-value 0.055 (95% level of confidence of rejecting the null hypothesis) and the higher distance 0.333 between them was shown to be suitable for PA at 60 min interval (for 10 min the distance was 0.1667 with *P*-value 0.760 and for 30 min the distance was 0.2000 with *P*-value was 0.537). So the decrease in of ferritin concentration was maximum in PA, as the time interval increases.

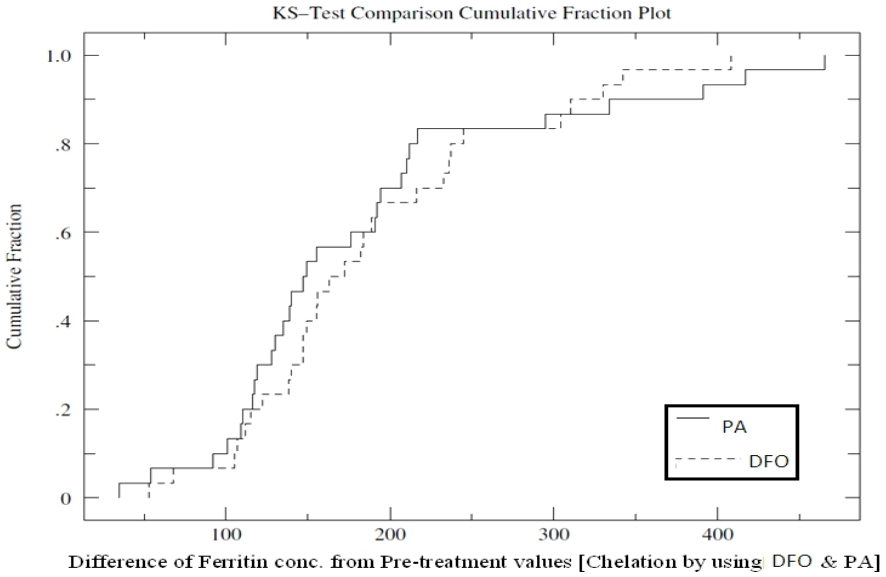

Fig. 1. Plots for 10 minutes values

The cumulative fraction plots were performed by using KS programme and the maximum difference between the distributions was determined to calculate the p-values for the validation of the hypothesis. When the time interval increased the p-value decreased. The results presented in Figs. 1, 2 and 3 showed that the distance was increased, which indicated that the PA is a better chelating agent than DFO, as the distribution line for PA is larger than DFO. While the results depicted in Fig. 4 showed the decreasing p-value of each pair of PA and DFO in all the three time intervals (10, 30 and 60 min). The decreased p-value leads to the increased level of confidence for rejecting the null hypothesis

In this preliminary study only ferritin chelation effect of 'PA' *was* evaluated *In vitro* in a battery of thalassaemic serum samples (both major and intermediate). The study showed that the PA can be used as a better iron chelator, as it can decrease the serum ferritin concentration significantly in all the samples studied. Moreover, *In vitro* decrease of ferritin level was observed in 30 selected cases, including both major and intermediate thalassaemia from male and female patients. We have used the PA in samples of thalassaemic patients of different age groups and observed its effect up to 60 min along with DFO, as control.

PA is a natural phytochemical with several pharmacological properties including antioxidative, antiinflammatory, anticancer and iron-chelation. In this study, PA was investigated for chelation in thalassaemic serum *In vitro* for the first time to show its potential as a better ferritin remover, compared with the widely used iron chelator, DFO.

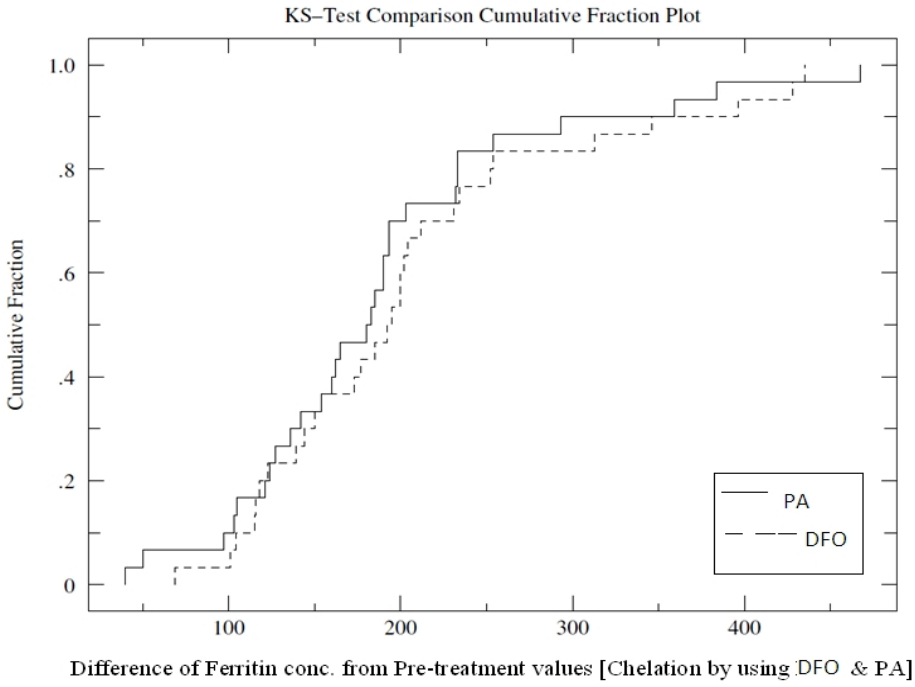

Fig. 2. Plots for 30 minutes values

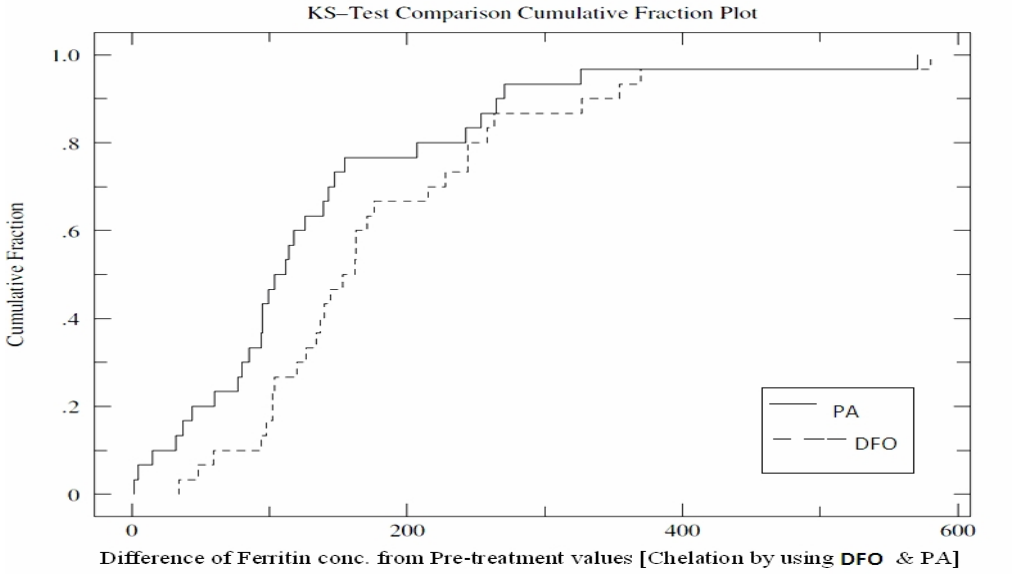

Fig. 3. Plots for 60 minutes values

Table 2. **Decreasing serum ferritin values (ng/ml) in two different conditions (treated with PA and DFO) from pretreated ferritin values at intervals 10, 30 and 60min.***

Patient Sl. No.	Pretreated ferritin value (ng/ml)	DFO treated Ferritin value (10 min) (ng/ml)	PA treated ferritin value (10 min) (ng/ml)	DFO treated ferritin value (30 min) (ng/ml)	PA treated ferritin value (30 min) (ng/ml)	DFO treated ferritin value (60 min) (ng/ml)	PA treated ferritin value (60 min) (ng/ml)
P1	637	54	53	40	69	2	34
P2	708	35	68	50	101	5	48
P3	1637	92	105	97	104	15	59
P4	628	101	107	103	115	32	94
P5	828	109	112	105	116	37	98
P6	655	110	115	121	118	44	102
P7	700	116	122	124	123	60	102
P8	525	117	138	127	139	77	104
P9	795	119	140	136	144	80	120
P10	850	128	147	142	150	85	127
P11	500	130	147	154	154	94	134
P12	515	135	149	160	173	95	137
P13	2500	139	155	162	177	95	140
P14	756	140	156	165	185	99	144
P15	625	147	163	180	192	104	153
P16	551	149	172	183	195	112	162
P17	1125	155	182	185	200	114	163
P18	721	176	184	1	200	118	163
P19	1778	191	189	190	202	126	171
P20	1352	192	192	193	204	139	176
P21	501	194	216	193	212	143	215
P22	461	207	233	203	231	147	228
P23	810	210	236	232	234	155	244
P24	972	212	237	233	252	265	244
P25	576	217	245	233	254	207	258
P26	1467	295	304	254	313	243	263
P27	2813	334	310	293	346	254	327
P28	740	391	330	384	396	271	354
P29	1005	417	342	359	428	326	370
P30	990	465	408	467	435	531	380

**DFO and PA treated Decreasing serum ferritin values (ng/ml) are the difference ferritin values from pretreated ferritin values at different time intervals 10, 30 and 60 min*

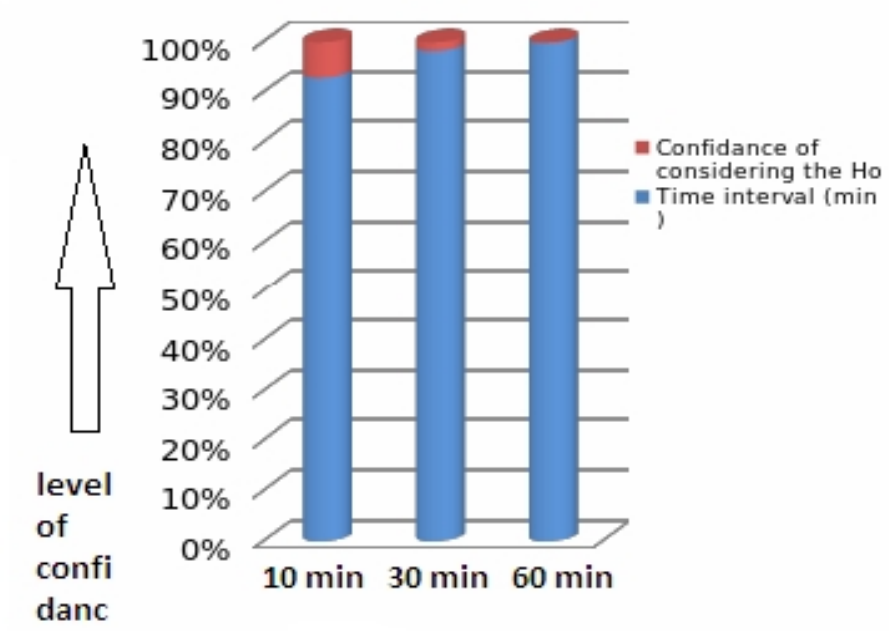

Fig. 4. Plots for 10, 30 and 60 minutes values

Here we have also discussed the *In vitro* mechanism of ELISA based ferritin (human) assay. The streptavidin coated micro plate well was exogenously added with biotinylated monoclonal anti-ferritin antibody. Upon mixing antibody and serum sample containing the native antigen, leads to a reaction that results an antibody-antigen complex. After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation or aspiration. Another antibody (directed at a different epitopes) labeled with horse radish peroxidase (HRP) is added to form an enzyme labeled antibody-antigen-biotinylated-antibody complex on the surface of the wells. The excess enzyme was then washed by an wash buffer and added with a substrate tetramethylbenzidine (TMB) that produce a colour, which was measured by a micro plate reader. The enzyme activity on the well is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve was generated from which the antigen concentration was measured.

The patient serum were mixed with DFO and PA separately and incubated for 10, 30 and 60 min and the serum ferritin level was recorded measuring OD at 450nm by using Accu Bind ELISA Ferritin Assay kit. The ferritin, if present, in the serum sample when incubated with DFO and PA (serum + DFO and serum + PA) for 10, 30 and 60 min will combine with antibody to form an antibody-antigen complex. So the change in ferritin level of the patient sample was due to the addition of DFO and PA, as per the time interval used.

It has been observed that PA can chelate a considerable amount of ferritin after 10 and 30 min, though slightly lesser amount than DFO, when added in equivalent concentrations. But after 60 min the removal of ferritin is much higher in PA treated samples than that of DFO. This indicated that PA is probably bound with ferritin by more specific chemical binding in time and concentration-dependent manner. However, the long term study is required to know

the desired effect. Temporarily PA can be used along with DFO as combination therapy for iron chelation management, because of its long-term chelation effect than DFO. However, further study and trials are needed to know its maintenance dose and effect in thalassaemic patients.

4. CONCLUSION

The aim of this study was to introduce a nontoxic, cost effective, oral iron chelator of natural origin that can be easily mobilize as iron-chelator complex, with a property of hexidentate binding of iron ions. Phytic acid is reported to have anticarcinogenic effect, and stimulate apoptosis of tumor cells. Similar to apoptosis, human erythrocytes undergo suicidal death or eryptosis, characterized by cell membrane scrambling and cell shrinkage, hence phytate intake could cause anemia, an effect attributed to iron complexation. It has been shown that phytic acid (≥1mM) does not cause significant hemolysis, but stimulates suicidal human erythrocyte death, an effect paralleling its pro-apoptotic effect on nucleated cells. Therefore, phytic acid is recommended to take before or along with the food to avoid gastro-intestinal iron absorption, as it can chelate polyvalent iron cations without producing any toxic products. The present study evaluated the efficacy of a herbal compound PA as new oral iron chelator, alternative to available drugs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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