ISSN 0975 - 6299 Vol 2 / Issue 4 / Oct - Dec 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

PHARMACOLOGY

ISOLATION, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF IRON CHELATOR FROM *TRITICUM AESTIVUM* (WHEAT GRASS)

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ABSTRACT

Triticum aestivum, is a one of the important species of wheatgrass, a cereal grass of Gramineae (Poaceae) family. Proponents of wheatgrass make many claims for its health properties, ranging from promotion of general well-being to cancer prevention and heavy metal detoxification. These claims have not been satisfactorily substantiated in scientific literature. Iron overload is one of the major causes of morbidity in all patients with severe forms of thalassemia and other iron overloaded disorders. Our previous study showed beneficial effects of methanol extracts in iron overloaded animals. Thus present study was planned to isolate and characterize iron chelating compound from methanol extract of wheatgrass. This isolated compound was subjected to determination of iron chelating activity in iron dextran induced acute iron overload animals. At the end of our study, we are able to characterize compound using LCMS and IR spectroscopy. Result inference is that isolated compound belonging to phenolic group and possesses in-vitro iron chelating activity. The isolated compound also possesses phenolic content which is confirmed using FC method. We have made the unexpected observation that this isolated compound from *T. aestivum* strikingly increase iron in urine in iron loaded animals. The chelating power or efficacy of the compound was found to be 34.5% to that of desferoxamine, a standard iron chelator used to reduce iron overload in thalassemia. In conclusion, our data suggest that isolated compound from *Triticum aestivum* possess iron chelator compound which is belonging to phenolic group.

ISSN 0975 - 6299 Vol 2 / Issue 4 / Oct - Dec 2011

KEYWORDS

Triticum aestivum, phenolic, iron overload, iron chelating property.

INTRODUCTION

Modern science has already accepted the potential of herbs as a source of new bio-active constituents. There are numerous plants derived drugs of unknown chemical structure that have been found clinically useful in different alternative system of medicine including Ayurveda, Homeopathy and Unani system of medicine. The plants are rich reservoir of potential leads for drug discovery against various disorders.

In medicine, iron overload indicates accumulation of iron in the body due to any cause. In normal subjects when a red cell is broken down into iron and protein, the iron released is recycled. Iron balance is maintained by limiting iron absorbtion from gut. Normal iron absorbtion is 1-1.5 mg/day; additional iron is absorbed only when it is required. Iron absorption is proportional to severity of anaemia, serum iron levels, erythropoesis (Formation of RBCs), amount of iron in food, presence of vitamin C, presence of sugars and other amino acids. While absorption of iron is inhibited by presence of high fiber diet, phytates, tannic which binds with iron etc. Iron overload is one of major causes of morbidity in all patients with severe forms of thalassemia, regardless of whether they are regularly transfused. A variety of other iron overload diseases are present. These are usually associated with chronic anemias. These are thalassemia, sideroblastic anemia, abnormal red cell production (dyseryphropoiesis), iron overload secondary to IV therapy, chronic liver disease secondary to alcohol, porphyria cutanea tarda[1].

In thalassemia major, body mistakes anemia for iron deficiency and absorbs iron as much as 3-4 mg/day depending upon severity of anemia from iron present in food. Absorption may increase up to 10 mg/day if iron tonics are administered to correct anemia. Iron absorbed from food adds to body iron stores. On an average each unit of blood contains 200-250 mg of iron. A patient receiving 30 units of blood/year receives 6 gm of elemental iron annually. Patients with chronic anemias such as thalassemia, require regular blood transfusions in order to improve both quality of life and survival. Humans are unable to eliminate iron released from breakdown of transfused red blood cells and excess iron is deposited as hemosiderin and ferritine in liver, spleen, endocrine organs and myocardium[2]. The accumulation of toxic quantities of iron causes tissue damage and leads to complications such as heart failure, endocrine abnormalities like diabetes, hypothyroidism, liver failure and ultimately early death[3, 4].

Wheat, (*Triticum* species) a cereal grass of the Gramineae (Poaceae) family, is the world's largest edible grain cereal-grass crop. The wheat plant is an annual grass. In early growth stages the wheat plant consists of a much-compressed stem or crown and numerous narrowly linear or linear-lanceolate leaves. For over fifty years, researchers have known that the cereal plant, at this young green stage, is many times richer in levels of vitamins, minerals and proteins as compared to seed kernel, or grain products of the mature cereal plant[5, 6].

Wheatgrass has been traditionally used, since ancient times, to treat various diseases and disorders. Presently, there are a number of wheatgrass suppliers, in almost all cities of India, supply fresh wheatgrass, on daily basis to their regular customers by home-delivery system for various ailments and as health tonic. Dr. Ann

Wigmore, U. S. A. founder director of the Hippocrates Health Institute, Boston, U.S.A. was one of the proponents of 'Wheatgrass Therapy'. Dr. Wigmore reported that "wheatgrass" used in her program contain abscisic acid and laetrile, both of which may have anti-cancer activity. It was also reported that young grasses and other chlorophyll-rich plants are safe and effective treatment for ailments such as high blood pressure, some cancers, obesity, diabetes, gastritis, ulcers, pancreas and liver problems, fatigue, anemia, asthma, eczema, hemorrhoids, skin problems, halitosis and constipation[7].

Scientific reports on nutritional analysis of wheatgrass have been published frequently in various journals. These reports and chemical analyses undertaken reveal that wheatgrass is rich in chlorophyll, minerals like magnesium, selenium, zinc, chromium, antioxidants like beta-carotene (pro-vitamin A), vitamin E, vitamin C, antianemic factors like vitamin B_{12}, iron, folic acid, pyridoxine and many other minerals, amino acids and enzymes, which have significant nutritious and medicinal value[8, 9]. Clinically it was proved that different varieties of wheatgrass extracts are therapeutically used in treatment of anemia, thalassemia (major), cancer and bacterial diseases[10]. There was a direct relation observed between iron chelating activity and phenolic content in plant. Some extracts with high phenol and flavanoid contents showed good chelating of Fe^{2+}. For example, *E. hirsutum* and *M. arvensis* that contained highest phenol and flavanoid contents showed the best chelating activity[11].

Synthetic agents like desferoxamine and deferiprone used for treatment of iron overload in thalassemia are accompanied by serious side effects and certain limitations including need for parenteral administration, arthralgia, nausea, gastrointestinal symptoms, fluctuating liver enzyme levels, leucopenia, agranulocytosis and zinc deficiency and obviously the heavy cost. In addition, they are not suitable for use during pregnancy. The poor oral bioavailability, short plasma half-life and severe side effects of available chelators are still not optimal[12, 13]. Within this context and taking into consideration the relative paucity of iron chelating agents, it is not surprising that clinical scientists put a great effort towards finding any potentially useful sources in order to obtain the maximum possible benefit with the least possible harm[14]. Compared to synthetic drugs, herbal preparations are frequently less toxic with fewer side effects. Therefore, the search for more effective and safer treatment of thalassemia and other blood disorders has become an area of current research activity. For thousands of years, mankind has known about the benefit of drugs from nature. Plant extracts, for the treatment of various ailments, were highly regarded by the ancient civilizations. Even today, plant materials remain an important resource for combating illnesses.

In the light of foregoing discussion, the present study is planned to isolate, characterize the iron chelator compound from Triticum aestivum for management of iron overload disorder. In order to narrow down work, we planned to find out biological evaluation of these iron chelator compound from Triticum aestivum responsible for these beneficial effects.

MATERIALS & METHODS

Certified samples of species of wheat *Triticum aestivum*, was acquired from Wheat Research Center, Gujarat Krushi University, Junagadh, Gujarat, India. The authenticity of these certified samples was also confirmed by comparing their morphological characters with description mentioned in different standard texts and floras[15]. These wheat varieties were grown in plastic trays as per standard procedure described by Wigmore, 1985[7]. As our previous study suggested that maximum iron chelating activity was detected in methanol extract in animals, so we tried to isolate these iron

chelating compound from methanol extract of *Triticum aestivum.*

(i) Isolation of iron chelating compound from methanol extracts

Methanol extract of *Triticum aestivum* was loaded on a glass column (60 X 3cm) packed with silica gel G (40g, 160-200 #, spectrochem Pvt. Ltd.) as a stationary phase. Gradient elution was performed using different proportion of methanol: water: acetone: glacial acetic acid (1:0-80:0.5:0.1). Total 115 fractions were collected. These fractions were analyzed by thin-layer chromatography to determine a) if fraction contains more than one component and b) if fractions can be combined without affecting the purity of those fractions. An IR spectrum was recorded on FT-IR-8400S, Shimadzu instrument. Mass spectrum was recorded on Shimbanzu GCMS-QP 2010 spectrophotometer. Isolated compound was subjected to determine in-vitro quantitative analysis of phenolic content and iron chelating activity. This isolated compound was also subjected for evaluation of iron chelation activity by in-vivo animal models.

(ii) Quantitative determination of total phenolic in isolated compound by FC method[16, 17]

Folin-Ciocalteau (FC) colorimetric method is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. The products of the metal oxide reduction have a blue colour that exhibits a broad light absorption with a maximum at 765 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols.

Procedure:

1. Put 50 µl sample, a gallic acid calibration standard, or blank (deionized or distilled water) into a 1-cm, 2-ml plastic or glass cuvette.
2. Add 1.58 ml water, followed by 100 µl FC reagent. Mix thoroughly by pipetting or inverting and incubate 1 to 8 min. The incubation must not be >8 min.
3. Add 300 µl sodium carbonate solution, mix, and incubate 2 hr at room temperature. A final volume of 2 ml must fill the cell adequately for a reading.
4. Measure sample absorbance at 765 nm and analyze in UV-visible spectrophotometer, Shimadzu instrument.

(iii) In-vitro metal chelating activity of isolated compound of Triticum aestivum[18]

The chelation of ferrous ions by isolated compound was estimated by method of Dinis et al., 1994. Briefly, 50 µl of 2 mM $FeCl_2$ was added to 1 ml of 1.0 mg/ml concentrations of isolated compound. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated as $[(A_0-A_S)/ A_S] X 100$, where A_0 was the absorbance of the control, and As was the absorbance of extract/standard. Na_2EDTA was used as positive control.

(iii)Determination of in-vivo iron chelating activity for isolated compound

All animals were housed at ambient temperature (22±10 ^{0}C), relative humidity (55±5 %) and 12h/12h light dark cycle. Animals had free access to standard pellet diet and water given ad libitum. The protocol of the experiment was approved by the institutional animal ethical committee as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry

of Social Justice and Empowerment, Government of India (Protocol No. RKCP/COL/RP/09/01 dated 7th March, 2009).

The S.D. rats were given single i.p. injections of iron-dextran (25 mg/l00 g body wt.) that results in condition of acute iron overload. Control rats were injected with an equal volume of dextran at the same time[19].

The experimental animals were divided into four groups, (n=6).

Group 1: Normal control received dextrose solution

Group 2: Disease control treated with iron dextran (25 mg/l00g body wt. for 2 days)

Group 3: Disease control treated with desferoxamine (40 mg/kg, p.o for 2 days)

Group 4: Disease control treated with isolated compound PI1 (40 mg/kg, p.o. for 2 days)

At the end of day two blood samples and urine samples were collected under fasting conditions. Urine sample was subjected for estimation of iron using fully automated biochemistry Dimension-Rx L Max-Dade Behring Analyzer.

RESULTS AND DISCUSSION

1. TLC Study :
   The identity of isolated compound PI_1 was confirmed by comparing the R_f 0.682 on TLC plate. These isolated compound gave black colour spot after spraying of 5 % ferric chloride solution on TLC plate that suggest the nature of these compound belonging to phenolic group.
2. Isolation and identification of active compound PI_1 :
   Column chromatography fraction 71-76 eluted using methanol: water: acetone: glacial acetic acid solvent system, upon concentration yielded brown crystals. On recrystalization from methanol these compound (PI_1) gave needles shape compound having melting point 215-218 ^{0}C.

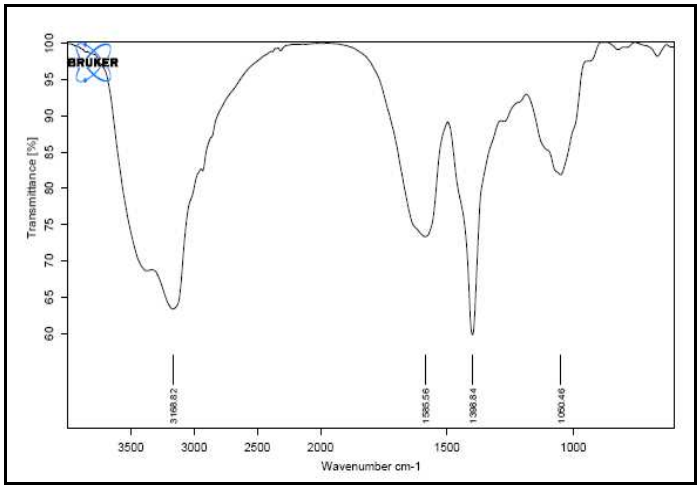


Figure 1

IR spectroscopy of isolated compound PI_1 from Triticum aestivum

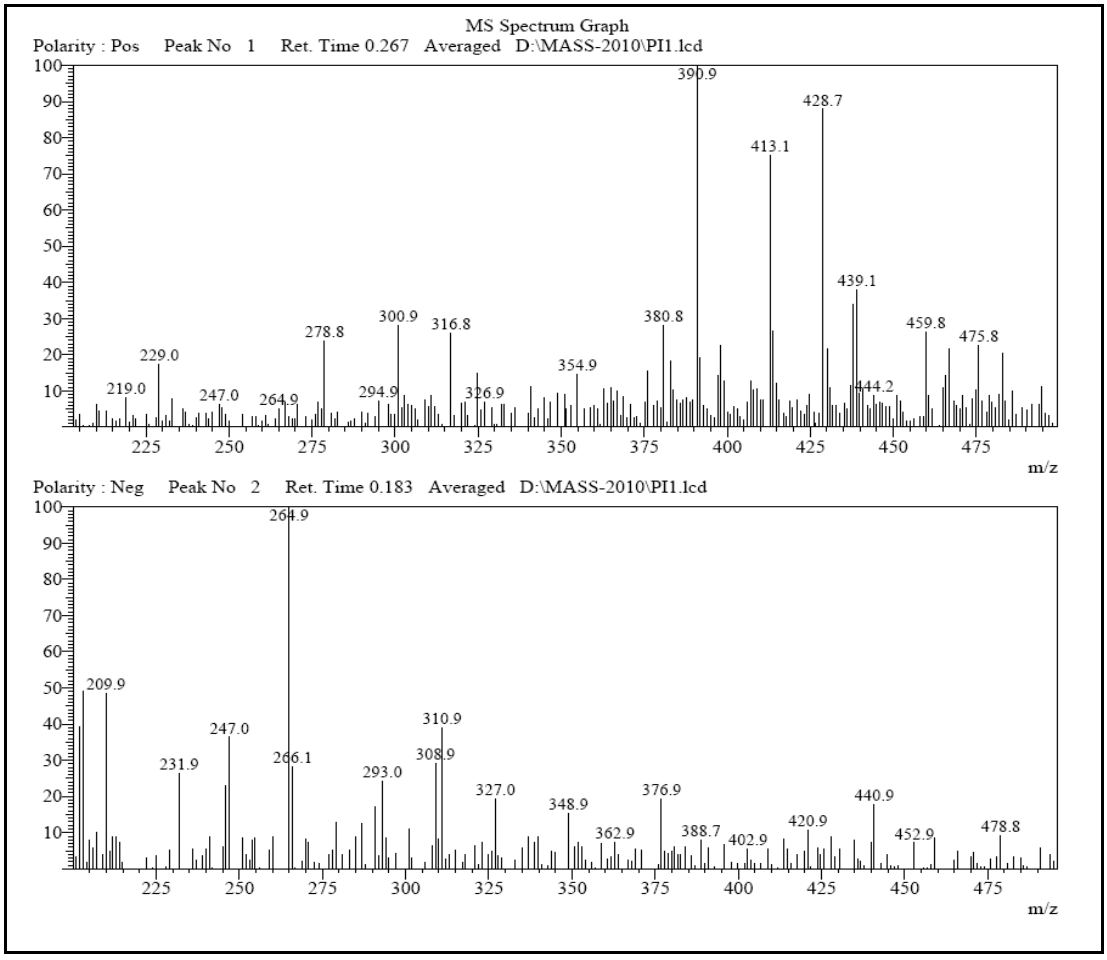


Figure 2
Mass spectroscopy of isolated compound PI_1 from Triticum aestivum Identification Parameters

The data of Spectral analysis, IR and GC-MS of isolated pure compound mentioned in table 1.

Table 1
IR and MASS spectral value of isolated pure compound PI_1.

Spectra	Spectral value	Inferences
IR	3168.82 Cm^{-1}	-OH (Phenolic) (stretching)
	1585.56 Cm^{-1}	aromatic group
	1060.46 Cm^{-1}	C-O again prove that OH is from phenol or alcohol not carboxylic acid
MASS	264.9 – 247=17	Removal of -OH group

Data from the mass and IR spectroscopes suggest that the nature of isolated compound is found to be aromatic in nature containing phenolic group.

3. Quantitative determination of total phenolic using Folin-Ciocalteau (FC) method :

It has been recognized that phenolic and flavanoid compounds show antioxidant and metal chelating activity and their effects on human nutrition and health are considerable. The mechanisms of action of phenolic compounds are through scavenging or chelating process[20, 21]. The phenolic content of extracts is calculated as gallic acid equivalent. The total phenolic content present in isolated compound from methanol fraction of *Triticum aestivum* are found to be 434.14 ± 28.02 µg Gallic acid equivalent of phenol was detected using Dinis et al., method.

4. In-vitro quantitative determination of Iron chelating activity :

Ferrozine can quantitatively form complexes with Fe^{2+}. However, in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. The transition metal ion, Fe^{2+} possesses the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals[22]. The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions. Isolated compound PI_1 from methanol extract of *T. aestivum*, is interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine.

Iron chelating property of isolated compound from *T. aestivum* are compared to standard iron chelator drug desferoxamine which is used in the treatment of iron overload patients of thalassemia at 1.0 mg/ml concentration level. % inhibition of complex formation between Fe^{2+} -ferrozine were found 61.18 ± 5.37 in desferoxamine and 30.27 ± 2.98 in isolated compound of *T. aestivum*. In vitro iron chelating power of the isolated compound is found to be 50% compared to standard iron chelator drug, desferoxamine.

5. Evaluation of iron chelating activity of isolated compound of *Triticum aestivum* for iron overload induce thalassemia model :

Intraperitoneal injections of iron-dextran (12.5 mg/l00 g body wt.) evenly distributed over a 2 days period results in condition of acute iron overload, which is very resemble to thalassemia. Control rats injected with an equal volume of dextran at the same time show normal level of iron in rat. At the end of day 2, urine sample was collected and subjected for estimation of iron. (Table 2)

Table 2

Comparison of excretion of iron in urine between desferoxamine and isolated compound $PI_1$of Triticum aestivum

Parameters	NC (n=6)	DC (n=6)	DCD (n=6)	Isolated compound PI_1
Urine Iron µg/dl	26.2 ± 6.4	34.25 ± 3.8	108.75 ± 7.45#	62.21 ± 9.45#

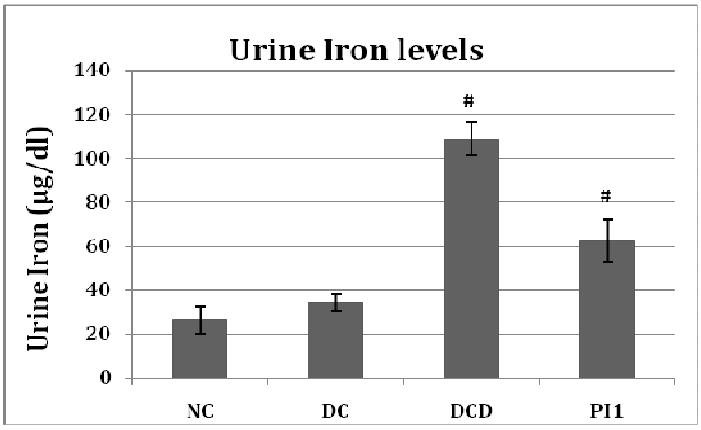
Patients with chronic anemias such as thalassemia, require regular blood transfusions in order to improve both quality of life and survival. Humans are unable to eliminate iron released from breakdown of transfused red blood cells and excess iron is deposited as hemosiderin and ferritine in liver, spleen, endocrine organs and myocardium[2]. The accumulation of toxic quantities of iron causes tissue damage and leads to complications such as heart failure, endocrine abnormalities like diabetes, hypothyroidism, liver failure and ultimately early death[3, 4].

There is significant increase in urine iron level in iron overloaded rats treated with desferoxamine (108.75 ± 7.45 µg/dl). Increase in urine iron level is due to iron chelating activity of desferoxamine, which is used to reduce iron overload and its complications in thalassemia.

Treatment of isolated compound on iron overloaded rats with PI_1 of *T. aestivum* results in significant increase in urine iron levels (66.21 ± 9.45 µg/dl) compared to diseases control (34.25 ± 3.8 µg/dl). Increased in urine iron in rats in isolated compound indicates its iron chelation property which are comparable to desferoxamine. The chelating power or efficacy of the compound is found to be 34.5% to that of desferoxamine.

Graph 1

Iron chelating property of isolated compound PI_1 compared to desferoxamine



 # - significantly different from diseases control (p < 0.05)
Group 1: Normal control received dextrose solution (NC)
Group 2: Disease control treated with iron dextran (12.5mg/l00g body wt.) (DC)
Group 3: Disease control treated with desferoxamine (40 mg/kg, p.o., per day) (DCD)
Group 5: Disease control treated with isolated compound PI_1 (100 mg/kg, p.o., per day) (DCMT)

CONCLUSION

Our investigation suggests, isolated compounds PI_1form methanol extract of *Triticum aestivum* possess in-vivo and in-vitro iron chelating activity. The Mass and IR spectroscopic studies indicates the nature of these isolated compound that is belonging to aromatic phenolic groups. Further characterization as well as detailed toxicological and clinical studies of this new iron chelator molecule, may provide a new chemical entity for better management of iron overload diseases like thalassemia.

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