INVESTIGATION INTO MECHANISM OF ACTION AND PHARMACOLOGICAL EVALUATION OF VARIOUS EXTRACTS OF *TRITICUM AESTIVUM* (WHEAT) GRASS WITH SPECIAL REFERENCE TO ITS BENEFICIAL EFFECTS ON IRON-OVERLOAD AND BLOOD DISORDERS

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By

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Certificate

This is to certify that the thesis entitled "Investigation into mechanism of action and pharmacological evaluation of various extracts of *Triticum aestivum* (wheat) grass with special reference to its beneficial effects on iron-overload and blood disorders" represents bonafide work of MR. PRAVIN RAMBHAI TIRGAR, carried out under my guidance and supervision. The work mentioned in this thesis was carried out at R. K. College of pharmacy, Rajkot during the period of years 2007-2011. The work is up to my satisfaction.

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DECLARATION BY THE CANDIDATE

I, hereby, declare that Saurashtra University, Rajkot shall have the right to preserve, use and disseminate this thesis, in print or electronic format for academic/research purpose.

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Statement under ordinance

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The contents of this thesis are my own work, carried out under supervision of Dr. T. R. Desai. It leads to some contribution in pharmacy, supported by necessary references.

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DECLARATION

I hereby declare that thesis entitled "**Investigation into mechanism of action and pharmacological evaluation of various extracts of *Triticum aestivum* (wheat) grass with special reference to its beneficial effects on iron-overload and blood disorders**" is a bonafide research work carried out by me, under the guidance of Dr. T. R. Desai. This work is original and has not been submitted in part or full for any degree/diploma to other University.

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Rajkot

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"I dedicate my dissertation work to my family and many friends.

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And to my wife Hetal, who has always stood by me."

1. ABSTRACT

Health is not merely absence of a disease. Rather, health is defined as positive state of well being in which, the harmonious development of physical and mental capacities of the individual lead to the enjoyment of a rich and full life. Health involves, primarily, the application of medical science for the benefit of individual and society. Health is thus, a vital part of a concurrent and integrated program of development of all aspects of community life. Considering the importance of health, WHO and UNICEF jointly organized an international conference on Primary Health Care at Alma Ata, USSR from 6th to 12th September, 1978 and took a momentous decision to achieve "Health for All" by the year 2000 AD. Now, we are entering in the year 2007, but the 'Health for All' by the year 2000 still continues to be a vision. There are various diseases like tropical diseases, herpes, AIDS, cancer, diabetes, thalassemia, certain blood disorders etc. for which the cure is yet to be found.

Iron is essential to life because of its unusual flexibility to serve as both an electron donor and acceptor. Iron can also be potentially toxic. Its ability to donate and accept electrons means that if iron is free within the cell, it can catalyze the conversion of hydrogen peroxide into free radicals. Free radicals can cause damage to cellular membranes, proteins, and DNA, a wide variety of cellular structures, and ultimately kill the cell. To prevent that kind of damage, all life forms that use iron, bind the iron atoms to proteins. This allows the cells to use the benefits of iron, but also limit its ability to do harm (Andrews NC 1995). Most well-nourished people have 4 to 5 grams of iron in their bodies. Of this, about 2.5 g is contained in the hemoglobin needed to carry oxygen through the blood, and most of the rest is contained in ferritin complexes that are present in all cells, but most commonly in bone marrow, liver, and spleen. The liver's stores of ferritin are the primary physiologic source of reserve iron in the body (Schrier SL 2005). The human body needs iron for oxygen transport. That oxygen is required for the production and survival of all cells in our bodies. Human bodies

tightly regulate iron absorption and recycling. Iron is such an essential element of human life, in fact, that humans have no physiologic regulatory mechanism for excreting iron (Schrier and Bacon 2005).

In medicine, iron overload disorders are diseases caused by the accumulation of iron in the body. Iron toxicity results when the amount of circulating iron exceeds the amount of transferrin available to bind it. The type of acute toxicity from iron ingestion causes severe mucosal damage in gastrointestinal tract, among other problems. Iron overload is one of the 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 thalassemia, sideroblastic anemia, abnormal red cell production (dyseryphropoiesis), iron overload secondary to IV therapy, chronic liver disease secondary to alcohol, porphyria cutanea tarda. Iron overload can be inherited (genetic) or acquired by receiving numerous blood transfusions, getting iron shots or injections, or consuming high levels of supplemental iron. Some of the genetic disorders that result in iron overload include are hereditary hemochromatosis (all types), African iron overload, sickle cell disease, thalassemia, X-linked sideroblastic anemia, enzyme deficiencies (pyruvate kinase; glucose-6-phosphate dehydrogenase) and very rare protein transport disorders aceruloplasminemia and atransferrinemia. None of these conditions should be confused with polycythemia vera (PV), which is not an iron disorder, but a condition where the bone marrow produces too many blood cells (red, white and platelet). People with PV have abnormally high hemoglobin and are at risk for a stroke and progressing to acute myelogenous leukemia (AML).

Excess iron in vital organs, even in mild cases of iron overload, increases the risk for liver disease (cirrhosis, cancer), heart attack or heart failure, diabetes mellitus, osteoarthritis, osteoporosis, metabolic syndrome, hypothyroidism, hypogonadism, numerous symptoms and in some cases premature death. Iron mismanagement resulting in overload can accelerate such neurodegenerative diseases as Alzheimer's, early-onset Parkinson's, Huntington's, epilepsy and multiple sclerosis. Synthetic agents like desferrioxamine and deferiprone used for the treatment of iron overload in thalassemia are accompanied by serious side effects and certain limitaions 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 (Hebbel et al., 1990; Grinberg et al., 1995; Kukongviriyapan et al., 2008). 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. The poor oral bioavailability, short plasma half-life and severe side effects of available chelators are still not optimal (Filburn et al., 2007; Rachmilewitz et al., 1979; Livrea et al., 1996). Within this context and taking in 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 (Loukopoulos, 2005; Ebrahimzadeh et al., 2007).

For thousands of years, mankind has known about the benefits of drugs from nature. Plant extracts like wheatgrass juice have been highly regarded for their curative effects by ancient civilizations. Even today, plant materials remain an important resource for combating illnesses. WHO has approved the use of traditional medicines as a part of health programme. To pursue research in these systems of medicine, several USA agencies and institutions such as FDA and National Institute of Health have setup separate wings. According to the WHO survey 80% of the populations living in the developing countries rely almost exclusively on traditional medicine for the primary health care needs. In almost all the traditional medicine, the medicinal plants play a major role and constitute the backbone of the traditional medicine. The potential of plant as a source for new drugs is yet to be unexplored systematically. Among the estimated 250,000-400,000 plant species, only 6% have been studied for biological activity and about 15% have been investigated phytochemically (Verpoorte et al., 1998; Cragg et al., 1997; Balandrin et al., 1985). India has an ancient heritage of traditional medicine. Materia Medica of India provides lots of information on the folklore practices and traditional aspects of therapeutically important natural products. Indian traditional medicine is based on various system including Ayurveda, Siddha and Unani. With the emerging interest in the world to adopt and study the traditional system and to exploit their potentials based on

different healthcare systems, the evaluation of the rich heritage of the traditional medicine is essential. Further, treatment with, one such traditional herbal drug viz. Wheatgrass, on patients with ß-thalassemia (major), has been reported to have beneficial effects by decreasing iron overload (Desai et al., 2005). Hence, in the present project, we planned to investigate iron chelating potential of wheatgrass and its various extracts, in iron overload condition.

Modern science has already, accepted the potential of the 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 a rich reservoir of potential leads for drug discovery against various disorders. Almost, half of the useful drugs today used for various diseases, are derived from natural sources. Only less than two percent of all the plants, available on the earth, have been subjected to pharmacological investigations. Research on the medicinal herbs can offer useful drugs, in time to come, for the treatment of chronic diseases like asthma and diabetes etc. The global market of herbal drugs is increasing very rapidly and it is expected to touch the $5 trillion by end of 2005 (Pharma Business, 2000). The recent development of the science of phyto-pharmaceuticals has generated new enthusiasm in herbal drug research to discover new medicines (Patel and Saluja, 2002). Looking at the dire need of a new safe and economical iron chelating molecule, we resolved to isolate probable active constituent of wheatgrass, responsible for its possible chelating activity.

Wheat (*Triticum* species) a cereal grass of the *Gramineae* (Poaceae) family, is the world's largest edible grain cereal-grass crop. Wheat has been a food crop for mankind since the beginning of agriculture. For over fifty years, researchers have known that the cereal plant, at this young green stage, is many times richer in the levels of vitamins, minerals and proteins as compared to seed kernel, or grain products of the mature cereal plant (Schnabel 1940). The young germinated plant is a factory of enzyme and growth activity. In the early stages of growth they store large amounts of vitamins and proteins in the young blades. After jointing stage, the nutritional level in the leaves drops rapidly while the fiber content increases rapidly (Kohler 1944). Agriculturally, important species of

Triticum include - *Triticum aestivum, Triticum durum* and *Triticum dicoccum.* Wheatgrass has been traditionally used, since ancient times, to treat various diseases and disorders. Presently, there are 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 a health tonic. Dr. Ann Wigmore, USA, founder director of the Hippocrates Health Institute, Boston, USA, was one of the proponents of the ''W*heatgrass Therapy*'. Dr. Wigmore claimed that wheatgrass is a safe and effective treatment for ailments such as high blood pressure, some cancers, obesity, diabetes, gastritis, ulcers, anemia, asthma and eczema. Scientific reports on nutritional analysis of wheatgrass have been published frequently in various journals (Kohler 1953, Hamilton et al., 1988, Laboratory Analyses 1989). These reports and the 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, phenol and flavonoid which have significant nutritious and medicinal value (Wigmore 1985). Since, iron overload induces considerable oxidative stress and wheatgrass is known to contain significant amount of antioxidants, we decided to investigate antioxidant benefits of wheatgrass in iron overload condition.

Platelets are made in the bone marrow similar to other cells in blood such as, white blood cells and red blood cells. Platelets originate from megakaryocytes which, are large cells found in the bone marrow. Platelets, in general, have a brief 7 to 10 days life in blood, after which they are removed from the blood circulation. The number of platelets in the blood is referred to as the platelet count and is normally between 150,000 to 450,000 per micro liter of blood. Platelet counts less than 150,000 are termed thrombocytopenia (Maton et al, 1993). There are a wide range of botanical sources and wide range of active constituents that might ultimately contribute to haemostatic action, including essential oils, flavonoids, saponins, and alkaloids. The possible mechanisms of action of the hemostatic herbs include: increasing the production of platelets, promoting the ability of platelets to aggregate when there is blood leakage, decreasing capillary permeability, contracting peripheral blood vessels,

inhibiting autoimmune attack against platelets. Dr. Wigmore in her programme the "wheatgrass" made several clinical trials on wheatgrass and reported that plants are a safe and effective treatment for anemia and various bleeding disorder like hemophilia and thrombocytopenia. These effects should be expected to be observed within a few days of administering the herbs (Wigmore 1985). In this context, we decided to investigate beneficial effects of wheatgrass in treatment of thrombocytopenia and other bleeding disorders.

The immune system is a remarkably effective structure that incorporates specificity, inducibility and adaptation. Failures of host defense do occur, however, and fall into three broad categories: immunodeficiencies, autoimmunity and hypersensitivities. The immune system is involved in the etiology as well as pathophysiological mechanisms of many diseases. Modulation of the immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles (Sharma P 1983). Indian medicinal plants are a rich source of substances which are claimed to induce paraimmunity, the non-specific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement functions (Sainis 1997). Ayurveda, the Indian traditional system of medicine, lays emphasis on promotion of health concept of strengthening host defenses against different diseases (Thatte 1986). Dr. Wigmore's opinions are based on her experiences. A few clinical studies, have verified that some disease conditions can be benefited by the use of wheatgrass. Remarkably, a relatively large number of studies indicate that food factors and nutrients found in wheatgrass may provide beneficial in immunological disorders. In the light of forgoing discussion, we made an attempt to assess immunomodulatory potential of wheatgrass, using various animal models.

In nutshell the objectives of the present project were –

1. To carry out pharmacognostic studies of *Triticum aestivum* (Wheatgrass).
2. To carry out phytochemical studies of *Triticum aestivum* and its various extracts.
3. To evaluate iron chelating activity of various extracts of *Triticum aestivum.*
4. To isolate iron chelating compound from extract of *Triticum aestivum,* using column chromatography.
5. To evaluate iron chelating activity of isolated compound of *Triticum aestivum*, using pre-clinical study.
6. To investigate anti-oxidant property of *Triticum aestivum* in iron overload condition.
7. To investigate therapeutic benefit of *Triticum aestivum* in thrombocytopenia.
8. To investigate immunomodulatory activity of *Triticum aestivum*.

In our investigation, certified samples of wheat viz. *Triticum aestivum,* was acquired from the Wheat Research Center, Gujarat Krushi University, Junagadh, Gujarat. Adequate quantities of unpolished wheat grain were soaked overnight in water, and were grown in plastic trays filled with soil, on the next day. Trays were watered adequately every day, for 8 days. On 9^{th} day the wheatgrass was harvested. For characterization, wheatgrass was subjected to microscopic study, which included transverse sections, surface preparations and powder study, using high-resolution microscope.

In conformation with the description in literature; the leaves were mainly near glabrous, auriculate, with blades narrowly to broadly linear; broad to narrow; 2–20 mm wide; flat; without cross venation (Percival 1974). Observations in microscopic studies of different species, also confirmed characteristics reported

in literature (Percival 1974). In transverse section, the wheatgrass leaf showed 1. elaborate epidermis with characteristic stomata and trichomes 2. green assimilating parenchyma, 3. conducting vascular bundles and 4. Longitudinal strands of fibrous stereome or supporting tissue. The upper surface of the leaf showed a series of longitudinal ridges or ribs, the lower surface being almost flat. At the summit of each ridge was a single row of elongated thick-walled and pitted cells alternating with hairs. The trichomes or hairs were always unicellular and very much in length and stoutness. On the leaves of *T. aestivum,* ample numbers of hair were present. Stomata were observed at the base of the ridge arranged in single or double lines. Each stoma on the leaf consisted of four cells; the two guard cells being narrow, with specially thickened walls round the stomata pore and thin-walled widely dilated ends. Pores of the stomata were seen to be in communication with large intracellular cavities in the mesophyll, called lacune. The ratio of the number of stomata on the upper and lower epidermis respectively was about 10:7, the number on the upper surface examined being 7000 per square centimeter. In the furrow between two ridges was a band of three to seven rows of motor cells. vascular bundles were collateral, with the xylem towards the upper surface of the leaf and the phloem bellow. In surface preparation, trichomes or hairs of various lengths were found scattered along the rows at more or less at regular intervals. The pharmacognostic characteristics observed in our study were in confirmation with that reported in the literature.

Successive solvent extracts of wheatgrass with petroleum ether, chloroform, acetone, methanol and water were prepared, employing soxhlet apparatus. Highest extractive value was that of water extract (5.3 %), followed by acetone (4.3 %), methanol (4.1 %), chloroform (3.4 %) and petroleum ether with lowest extractive value of 2.8 %.

There is a direct relation beteween iron chelatory activity and the contents of phenolic and flavonoids in some extracts as reported by Ebrahimzadeh et al., 2008. Hence, the extracts obtained from successive solvent extraction process were subjected to shinoda and $FeCl_3$ tests to determine the presence of phenolics, tannins and flavanoids. Methanol and water extracts showed presence

of phenolics, tannins and flavanoids, while petroleum ether, chloroform and acetone extracts had no phenolic and flavanoids components.

We also made attempt for in-vitro quantitative determination of phenolic contents and iron chelating property of various extracts of wheatgrass using Folin-Ciocalteau (FC) and Dinis et al. 1994, methods respectively. In methanol and water extracts 506.92 ± 16.36 and 198.5 ± 10.61 µg Gallic acid equivalent of phenol content were detected. Phenolic content of methanol extract was found to be higher compared to water extract. In-vitro iron chelating activities of EDTA, desferoxamine and both extracts were found to be increased with increase in their concentration with highest activity at concentration of 2 mg/ml. The chelating activity of methanol extract was found to be significantly higher compared to water extract.

Intraperitoneal injections of iron-dextran (12.5 mg/l00 g body wt.) evenly distributed over a 30 days period on Sprague dwaley rats resulted in condition of chronic iron overload (serum iron - 6099 ± 252 µg/dl). Control group rats injected with an equal volume of dextran showed normal level of iron (serum iron - 203 ± 17 µg/dl). There was significant increase in serum ferritin level in iron overloaded group rats (1.13 ± 0.07 mg/dl) compared to normal control group rats (4.83 ± 0.51 mg/dl). All the studies were carried out for a period of 30 days. Blood, urine and fecal samples were collected on 15^{th} and 30^{th} days under fasting conditions and were subjected for various biochemical parameters.

After 15 days of treatment, there were significant reductions in serum iron and ferritin levels in desferoxamine group (serum iron - 2876 ± 281µg/dl, serum ferritin - 2.74 ± 0.42 mg/dl). There were significant reduction in serum iron and ferritin levels after treatment with water extract group (serum iron - 3510 ± 264 µg/dl, serum ferritin - 3.32 ± 0.19 mg/dl) and methanol extract group (serum iron - 4636 ± 142 µg/dl, serum ferritin - 3.97 ± 0.29 mg/dl) of wheatgrass compared to disease group. Treatment with acetone extract did not significantly reduce serum iron or ferritin levels (serum iron - 5222 ± 314µg/dl, serum ferritin – 4.64 ± 0.38 mg/dl) compared to disease control.

No changes were observed in urine and faces iron in iron overloaded group rats (urine iron – 69.2 ± 7.7 µg/dl, faces iron – 9.17 ± 2.5 µg/dl) and placebo group (urine iron – 26.2 ± 6.4 µg/dl, faces iron – 2.83 ± 0.3 µg/dl). There was significant increase in urine iron and faces iron levels in desferoxamine group (urine iron - 422.5 ± 79.1 µg/dl, faces iron - 31.0 ± 5.3 µg/dl), water extract group (urine iron - 256.0 ± 32.6 µg/dl, faces iron - 17.67 ± 2.1 µg/dl) and methanol extract group (urine iron - 296.5± 33.8 µg/dl, faces iron - 30.33 ± 2.5 µg/dl) compared to iron overloaded group rats (urine iron - 69.2 ± 7.7 µg/dl, faces iron - 9.17 ± 2.5 µg/dl). Increase in urine and fecal excretion of iron in rats treated with water and methanol extracts of wheatgrass indicate iron chelating property of wheatgrass that was comparable to desferoxamine. Similarly there were beneficial effects observed after 30 days treatment period with wheatgrass in iron overloaded rats group. Treatment of acetone extracts did not produce any significant increase in urine iron (94.0 ± 11.9 µg/dl) and faces iron (12.0 ± 1.01 µg/dl) levels compared to diseases control group. These data suggest effectiveness of water and methanol extracts in reduction of iron overload in rats by increase iron excretion in urine and faeces.

Excess iron in vital organs, even in mild cases of iron overload, increases the risk for liver disease (cirrhosis, cancer), kidney diseases, heart attack or heart failure, diabetes mellitus etc. and in some cases, premature death.

There were significant increases in SGPT (101.9 ± 8.7 µg/l) and SGOT (170.9 ± 11.3 µg/l) levels in iron overloaded group as compared to normal control group (SGPT – 12.4 ± 1.9 µg/l, SGOT - 46.28 ± 5.2 µg/l). After treatment with water and methanol extracts of wheatgrass there was significant reduction in these enzyme levels (water extract SGPT – 95.65 ± 6.9 µg/l, SGOT - 148.0 ± 6.5 µg/l; methanol extract SGPT – 81.9 ± 5.8 µg/l, SGOT - 132.4 ± 8.8 µg/l) indicating protective effects of extracts in liver complications due to iron overload.

Serum creatinine and creatinine kinase levels were significant increased in iron overloaded group rats (serum creatinine- 1.76 ± 0.08 mg/dl and creatinine kinase- 398.2 ± 23.7 µg/l) as compared to placebo group (serum creatinine- 0.67 ± 0.08 mg/dl and creatinine kinase- 91.8 ± 8.76 µg/l). Methanol and water extracts treated animals showed reduction in levels of these enzymes (water

extract, serum creatinine- 1.59 ± 0.05 mg/dl and creatinine kinase- 335.7 ± 17.9 µg/l; methanol extract, serum creatinine- 1.45 ± 0.07 mg/dl and creatinine kinase- 316.5 ± 11.5 µg/l) indicating that these extracts prevent damage to vital organs like kidney and heart in iron overload complications.

Results of histopathological study of liver suggested that chronic treatment with desferoxamine and water; methanol and acetone extracts of wheatgrass reduce iron pigmentation, pleomorphism, vaculation, fibrosis, disarrangement and degeneration of hepatocytes as compared to iron overloaded group animals. The degree of protection was found to be minimal with acetone extract group. Histological findings of kidney (injured brush-border microvilli and swollen proximal convoluted tubular cells) in iron overloaded group rats suggested protective effects of methanol, water and acetone extracts of wheatgrass in iron overload kidney complications. Protective effects were much better in methanol extract treated group and lesser in acetone extract treated group. Treatment with desferoxamine and methanol and water extracts of wheatgrass showed protective effects on myocytes and fibrosis. Vascular hemorrhages were also reduced in iron overloaded group rats treated with methanol and water extracts. Treatment with acetone extract produced less protective effects as compared to water and methanol extracts.

Accumulation of iron in body leads to suppression of bone marrow resulting in reduction of total and differential leucocytes counts. This was observed in iron overloaded rats of disease control group (3.89 ± 0.24 10^3/πl) as compared to placebo group (5.98 ± 0.41 10^3/πl). After 15 and 30 days treatment with desferoxamine (4.01 ± 0.23 10^3/πl), water extract (4.93 ± 0.27 10^3/πl) and methanol extract (4.53 ± 0.32 10^3/πl) of wheatgrass in iron over loaded rats total WBC count was significant increased. The increase in leukocyte count indicates that wheatgrass may have stimulating effect on bone marrow and also on synthesis of all types of leucocytes. Treatment with acetone extract in iron overload rats did not produce any significant increase in total and differential leucocytes counts as compared to iron overloaded group rats. These results indicate beneficial effect of wheatgrass on immune system.

Iron overloaded group rats exhibited significant decrease in Hb count (10.78 ± 0.84 gm/dl) and RBC count (6.77 ± 0.29 m/cmm) as compared to normal control group rats (Hb-14.57 ± 0.61 gm/dl, RBC- 8.65 ± 0.49 m/cmm) suggesting toxic effect of iron excess on Hb and RBC synthesis. 15 days treatment with desferoxamine (Hb-13.5 ± 0.51 gm/dl, RBC- 7.86 ± 0.58 m/cmm), water extract (Hb-13.83 ± 0.31 gm/dl, RBC- 7.69 ± 0.52 m/cmm) and methanol extract (Hb-14.03 ± 0.68 gm/dl, RBC- 8.13 ± 0.87 m/cmm) in iron over loaded rats produced significant increase in Hb levels and RBC counts. The increase in RBC count indicates that wheatgrass may have stimulated haemopoietic process while rise in hemoglobin content indicates stimulation of hemoglobin synthesis in individual RBC. Treatment with acetone extract in iron overload rats did not produce any significant increase in Hb level (10.67 ± 0.68 gm/dl) and RBC count (7.13 ± 0.44 m/cmm) as compared to iron overloaded rats. Over all, there was improvement in Hb level and RBC count after treatment with wheatgrass extracts after 15 and 30 days in iron overload rats.

Similarly, treatment with methanol extract (699.6 ± 38.6 10^3/πl) and water (793.6 ± 43.5 10^3/πl) extract of wheatgrass, significantly increased platelet counts in iron over loaded group rats as compared to diseases control group rats (530.2 ± 32.6 10^3/πl). Acetone extract did not produce any significant increase in platelet count (590.5 ± 59.5 10^3/πl) as compared to disease control group. These data indicate beneficial effect of wheatgrass in platelet deficiency disorders.

In iron overload condition, oxidative stress is ultimately involved in dysfunction of vital organs including cardiovascular system (Shinar and Rachmilewitz, 1990; Hebbel et al., 1990; Grinberg et al., 1995). Antioxidant and other supportive therapies protect RBC against oxidative damage (Kukongviriyapan et al., 2008; Filburn et al., 2007). Also, a higher rate of LDL oxidation in thalassemia patients is, due to a lower concentration of vitamin E and C in the LDL particles. Enrichment with vitamins E and C was effective in preventing LDL oxidation in patients with thalassemia (Rachmilewitz et al., 1979; Livrea et al., 1996). Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications and thereby improves quality of life and overall survival (Shinar and Rachmilewitz, 1990; Hebbel et al., 1990).

In our study, at the end of 30 days treatment periods, liver homogenates of iron overloaded group rats showed significant increase in OFRs, MDA level (2.56 ± 0.18 nmoles/mg protein) and decrease in SOD (0.73 ± 0.07 units/min/mg protein), catalase (0.98 ± 0.14 units/min/mg protein) and glutathione levels (7.22 ± 1.75 µgm/mg protein) compared to normal group rats (MDA- 0.74 ± 0.11 nmoles/mg protein, SOD- 1.42 ± 0.1 units/min/mg protein, Catalase- 4.59 ± 1.11 units/min/mg protein , GSH- 12.69 ± 1.04 µgm/mg protein). Treatment with methanol and water extracts of wheatgrass significantly reduced MDA level (1.24 ± 0.06 and 1.16 ± 0.08 nmoles/mg protein) in the rat liver homogenates. There were significant improvements in GSH, SOD and catalase levels in iron overloaded group rats treated with methanol (SOD- 1.29 ± 0.13 units/min/mg protein, Catalase- 3.47 ± 0.17 units/min/mg protein, GSH- 10.8 ± 0.7 µgm/mg protein) and water extracts (SOD- 1.35 ± 0.11 units/min/mg protein, Catalase- 3.78 ± 0.69 units/min/mg protein, GSH- 11.82 ± 0.51 µgm/mg protein). No significant changes were observed in MDA, SOD, catalase and glutathione levels in acetone extract treated group. These data indicate strengthening of antioxidant defense by wheatgrass in iron overload condition.

Since, the results, obtained so far, revealed maximum chelating activity in methanol extract group, we decided to process the methanol extract further, for the purpose of isolation of active iron chelator constituent.

Column chromatography fraction 71-76 eluted using methanol: water: acetone: glacial acetic acid (1:0-80:0.5:0.1) solvent system followed by concentration yielded brown crystals. On recrystallization with methanol produced needle shaped crystals of the active compound (PI_1), having melting point of 215-218 ^{0}C. The identity of the isolated compound PI_1, was confirmed by comparing the R_f value 0.682 on TLC plate. The isolated compound gave black colored spot on spraying 5 % ferric chloride solution on TLC plate suggesting phenolic nature.

The isolated iron chelator compound was subjected to LCMS and IR spectroscopic analyses, for its molecular characterization. The compound was found to be aromatic in nature containing phenolic group.

The total phenolic content in isolated compound from methanol fraction of *Triticum aestivum,* using Folin-Ciocalteau (FC) method, was found to be 434.14 ± 28.02 µg Gallic acid equivalent of phenol. In-vitro iron chelating activity of isolated compound was compared with standard iron chelator drug, desferoxamine 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 PI_1.

Intraperitoneal injections of iron-dextran (12.5 mg/l00g of body wt.), evenly distributed over a 2 days period, resulted in condition of acute iron overload, in SD rats. Control group rats injected with an equal volume of dextran, showed normal serum level of iron. At the end of day 2, urine samples were collected and analyzed for iron content. No significant changes in urine iron levels were observed in iron overloaded rats (34.25 ± 3.8 µg/dl) and normal control rats (26.2 ± 6.4 µg/dl). 2 day's treatment with desferoxamine (urine iron- 108.75± 7.4 µg/dl) and isolated compound (urine iron- 62.21 ± 9.4 µg/dl) in iron overloaded rats produced significant increase in urine iron levels compared to diseases control rats. The chelating power or efficacy of the isolated compound was 34.5% compared to that of desferoxamine.

For evaluation of effects of wheatgrass in thrombocytopenia, busulfan was used to induce experimental thrombocytopenia. Busulfan is an alkylating agent with myeloablative properties and activity against non-dividing marrow cells and possibly, non-dividing malignant cells. Busulfan solution, at concentration of 10 mg/ml in polyethylene glycol, was prepared and infused in wistar rats at doses of 25 mg busulfan/kg body weight each, at 1, 5, 10 and 15 days of interval produced pancytopenia with significant reduction in platelet count mimicking severe bleeding conditions as found in thrombocytopenia.

Disease control group rats which, received busulfan showed significant reduction in Hb (8.1 ± 0.75 gm/dl) and RBC count (5.4 ± 0.5 m/cmm) compared to normal healthy group rats (Hb- 10.9 ± 0.98 gm/dl, RBC- 6.8 ± 0.89 m/cmm) indicating anemia in iron overloaded rats. Treatment with fresh wheatgrass juice (Hb- 10.5 ± 1.1 gm/dl, RBC- 6.7 ± 0.59 m/cmm), methanol extract (Hb- 10.2 ± 1.2 gm/dl, RBC- 6.1 ± 0.47 m/cmm) and acetone extract (Hb- 9.7 ± 0.89 gm/dl, RBC-

6.3 ± 0.7 m/cmm) of wheatgrass, produced significant increase in Hb levels and RBC counts in diseased rats. Decrease in blood Hb level and RBC count in rats was significantly prevented by treatment with fresh juice, methanol and acetone extracts of wheat grass. Thus, wheatgrass seems to help improve blood purification and also, to increase hemoglobin level and RBC count near to normal.

In disease control group rats which received busulfan, there was significant reduction in platelet count (523 $\pm$ 46 $10^3/\pi l$) compared to normal healthy group rats (905 $\pm$ 82 $10^3/\pi l$) indicating thrombocytopenia. Treatment with fresh wheatgrass juice (804 $\pm$ 72 $10^3/\pi l$), methanol extract (761 $\pm$ 58 $10^3/\pi l$) and acetone extract (708 $\pm$ 63 $10^3/\pi l$) produced significant increase in platelet count as compared to disease control group rats. Decrease in platelet count in these rats was significantly prevented by treatment with fresh wheatgrass juice, methanol and acetone extracts of wheatgrass.

Disease control group rats which received busulfan showed significant increase in bleeding (190 $\pm$ 18 sec) and clotting time (390 $\pm$ 35 sec) as a result of reduction in platelet counts compared to normal healthy group rats (bleeding time- 80 $\pm$ 12 sec, clotting time- 130 $\pm$ 22 sec) indicating hemophilia and thrombocytopenia in animals. Treatment with fresh wheatgrass juice (bleeding time- 98 $\pm$ 13 sec, clotting time- 150 $\pm$ 23 sec), methanol extract (bleeding time- 106 $\pm$ 17 sec, clotting time- 196 $\pm$ 24 sec) and acetone extract (bleeding time- 125 $\pm$ 15 sec, clotting time- 214 $\pm$ 30 sec) produced significant reduction in bleeding and clotting time in disease suffering rats. Increases in bleeding and clotting time in rats were significantly prevented by treatment with fresh juice, methanol and acetone extract of wheatgrass. Thus wheatgrass seems to help in reducing bleeding and clotting time, near to normal.

Disease control group rats which received busulfan showed significant reduction in total WBC and differential WBC counts compared to normal healthy group rats. Treatment with fresh wheatgrass juice, methanol extract produced significant increase in total WBC counts and differential WBC counts, in busulfan induced pancytopenic rats. Treatment with acetone extract did not produce significant increase in total WBC counts. Disease control group rats showed

pancytopenia (reduction in all blood cells count) compared to normal healthy control group rats. Treatment with fresh wheatgrass juice and different extracts showed increase in WBC counts compare to disease control group.

For investigation of beneficial effects of wheatgrass on immune system, reduction in cyclophosphamide-induced neutropenia and carbon clearance test, were used in our study. Cyclophosphamide belongs to nitrogen mustard subclass of alkylating agents and acts as an immunosuppressive agent by causing alkylation of DNA, in turn by interfering in DNA synthesis and function. It is also used extensively as an immunosuppressant (Thatte UM et al., 1987). Administration of cyclophosphamide (200 mg/kg, sc) produced a decrease in neutrophil count in all groups. Water extract of *Triticum aestivum* decreased neutrophil count significantly compared to control group.

There was significant increase in neutrophil adhesion to nylon fibres and increase in macrophage induced phagocytosis in carbon clearance test along with reduction in cyclophosphamide induced neutropenia. The methanol extract of wheatgrass was more effective than the water extract. The increase in adhesion of neutrophil to nylon fibres indicates migration of cells from blood vessels and the number of neutrophils reaching the site of inflammation (Shinde UA et al., 1999). Increase in neutrophil adhesion to nylon fibres may be due to up regulation of β_2 integrins that are present on surface of neutrophils through which; they adhere firmly to nylon fibres. Hence, it can be inferred that wheatgrass causes stimulation of neutrophil migration towards the site of inflammation. Results of the present study also suggest that wheatgrass may stimulate cell mediated immunity.

Carbon clearance test was carried out to evaluate effect of drugs on the reticulo-endothelial system (RES). It is a diffuse system of phagocytic cells, comprising of fixed tissue macrophages and mobile macrophages. The phagocytic cells in this system comprise of mononuclear phagocyte system (MPS). Macrophages are the major differentiated cell in MPS. Cells of the RES and MPS are known to be important in the clearance of particles from bloodstream. When colloidal ink containing carbon particles is injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an

exponential equation (Das M et al., 1998). Water and methanol extracts of wheatgrass showed significant increase in phagocytic index. Hence, we infer that wheatgrass may stimulate the reticuloendothelial system.

Thus, our investigation indicates beneficial effects of wheatgrass in iron overload diseases, immnocompromised conditions and thrombocytopenia. We have also, isolated a new iron chelator compound from wheatgrass. 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.

2. INTRODUCTION

The disease-preventive and health-promotive approach of 'Ayurveda', which takes into consideration the whole body, mind and spirit while dealing with the maintenance of health, promotion of health and treating ailments, is holistic and finds increasing acceptability in many regions of the world. Ancient Ayurvedic physicians had developed certain dietary and therapeutic measures to arrest / delay ageing and rejuvenating whole functional dynamics of the body system. Health is not merely absence of a disease. Rather, health is defined as positive state of well being in which the harmonious development of physical and mental capacities of the individual lead to the enjoyment of a rich and full life. Health involves primarily the application of medical science for the benefit of individual and society. Health is thus, a vital part of a concurrent and integrated program of development of all aspects of community life. Considering the importance of health, WHO and UNICEF jointly organized an international conference on Primary Health Care at Alma Ata, USSR from 6th to 12th September 1978 and took a momentous decision to achieve "Health for All" by the year 2000 AD. Now we are entering in the year 2007 but the Health for All by the year 2000 still continues to be a vision. There are various diseases like tropical diseases, herpes, AIDS, cancer, diabetes, thalassemia, certain blood disorders etc. for which the cure is yet to be found.

Iron is an absolute requirement for most forms of life, including humans and most bacterial species, because plants and animals all use iron. Iron is essential to life because of its unusual flexibility to serve as both an electron donor and acceptor. Iron can also be potentially toxic. Its ability to donate and accept electrons means that if iron is free within the cell, it can catalyze the conversion of hydrogen peroxide into free radicals. Free radicals can cause damage to cellular membranes, proteins, and DNA, a wide variety of cellular structures, and ultimately kill the cell. To prevent that kind of damage, all life forms that use iron bind the iron atoms to proteins. That allows the cells to use the benefits of iron, but also limit its ability to do harm (Andrews NC, 1995). Most well-nourished

people have 4 to 5 grams of iron in their bodies. Of this, about 2.5 g is contained in the hemoglobin needed to carry oxygen through the blood, and most of the rest is contained in ferritin complexes that are present in all cells, but most common in bone marrow, liver, and spleen. The liver's stores of ferritin are the primary physiologic source of reserve iron in the body (Schrier SL 2005). The human body needs iron for oxygen transport. That oxygen is required for the production and survival of all cells in our bodies. Human bodies tightly regulate iron absorption and recycling. Iron is such an essential element of human life, in fact, that humans have no physiologic regulatory mechanism for excreting iron (Schrier and Bacon 2005).

In medicine, iron overload disorders are diseases caused by the accumulation of iron in the body. Iron toxicity results when the amount of circulating iron exceeds the amount of transferrin available to bind it. The type of acute toxicity from iron ingestion causes severe mucosal damage in gastrointestinal tract, among other problems. Iron overload is one of the 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 thalassemia, sideroblastic anemia, abnormal red cell production (dyseryphropoiesis), iron overload secondary to IV therapy, chronic liver disease secondary to alcohol, porphyria cutanea tarda. Iron overload can be inherited (genetic) or acquired by receiving numerous blood transfusions, getting iron shots or injections, or consuming high levels of supplemental iron. Some of the genetic disorders that result in iron overload include are hereditary hemochromatosis (all types), African iron overload, sickle cell disease, thalassemia, X-linked sideroblastic anemia, enzyme deficiencies (pyruvate kinase; glucose-6-phosphate dehydrogenase) and very rare protein transport disorders aceruloplasminemia and atransferrinemia. None of these conditions should be confused with polycythemia vera (PV), which is not an iron disorder, but a condition where the bone marrow produces too many blood cells (red, white and platelet). People with PV have abnormally high hemoglobin and are at risk for a stroke and progressing to acute myelogenous leukemia (AML).

Excess iron in vital organs, even in mild cases of iron overload, increases the risk for liver disease (cirrhosis, cancer), heart attack or heart failure, diabetes

mellitus, osteoarthritis, osteoporosis, metabolic syndrome, hypothyroidism, hypogonadism, numerous symptoms and in some cases premature death. Iron mismanagement resulting in overload can accelerate such neurodegenerative diseases as Alzheimer's, early-onset Parkinson's, Huntington's, epilepsy and multiple sclerosis. Iron overload is major problem found in thalassemia major patients. In untransfused patients with severe ß-thalassemia, abnormally regulated iron absorption results in increases in body iron burden that may, depending on the severity of erythroid expansion, vary between 2 and 5 grams per year (Pippard et al., 1997, Pootrakul et al., 1988). Regular transfusions may double this rate of iron accumulation. Although most clinical manifestations of iron loading do not appear until the second decade of life in inadequately chelated individuals, evidence from serial liver biopsies in young patients indicates that the deleterious effects of iron are mediated much earlier. After approximately one year of transfusions, iron is deposited in parenchymal tissues, where it may cause significant toxicity as compared to that within reticuloendothelial cells (Hershko et al., 1998). As iron loading progresses, the capacity of serum transferrin, the main transport protein of iron, to bind and detoxify iron may be exceeded. Thereafter, the non-transferrin-bound fraction of iron within plasma may promote generation of free hydroxyl radicals, propagators of oxygen-related damage (Hershko et al., 1998). The effectiveness of an iron-chelating agent depends in part on its ability to bind non-transferrin bound plasma iron over sustained periods of time, thereby ameliorating iron-catalyzed toxicity of free radicals.

Synthetic agents like desferrioxamine and deferiprone used for the treatment of iron overload in thalassemia are accompanied by serious side effects and certain limitaions 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 (Hebbel et al., 1990; Grinberg et al., 1995; Kukongviriyapan et al., 2008). 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. The poor oral bioavailability, short plasma half-life and severe side effects of available

chelators are still not optimal (Filburn et al., 2007; Rachmilewitz et al., 1979; Livrea et al., 1996). Within this context and taking in 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 (Loukopoulos, 2005; Ebrahimzadeh et al., 2007; Mahmoudi et al., 2007; Pourmorad et al., 2007).

Despite the advances of combinatorial chemistry, natural products remain a major source of innovative therapeutic agents for numerous ailments including infectious diseases, cancer, lipid disorders, thalassema, and several blood disorders. Further, they serve as lead compounds for drug development and are proven to be invaluable biochemical tools for the identification of novel biological targets. For thousands of years, mankind has known about the benefits of drugs from nature. Plant extracts like wheatgrass juice have been highly regarded for their curative effects by ancient civilizations. Even today, plant materials remain an important resource for combating illnesses. WHO has approved the use of traditional medicines as a part of health programme. To pursue research in these systems of medicine, several USA agencies and institutions such as FDA and National Institute of Health have setup separate wings. According to the WHO survey 80% of the populations living in the developing countries rely almost exclusively on traditional medicine for the primary health care needs. In almost all the traditional medicine, the medicinal plants play a major role and constitute the backbone of the traditional medicine. The potential of plant as a source for new drugs is yet to be unexplored systematically. Among the estimated 250,000-400,000 plant species, only 6% have been studied for biological activity and about 15% have been investigated phytochemically (Verpoorte et al., 1998; Cragg et al., 1997; Balandrin et al., 1985). India has an ancient heritage of traditional medicine. Materia Medica of India provides lots of information on the folklore practices and traditional aspects of therapeutically important natural products. Indian traditional medicine is based on various system including Ayurveda, Siddha and Unani. With the emerging interest in the world to adopt and study the traditional system and to exploit their potentials based on different healthcare systems, the evaluation of the rich heritage of the traditional medicine is essential. Further, treatment with, one such traditional herbal drug viz. Wheatgrass, on patients with ß-

thalassemia (major) has been reported to have beneficial effects by decreasing iron overload (Desai et al., 2005). *Hence, in the present project we planned to investigate iron chelating potential of wheatgrass and its various extracts, in iron overload condition.*

Modern science has already accepted the potential of the 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 a rich reservoir of potential leads for drug discovery against various disorders. Almost half of the useful drugs today used for various diseases are derived from natural sources. Only less than two percent of all the plants available on the earth have been subjected to pharmacological investigations. Research on the medicinal herbs can offer useful drugs in time to come for the treatment of chronic diseases like asthma and diabetes etc. The global market of herbal drugs is increasing very rapidly and it is expected to touch the $5 trillion by end of 2005 (Pharma Business, 2000). The recent development of the science of phyto-pharmaceuticals has generated new enthusiasm in herbal drug research to discover new medicines (Patel and Saluja, 2002). *Looking at the dire need of a new safe and economical iron chelating molecule, we resolved to isolate probable active constituent of wheatgrass responsible for its possible chelating activity.*

Wheat (*Triticum* species) a cereal grass of the Gramineae (Poaceae) family is the world's largest edible grain cereal-grass crop. Wheat has been a food crop for mankind since the beginning of agriculture. For over fifty years, researchers have known that the cereal plant, at this young green stage, is many times richer in the levels of vitamins, minerals and proteins as compared to seed kernel, or grain products of the mature cereal plant (Schnabel 1940). The young germinated plant is a factory of enzyme and growth activity. In the early stages of growth they store large amounts of vitamins and proteins in the young blades. After jointing stage, the nutritional level in the leaves drops rapidly while the fiber content increases rapidly (Kohler 1944). Agriculturally, important species of *Triticum* include - *Triticum aestivum, Triticum durum* and *Triticum dicoccum.* Wheatgrass has been traditionally used, since ancient times, to treat various

diseases and disorders. Presently, there are 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 a 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 the ‘‘Wheatgrass Therapy”. Dr. Wigmore claimed that wheatgrass is a safe and effective treatment for ailments such as high blood pressure, some cancers, obesity, diabetes, gastritis, ulcers, anemia, asthma and eczema. Scientific reports on nutritional analysis of wheatgrass have been published frequently in various journals (Kohler 1953, Hamilton et al., 1988, Laboratory Analyses 1989). These reports and the 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, phenol and flavonoid which have significant nutritious and medicinal value (Wigmore 1985). *Since, iron overload induces increased oxidative stress and wheatgrass is known to contain considerable amount of antioxidants, we decided to investigate antioxidant benefits of wheatgrass in iron overload condition.*

Platelets are made in the bone marrow similar to other cells in blood such as, white blood cells and red blood cells. Platelets originate from megakaryocytes which are large cells found in the bone marrow. Platelets, in general, have a brief 7 to 10 days life in blood, after which they are removed from the blood circulation. The number of platelets in the blood is referred to as the platelet count and is normally between 150,000 to 450,000 per micro liter of blood. Platelet counts less than 150,000 are termed thrombocytopenia (Maton et al, 1993). There are a wide range of botanical sources and wide range of active constituents that might ultimately contribute to haemostatic action, including essential oils, flavonoids, saponins, and alkaloids. The possible mechanisms of action of the hemostatic herbs include: increasing the production of platelets, promoting the ability of platelets to aggregate when there is blood leakage, decreasing capillary permeability, contracting peripheral blood vessels, inhibiting autoimmune attack against platelets. Dr. Wigmore in her programme the “wheatgrass” made several clinical trials on wheatgrass and reported that plants are a safe and effective treatment for anemia and various bleeding

disorder like hemophilia and thrombocytopenia (Wigmore 1985). These effects should be expected to be observed within a few days of administering the herbs. *In this context, we decided to investigate beneficial effects of wheatgrass in treatment of thrombocytopenia and other bleeding disorders.*

The immune system is a remarkably effective structure that incorporates specificity, inducibility and adaptation. Failures of host defense do occur, however, and fall into three broad categories: immunodeficiencies, autoimmunity and hypersensitivities. The immune system is involved in the etiology as well as pathophysiological mechanisms of many diseases. Modulation of the immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles (Sharma P 1983). Indian medicinal plants are a rich source of substances which are claimed to induce paraimmunity, the non-specific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement functions (Sainis 1997). Ayurveda, the Indian traditional system of medicine, lays emphasis on promotion of health concept of strengthening host defenses against different diseases (Thatte 1986). Dr. Wigmore's opinions are based on her experiences. A few clinical studies, have verified that some disease conditions can be benefited by the use of wheatgrass. Remarkably, a relatively large number of studies indicate that food factors and nutrients found in wheatgrass may provide beneficial in immunological disorders. *Hence, we made an attempt to assess immunomodulatory potential of wheatgrass, using various animal models.*

In nutshell the objectives of the present project were –

1. To carry out pharmacognostic study of *Triticum aestivum.*
2. To carry out phytochemical studies of *Triticum aestivum* (Wheatgrass) and it's various extracts.
3. To evaluate iron chelating activity of various extracts of *Triticum aestivum.*
4. Isolation of iron chelating compound from extract of *Triticum aestivum* using column chromatography.
5. To evaluate iron chelating activity of isolated compound of *Triticum aestivum* using pre-clinical study.
6. To investigate anti-oxidant property of *Triticum aestivum* in iron overload condition.
7. To investigate therapeutic benefit of *Triticum aestivum* in thrombocytopenia.
8. To investigate immunomodulatory activity of *Triticum aestivum.*

3. REVIEW OF LITERATURE

3.1 Iron overload diseases

Physiology of iron in human body

Iron is an absolute requirement for most forms of life, including humans and most bacterial species, because plants and animals all use iron. Iron is essential to life because of its unusual flexibility to serve as both an electron donor and acceptor. Iron can also be potentially toxic. Its ability to donate and accept electrons means that if iron is free within the cell, it can catalyze the conversion of hydrogen peroxide into free radicals. Free radicals can cause damage to cellular membranes, proteins, and DNA, a wide variety of cellular structures, and ultimately kill the cell. To prevent that kind of damage, all life forms that use iron bind the iron atoms to proteins. That allows the cells to use the benefits of iron, but also limit its ability to do harm (Andrews NC 1995).

Body iron and its physiological role

Most well-nourished people have 4 to 5 grams of iron in their bodies. Of this, about 2.5 g is contained in the hemoglobin needed to carry oxygen through the blood, and most of the rest (approximately 2 grams in adult men, and somewhat less in women of childbearing age) is contained in ferritin complexes that are present in all cells, but most common in bone marrow, liver, and spleen. The liver's stores of ferritin are the primary physiologic source of reserve iron in the body. The reserves of iron in adults tend to be lower in children and women of child-bearing age, than in men and in the elderly. Women who must use their stores to compensate for iron lost through menstruation, pregnancy or lactation, have lower body stores, which may consist of 500 mg or even less (Schrier SL 2005).

Of the body's total iron content, about 400 mg is devoted to cellular proteins that use iron for important cellular processes like storing oxygen (myoglobin), or

performing energy-producing redox reactions (cytochromes). A relatively small amount (3-4 mg) circulates through the plasma, bound to transferrin (Fleming and Bacon, 2005). Because of its toxicity, free soluble iron (soluble ferrous ions Fe (II)) is kept in low concentration in the body. The most important group of iron-binding proteins contains the heme molecules, all of which contain iron at their centers. Humans and most bacteria use variants of heme to carry out redox reactions and electron transport processes. These reactions and processes are required for oxidative phosphorylation. That process is the principal source of energy for human cells; without it, most types of cells would die. The iron-sulfur proteins are another important group of iron-containing proteins. Some of these proteins are also essential parts of oxidative phosphorylation. Humans also use iron in the hemoglobin of red blood cells, in order to transport oxygen from the lungs to the tissues and to export carbon dioxide back to the lungs. Iron is also an essential component of myoglobin to store and diffuse oxygen in muscle cells (Fleming and Bacon, 2005).

The human body needs iron for oxygen transport. That oxygen is required for the production and survival of all cells in our bodies. Human bodies tightly regulate iron absorption and recycling. Iron is such an essential element of human life, in fact, that humans have no physiologic regulatory mechanism for excreting iron. Most humans prevent iron overload solely by regulating iron absorption. Those who cannot regulate absorption well enough get disorders of iron overload. In these diseases, the toxicity of iron starts overwhelming the body's ability to bind and store it (Schrier and Bacon, 2005).

Absorption of iron from diet

The absorption of dietary iron is a variable and dynamic process. The amount of iron absorbed compared to the amount ingested is typically low, but may range from 5% to as much as 35% depending on circumstances and type of iron. The efficiency with which iron is absorbed varies depending on the source. Generally the best-absorbed forms of iron come from animal products. Absorption of dietary iron in iron salt form (as in most supplements) varies somewhat according to the body's need for iron, and is usually between 10% and 20% of iron intake. Absorption of iron from animal products, and some plant products, is

in the form of heme iron, and is more efficient, allowing absorption of from 15% to 35% of intake. Heme iron in animals is from blood and heme containing proteins in meat and mitochondria, whereas in plants, heme iron is present in mitochondria in all cells that use oxygen for respiration (Andrews NC 1995).

Like most mineral nutrients, the majority of the iron absorbed from digested food or supplements is absorbed in the duodenum by enterocytes of the duodenal lining. These cells have special molecules that allow them to move iron into the body. To be absorbed, dietary iron can be absorbed as part of a protein such as heme protein or must be in its ferrous Fe^{2+} form. A ferric reductase enzyme on the enterocytes brush border, Dcytb, reduces ferric Fe^{3+} to Fe^{2+}. A protein called divalent metal transporter DMT_1, which transports all kinds of divalent metals into the body, then transports the iron across the enterocyte's cell membrane and into the cell. These intestinal lining cells can then either store the iron as ferritin, which is accomplished by Fe^{3+} binding to apoferritin (in which case the iron will leave the body when the cell dies and is sloughed off into feces) or the cell can move it into the body, using a protein called ferroportin. The body regulates iron levels by regulating each of these steps. For instance, cells produce more Dcytb, DMT_1 and ferroportin in response to iron deficiency anemia (Berg J et al., 2001).

Our body's rates of iron absorption appear to respond to a variety of interdependent factors, including total iron stores, the extent to which the bone marrow is producing new red blood cells, the concentration of hemoglobin in the blood, and the oxygen content of the blood. We also absorb less iron during times of inflammation. Recent discoveries demonstrate that hepcidin regulation of ferroportin is responsible for the syndrome of anemia of chronic disease. While Dcytb is unique to iron transport across the duodenum, ferroportin is distributed throughout the body on all cells which store iron. Thus, regulation of ferroportin is the body's main way of regulating the amount of iron in circulation. Hephaestin, a ferroxidase that which can oxidize Fe^{2+} to Fe^{3+} and is found mainly in the small intestine, helps ferroportin transfer iron across the basolateral end of the intestine cells. Iron absorption from diet is enhanced in the presence of vitamin C and diminished in the presence of calcium. (Berg J et al., 2001)

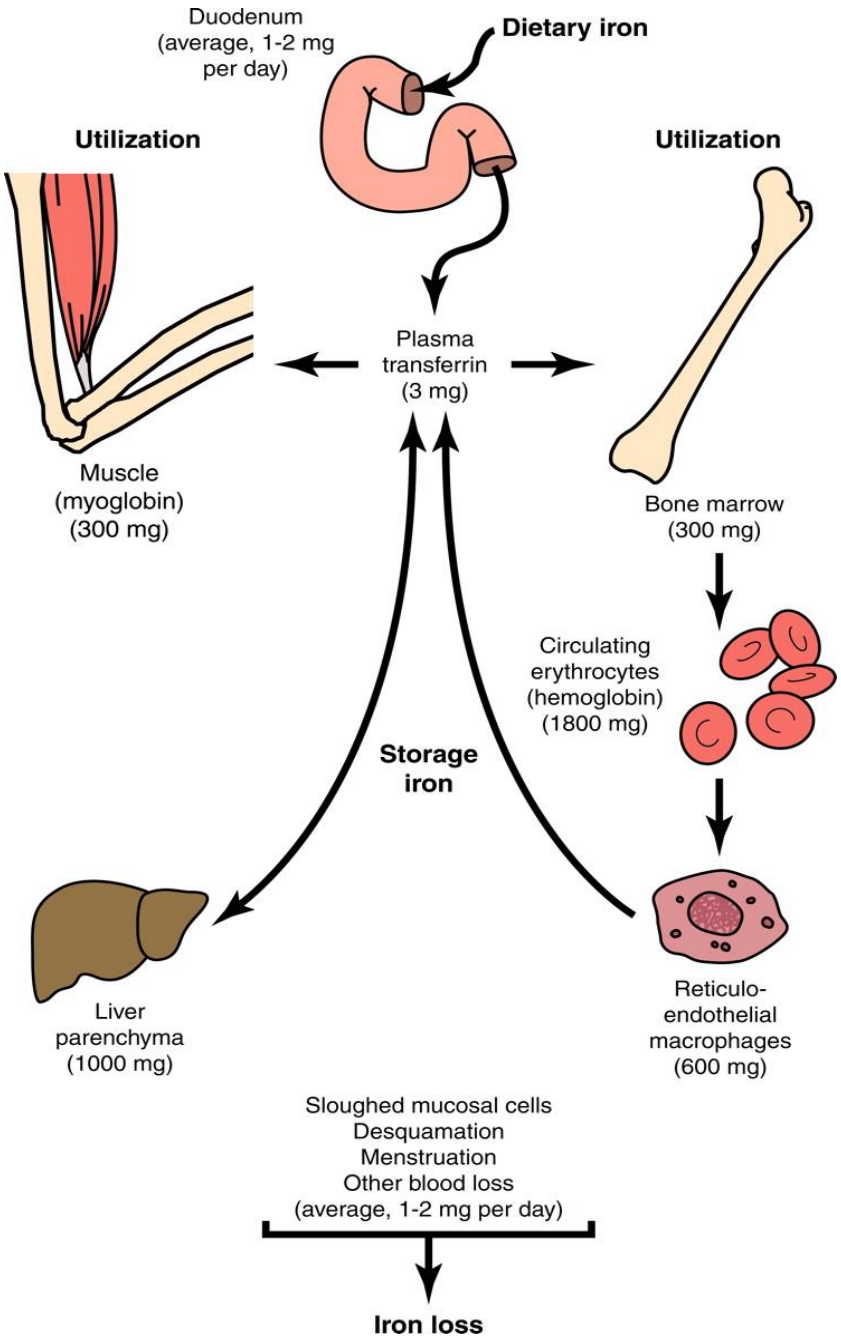


Figure 3.1: Iron balance in human body (Jonathan Himmelfarb, 2007)

Regulation of iron levels is a task of the whole body, as well as for individual cells. When body levels of iron are too low, then hepcidin in the duodenal epithelium is decreased. This causes an increase in ferroportin activity, stimulating iron uptake in the digestive system. The reverse occurs when there is an iron surplus. In individual cells, an iron deficiency causes responsive element binding protein to iron responsive elements on mRNA for transferrin receptors,

resulting in increased production of transferrin receptors. These receptors increase binding of transferrin to cells, and therefore stimulating iron uptake.

Iron transfer/recycling

Iron is not free in circulation but exists as transferrin (bound to apotransferrin). Most of iron used for red blood cell hemoglobin production is obtained from hemoglobin breakdown of senescent RBCs (called recycling). When red blood cells reach end of their lifespan (senescent), they are phagocytized by macrophages (in the spleen, liver, bone marrow). Hydrolytic enzymes in macrophages degrade the ingested RBCs and release hemoglobin. Proteolytic digestion of hemoglobin liberates heme and globins. Globins are broken down to amino acids which can be used for protein production. The iron is released from heme, leaving a porphyrin ring which is converted to bilirubin. For more information on this, refer to the page on extravascular hemolysis). Once iron is released from the heme, it is utilized by the cell (iron is an essential component of many enzymes), exported (via ferroportin), or stored as ferritin (like enterocytes). In macrophages, ceruloplasmin (which like hephaestin in intestinal cells also requires copper) is a ferroxidase and facilitates transfer of macrophage iron to transferrin. So copper deficiency decreases iron release from macrophages and affects iron absorption. Like enterocytes, hepcidin down regulates ferroportin causing iron sequestration in macrophages.

Most of the iron in the body is hoarded and recycled by the reticuloendothelial system, which breaks down aged red blood cells. However, people lose a small but steady amount by sweating and by shedding cells of the skin and the mucosal lining of the gastrointestinal tract. The total amount of loss for healthy people in the developed world amounts to an estimated average of 1 mg a day for men and 1.5–2 mg a day for women with regular menstrual periods. People with gastrointestinal parasitic infections, more commonly found in developing countries, often lose more (Baker MD 2003). This steady loss means that people must continue to absorb iron. They do so via a tightly regulated process that under normal circumstances protects against iron overload.

Iron uptake by eythroid progenitors

Transferrin-bound iron (from absorption of dietary iron in the intestine or released by macrophages) binds to transferrin receptors, which are highly expressed on the surface of red cell precursors, and is taken up into the cells where it is used to form hemoglobin. Erythroid progenitors cluster around macrophages in the bone marrow and spleen (figure 3.2), because they are obtaining their iron (required for hemoglobin synthesis) from these iron-storing cells, as well as from circulating transferrin.

Excess iron is dangerous, because it promotes free radical production. Whole body iron levels are regulated primarily at the level of absorption by enterocytes, there is no regulated pathway for active excretion of iron (can only occur by bleeding or sloughing of iron-laden enterocytes). Regulation of iron uptake by enterocytes and release of iron stores from macrophages and hepatocytes is mediated by the hormone hepcidin, and its effect on ferroportin. Hepcidin decreases serum iron by decreasing iron absorption and preventing macrophages from releasing iron (causing iron sequestration). Hepcidin is regulated by iron levels and erythropoiesis. Increased iron will unregulated hepcidin which then decreases iron and vice versa. Active erythropoiesis inhibits hepcidin (allowing iron to be absorbed/released for hemoglobin synthesis). Hepcidin is increased by inflammatory cytokines, particularly IL_6, and reduces available iron during inflammatory processes. Inflammation thus causes a "functional" iron deficiency because iron is not released from macrophages (results in increased iron stores). This contributes to the anemia of inflammatory disease.

Iron absorbed from the intestine is stored as ferritin in intestinal epithelium or transported in plasma as transferrin. Erythroid progenitors obtain iron for hemoglobin synthesis from plasma transferrin or from recycling of senescent erythrocytes by macrophages in bone marrow, spleen and liver. Iron that is in excess for that required for hemoglobin production is stored in macrophages as ferritin, which is oxidized to hemosiderin. These stores can be released from macrophages in times of need (increased erythropoiesis).

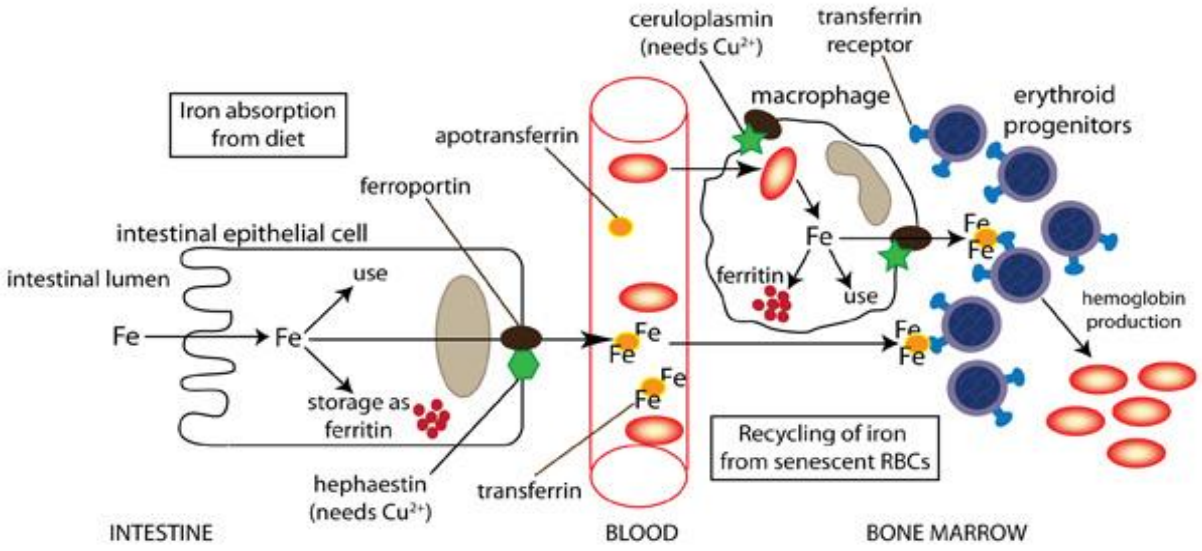


Figure 3.2: Iron absorption and recycle in human body

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 absorption from gut. Normal iron absorption is 1-1.5 mg/day; additional iron is absorbed only when it is required. Iron absorption is proportional to severity of anemia, serum iron levels, erythropoesis (Formation of RBCs), amount of iron in the 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 deficiency disorders

Iron deficiency anemia (or iron deficiency anaemia) is a common anemia that occurs when iron loss (often from intestinal bleeding or menses) occurs, and/or the dietary intake or absorption of iron is insufficient. In iron deficiency, hemoglobin, which contains iron, cannot be formed. (Brady PG, 2007)

Iron deficiency is the most common single cause of anemia worldwide, accounting for about half of all anemia cases. It is more common in women than men. Estimates of iron deficiency world wide range very widely, but the number almost certainly exceeds one billion persons globally. Worldwide, the most important cause of iron deficiency anemia is parasitic infection caused by

hookworms, whipworms, and roundworms, in which intestinal bleeding caused by the worms may lead to undetected blood loss in the stool. These are especially important problems in growing children (Calis JC et al., 2008). Malaria infections that destroy red blood cells (although the iron is recycled) and chronic blood loss caused by hookworms (where the iron is lost) contribute to anemia during pregnancy in most developing countries (Dreyfuss ML et al., 2000). In adults of post-menopausal age (over 50 years old) the most common cause of iron-deficiency anemia is chronic gastrointestinal bleeding from nonparasitic causes, such as from gastric ulcer, duodenal ulcer or a gastrointestinal cancer.

Causes of iron deficiency

- Chronic bleeding (hemoglobin contains iron)
  - Excessive menstrual bleeding
  - Non-menstrual bleeding
  - Bleeding from the gastrointestinal tract (ulcers, hemorrhoids, etc.)
  - Rarely, laryngological bleeding or from the respiratory tract
- Inadequate intake (special diets low in dietary iron)
- Substances (in diet or drugs) interfering with iron absorption
- Malabsorption syndromes
- Fever where it is adaptive to control bacterial infection
- Blood donation

Though genetic defects causing iron deficiency have been studied in rodents, there are no known genetic disorders of human iron metabolism that directly cause iron deficiency.

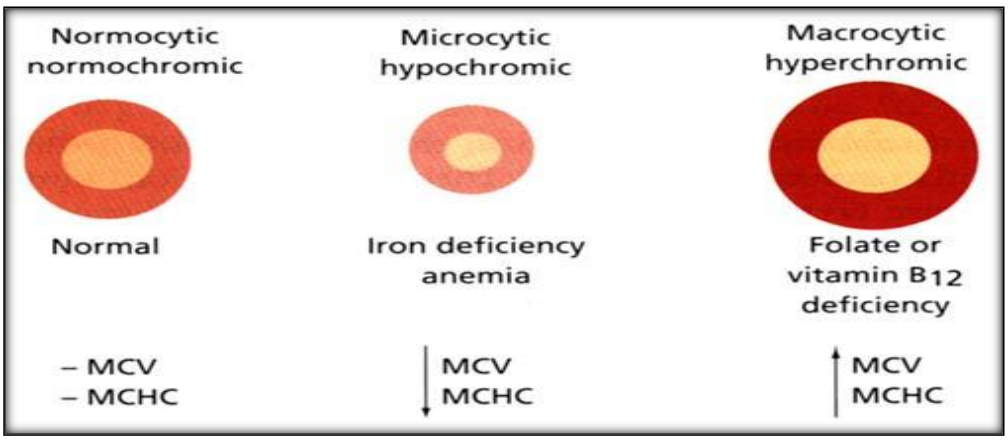


Figure 3.3: Different types of anemia

Iron deficiency anemia is characterized by pallor (reduced amount of oxyhemoglobin in skin or mucous membrane), fatigue and weakness. Because it tends to develop slowly, adaptation occurs and the disease often goes unrecognized for some time. In severe cases, dyspnea (trouble breathing) can occur. Unusual obsessive food cravings, known as pica, may develop. Pagophagia or pica for ice is a very specific symptom and may disappear with correction of iron deficiency anemia. Hair loss and lightheadedness can also be associated with iron deficiency anemia. Other symptoms and signs of iron deficiency anemia include anxiety often resulting in OCD type compulsions and obsessions, irritability or a low feeling, angina, constipation, sleepiness, tinnitus, mouth ulcers, palpitations, hair loss, fainting or feeling faint, depression, breathlessness on exertion, twitching muscles, tingling, numbness, or burning sensations, missed menstrual cycle, heavy menstrual period, slow social development, glossitis (inflammation or infection of the tongue), angular cheilitis (inflammatory lesions at the mouth's corners), koilonychia (spoon-shaped nails) or nails that are weak or brittle, poor appetite, pruritus (itchiness), dysphagia due to formation of esophageal webs (Plummer-vinson syndrome), Restless Legs Syndrome. (Rangarajan et al., 2007)

Iron deficiency anemia affects neurological development in infant by decreasing learning ability, altering motor functions, and permanently reducing the number of dopamine receptors and serotonin levels. Iron deficiency during development can lead to reduced myelination of the spinal cord, as well as a change in myelin composition. Additionally, iron deficiency anemia has a negative effect on physical growth. Growth hormone secretion is related to serum transferrin levels, suggesting a positive correlation between iron-transferrin levels and an increase in height and weight.

Anemia can easily diagnose by routine blood tests, which generally include a complete blood count (CBC) which is performed by an instrument which gives an output as a series of index numbers. Sufficiently low hemoglobin (HGB) by definition makes the diagnosis of anemia, and a low hematocrit (HCT) value is also characteristic of anemia. If anemia is due to iron deficiency, one of first abnormal values to be noted on a CBC, as body's iron stores begin to be depleted, will be a high red blood cell distribution width (RDW), reflecting an increased

variability in size of red blood cells (RBCs). In the course of slowly depleted iron status, an increasing RDW normally appears even before anemia appears. A low mean corpuscular volume (MCV) often appears next during the course of body iron depletion. It is the result of many red blood cells which are abnormally small. A low MCV, a low mean corpuscular hemoglobin (MCH) and/or Mean corpuscular hemoglobin concentration (MCHC), and the appearance of the RBCs on visual examination of a peripheral blood smear narrows the problem to a microcytic anemia (literally, a "small red blood cell" anemia). (NPS News 70)

Change in lab values in iron deficiency anemia	
Change	**Parameter**
Decrease	ferritin, hemoglobin, MCV
Increase	TIBC, transferrin, RDW

The blood smear of a patient with iron deficiency shows many hypochromic (pale and relatively colorless) and rather small RBCs, and may also show poikilocytosis (variation in shape) and anisocytosis (variation in size). With more severe iron deficiency anemia the peripheral blood smear may show target cells, hypochromic pencil-shaped cells, and occasionally small numbers of nucleated red blood cells (Mazza, J et al., 1978). Very commonly, the platelet count is slightly above the high limit of normal in iron deficiency anemia (this is mild thrombocytosis). This effect which was classically postulated to be to high erythropoietin levels in the body as a result of anemia, cross-reacting to activate thrombopoietin receptors in precursor cells which make platelets; however, this mechanistic effect has been searched for and not corroborated. Such slightly increased platelet counts present no danger, but remain valuable as an indicator even if their mechanistic origin is not yet known.

If the cause is dietary iron deficiency, eating more iron-rich foods such as beans and lentils or taking iron supplements, usually with iron(II) sulfate, ferrous gluconate, or iron amino acid chelate ferrous bisglycinate, synthetic chelate NaFerredetate EDTA will usually correct the anemia. If anemia does not respond to oral treatments, it may be necessary to administer iron parenterally (e.g. as iron dextran) using a drip or hemodialysis. Parenteral iron involves risks of

fever, chills, backache, myalgia, dizziness, syncope, rash and anaphylactic shock. A follow up blood test is essential to demonstrate whether the treatment has been effective. Iron supplements should be kept out of the reach of children, as iron-containing supplements are a frequent cause of poisoning in children.

Iron overload in various diseases

In medicine, iron overload disorders are diseases caused by the accumulation of iron in the body. Iron overload is one of the 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. Iron toxicity results when the amount of circulating iron exceeds the amount of transferrin available to bind it, but the body is able to vigorously regulate its iron uptake. Thus, iron toxicity from ingestion is usually resulting of extraordinary circumstances like iron tablet overdose rather than variations in diet. The type of acute toxicity from iron ingestion causes severe mucosal damage in gastrointestinal tract, among other problems.

List of iron overload disorders

Primary iron overload

- Type 1: Haemochromatosis
- Type 2: Juvenile hereditary iron overload
  (Mendelian Inheritance in Man (OMIM))
- Type 3: Hereditary iron overload associated to mutations in transferrin receptor 2 genes (Mendelian Inheritance in Man (OMIM))
- Type 4: Hereditary iron overload associated to mutations in the SLC11A3 gene (Ferroportin/MTP/IREG-1) (Mendelian Inheritance in Man (OMIM))
- Aceruloplasminaemia (rare disease)
  (Mendelian Inheritance in Man (OMIM))
- Congenital atransferrinaemia (rare disease)
  (Mendelian Inheritance in Man (OMIM))
- Others (unidentified genes)

- ✓ Juvenile haemochromatosis (Mendelian Inheritance in Man (OMIM))
- ✓ Neonatal haemochromatosis (Mendelian Inheritance in Man (OMIM))

Secondary iron overload

- Dietary iron overload
- Transfusional iron overload
- Long term haemodialysis
- Chronic liver disease
  - ✓ Hepatitis C
  - ✓ Alcoholic cirrhosis, especially when advanced
  - ✓ Non-alcoholic steatohepatitis
- Porphyria cutanea tarda
- Post-portacaval shunting
- Dysmetabolic iron overload syndrome

Excess iron in vital organs, even in mild cases of iron overload, increases the risk for liver disease (cirrhosis, cancer), heart attack or heart failure, diabetes mellitus, osteoarthritis, osteoporosis, metabolic syndrome, hypothyroidism, hypogonadism, numerous symptoms and in some cases premature death. Iron mismanagement resulting in overload can accelerate such neurodegenerative diseases as Alzheimer's, early-onset Parkinson's, Huntington's, epilepsy and multiple sclerosis.

Iron overload can be inherited (genetic) or acquired by receiving numerous blood transfusions, getting iron shots or injections, or consuming high levels of supplemental iron. Some of the genetic disorders that result in iron overload include are hereditary hemochromatosis (all types), African iron overload, sickle cell disease, thalassemia, X-linked sideroblastic anemia, enzyme deficiencies (pyruvate kinase; glucose-6-phosphate dehydrogenase) and very rare protein transport disorders aceruloplasminemia and atransferrinemia. None of these conditions should be confused with polycythemia vera (PV), which is not an iron disorder, but a condition where the bone marrow produces too many blood cells (red, white and platelet). People with PV have abnormally high hemoglobin and

are at risk for a stroke and progressing to acute myelogenous leukemia (AML). Part of the therapy for PV is phlebotomy.

Symptoms, signs and diseases resulting from too much iron (iron overload):

- chronic fatigue
- joint pain
- abdominal pain
- liver disease (cirrhosis, liver cancer)
- diabetes mellitus
- irregular heart rhythm
- heart attack or heart failure
- skin color changes (bronze, ashen-gray green)
- loss of period
- loss of interest in sex
- osteoarthritis
- osteoporosis
- hair loss
- enlarged liver or spleen
- impotence
- infertility
- hypogonadism
- hypothyroidism
- hypopituitarism
- depression
- adrenal function problems
- early onset neurodegenerative disease
- elevated blood sugar
- elevated liver enzymes like SGPT, SGOT
- elevated iron (serum iron, serum ferritin)

Haemochromatosis or haemosiderosis

Historically, the term haemochromatosis was initially used to refer to what is now more specifically called haemochromatosis type 1 (or HFE-related hereditary haemochromatosis). Currently, haemochromatosis (without further

specification) is mostly defined as iron overload with a hereditary/primary cause or originating from a metabolic disorder (American Heritage Medical Dictionary, 2004). However, the term is currently also used more broadly to refer to any form of iron overload, thus requiring specification of the cause, for example, hereditary haemochromatosis. Hereditary haemochromatosis is an autosomal recessive disease with estimated prevalence in the population of 2 in 1,000 in Caucasians, with lower incidence in other races. The gene responsible for hereditary haemochromatosis (known as HFE gene) is located on chromosome 6; the majority of hereditary haemochromatosis patients have mutations in this HFE gene. Hereditary haemochromatosis is characterized by an accelerated rate of intestinal iron absorption and progressive iron deposition in various tissues that typically begins to be expressed in the third to fifth decades of life, but may occur in children. The most common presentation is hepatic cirrhosis in combination with hypopituitarism, cardiomyopathy, diabetes, arthritis, or hyperpigmentation. Because of the severe sequelae of this disease if left untreated, and recognizing that treatment is relatively simple, early diagnosis before symptoms or signs appear is important (Pietrangelo and Antonello 2010).

In general, the term haemosiderosis is used to indicate the pathological effect of iron accumulation in any given organ, which mainly occurs in the form of haemosiderin. Sometimes, the simpler term siderosis is used instead.

Other definitions distinguishing haemochromatosis or haemosiderosis that are occasionally used include: (American Heritage Medical Dictionary, 2004)

- Haemosiderosis is haemochromatosis caused by excessive blood transfusions, that is, haemosiderosis is a form of secondary haemochromatosis.
- Haemosiderosis is hemosiderin deposition within cells, while haemochromatosis is hemosiderin within cells and interstitium.
- Haemosiderosis is iron overload that does not cause tissue damage, while haemochromatosis does.

- Haemosiderosis is arbitrarily differentiated from haemochromatosis by the reversible nature of the iron accumulation in the reticuloendothelial system.

Haemochromatosis may present with the following clinical syndromes include cirrhosis of the liver, diabetes due to pancreatic islet cell failure, cardiomyopathy, arthritis (iron deposition in joints), testicular failure, tanning of the skin.

The causes can be distinguished between primary cases (hereditary or genetically determined) and less frequent secondary cases (acquired during life)(Pietrangelo A, 2003). People of Celtic (Irish, Scottish, Welsh) origin have a particularly high incidence of whom about 10% are carriers of the gene and 1% sufferers from the condition.

Primary haemochromatosis

The fact that most cases of haemochromatosis were inherited was well known for most of 20th century, though they were incorrectly assumed to depend on a single gene (Cam Patterson 2006). The overwhelming majority actually depends on mutations of HFE gene discovered in 1996, but since then others have been discovered and sometimes are grouped together as "non-classical hereditary haemochromatosis" "non-HFE related hereditary haemochromatosis" or "non-HFE haemochromatosis". (Mendes and Ana Isabel, 2008)

Table 3.1: Genetic mutation in various types of haemochromatosis (Franchini and Massimo, 2006)

Description	Mutation
Haemochromatosis type 1: "classical"-haemochromatosis	HFE
Haemochromatosis type 2A: juvenile haemochromatosis	Haemojuvelin ("HJV", also known as RGMc and HFE2)
Haemochromatosis type 2B: juvenile haemochromatosis	Hepcidin antimicrobial peptide (*HAMP*) or HFE_2B
Haemochromatosis type 3	Transferrin receptor-2

	(TFR$_2$ or HFE$_3$)
Haemochromatosis type 4/ African iron overload	Ferroportin (SLC$_{11}$A$_3$/SLC$_{40}$A$_1$)
Neonatal haemochromatosis	(unknown)
Acaeruloplasminemia (very rare)	Caeruloplasmin
Congenital atransferrinaemia (very rare)	Transferrin
GRACILE syndrome (very rare)	BCS$_1$L

Most types of hereditary haemochromatosis have autosomal recessive inheritance, while type 4 has autosomal dominant inheritance.

Secondary haemochromatosis

- Severe chronic hemolysis of any cause, including intravascular hemolysis and ineffective erythropoiesis (hemolysis within the bone marrow).
- Multiple frequent blood transfusions (either whole blood or just red blood cells), which are usually needed either by individuals with hereditary anaemias (such as beta-thalassaemia major, sickle cell anaemia, and Diamond–Blackfan anaemia) or by older patients with severe acquired anaemias such as in myelodysplastic syndromes.
- Excess parenteral iron supplements, such as can acutely happen in iron poisoning
- Excess dietary iron
- Some disorders do not normally cause haemochromatosis on their own, but may do so in the presence of other predisposing factors. These include cirrhosis (especially related to alcohol abuse), steatohepatitis of any cause, porphyria cutanea tarda, prolonged haemodialysis, post-portacaval shunting.

Treatment

Routine treatment in an otherwise-healthy person consists of regularly scheduled phlebotomies (bloodletting). When first diagnosed, the phlebotomies may be fairly frequent, perhaps as often as once a week, until iron levels can be brought to within normal range. Once iron and other markers are within the normal range, phlebotomies may be scheduled every other month or every three months depending upon the patient's rate of iron loading.

For those unable to tolerate routine blood draws, there is a chelating agent available for use. The drug desferoxamine binds with iron in the bloodstream and enhances its elimination via urine and faeces. Typical treatment for chronic iron overload requires subcutaneous injection over a period of 8–12 hours daily. Two newer iron chelating drugs that are licensed for use in patients receiving regular blood transfusions to treat thalassemia (and, thus, who develop iron overload as a result) are deferasirox and deferiprone.

Thalassemia

Thalassemia is one of the most common groups of genetic blood disorder. The word thalassemia was derived from two Greek words – 'Thalassa' meaning the sea and 'haima' meaning blood. Thomas Benton Cooley (1871-1945) was the first American physician to describe the clinical presentation and features of unexplained severe anemia and hence it was coined Cooley's anemia. Countries like Italy, Greece and Cyprus have the highest frequency of thalassemia cases in the world. There exists a thalassemic "belt" that includes the Mediterranean passing through West and Central Asian countries like Turkey, Iran, Afghanistan onto Pakistan and India and passes on to the South East Asian countries like Indonesia, Burma and Thailand. There are an estimated 240 million carriers of thalassemia in the world. India has the largest pool of numbering around 30 million (every 8th carrier of thalassemia is an Indian). The distribution of ß-thalassemia gene is not uniform in the Indian subcontinent. The highest frequency of ß-thalassemia trait is reported in Gujarat (10-15%), followed by Sindh (10%), Tamil Nadu (8.4%), Maharashtra (7.04%), Punjab (6.5%) and South India (4.3%) (Varawalla et al., 1991, Balgir, 1996, Sukumaran and Master, 1974). The condition is uncommon south of Vindhyas. Very high rates have been

found in certain communities such as Sindhis 12.4% and in Lohana Gujratis 13.6 %. The reason is attributed to intra-caste and intra-community marriages. It is estimated that every year about 10,000 children are born in India with the disorders. A staggering sum of Rs. 150 crores is required to be spent every year, in terms of cost of ideal maintenance of these children (Varawalla et al., 1991).

The thalassemias are characterized by impaired production of one or more polypeptide chains of globin. Any of the four polypeptides (α, β, γ, ∂) that occur in normal hemoglobin may be involved. However, the most prevalent thalassemia syndromes are those that involve diminished or absent synthesis of the α- or β-globin chains of HbA_1 (Weatherall 1997, Jandl 1987, Steinberg 1988). Two distinct types of globin chains (each with its individual heme molecule) combine to form hemoglobin. One of the chains is alpha (α). The second chain is "non-alpha". With the exception of the very first weeks of embryogenesis, one of the globin chains is always alpha. A number of variables influence the nature of the non-alpha chain in the hemoglobin molecule. The fetus has a distinct non-alpha chain called gamma (γ). After birth, a different non-alpha globin chain, called beta (β), pairs with the alpha chain. The combination of two alpha chains and two non-alpha chains produces a complete hemoglobin molecule (a total of four chains per molecule). The combination of two alpha chains and two gamma chains form "fetal" hemoglobin, termed "hemoglobin F" i.e. (α_2 β_2). With the exception of the first 10 to 12 weeks after conception, fetal hemoglobin is the primary hemoglobin in the developing fetus. The combination of two alpha chains and two beta chains form "adult" hemoglobin, also called "hemoglobin A" i.e. (α_2 β_2). Although hemoglobin A is called "adult", it becomes the predominant hemoglobin within about 18 to 24 weeks of birth.

The pairing of one alpha chain and one non-alpha chain produces a hemoglobin dimer (two chains). The hemoglobin dimer does not efficiently deliver oxygen, however. Two dimers combine to form a hemoglobin tetramer, which is functional form of hemoglobin. Complex biophysical characteristics of hemoglobin tetramer permit exquisite control of oxygen uptake in the lungs and release in the tissues that is necessary to sustain life. The genes that encode the alpha globin chains are on chromosome 16. Those that encode the non-alpha globin chains are on chromosome 11. Multiple individual genes are expressed at

each site. Pseudogenes are also present at each location. The alpha complex is called the "alpha globin locus", while the non-alpha complex is called the "beta globin locus". The expression of the alpha and non-alpha genes is closely balanced by an unknown mechanism. Balanced gene expression is required for normal red cell function.

Alpha Thalassemia -

Alpha thalassemia occurs when one or more of the four alpha chain genes fails to function. Alpha chain protein production, for practical purposes, is evenly divided among the four genes. With alpha thalassemia, the "failed" genes are almost invariably lost from the cell due to a genetic accident. α-thalassemia has four manifestations, which correlate with the number of defective genes. (i) Silent carrier state. (ii) Mild alpha-thalassemia. (iii) Hemoglobin H disease. (iv) Hydrops fetalis. Since the gene defect is almost invariably a loss of the gene, there are no "shades of function" to obscure the matter as occurs in beta thalassemia.

Silent carrier state -

This is the one-gene deletion alpha thalassemia condition. People with this condition are hematologically normal. They are detected only by sophisticated laboratory methods.

Mild alpha-thalassemia –

These patients have lost two alpha globin genes. They have small red cells and a mild anemia. These people are usually asymptomatic. Often, physicians mistakenly diagnose people with mild alpha-thalassemia as having iron deficiency anemia. Iron therapy, of course, does not correct the anemia.

Hemoglobin H disease –

These patients have lost three alpha globin genes. The result is a severe anemia, with small, misshapen red cells and red cell fragments. These patients typically have enlarged spleens. Bony abnormalities particularly involving the cheeks and forehead are often striking. The bone marrow works at an extraordinary pace in an attempt to compensate for the anemia. As a result, the marrow cavity within

the bones is stuffed with red cell precursors. These cells gradually cause the bone to "mold" and flair out. Patients with hemoglobin H disease also develop large spleens. The spleen has blood-forming cells, the same as the bone marrow. These cells become hyperactive and over expand, just as those of the bone marrow. The result is a spleen that is often ten-times larger than normal. Patients with hemoglobin H disease often are small and appear malnourished, despite good food intake. This feature results from the tremendous amount of energy that goes into the production of new red cells at an extremely accelerated pace. The constant burning of energy by these patients mimics intense aerobic exercise; exercise that goes on for every minute of every day.

Hydrops fetalis –

This condition results from the loss of all four alpha globin genes. The affected individual usually succumbs to the severe anemia and complications before birth.

Beta thalassemia

The fact that there are only two genes for the beta chain of hemoglobin makes ß-thalassemia simpler to understand than α-thalassemia (Rund and Rachmilewitz 1995). Unlike α-thalassemia, ß-thalassemia rarely arises from the complete loss of a β-globin gene. The β-globin gene is present, but produces little β-globin protein. The degree of suppression varies. The type of thalassemia usually carries the name of the under-produced chain(s). The reduction varies from a slight decrease to complete absence of production. For example, when beta chains are produced at a lower rate, the thalassemia is termed beta+, whereas beta0 thalassemia indicates a complete absence of production of beta chains from the involved allele. As per the clinical manifestations, ß-thalassemia is classified in to three categories, (i) Thalassemia minor, or thalassemia trait (ii) Thalassemia intermedia (iii) Thalassemia major.

Thalassemia minor or thalassemia trait -

These terms are used interchangeably for people who have small red cells and mild (or no) anemia due to thalassemia. These patients are clinically well, and are usually only detected through routine blood testing. Physicians often

mistakenly diagnose iron deficiency in people with thalassemia trait. Iron replacement does not correct the condition. The primary caution for people with beta-thalassemia trait involves the possible problems that their children could inherit if their partner also has beta-thalassemia trait. These more severe forms of beta-thalassemia trait are outlined below.

Thalassemia intermedia –

Thalassemia intermedia is a confusing concept. The most important fact to remember is that a thalassemia intermedium is a description, and not a pathological or genetic diagnosis. Patients with thalassemia intermedia have significant anemia, but are able to survive without blood transfusions.

Thalassemia major

This is the condition of severe thalassemia in which chronic blood transfusions are needed. In some patients the anemia is so severe, that death occurs without transfusions. Other patients could survive without transfusions, for a while, but would have terrible deformities. While transfusions are life saving in patients with thalassemia major, transfusions ultimately produce iron overload. Chelation therapy, usually with the iron-binding agent, desferoxamine (Desferal), is needed to prevent death from iron-mediated organ injury.

Cellular pathophysiology

As discussed earlier, the basic defect in all types of thalassemia is imbalanced globin chain synthesis. However, the consequences of accumulation of the excessive globin chains in the various types of thalassemia are quite different. In beta thalassemia, excessive alpha chains, unable to form Hb tetramers, precipitate in the RBC precursors and, in one way or another, produce most of the manifestations encountered in all of the beta thalassemia syndromes. This is not the situation in alpha thalassemia. The excessive chains in alpha thalassemia are gamma chains earlier in life and beta chains later on. Both are able to form homotetramers that, although relatively unstable, nevertheless remain viable and able to produce soluble Hb molecules such as Bart (4 gamma chains) and H (4 beta chains). These basic differences in the 2 main types of thalassemia are responsible for the major differences in their clinical manifestations and severity.

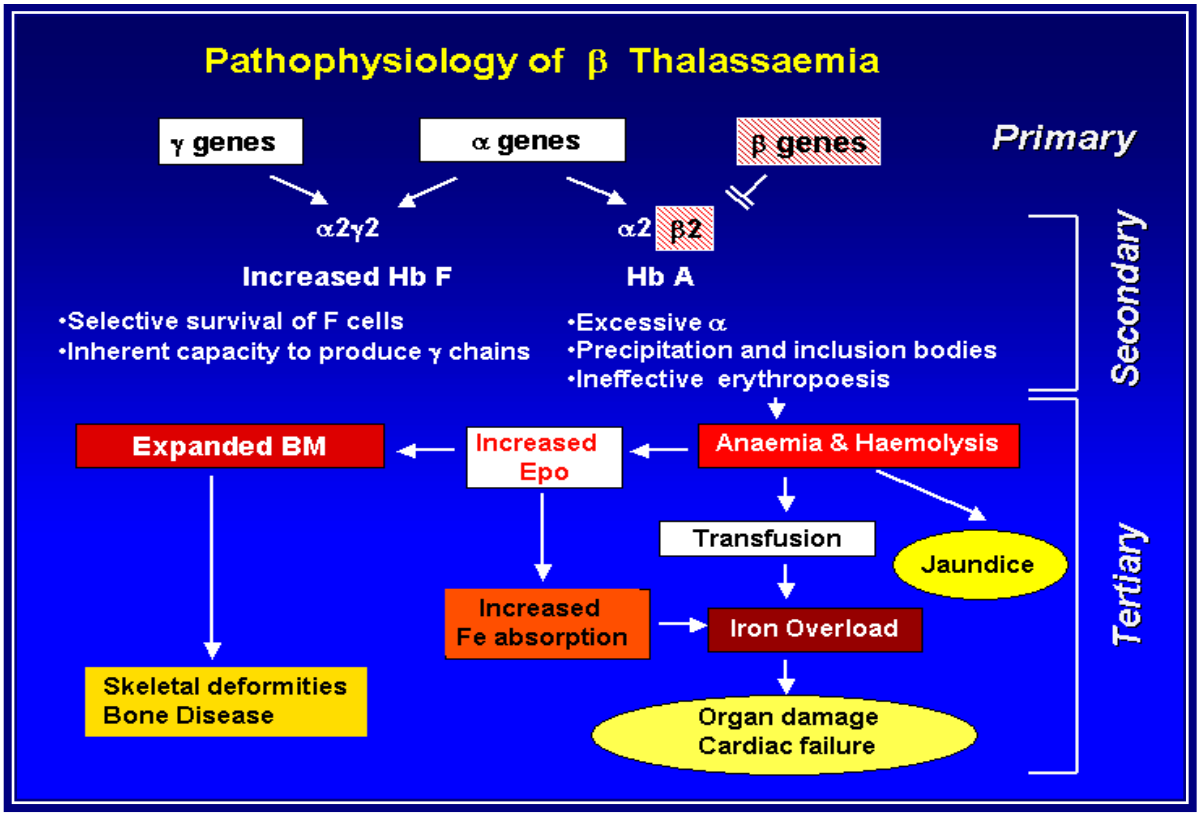


Figure 3.4: Pathophysiological features of hemolysis and hypercoagulability and accumulation of iron in organ in thalassemia

In β-thalassemia, the synthesis and accumulation of excess normal α-globin chain within the red cell, lead to the formation of unstable aggregates, which upon oxidation, due to oxidative stress generated by iron overload, may precipitate and cause cell membrane damage. These deformed cells undergo premature destruction either in the bone marrow (extravascular hemolysis) or the peripheral circulation (intravascular hemolysis) (Weatherall 1997, Festa 1985). This means that both hemolysis and ineffective erythropoiesis cause anemia in the patient with beta thalassemia. The ability of some red cells to maintain the production of gamma chains, which are capable of pairing with some of the excessive alpha chains to produce HbF, is advantageous. Binding some of the excess alpha chains undoubtedly reduces the symptoms of the disease. The elevated HbF increases oxygen affinity leading to hypoxia, which, together with the profound anemia, stimulates the production of erythropoietin. As a result, severe expansion of the ineffective erythroid mass occurs leading to severe bone expansion and deformities. Both iron absorption and metabolic rate increase, adding more symptoms to the clinical and laboratory manifestations of the

disease. The large numbers of abnormal red cells processed by the spleen, together with its hematopoietic response to the anemia if untreated, results in massive splenomegaly, leading to manifestations of hypersplenism.

Iron overload in thalassemia -

In untransfused patients with severe ß-thalassemia, abnormally regulated iron absorption results in increases in body iron burden that may, depending on the severity of erythroid expansion, vary between 2 and 5 grams per year (Pippard et al., 1997, Pootrakul et al., 1988). Regular transfusions may double this rate of iron accumulation. Although most clinical manifestations of iron loading do not appear until the second decade of life in inadequately chelated individuals, evidence from serial liver biopsies in young patients indicates that the deleterious effects of iron are mediated much earlier. After approximately one year of transfusions, iron is deposited in parenchymal tissues, where it may cause significant toxicity as compared to that within reticuloendothelial cells (Hershko et al., 1998). As iron loading progresses, the capacity of serum transferrin, the main transport protein of iron, to bind and detoxify iron may be exceeded. Thereafter, the non-transferrin-bound fraction of iron within plasma may promote generation of free hydroxyl radicals, propagators of oxygen-related damage (Hershko et al., 1998). The effectiveness of an iron-chelating agent depends in part on its ability to bind non-transferrin bound plasma iron over sustained periods of time, thereby ameliorating iron-catalyzed toxicity of free radicals.

Management of ß-thalassemia major –

The management requires patients to have life-long regimen of regular blood transfusions coupled with iron chelation therapy (Modell 1994, Cao et al., 1997). The frequency of blood transfusion requirement increases with growing age. On an average transfusion is required every fortnight. Blood transfusion produces on long term, serious and unavoidable side-effects because with each unit of blood transfused 200 to 250 mg of iron gets deposited in the heart, liver, pancreas and other glands in the body. This may lead to heart failure, cirrhosis of liver, diabetes mellitus and malfunctioning of other glands. Generally, regular blood transfusions and iron chelation treatment with desferoxamine are initiated

early in life; therefore, the patients and their families have to sustain regular treatment throughout their childhood, adolescent, and adult years (Olivieri et al., 1994, Zurlo et al., 1989).

Clinical impact of iron overload

Cardiac complications –

The commonest cause of death in cases of thalassemia major is heart failure. In the absence of chelating therapy, iron accumulation results in progressive dysfunction of the heart, liver and endocrine glands (Olivieri and Brittenham 1997). In response to iron loading, human myocytes in vitro upregulate the transport of non-transferrin-bound iron (Parkes et al., 1993), thereby possibly aggravating cardiac iron loading. Extensive iron deposits are associated with cardiac hypertrophy and dilatation, myocardial fiber degeneration and, rarely, fibrosis (Buja and Roberts 1971). In many patients abnormal function is observed in the absence of symptoms (Fiorillo et al., 2000). In transfused, unchelated patients, symptomatic cardiac disease is observed after about ten years following the start of transfusions (Wolfe et al., 1985) and may be aggravated by myocarditis (Kremastinos et al., 1996) and pulmonary hypertension (Aessopos et al., 1995, Du et al., 1997). Survival is determined by the magnitude of iron loading within the heart (Brittenham et al., 1994, Olivieri et al., 1994).

Liver damage -

Iron-induced liver disease is a common cause of death in transfused patients (Zurlo et al., 1989). Within two years following the start of transfusions, collagen formation (Iancu et al., 1977) and portal fibrosis (Thakerngpol et al., 1996) are observed; in the absence of chelating therapy, cirrhosis may develop in the first decade of life (Jean et al., 1984). As in cultured heart cells, upregulation of transport of non-transferrin-bound iron is observed in cultured hepatocytes (Parkes et al., 1995), possibly aggravating iron loading in vivo.

Endocrinal disorders -

In cases of thalassemia major, increasingly severe impairment of the endocrine system can be observed as the years go by and as more blood transfusions are administered. Iron deposition in the interstitial tissues and in the secreting cells of the endocrine glands results in disorders such as diabetes mellitus, hypothyroidism, hypoparathyroidism, hypoadrenalism, and hypogonadism. Already during the early stages of the disease, involvement of the α and β cells in the pancreas manifests itself in the form of disorders affecting insulin secretion and glucose tolerance. In transfused patients, iron loading within the anterior pituitary results in disturbed sexual maturation in approximately 50% of males and females. Diabetes mellitus is observed in about 5% of adults (Cavallo-Perin et al., 1995). Chronic iron deposition also damages the thyroid, parathyroid, adrenal glands, and exocrine pancreas (Magro et al., 1990, Sklar et al., 1987, Gullo et al., 1993) and may provoke pulmonary hypertension, right ventricular dilation, and restrictive lung disease (Tai et al., 1996, Piatti et al., 1997). Hypofunction of the adrenal cortex may give rise quite early on to an increase in melanin production and skin pigmentation, as well as to the appearance of low voltage waves in the ECG, which, however, are unaccompanied by any evidence of organic damage to the heart. Growth retardation in patients with thalassemia major can occur for a number of reasons, depending on how the patient is managed. A poorly transfused or non-transfused patient has severe anemia, with or without multiple infections and accompanied by failure to thrive. A child on regular transfusions without iron chelation therapy will have stunting as a result of the iron overload. In this case, the poor growth results from the effect of iron on pituitary function, leading to a lack of puberty, and/or from poor liver function and a consequent interference with somatomedin metabolism, leading to short stature. Desferal treatment itself, if too intensive, has been reported to result in short stature.

Hyperbilirubinaemia and gallstones -

The severe hemolysis occurring in thalassemia major gives rise to hyperbilirubinaemia, which in turn leads to bilirubinuria, a condition causing dark brown discoloration of the urine; after the fourth year of life it may also result in the formation of gallstones, the incidence of which rises to 75% by the

15th year of life. In addition, this hemolysis leads to an increase in the serum concentrations of various substances released as the erythrocytes break down, e.g. lactic dehydrogenase, alkaline phosphatase, glutamic acid, uric acid, and iron. One consequence of the damage sustained by the liver is deficiencies affecting blood coagulation factors.

Splenomegaly -

The important functions of the spleen are summarized below: A) Immune functions, including clearance of particulate matter and microorganisms and generation of humoral and cellular response to foreign antigens. B) Sequestration of normal and abnormal cells. C) Regulation of portal blood flow. D) Site of extramedullary erythropoiesis. Increased erythrocyte production, coupled with increased phagocytosis and storage of stroma from broken down erythrocytes, leads to hyperplasia of the reticulo-endothelial system. Splenomegaly produces enlargement of the abdomen, which may eventually assume massive proportions. Hypersplenism is defined as a condition in which the spleen removes circulating red blood cells, granulocytes and platelets in excessive quantity. Hypersplenism is diagnosed if the following criteria are fulfilled: 1) pancytopenia 2) normal or hypercellular bone marrow 3) splenomegaly and 4) correction of cytopenia after splenectomy.

Sexual development -

Sexual development in these patients shows a stronger correlation with bone age rather than with chronological age. Delayed sexual development – like the other complications such as cardiac lesions, diabetes mellitus, and liver cirrhosis – seems to be largely caused by the hemosiderosis; however, menstruation and spermatogenesis appear to be normal in some patients.

Skeletal changes and facial appearance -

Changes occurring in the skeleton are due to chronic over activity of the bone marrow, which results in widening of the medullary spaces. Included among these changes are osteoporosis, thinning of the cortex, and trabecular atrophy. Owing to such lesions, deformities of the spinal column tend to occur, as well as pathological bone fractures in response to minor injuries25. Excessive

prominence of the cheekbones, forehead, and parietal eminence, coupled with depression of the root of the nose, gives the face a characteristic Asiatic appearance. Premature fusion of the epiphyses in the long bones often leads to shortening or angulation of the limbs. Arthropathy, synovitis, and arthralgia of the knees and ankles, together with oedema of the ankles and feet, are attributable to a combination of secondary haemosiderosis and hyperuricaemia.

Assessment of body iron burden

Both direct and indirect means for the assessment of body iron are available; no single indicator or combination of indicators is ideal for the evaluation of iron status in all clinical circumstances. The measurement of plasma or serum ferritin is the most commonly used indirect estimate of body iron stores (Brittenham et al., 2001). Interpretation of ferritin levels may be complicated by a variety of conditions that alter concentrations independently of changes in body iron burden, including ascorbate deficiency, fever, acute infection, chronic inflammation, acute and chronic hepatic damage, hemolysis and ineffective erythropoiesis (Brittenham et al., 2001). Serum iron, transferrin, transferrin saturation and transferrin receptor concentration, or urinary iron excretion following an infusion of DFO do not quantitatively reflect body iron stores. The availability of a simple assay for monitoring non-transferrin-bound plasma iron could provide a useful measurement of iron status but has not been applied widely (Breuer et al., 2001). A variety of studies have been directed at imaging tissue iron by computed tomography, nuclear resonance scattering, and magnetic resonance imaging. Measurement of hepatic iron concentration is the most quantitative, specific, and sensitive method for determining body iron burden Breuer et al., 2001). Iron in biopsy samples is assessed most frequently using atomic absorption spectroscopy.

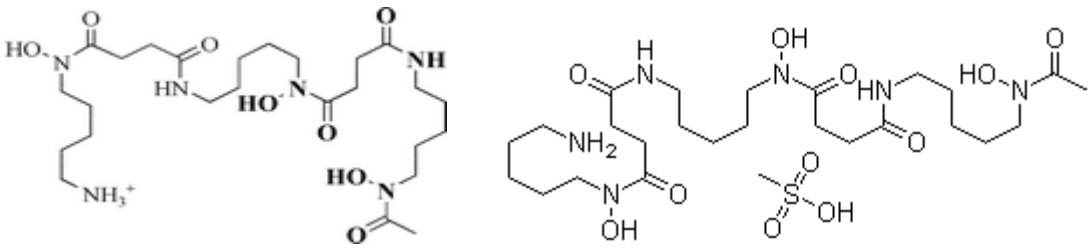
Treatment of iron overload

Iron chelating therapy -

Iron overload may be treated or prevented with a chelating agent capable of complexing with iron and promoting its excretion. The only iron-chelating agent presently available for clinical use in the US is desferrioxamine (Desferal).

Desferoxamine (Desferal) –

Desferoxamine is a chelating agent with a strong affinity for iron. Discovered in 1960 as a result of work undertaken conjointly in the laboratories of Ciba and of the Swiss Federal Institute of Technology in Zürich, it was introduced in 1963. The active substance of Desferal is desferrioxamine B, an iron-chelating compound derived from ferrioxamine B, which is an iron-bearing metabolite produced by Actinomycetes (Streptomyces pilosus) and belongs to the group of the sideramines. Desferrioxamine B is present in Desferal in the form of the methane sulphonate, a chain molecule of the following structure:



Desferoxamine B Desferal

Figure 3.5: Structure of Iron chelator used in iron overload conditions

Trivalent iron reacts with desferrioxamine B to form ferrioxamine B, an octahedral iron complex: Subcutaneous infusion at a daily dose 40 mg/kg over 8-12 hours has become the standard schedule of delivery for over 20 years.

Biodistribution and metabolism -

DFO is hydrophilic, and this property together with its high molecular weight means that uptake into cells and subcellular compartments is generally slow relative to hydroxypyridinones (such as deferiprone), taking several hours for equilibration (Hoyes et al., 1993, Cooper et al., 1996). Uptake into hepatocytes is rapid, however, probably by a facilitated process, making access to intrahepatic iron relatively efficient (Porter et al., 1988). Due to the positive charge of both DFO and FO, egress from cells by diffusion will be slow (Singh et al., 1990). DFO is relatively stable for several hours in plasma, and the majority of metabolism takes place within hepatocytes by oxidative deamination of the N-terminus, yielding metabolite B as the predominant product (Singh et al., 1990). The iron free form of metabolite B can diffuse back into plasma rapidly, due to its negative

charge (Singh et al., 1990), where it is rapidly cleared from plasma ($t_{1/2}$ = 1.3 hours) and eliminated in the urine (Lee et al., 1993).

Clinical toxicity -

Toxicity from DFO in thalassemia major is unlikely provided that doses do not exceed 40 mg/kg/day, that DFO is not introduced at too young an age (see below), and that the dose is reduced as iron loading falls. Systemic reactions with fever, muscle aches and arthralgia are uncommon and anaphylaxis may occasionally occur.

Infections –

There is an increased risk of Yersinia infection in iron overload, and this risk increases further with DFO treatment as Yersinia does not make a natural siderophore and uses iron from FO to facilitate its growth (Robins-Browne and Prig, 1985). Patients who present with diarrhea, abdominal pain or fever should stop DFO until appropriate stool samples, blood cultures and serological testing can reasonably exclude Yersinia infection. The growth of other organisms (e.g. Klebsiella) may also be facilitated by FO and it is wise to withhold DFO in a febrile patient until the source of the fever has been identified.

Deferiprone -

Pharmacology and pharmacokinetics –

Deferiprone (1,2 dimethy-3-hydroxypyridin-4-one) is a member of a family of hydroxypyridin-4-one (HPO) chelators (Hider et al., 1982) that require three molecules fully to bind Fe^{+3}, each molecule providing two co-ordination sites (bidentate chelation). The pM of deferiprone for Fe^{+3} (pM = 20) is less than that of DFO, which reflects the lower stability of the iron-chelate complex. Other metals such as Ga, Al^{3+} and In 3+ are bound tightly with relative affinities for other metals being $Cu^{2+} > Zn^{2+} > Ca^{2+} > Mg^{2+}$ (Hider et al., 1994). The molecular weight of deferiprone is approximately one-third that of DFO and this together with its neutral charge and relative lipophilicity account for its rapid absorption from the gut. These same properties also allow more rapid access by deferiprone and related HPOs to intracellular iron (Porter et al., 1988), to labile intracellular iron (Zanninelli et al., 1997) as well as more rapid access to intra-lysosomal iron

pools and to iron containing enzymes (Abeysinghe et al., 1996, Cooper et al., 1996).

Deferiprone appears in plasma within 5 to 10 minutes of ingestion, the peak concentrations (C_{max}) occurring within 1 hour, reaching levels in excess of 300 μM after oral ingestion of a 50 mg/kg dose (Kontoghiorghes et al., 1990, Al-Refaie et al., 1995). However, these levels are short-lived with an elimination $t_{1/2}$ of 1.52 hours. Unlike DFO, where approximately half of iron excretion is fecal, there is little fecal iron excretion with deferiprone (Kontoghiorghes et al., 1988, Collins et al., 1994, Olivieri et al., 1990).

Metabolism -

Deferiprone is metabolized to the inactive glucuronide that is the predominant form recovered in the urine (Lange et al., 1993).

Toxicities –

Neutropenia and agranulocytosis: Agranulocytosis is the most serious unwanted effect of this drug so far identified. This was initially reported in 3-4% of patients treated with deferiprone; mild neutropenia was found in an additional 4% (Al-Refaie et al., 1992, Al-Refaie et al., 1995).

Arthropathy: Painful swelling of the joints, particularly the knees, occurs in 6-39% of patients (Agarwal et al., 1992). This complication usually but not always resolves after stopping therapy. Arthritis was the most common side effect in the Indian study in which many of the patients were heavily iron overloaded (Agarwal et al., 1992). Other unwanted effects include nausea (8%), zinc deficiency (14%) and fluctuation in liver function tests (44%) (Al-Refaie et al., 1995).

Liver fibrosis: In a retrospective analysis, fibrosis was reported to progress in 5 of 12 patients on deferiprone and in none of 12 age-matched control subjects receiving regular DFO (Olivieri et al., 1998).

3.2 Thrombocytopenia

Thrombocytopenia is a lower than normal number of platelets in the blood. Platelets play an important role in clotting and bleeding. Platelets are made in the bone marrow similar to other cells in blood such as, white blood cells and red blood cells. Platelets originate from megakaryocytes which are large cells found in the bone marrow. Platelets, in general, have a brief 7 to 10 days life in blood, after which they are removed from the blood circulation. The number of platelets in the blood is referred to as the platelet count and is normally between 150,000 to 450,000 per micro liter (one millionth of a liter) of blood. Platelet counts less than 150,000 are termed thrombocytopenia. Platelet counts greater that 450,000 are called thrombocytosis. (Maton et al., 1993)

The function of platelets is very important in clotting system. They circulate in blood vessels and become activated if there is any bleeding or injury in body. Certain chemicals are released from injured blood vessels or other structures that signal platelets to become activated and join the other components of the system to stop the bleeding. When activated, platelets become sticky and adhere to one another and to blood vessel wall at the site of injury to slow down and stop bleeding by plugging up the damaged blood vessel or tissue (hemostasis).

Low platelet count in severe cases may result in spontaneous bleeding or may cause delay in normal process of clotting. In mild thrombocytopenia, there may be no adverse effects in the clotting or bleeding pathways.

Pathophysiology of thrombocytopenia

Some of the most common and important causes or thrombocytopenias are outlined below.

1. Decreased platelet production

Decreased platelet production is usually related to a bone marrow problem (agranulocytosis). In most of these conditions, red blood cell and white blood cell productions may also be affected. (http://archive.rubicon-foundation.org)

- Viral infections affecting the marrow for example: parvovirus, rubella, mumps, varicella (chickenpox), hepatitis C, Epstein-Barr virus, and HIV.
- Aplastic anemia is a general term used when the bone marrow fails to produce any blood cells (red cells, white cells, and platelets), also called pancytopenia. This can be caused by some viral infections (parvovirus or HIV), medications or radiation, or rarely, it can be congenital (Fanconi's anemia).
- Chemotherapy drugs and thiazide diuretics frequently cause bone marrow suppression resulting in thrombocytopenia.
- Cancers of the bone marrow and blood (leukemia) or cancers of the lymph nodes (lymphoma) can cause various degrees of thrombocytopenia.
- Cancers from other organs can sometimes infiltrate (invade) the bone marrow and result in impaired production of platelets.
- Long term alcohol can cause direct toxicity of the bone marrow.
- Deficiency of vitamin B_{12} and folic acid can result in low platelet production by the bone marrow.

2. Increased platelet destruction or consumption

Increased platelet destruction or consumption can be seen a number of medical conditions. They can be divided into immune related and non-immune related causes. Many medications can cause low platelet count by causing immunologic reaction against platelets, called drug-induced thrombocytopenia. Some examples may include:

- Sulfonamide antibiotics, carbamazepine anti-seizure drug, digoxin, quinine, quinidine, acetaminophen, and rifampin.
- Heparin, a commonly used blood thinner, and similar medications like enoxaparin can occasionally induce an immune response against platelets resulting in rapid destruction of platelets. This condition is termed heparin-induced thrombocytopenia or HIT.

- Idiopathic thrombocytopenic purpura (ITP) is a condition where the immune system attacks platelets. In severe conditions, ITP can result in very low platelet counts.
- Some rheumatologic condition, such as systemic lupus erythematosus (SLE) or other autoimmune conditions (connective tissue diseases), can cause platelet destruction.
- Transfusion of blood products and organ transplantation can sometimes cause immunologic disturbances resulting in thrombocytopenia.
- Thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) are similar conditions that can cause non-immune related consumptive thrombocytopenia resulting from some viral illnesses, pregnancy, some metastatic cancers, or chemotherapy. Other manifestations of these conditions include kidney insufficiency, confusion, anemia (hemolytic), and fever.
- HELLP syndrome (hemolysis elevated liver tests low platelets) is another non-immune thrombocytopenia that may occur during pregnancy and can include elevation of liver enzyme and anemia (specifically, hemolytic anemia or rupturing of red blood cells).
- Disseminated intravascular coagulopathy (DIC) is a rare but severe condition that may be a complication of overwhelming infections, traumas, burns, or pregnancy.
- Injury to or inflammation of blood vessels (vasculitis) and, sometimes, artificial heart valves can cause increased destruction of platelets as they pass by.
- Severe infections (sepsis) or trauma can sometimes cause consumptive thrombocytopenia (without DIC).

3. Splenic sequestration

- Common causes of thrombocytopenia due to splenic enlargement may include advanced liver disease (cirrhosis, for example, from chronic hepatitis B or C) and blood cancers (leukemias or lymphomas).

- Pseudo thrombocytopenia (false thrombocytopenia) is also one of a commonly encountered condition where the number of platelets seen on a complete blood count analysis (CBC) may falsely appear low because of the clumping of platelets together. This can lead to a smaller number of platelets seen throughout the slide reviewed by the technician. If this is suspected, the blood can be redrawn in a tube with a material that prevents clumping of platelets for repeat analysis.
- Thrombocytopenia can also be present at birth, called neonatal thrombocytopenia. Most of these cases can be caused by processes similar to above, although, they are occasionally related to rare genetic conditions.

Symptoms of thrombocytopenia

In many instances, thrombocytopenia may have no symptoms, especially if mild, and it can be detected only incidentally on routine blood work done for other reasons. If thrombocytopenia is severe, for example less than 20 per micro liter, it can potentially manifest as increase bleeding when a person is cut or injured or increased bleeding during menstrual period.

Spontaneous bleeding can also happen with severe thrombocytopenia (less than 10,000 to 20,000 platelets). This type of bleeding usually occurs under the skin or the mucus membrane (the inner lining of the oral cavity, gastrointestinal tract, or the nasal cavity). Petechiae may be seen in patients with very low platelet counts. Petechiae are small (pin head size) red, flat spots seen under the skin on the dependent parts of the body because of increased pressure due to gravity, for example, on the lower legs. These happen because of bleeding out of the tiny blood vessels under the skin or the mucus membrane. Petechiae are generally not palpable or painful.

Other rashes or bruises seen in thrombocytopenia are called purpura, which are small, purple spots under the skin as a result of hemorrhage. These are typically greater than 3 millimeters in diameter and may represent a confluence of petechiae.

Diagnosis of thrombocytopenia

Thrombocytopenia is usually detected incidentally from routine complete blood count (CBC). Platelet counts less than 150,000 are termed thrombocytopenia. If thrombocytopenia is seen for the first time, it is prudent to repeat the complete blood count in order to rule out pseudo thrombocytopenia. If the repeat CBC confirms low platelet counts, then further evaluation can begin. Once detected, the cause of thrombocytopenia may be investigated by the doctor. The most essential part of this evaluation includes a thorough physical examination and medical history of the patient. In the medical history, the complete list of all medications is routinely reviewed. Some of the other important components of the history include reviewing previous known history of low platelet count, family history of thrombocytopenia, recent infections, any previous cancers, other autoimmune disorders, or liver disease.

A review of the symptoms related to excessive bleeding or bruising can also provide additional information. As a part of a thorough physical examination, special attention may be given to the skin and mucus membrane in the oral cavity for petechiae or purpura or other signs of bleeding. On the abdominal examination, an enlarged spleen (splenomegaly) can provide important diagnostic clues. The urgency to perform additional testing and evaluation is largely dependent on how low the platelet count is on the blood count, and what the clinical situation may be. For instance, in a person who needs a surgery and has a platelet count of less than 50 the investigation will take precedence over one whose thrombocytopenia was detected on a yearly blood work with a platelet of 100.

A comprehensive review of the other components of the CBC is one of the most important steps in the evaluation of low platelet count. The CBC can tell us whether other blood disorders may be present, such as, anemia (low red cell count or hemoglobin), erythrocytosis (high red blood cell count or hemoglobin), leukopenia (low white cells count), or leukocytosis (elevated white blood cell count). These abnormalities may suggest bone marrow problems as the potential cause of thrombocytopenia. Abnormally shaped or ruptured red cells (schistocytes) seen on the blood smear may suggest evidence of HELLP, TTP, or

HUS. Another clue in the CBC is the mean platelet volume or MPV, which is an estimate of the average size of platelets in the blood. A low MPV number may suggest platelet production problem, whereas, a high number may indicate increased destruction.

It is important to also review other blood work including the complete metabolic panel, coagulation panel, and urinalysis. Certain abnormalities in these tests can suggest advanced liver disease (cirrhosis), kidney problems (renal failure), or other pertinent underlying medical conditions. In some causes of thrombocytopenia, such as HIT or ITP, additional testing with antibodies or assays may be done. Bone marrow biopsy can sometimes be performed if a bone marrow problem is suspected.

Treatment of thrombocytopenia

Treatment is guided by etiology and disease severity. The main concept in treating thrombocytopenia is to eliminate the underlying problem, whether that means discontinuing suspected drugs that cause thrombocytopenia, or treating underlying sepsis. Diagnosis and treatment of serious thrombocytopenia is usually directed by a hematologist.

Specific treatment plans often depend on the underlying etiology of the thrombocytopenia.

Condition	Treatment
Thrombotic thrombocytopenic purpura (is caused by spontaneous aggregation of platelets and activation of coagulation in the small blood vessels. Platelets are consumed in the coagulation process, and bind fibrin, the end product of the coagulation pathway. These platelet-fibrin complexes form microthrombi which circulate in the vasculature and cause shearing of red blood cells, resulting in hemolysis.) (Moake JL 2002)	Application of plasmapheresis. this treatment theoretically works by removing antibodies directed against the von Willebrand factor cleaving protease, ADAMTS-13. The plasmapheresis procedure also adds active ADAMTS-13 protease proteins to the patient, restoring a more physiological state of von Willebrand factor multimers.

Idiopathic thrombocytopenic purpura ITP	Prednisone and other corticosteroids Intravenous immune globulin Splenectomy Danazol, Rituximab, Romiplostim
Heparin-induced thrombocytopenia	patients are usually treated with a type of blood thinner called a direct thrombin inhibitor such as lepirudin or argatroban, bivalirudin and fondaparinux. Platelet transfusions
Congenital amegakaryocytic thrombocytopenia	Bone Marrow/Stem Cell Transplant is the only thing that ultimately cures this genetic disease.

Prevention of thrombocytopenia

In general, thrombocytopenia can be prevented if the cause is known and it is preventable. If a certain medication is found to induce low platelet count in an individual, then its future use needs to be avoided. Alcohol avoidance should be encouraged in people with known alcohol-induced thrombocytopenia. Current and future use of all heparin products must be avoided in people diagnosed with heparin-induced thrombocytopenia.

Herbs for thrombocytopenia

There are a wide range of botanical sources and wide range of active constituents that might ultimately contribute to hemostatic action, including essential oils, flavonoids, saponins, and alkaloids. The possible mechanisms of action of the hemostatic herbs include:

- Increasing the production of platelets
- Promoting the ability of platelets to aggregate when there is blood leakage
- Decreasing capillary permeability
- Contracting peripheral blood vessels
- Inhibiting autoimmune attack against platelets

These effects should be expected to be observed within a few days of administering the herbs. In most of the Chinese medical reports, improvements in symptoms (such as spontaneous bleeding and petechia) were observed within about 10 days. Changes in bone-marrow functions and autoimmune processes may require somewhat longer therapy, at least several weeks (typically one to three months treatment time), with increasing effect in responsive patients. The reported changes include higher platelet counts and lower IgG levels. Three groups of active constituents are known to have some hemostatic effects and may influence autoimmune processes:

- Anthraquinones, found in rubia and rumex and also an ingredient of rhubarb root (which has hemostatic effects, but is not included in the ITP formulas)
- Flavanoids, found in eclipta and agrimony, and also in scute (used to inhibit bleeding but rarely in the ITP formulas)
- Alkaloids, found in lotus (all plant parts), eclipta, and san-chi.

The role of essential oils (which usually dilate vessels; some might increase bleeding), triterpenes, and saponins found in several of the herbs remains unknown. One of the most frequently-used herbs in the formulas, raw rehmannia, contains iridoid glycosides that have hemostatic effects (see: Rehmannia). The same active constituents are found in gardenia, which is mentioned in a few of the ITP treatments, as well as in scrophularia and cornus (only rarely mentioned in the ITP formulas).

Table 3.2: Hemostatic herbs used for thrombocytopenia.

All of the herbs listed here are reported to shorten bleeding time in laboratory testing.

Common Name Pinyin	Botanical Name Active Constituents	Comments
Agrimony *xianhecao*	*Agrimonia pilosa*; agrimonin (essential oil); agriminolide (flavonoid)	Agrimonin has been developed into a hemostatic drug in China, but pharmacology studies give conflicting results. The clinical effectiveness is not confirmed.
Biota tops *ceboye*	*Biota orientalis* essential oils: juniperic acid, thujone	Biota leaves are frequently used (applied topically and taken internally) to treat alopecia, which is thought to involve an autoimmune disorder.
Eclipta *hanliancao*	*Eclipta prostrata* (ecliptine,	Though classified as a yin tonic, it is often used to control bleeding. The flavonoids

Common Name Pinyin	Botanical Name Active Constituents	Comments
	wedelolactone)	may reduce capillary permeability.
Imperata *maogen*	*Imperata cylindrica* triterpenes: simiarenol, fernenol	The triterpenes reduce inflammation; there may be flavonoids in the flower that reduce capillary permeability.
Lotus node *oujie*	*Nelumbo nucifera* alkaloids: nuciferine, liriodenine	The alkaloids shorten bleeding time.
Rubia *qiancaogen*	*Rubia cordifolia* alizarin, purpurin anthraquinones	The herb extract dilates vessels and shortens bleeding time.
Rumex (*yangdi*; *suanmo*)	*Rumex spp.* anthraquinones: emodin	Although not frequently mentioned in the Chinese literature, the rumex plants are recommended for bleeding in association with blood stasis.
San-chi *sanqi*	*Panax notoginseng* Dencichine	This is the key ingredient in the popular hemostatic remedy *Yunnan Baiyao*.
Sanguisorba *diyu*	*Sanguisorba officinalis* saponins: sanguisorbin	Sanguisorba is especially used in cases of rectal bleeding.

Hemophilia is a bleeding disorder caused by a deficiency in one of two blood clotting factors: factor VIII or factor IX. There are two forms of hemophilia. Hemophilia A, which accounts for about 80% of all cases, is a deficiency in clotting factor VIII. Hemophilia B is a deficiency in clotting factor IX. The bleeding patterns and consequences of these two types of hemophilia are similar. Deficiency of clotting factor XI also causes a hereditary bleeding disorder. About 50% of cases of factor XI deficiency occur among people of Eastern European Jewish ancestry. Factor XI deficiency affects both males and females and may cause bleeding after injury or surgery. Spontaneous bleeding episodes are usually less frequent and milder than in hemophilia A or B. Hemophilia is caused by several different gene abnormalities. They are sex-linked, which means that the gene abnormalities are inherited through the mother and that nearly everyone with hemophilia is male. If you have hemophilia, your blood will not clot normally and you may bleed for longer than normal, or you may bleed internally, particularly into joints such as your knees, elbows and ankles.

3.3 Diseases of Immune system

The immune system is a remarkably effective structure that incorporates specificity, inducibility and adaptation. Failures of host defense do occur, however, and fall into three broad categories: immunodeficiencies, autoimmunity, and hypersensitivities. Components of Immune system involve antigen, antibody (produced by lymphocytes), and antigen presenting cells (APCs) like macrophages, basophils, monocytes and direct killer cells like neutrophills.

Cell-mediated immunity and Humoral immunity

Macrophages engulf antigens, process them internally, then display parts of them on their surface together with some of their own proteins. This sensitizes the T cells to recognize these antigens. All cells are coated with various substances. CD stands for cluster of differentiation and there are more than one hundred and sixty clusters, each of which is a different chemical molecule that coats the surface. CD_{8+} is read "CD_8 positive." Every T and B cell has about 105 = 100,000 molecules on its surface. B cells are coated with CD_{21}, CD_{35}, CD_{40}, and CD_{45} in addition to other non-CD molecules. T cells have CD_2, CD_3, CD_4, CD_{28}, CD_{45}, and other non-CD molecules on their surfaces.

The large number of molecules on the surfaces of lymphocytes allows huge variability in the forms of the receptors. They are produced with random configurations on their surfaces. There are some 1018 different structurally different receptors. Essentially, an antigen may find a near-perfect fit with a very small number of lymphocytes, perhaps as few as one. T cells are primed in the thymus, where they undergo two selection processes. The first positive selection process weeds out only those T cells with the correct set of receptors that can recognize the MHC molecules responsible for self-recognition. Then a negative selection process begins whereby T cells that can recognize MHC molecules complexed with foreign peptides are allowed to pass out of the thymus. Cytotoxic or killer T cells ($CD8^+$) do their work by releasing lymphotoxins, which cause cell lysis. Helper T cells ($CD4^+$) serve as managers, directing the immune response.

They secrete chemicals called lymphokines that stimulate cytotoxic T cells and B cells to grow and divide, attract neutrophils, and enhance the ability of macrophages to engulf and destroy microbes. Suppressor T cells inhibit the production of cytotoxic T cells once they are unneeded, lest they cause more damage than necessary. Memory T cells are programmed to recognize and respond to a pathogen once it has invaded and been repelled.

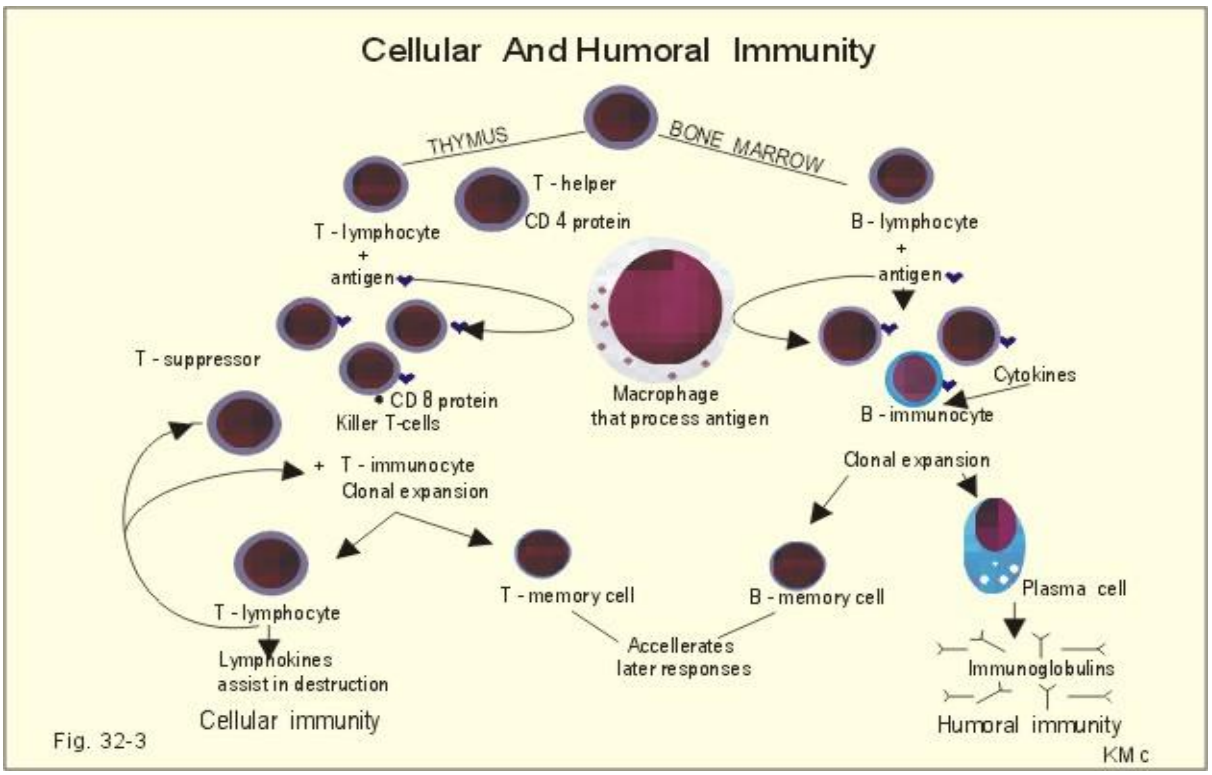


Figure 3.6: Cell mediated and Humoral immunity

Humoral immunity

An immuno competent but as yet immature B-lymphocyte is stimulated to maturity when an antigen binds to its surface receptors and there is a T helper cell nearby (to release a cytokine). This sensitizes or primes the B cell and it undergoes clonal selection, which means it reproduces asexually by mitosis. Most of the family of clones becomes plasma cells. These cells, after an initial lag, produce highly specific antibodies at a rate of as many as 2000 molecules per second for four to five days. The other B cells become long-lived memory cells.

Antibodies, also called immunoglobulins or Igs [with molecular weights of 150–900 Md], constitute the gamma globulin part of the blood proteins. They are soluble proteins secreted by the plasma offspring (clones) of primed B cells. The antibodies inactivate antigens by, (a) complement fixation (proteins attach to antigen surface and cause holes to form, i.e., cell lysis), (b) neutralization (binding to specific sites to prevent attachment—this is the same as taking their parking space), (c) agglutination (clumping), (d) precipitation (forcing insolubility and settling out of solution), and other more arcane methods.

Constituents of gamma globulin are: IgG-76%, IgA-15%, IgM-8%, IgD-1%, and IgE-0.002% (responsible for autoimmune responses, such as allergies and diseases like arthritis, multiple sclerosis, and systemic lupus erythematosus). IgG is the only antibody that can cross the placental barrier to the fetus and it is responsible for the 3 to 6 month immune protection of newborns that is conferred by the mother. IgM is the dominant antibody produced in primary immune responses, while IgG dominates in secondary immune responses. IgM is physically much larger than the other immunoglobulins.

Immune response is a reaction or response that occurs in the body against a great variety of foreign organic macromolecules. The immune response is referred to as allergy or hypersensitivity in many instances.

Immunomodulators: These are substances that modulate the intensity of immune responses by inducing maturation of potentially immuno-competent cells, expression of epitopes on the cell membranes, differentiation of responsiveness to specific antigens or regulation of cell metabolism.

Immunoenhancers: These are agents that increase the immunity.

Immunosuppressant: These are agents that suppress the immune system. They are useful in organ transplantation, in treatment of autoimmune diseases like myasthenia gravis, rheumatoid arthritis, systemic lupus erythematosus, psoriasis, ulcerative colitis, etc. and in treatment of Rh-hemolytic disease.

Studies have indicated that immunomodulator drugs also contain antioxidant components, which can slow down the cellular ageing process and booster the immune system as well.

Immunodeficiencies disorders

Immunodeficiencies occur when one or more of the components of the immune system are inactive. The ability of the immune system to respond to pathogens is diminished in both the young and the elderly, with immune responses beginning to decline at around 50 years of age due to immunosenescence (Silva 2007, Chandra RK 1997). In developed countries, obesity, alcoholism, and drug use are common causes of poor immune function (Chandra RK 1997). However, malnutrition is the most common cause of immunodeficiency in developing countries (Chandra RK 1997). Diets lacking sufficient protein are associated with impaired cell-mediated immunity, complement activity, phagocyte function, IgA antibody concentrations, and cytokine production. Deficiency of single nutrients such as iron; copper; zinc; selenium; vitamins A, C, E, and B6; and folic acid (vitamin B9) also reduces immune responses (Chandra RK 1997). Additionally, the loss of the thymus at an early age through genetic mutation or surgical removal results in severe immunodeficiency and a high susceptibility to infection (Miller JF 2002).

Immunodeficiency can also be inherited or 'acquired'. Chronic granulomatous disease, where phagocytes have a reduced ability to destroy pathogens, is an example of an inherited, or congenital, immunodeficiency. AIDS and some types of cancer cause acquired immunodeficiency (Joos L 2005).

Autoimmunity

Overactive immune responses comprise the other end of immune dysfunction, particularly the autoimmune disorders. Here, the immune system fails to properly distinguish between self and non-self, and attacks part of the body. Under normal circumstances, many T cells and antibodies react with “self” peptides (Miller JF 1993). One of the functions of specialized cells (located in the thymus and bone marrow) is to present young lymphocytes with self antigens

produced throughout the body and to eliminate those cells that recognize self-antigens, preventing autoimmunity (Sproul TW 2000).

Hypersensitivity

Hypersensitivity is an immune response that damages the body's own tissues. They are divided into four classes (Type I–IV) based on the mechanisms involved and the time course of the hypersensitive reaction. Type I hypersensitivity is an immediate or anaphylactic reaction, often associated with allergy. Symptoms can range from mild discomfort to death. Type I hypersensitivity is mediated by IgE, which triggers degranulation of mast cells and basophils when cross-linked by antigen (Ghaffar 2006). Type II hypersensitivity occurs when antibodies bind to antigens on the patient's own cells, marking them for destruction. This is also called antibody-dependent (or cytotoxic) hypersensitivity, and is mediated by IgG and IgM antibodies (Ghaffar 2006). Immune complexes (aggregations of antigens, complement proteins, and IgG and IgM antibodies) deposited in various tissues trigger Type III hypersensitivity reactions. Type IV hypersensitivity (also known as cell-mediated or delayed type hypersensitivity) usually takes between two and three days to develop. Type IV reactions are involved in many autoimmune and infectious diseases, but may also involve contact dermatitis (poison ivy). These reactions are mediated by T cells, monocytes, and macrophages (Ghaffar 2006).

Another important role of the immune system is to identify and eliminate tumors. The transformed cells of tumors express antigens that are not found on normal cells. To the immune system, these antigens appear foreign, and their presence causes immune cells to attack the transformed tumor cells. The antigens expressed by tumors have several sources; (Andersen MH 2006) some are derived from oncogenic viruses like human papillomavirus, which causes cervical cancer, (Andersen MH 2006) while others are the organism's own proteins that occur at low levels in normal cells but reach high levels in tumor cells. One example is an enzyme called tyrosinase that, when expressed at high levels, transforms certain skin cells (e.g. melanocytes) into tumors called melanomas (Castelli C 2000). A third possible source of tumor antigens are proteins normally important for regulating cell growth and survival, that

commonly mutate into cancer inducing molecules called oncogenes (Renkvist N 2001).

The immune system is involved in the etiology as well as pathophysiological mechanisms of many diseases. Modulation of the immune responses to alleviate the diseases has been of interest for many years and the concept of 'rasayana' in Ayurveda is based on related principles. (Sharma P 1983) Indian medicinal plants are a rich source of substances which are claimed to induce paraimmunity, the non-specific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement functions (Sainis 1997). Ayurveda, the Indian traditional system of medicine, lays emphasis on promotion of health concept of strengthening host defenses against different diseases (Thatte 1986).

Immunology plays an important and increasing role in the understanding and diagnosis of disease. Also it is involved in the etiology as well as the pathophysiologic mechanisms of many diseases. Immunology is not only the most rapidly developing area of biomedical research but has the great promise of major advances in the prevention and treatment of a wide range of disorders. Arthritis, ulcerative colitis, asthma, allergy, parasitic and infectious diseases are now primarily considered to be immunological disorders, while immune mechanisms are also involved in variety of other diseases such as diabetes mellitus, cancer, myocardial diseases, cirrhosis and atherosclerosis.

A significant development has been the new approach to control and manipulate the immunological mechanisms by drugs causing immunostimulation or immunosupression and their application in therapy. Immunity is the ability of the living body or the processes thereof to resist various types of organisms or toxins that tend to damage the tissues and organs.

Free radicals are natural by-products of our own metabolism, such as in respiration and some cell mediated immune functions. They are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, air-pollution, pesticides etc. These are electrically charged molecules that attack our cells, tearing through cellular membranes to react and create

havoc with the nucleic acids, proteins and enzymes present in the body. These attacks by free radicals, collectively known as oxidative stress, are capable of causing cells to loose their structure, function and can eventually destroy them.

A majority of the present day diseases are reported to be due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic phenomena in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals caused by excessive oxidative stress of the present day life or due to the poor scavenging/quenching in the body caused by depletion of the dietary antioxidants. Free radicals may be designated as molecular sharks that damage molecules in cell membranes, mitochondria, DNA and are very unstable, tend to rob electrons from the molecules in the immediate surroundings in order to replace their own losses. Reactive oxygen species (ROS) is collective term, which includes not only the oxygen radicals ($O^{\cdot}_2$, $^{\cdot}OH$ and $NO^{\cdot}$) but also some non-radical oxygen derivatives like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3).

Over about 100 disorders like rheumatoid arthritis, hemorrhagic shock, CVS disorders, cystic fibrosis, metabolic disorders, neurodegenerative disorders, gastrointestinal ulcerogenesis and AIDS have been reported as ROS mediated. Some specific ROS mediated diseases include alzheimer's disease, parkinson's disease, atherosclerosis, cancer, down's syndrome and ischemic reperfusion injury in different tissues including heart, liver, brain, kidney and gastrointestinal tract. The role played by ROS in stress induced gastric ulcer and inflammatory bowl diseases have been well established.

Antioxidant defenses include the antioxidant enzymes like superoxide dismutase (SOD), CAT, and glutathione peroxidase (GSH-px) etc. It also includes proteins that minimize the availability of peroxidase such as iron ions, copper ions and heame and law molecular mass agents visually ascorbic acid, α- tocopherol that scavenge ROS (Singh VK et al,1996).

Table 3.3: Indigenous rasayana plants with suggested immunomodulatory properties: (Gulati K et al., 2002)

Botanical Name	Ayurvedic Name
Acorus calamus	Vacha
Abutilon indicum	Atibala
Aloe vera	Ghrit-Kumari
Argyreia speciosa	Vriddhadara
Asparagus racemosus	Satavari
Azadirachta indica	Nimba
Bacopa monnieri	Nir-brahmi
Boerhaavia diffusa	Sothaghni
Cissampelos pareira	Paatha
Commiphora mukul	Guggulu
Convolvulus pluricaulis	Shankhpushpi
Curculigo orchioides	Krishna Musalee
Curcuma longa	Haridra
Desmodium gangeticum	Shalaparni
Dioscorea bulbifera	Raktalukum
Emblica officinalis	Aamalaki
Embelia ribes	Vidanga
Glycerrhiza glabra	Yashtimadhu
Gmelina arborea	Kasmari
Hemidesmus indicus	Anantamula
Ipomoea digitata	Vidaree
Leptadenia reticulate	Jeevantee
Piper longum	Pippali
Plumbago zeylanica	Chitrak
Psoralea corylifolia	Vakuchibheda
Pterocarpus marsupium	Bijasar
Semecarpus anacardium	Bhallatak
Sida spinosa	Nagbala
Sida cordifolia	Bala
Solanum nigrum	Kakamachi
Sphaeranthus indicus	Mundi
Terminalia bellirica	Bibhitaka
Terminalia chebula	Haritaki

Tinospora cordifolia	Guduchi
Withania somnifera	Ashwagandha

Oxidative stress -

Free radicals and oxidative stress -

Free radicals and reactive oxygen species (ROS) are short-lived reactive chemical species having one or more electrons with unpaired spins. The roles for such electronically activated species (e.g., superoxide and singlet oxygen) in the normal function of cells and tissues and in the etiology of certain diseases in man have been extensively studied. Radical generating processes may be key components in the toxicity of many drugs (Reynolds et al., 1980, Reynolds et al., 1981, Pryor 1980, McGinness et al., 1978, McGinness et al., 1982) in antimicrobial defense (Babior 1982, Hammerschmidt and Jacob 1982) and in inflammation (Kuehl et al., 1982). The generation of reactive oxygen species (ROS) is a steady-state cellular event in respiring cells.

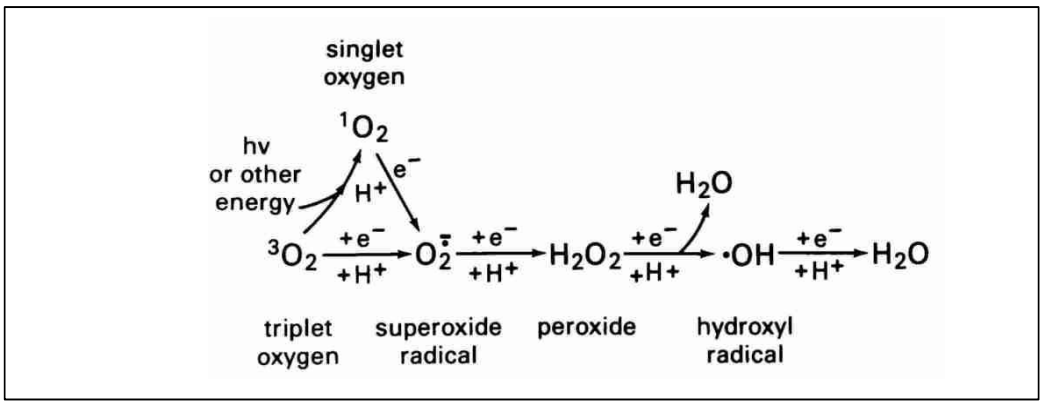


Figure 3.7 - Formation of reactive oxygen species.

In biologic systems molecular oxygen is reduced to water in four one-electron steps. Reduction of molecular oxygen to superoxide, and of peroxide to hydroxyl radical are "spin forbidden" and thus are slow unless catalyzed by a heavy ion. Alternative spin-permitted pathways for the reduction of 0_2 include interaction of molecular oxygen with the excited triple state of another molecule to produce singlet oxygen from light or an excited state molecule and jumps to a higher energy orbital on the same atom (Kuel et al., 1979). Excited-state species produced may be highly reactive and participate in reactions not unlike those of

free radicals. Singlet oxygen, itself a strong oxidizing agent, may be responsible for some of the effects assigned to other active oxygen species such as superoxide. Roles for singlet oxygen have also been postulated in photosensitized reactions, and in antimicrobial defense (Foote 1976).

Oxidative stress in pathological processes -

In addition to environmental causes such as oxygen (McLennon and Autor 1982), light, or ionizing radiation (Foote 1976) three physiological circumstances result in extraordinarily high local fluxes of radical species: (1) activation of the P-450-centered mixed function oxidase systems of endoplasmic reticulum, (2) activation of NADPH oxidase in phagocytes in response to antimicrobial defense and inflammation and (3) the presence of extraordinarily high levels of compounds which can reduce oxygen directly in auto oxidation reactions. Under such circumstances, the rate of active species generation may exceed the local capacity of the antioxidant defense and may contribute to injury.

Electron donors act as pro-oxidants by reducing less reactive species, such as molecular oxygen and peroxide, to more reactive species via reactions, which are typically mediated by the cyclical reduction/oxidation of transition-metal ions. The reduction of peroxide to hydroxyl radical by ferrous iron is known as Fenton's reaction." Peroxide and superoxide can also react in the presence of a metal ion to produce hydroxyl radical and molecular oxygen. This latter reaction is called the "Haber Weiss Reaction" (Crichton 1979). Transition metal ions are remarkably good promoters of free radical reactions (Hill 1981). Organisms use superoxide dismutases, catalase, and glutathione peroxidase as protection against generation of reactive oxygen species. Organisms also keep as many iron and copper ions as possible safely bound in storage or transport proteins (Halliwell et al., 1988, Halliwell and Gutteridge 1986, Aruoma and Haliwell 1987). There is three times as much transferrin iron-binding capacity in plasma as iron needing to be transported, so that there are essentially no free iron ions in the plasma (Halliwell and Gutteridge 1986). Iron ions bound to transferrin cannot stimulate lipid peroxidation or formation of free .OH radicals. The same is true of copper ions bound to the plasma proteins ceruloplasmin or albumin (Halliwell et al., 1988, Halliwell and Gutteridge 1986, Aruoma and Haliwell 1987,

Grootveld et al., 1989). The value of this sequestration is shown by an inspection of the pathology suffered by patients with iron-overload disease, in whom iron ion-citrate chelates circulate in the blood (Grootveld et al., 1989). These patients can suffer liver damage, diabetes, joint inflammation, and hepatoma, among other problems (McLaren et al., 1983). Metal ion sequestration is an important antioxidant defense. For example, recent papers have referred to ascorbic acid as a major antioxidant in plasma. However, ascorbate can only exert antioxidant properties in the absence of transition metal ions (Halliwell 1990).

Oxidative stress in thalassemia -

Sickle cell anemia, thalassemia, and glucose-6-phosphate-dehydrogenase deficiency are all hereditary disorders with higher potential for oxidative damage due to chronic redox imbalance in red cells that often results in clinical manifestation of mild to serve hemolysis in patients with these disorders. The release of hemoglobin, during hemolysis, and the subsequent therapeutic transfusion lead to systemic iron overloading that further potentiates the generation of ROS in thalassemia (Chan et al., 1999). The synthesis and accumulation of excess normal globin chain (i.e. β-chain in α-thalassemia and α-chain in β-thalassemia), within the red cell, lead to the formation of unstable aggregates, which upon oxidation, due to oxidative stress generated by iron overload, may precipitate and cause cell membrane damage. These deformed cells undergo premature destruction either in the bone marrow (extravascular hemolysis) or the peripheral circulation (intravascular hemolysis) (Weatherall 1997, Festa 1985). There is also an increase in erythropoietic activity in the bone marrow and in extramedullary sites (i.e. liver, spleen and lymph nodes) in several forms of thalassemias (e.g. β-thalassemia major) (Weatherall 1997, Festa 1985). The ineffective erythropoiesis and microcytic hypochromic anemia, described earlier, are associated with a compensatory increase in the absorbance of dietary iron. This may contribute to the iron overload that often results from blood transfusion therapy. Further, our results point out that the iron-induced liver damage in thalassemia may play a major role in the depletion of lipid-soluble antioxidants (Livrea et al., 1996).

Role of antioxidants -

Reactive oxygen species are constantly formed in the human body and removed by antioxidant defenses. An antioxidant is a substance that when present at low concentrations compared to that of an oxidizable substrate significantly delays or prevents oxidation of that substrate. Antioxidants can act by scavenging biologically important reactive oxygen species ($[O_2]$.,$[H_2][O_2]$, .OH, HOCl, ferryl, peroxyl, and alkoxyl), by preventing their formation, or by repairing the damage that they do. In biological systems the sources of the electrons are generally enzymes (e.g., NAD(P)H oxidase) and reducing substances (electron-donors). Simplistically, electron-donors act as antioxidants by (e.g.) reducing more reactive species such as trichloromethyl, superoxide, or hydroxyl radicals to less reactive species such as chloroform, peroxide, or water. Antioxidants come only from two sources - both natural: endogenous or exogenous.

Endogenous Antioxidants -

Three important intracellular enzymes constitute antioxidant defense; superoxide dismutase (SOD), catalase, and the GSH peroxidase/GSSG reductase system. SOD catalyzes the dismutation of superoxide, catalase the conversion of hydrogen peroxide to H_2O and O_2 (Willson 1979). Various organelle specific isoenzymes of superoxide dismutase exist (Fridovich 1979). The Zn-Cu SOD is cytoplasmic, while the Zn-Mn enzyme is chiefly mitochondrial. Neither isoenzyme is found in high concentrations in extracellular fluids (Fridovich 1979). GSH peroxidase transfers electrons from GSH to reduce peroxides to water. The oxidized glutathione produced (GSSG) is re-reduced back to GSH by glutathione reductase utilizing NADPH produced by the HMP shunt acting as an enzyme cofactor (Carrell et al., 1978).

The enzymatic production of active oxygen species by inflammatory cells may contribute to the pathophysiology of leukocyte dependent inflammatory processes (Huber 1980). Further, peroxidase release from eosinophils may play a similar role in inhibition of the inflammatory response while the antioxidant properties of ceruloplasmin may also give this compound antiinflammatory property. Production of active oxygen species by activated phagocytes may also

have a role in vascular (and other) damage following endotoxin shock, burn-induced plasma volume loss and even in atherosclerosis (Huber 1980).

It is not surprising that antioxidants, SOD (Huber 1980) and catalase (McCord et al., 1982) should ameliorate inflammatory symptoms in human and animal systems. This is of some clinical importance, since an antiinflammatory pharmaceutical preparation rich in SOD ("orgotein") is used in veterinary medicine and recently has been shown to be both effective and apparently safe in the treatment of various inflammatory lesions in man. Catalase has also been used in the treatment of arthritic disease in man with reported success (Riu et al., 1971).

3.4 *Triticum aestivum* (Wheatgrass)

Pharmacognosy and phytopharmacology of wheatgrass

Taxonomical details -

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Liliopsida – Monocotyledons
Subclass	Commelinidae –
Order	Cyperales –
Family	Poaceae – Grass family
Genus	*Triticum L.* – wheat
Species	*T. aestivum*

Wheat, a cereal grass of the Gramineae (Poaceae) family and of the genus *Triticum* and its edible grain, is the world's largest cereal-grass crop. It has been a food crop for mankind since the beginning of agriculture. The Middle East is probably the area of origin, and wheat apparently spread throughout Europe not later than the Stone Age. Historians believe it has been growing since Paleolithic times and cultivated for at least 6,000 years. Its status as a staple is second only

to rice. One reason for its popularity is that, unlike other cereals, wheat contains a high amount of gluten, the protein that provides the elasticity necessary for excellent bread making. Although over 30,000 varieties of wheat exist, the two major types are bread wheat and durum wheat. Global production of wheat is approximately 600 million tons; with international trade approximately 100 million tons annually. Wheat is Asia's second most important staple and has been growing much faster than rice. Wheat now provides one-fifth of total developing country food supply, up from 15 % in the early 1970s. In 1992-94, developing countries accounted for 45 % of world wheat production (551 million tons) and 46 % of world wheat area (219 million hectors).

The wheat plant is an annual grass. It is mainly grown as a winter annual in milder climates, with seeding in the fall and harvest from June through August depending on the length of the winter. In areas with rigorous winter climates it is mainly spring seeded. Planting is as early as soil can be worked, and harvest is in late summer and early fall. In early growth stages the wheat plant consists of a much-compressed stem or crown and numerous narrowly linear or linear-lanceolate leaves. Leaves are mainly near glabrous. Buds in the leaf axils below the soil surface grow into lateral branches termed tillers. From both the main crown and the tillers, elongated stems develop later and terminate in a spike or head in which the flowers, and finally the seed or grain, develop. Stems of wheat reach from 18 inches to 4 or more feet in height depending on kind and growing conditions.

Although over 30,000 varieties of wheat exist, they are of two major types: bread wheat and durum wheat. In U. S. Dept. of Agriculture- Technical Bulletin 1287 has classified wheat into 10 species of Triticum. Six of these are cultivated and four are non-cultivated, or rarely so. Agriculturally, important species of Triticum include -

1. *Triticum aestivum* (Common wheat, bread wheat, local varieties - Lok1, GW273) - Triticum aestivum comprises nearly 95 percent of the wheat grown. Its principal use is for flour. It is the most important variety for agriculture.

Figure 3.8: *Triticum aestivum* (Wheat grass) and wheat grass juice

2. *Triticum durum* (Durum wheat, local variety Raj 1555) - *Triticum durum* is used mainly for the manufacture of semolina, which is made into macaroni, spaghetti and related products. It is next in importance to *Triticum aestivum*.

3. *Triticum dicoccum* (Emmer wheat, local variety DDK) - *Triticum dicoccum* is one of the most ancient of cultivated cereals. Emmer was formerly grown in the United States for feed on a limited acreage but now has substantially disappeared from cultivation.

Macroscopy (*Triticum aestivum*)

Triticum aestivum is a bisexual plant with bisexual spikelets and hermaphrodite florets. Culm nodes are hairy, or glabrous. Culm internodes are solid, or hollow. Leaves are auriculate with blades narrowly to broadly linear. Leaves are 2–20 mm wide, flat, without cross venation and persistent. Inflorescence is a single elongated spike. Rachides are hollow. Spikelets are solitary; distichous and sessile. Female-fertile spikelets are 9–16 mm long, laterally compressed and disarticulating above the glumes. Rachilla are prolonged beyond the uppermost female-fertile floret. The rachilla extension is incomplete florets. Hairy callus is absent. Callus is very short and blunt. Glumes are two and more or less equal in size. They are shorter than the adjacent lemmas and lateral to the rachis; without conspicuous tufts or rows of hairs. Lower glume is 5–11 nerved. Upper glume is 5–11 nerved. Spikelets are usually with incomplete florets. The incomplete florets are distal to the female-fertile florets. The distal incomplete florets are usually 1, or 2 in number. Female-fertile florets have lemmas similar in texture to

the glumes. Awns when present are much shorter than the body of the lemma or much longer than the body of the lemma. Lemmas are hairy or hairless but scabrid. Palea is present and is relatively long, entire or apically notched. Palea keels are somewhat winged. Lodicules are membranous and ciliate. 3 stamens are present. Anthers are not penicillate with 2–4.5 mm length. Ovary is hairy. Styles are free to their bases. Stigmas are 2 in number and white in color. Fruit is free from both lemma and palea. It is medium sized or large i.e. up to 11 mm long, ellipsoid, longitudinally grooved, compressed dorsiventrally and with hairs confined to a terminal tuft. Hilum is long and linear. Embryo is large to small. Endosperm is hard; without lipid and contains only simple starch grains. Embryonic leaf margins are meeting. Seedling has a short mesocotyl and a tight coleoptile. First seedling leaf has a well-developed lamina. The lamina is narrow and erect.

Microscopy (*Triticum aestivum*)

Leaf-blade epidermis has conspicuous costal zonation. Papillae are absent. There are long-cells of similar wall thickness. Mid-intercostal long-cells are rectangular and fusiform, having markedly sinuous walls. Microhairs are absent. Stomata are common with 63–69 microns length. Subsidiaries are parallel-sided or dome-shaped. Guard cells are overlapped by the interstomatals. Intercostal short-cells are common (e.g. *T. polonicum*) or absent or very rare. Crown cells are present. Costal zones have short cells. Costal short-cells are predominantly paired (*T. polonicum*) or neither distinctly grouped into long rows nor predominantly paired. Costal silica bodies are horizontally elongated.

Transverse section of leaf blade shows parenchymatous cells without a suberised lamella. Mesophyll has non-radiate chlorenchyma. Leaf blade has distinct and prominent abaxial ribs of more or less constant size. Midrib is conspicuous with one bundle only or has a conventional arc of bundles. The lamina is symmetrical on either side of the midrib. Bulliform cells are present in discrete and regular abaxial groups in the furrows). Many of the smallest vascular bundles are unaccompanied by sclerenchyma. Combined sclerenchyma girders are present (rarely) or are absent. Sclerenchyma is all associated with vascular bundles.

Nutritional analysis of wheatgrass

Scientific reports on nutritional analysis of wheatgrass have been published frequently in various journals (Kohler 1953, Hamilton et al., 1988, Laboratory Analyses 1989). Also, several reputed companies involved in growing and selling of wheatgrass have published analyses of wheatgrass. As is evident from table 3.5 and table 3.6, wheatgrass is a rich source of chlorophyll, various minerals like iron, magnesium, calcium, phosphorus, antioxidants like beta carotene, insoluble dietary fibers while being low in fat content.

Table 3.4: Nutritional analysis of wheatgrass

Protein	1.959	Calories	21.0 Cal
Carbohydrates total	2.09	Moisture	95.9
Ash	0.0489	Magnesium	24 mg
Selenium	<1 ppm	Potassium	1 479
Zinc	0.33g	Phosphorus	75.2g
Calcium	24.2 mg	Sodium	10.3 mg
Iron	0.61 mg	Vitamin A	427 ILI
Vitamin B1	0.8 mg	Vitamin B2	0.13 mg
Vitamin B	0.11 mg	Vitamin B5	6.0 mg
Vitamin	96.0.2 mg	Vitamin B12	<1 mcg
Vitamin C	3.65 mg	Vitamin E	15.2 IU
Folic Acid	29 mcg	Biotin	10 mcg
Dietary Fiber (total)	0.1g	Lecithin	0.03 gm
Chlorophyll	42.2 mg	Choline	92.4 mg
Aspartic: Acid	260 mg	L-Arginine	135 mg

All above constituents are present in per 100g juice.
Data based on scientific laboratory analysis by Irvine Analytical Laboratories Inc., Irvine, CA, USA.

Table 3.5: Major ingredients in wheatgrass juice powder

Main Ingredients	**Content %**		
Carbohydrates	23.5	Fat	3.7
Moisture	0	Protein	46.7
		Ash (Minerals)	26.1
Mineral & trace minerals mg/gm			
Boron	.0055	Calcium	4.9
Chloride	.49	Chromium	.0012
Cobalt	<0.0005	Copper	.027
Fluoride	.0065	Germanium	<.011
Iodine	<0.0005	Iron	.051
Magnesium	4.4	Manganese	.026
Molybdenum	<0.0005	Nickel	<0.0005
Phosphorous	29	Potassium	2.8
Selenium	<0.0005		
Silicon	.16	Sodium	.11
Tin	<0.0005	Titanium	<0.0005
Vanadium	<0.0005	Zinc	.066
Vitamins mg/g			
Biotin	.00011	Choline	.0011

Cyanocobalamin (B12)	.00001	Folic Acid	.0012
Inositol	<0.011	Niacin (B3)	.09
Panotothenic Acid	.0196	Pyridoxine HCL B6	.0065
Riboflavin	.0031	Thiamin (B1)	.0098
Vitamin A (Retino)l	501 IU/Gm	Vitamin C	.185
Vitamin D	<0.1 IU/Gm	Vitamin E	.02 IU/Gm
Others mg/g		Chlorophyll	1.2
Cholesterol	<0.01	Sugars	48
Essential Amino Acids mg/g			
Isoleucine	15.8	Leucine	31.5
Lysine	22.6	Methionine	3.5
Phenylalanine	19.8	Proline	17.1
Threonine	14.8	Valine	22.1
Non-Essential Amino Acids mg/g			
Alanine	24.8	Arginine	22.1
Aspartic Acid	46.9	Glutamine	77.4
Glycine	20.4	Histidine	7.4
Serine	15.9	Tyrosine	6.9

Source – EPA, USDA

Table 3.6: Comparison of contents of wheatgrass with other vegetables.

	Wt.	Protein	Fiber	Calcium	Vit. A	Iron	Selenium	Magnesium	Potassium
Vegetable	gr.	gr.	gr.	mg.	IU	mg.	mcg.	Mg.	mg.
Dehydrated Wheat grass	100	25	17	515	66,08	57.1	99.7	197.5	1,425
Beets (raw)	100	1.7	0.8	17	22	0.7	-	23.3	339
Bib Lettuce (raw)	100	1.3	0.5	35	964	2.1	-	9	264
Broccoli (raw)	100	3.6	1.5	103	2,500	1.1	-	24	380
Cabbage (raw)	100	0.9	0.8	34	90	0.3	1.5	13	163
Cauliflower	100	2.7	1	25	60	1.1	0.7	24	295
Celery, raw	100	0.9	0.6	39	266	0.3	-	21.6	39
Collards (raw)	100	3.6	0.9	401	6,500	1	-	57	401
Corn (cooked)	100	3.2	0.7	163	396	3	-	20	163
Cucumber (raw)	100	0.9	0.6	25	245	1.1	0.1	11.2	158
Eggplant (raw)	100	1.2	0.9	12	10	0.7	-	16	214
Green Pepper (raw)	100	1.3	1.4	9	425	0.8	0.6	18	213
Kale (raw)	100	4.2	1.3	179	8,900	0.5	-	37	318
Mushrooms (raw)	100	2.7	0.8	6	5	0.8	12	10.8	406
Okra (raw)	100	2.4	1	249	520	0.6	-	41	-
Onions (raw)	100	1.5	0.6	27	41	0.5	1.5	11.8	155
Peas (raw)	100	6.3	2	26	632	1.9	-	34.5	311
Potato (raw)	100	2.2	0.8	7	5	0.6	-	-	409
Radish (raw)	100	1	0.7	28	5	1	4.2	14	290
Spinach (raw)	100	3.5	0.6	97	8,109	3.2	-	80	471
Sweet Potato (baked)	100	1.6	1.2	31	3,400	0.7	-	-	233
Tomato (raw)	100	1.1	0.5	10	905	0.6	0.5	14.1	245
Turnips (raw)	100	1	0.8	38	5	0.5	0.6	18.8	261

Source: *Nutrition Almanac* and published scientific papers on cereal grass by Dr. George Kohler (Kohler 1944)

Therapeutic uses of wheatgrass

Traditionally, wheatgrass has been used as a medicinal herb in India. In western countries neutraceutical science is gaining momentum and wheatgrass is now being advocated as a food supplement. A closer look at table - 3.3 reveals that compared to most of the vegetables, which are part of our daily food, wheatgrass has higher contents of all the nutritional factors and minerals. Thus, wheatgrass is not just an herb or a food supplement, but it is a complete food and may be taken daily, even by healthy people.

In today's fast lifestyle and fast-food world, deficiency of any or many of these biochemical factors could easily occur culminating into a disease or disorder. For example, as reported by Altura and Altura (1995), 'It is now becoming clear that a lower than normal dietary intake of magnesium can be a strong risk factor for hypertension, cardiac arrhythmias, ischemic heart disease, atherogenesis and sudden cardiac death. Deficiency in serum magnesium is often associated with arrhythmias, coronary vasospasm and high blood pressure'. Wheatgrass juice, being a rich source of magnesium, may replenish the deficiency of magnesium and improve the clinical picture.

Conversely, a disease state may cause deficiency of a nutrient. According to Rude (1993), 'A large body of evidence demonstrates the prevalence and adverse clinical consequences of magnesium deficiency in patients with diabetes mellitus. It would be prudent for physicians who treat these patients to consider magnesium deficiency as a contributing factor in many diabetic complications and in exacerbation of the disease itself. Repletion of the deficiency or prophylactic supplementation with oral magnesium may help avoid or ameliorate such complications as arrhythmias, hypertension, and sudden cardiac death and may even improve the course of the diabetic condition'. Regular intake of wheat grass juice could correct the secondary magnesium deficiency and thus, may be helpful in averting long-term clinical complications of diabetes mellitus.

The movement for the human consumption of wheatgrass began in the western world in the 1930's and was initiated by Charles F. Schnabel, known as the father of wheatgrass (Anderson 1986). He said 'Fifteen pounds of wheatgrass is

equivalent to 350 pounds of the choicest vegetables.' Later Wigmore (1940) healed herself of cancer from the weeds she found in vacant lots in Boston. She began a study of natural healing modalities—and with the help of a friend, Dr. Earp Thomas, she found that there are 4700 varieties of grass in the world and all are good for man.

Dr. Wigmore reported that the "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 a 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, body odor and constipation (Wigmore 1985). Dr. Wigmore's opinions are based on her experiences with her guests at Hippocrates. A few clinical studies have verified that some disease conditions can be benefited by the use of wheatgrass. Remarkably, a relatively large number of studies indicate that food factors and nutrients found in wheatgrass may provide relief from many of the conditions claimed by Ann Wigmore.

Clinical studies on wheatgrass

Ulcerative colitis is a common and sometimes serious disorder of the large intestine that can cause abdominal pain, diarrhea, and bleeding. Ben-Ayre et al., (2002) reported that people taking wheat grass juice experience a significant improvement of their ulcerative colitis symptoms on a scale that measured overall disease activity, compared with people taking a placebo. Wheat grass juice also significantly reduced the severity of rectal bleeding and abdominal pain. The initial dose of wheatgrass juice, 20 ml (two-thirds of an ounce) per day, was increased over a period of several days to a maximum of 100 ml (3.5 ounces) per day. In addition to the positive results mentioned above, an examination of the colon (sigmoidoscopy) showed improvement in 78% of the people receiving wheat grass juice, compared with only 30% of those receiving placebo. No serious side effects were seen. Although nausea was reported by 33% of the participants receiving wheat grass juice, 41% noted an increase in vitality while taking the supplement.

A clinical pilot study was carried out by Marwaha et al., (2004) at the Advanced Pediatric Centre, Postgraduate Institute of Medical Education and Research, Chandigarh, India. It was reported that during period of wheatgrass juice ingestion: all participants experienced lower blood transfusion requirements, 50% had at least 25% reduction in transfusion requirements, the mean interval between transfusions increased 29.5%, overall, hemoglobin levels were not compromised by reduced transfusion volumes.

Wheatgrass and iron overload

It has been recognized that flavanoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavanoids are through scavenging or chelating process (Kessler et al., 2003; Cook and Samman, 1996). Phenolic compounds are a class of antioxidant compounds which act as free radical terminators (Shahidi and Wanasundara, 1992). The compounds such as flavanoids, which contain hydroxyl functional groups, are responsible for antioxidant effect in the plants (Das and Pereira, 1990; Younes, 1981). There was a direct relation between chelatory activity and the content of active compounds, phenol and flavanoids in some extracts in this study. Some extracts with high phenol and flavonoid contents showed good chelating of Fe^{2+}. For example, *E. hirsutum* and *M. arvensis* that contained highest phenol and flavanoids contents showed the best chelating activity.

Patients with chronic anemia such as thalassemia, require regular blood transfusions in order to improve both quality of life and survival. Humans are unable to eliminate the iron released from the breakdown of transfused red blood cells and the excess iron is deposited as hemosiderin and ferritin in the liver, spleen, endocrine organs and myocardium. 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 (Taher et al., 2006; Rund and Rachmilewitz, 2005; Loukopoulos, 2005). Thalassemia major is characterized by anemia, iron overload, further potentiation of reactive oxygen species (ROS) and damage to major organs, especially the cardiovascular system. Oxidative stress is ultimately involved in endo-thelial dysfunction, a condition which is evident in adults

suffering from various cardiovascular diseases including thalassemia (Shinar and Rachmilewitz, 1990; Hebbel et al., 1990; Grinberg et al., 1995). Antioxidant and other supportive therapies protect red blood cells (RBC) against oxidant damage (Kukongviriyapan et al., 2008; Filburn et al., 2007). Also a higher rate of LDL oxidation in thalassemia patients is due to a lower concentration of vitamin E and C in the LDL particles. Enrichment with vitamins E and C was effective in preventing LDL oxidation in patients with thalassemia (Rachmilewitz et al., 1979; Livrea et al., 1996). Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications and thereby improves quality of life and overall survival (Shinar and Rachmilewitz, 1990; Hebbel et al., 1990). The poor oral bioavailability, short plasma half-life and severe side effects makes available chelators suboptimal (Hebbel et al., 1990; Grinberg et al., 1995, Kukongviriyapan et al., 2008; Filburn et al., 2007).

Clinically it was proved that different varieties of wheatgrass extracts are therapeutically used in treatment of anemia, thalassemia (major), cancer and bacterial diseases (Desai et al., 2005). It was reported that aqueous extract of wheatgrass have mild to moderate antibacterial activity against four common opportuinistic pathogenic bacteria viz. Staphylococcus aureus, Bacillus cereus, Salmonella typhimurium and Kleibsella pneumoneae (Desai et al., 2005).

Potential benefits of *Triticum aestivum* in iron overload in thalassemia and other blood disorders

Some research workers have studied chlorophyll, one of the major ingredients present in wheatgrass. Chlorophyll is not so unique in its chemical structure. It is built around a porphyrin ring, which occurs in a variety of natural organic molecules. The most interesting group of molecules which contain porphyrin rings are those involved in cellular respiration, or the transportation and consumption of oxygen. These include hemoglobin, myoglobin, and the cytochromes. The chemical similarity between hemoglobin and chlorophyll was first suggested by Verdel in 1855 (Carpenter 1949), and specifically demonstrated in the early 1920s. One of the major differences between chlorophyll and hemin is that chlorophyll contains magnesium while the hemin

molecule contains iron as its central atom. Owing to the close molecular resemblance between chlorophyll and hemoglobin, it was hypothesized that chlorophyll is nature's blood-building element for all herbivorous animals and humans.

Some studies have indicated that feeding chlorophyll-rich foods to rats stimulates the regeneration of red blood cells (Scott and Delor 1933). Researchers were able to demonstrate that this effect was not due to the iron or copper in the green foods (Rothemund et al., 1934). Hughes and Latner (1936) fed several doses and forms of chlorophyll to anemic rabbits and found that very small doses of purified chlorophyll or large doses of a crude chlorophyll extract produced a very favorable effect on hemoglobin regeneration. They suggested that the chlorophyll is acting as a physiological stimulant of the bone marrow and is not really concerned with the actual chemistry of regeneration of the porphyrin.

Synthetic agents like desferrioxamine and deferiprone used for the treatment of iron overload in thalassemia are accompanied by serious side effects and certain limitaions including the 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. 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.

consideration the relative paucity of iron chelating agents, it is not surprising that clinical scientists are putting a great effort towards finding any potentially useful sources in order to obtain the maximum possible benefit with the least possible harm (Loukopoulos, 2005; Ebrahimzadeh et. al., 2006; Pourmorad et al., 2006; Hosseinimehr et al., 2007; Pourmorad et al., 2007). 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. Some medicinal plants traditionally used for management of

diseases were selected and their phenol and flavanoids content and iron chelating activities were evaluated in this study.

Table 3.7: Total phenol and flavonoid content and iron chelating IC_{50} of the herbs.

Plant	Common name	Part of plant tested	Medical use/disease treated
Myrtaceae *Feijoa sellowiana*	Feijoa, Pineapple Guava, Guavasteen	Fruits peels and leaves	Human food
Caprifoliaceae *Sambucus ebulus*	Danewort, Dwarf Elder,	Fruits	Antinociceptiv; anti inflammatory activity Antiphlogistic;Cholagogue; Diaphoretic; Diuretic; Expectorant; Homeopathy; Poultice; Purgative.
Rosaceae *Crataegus pentagyna*	-	Fruits	Hypotensive; cardiotonic
Juglandaceae *Pterocarya fraxinifolia*	Caucasian wingnut, Pterocarya caucasica	Fruits and stem barks	Diaphoretic
Anacardiaceae *Pistacia lentiscus*	Mastic gum	Gum	Antimicrobial;antioxidant;hepatoprotective; Analgesic; Antitussive; Carminative; Diuretic; Expectorant; Odontalgic; Sedative; Stimulant
Fabaceae *Melilotus arvensis*	Yellow Melilot	Arial parts	Antispasmodic; Aromatic; Carminative; Diuretic; Emollient; Expectorant; Ophthalmic; Vulnerary
Onagraceae *Epilobium hirsutum*	Great Willowherb, Greater Hairy Willowherb	Leaves	Antimotility;antibacterial; anti-inflammatory; analgesic activity
Graminaceae, Corn silk (*Zea mays*)	Maize silk, mealie silk and Yu mi shu.	The silk on the cob are used for making the brew	Diuretic; kidney Stones; cystitis; demulcent;anti-inflammatory; tonic;anti diarrhea;anti itching;

			prostateproblems; blood sugar decreasing; intestinal and liver function regulatory effect
Ebenaceae Diospyros lotus	Persimmon	Fruit	Anticeptic, sedative, anti fever, antidiabetic, antitumor
Rosaceae *Pyrus boissieriana*	Pear	Fruit	Antioxidant
Lamiaceae *Salvia glutinosa*	Jupiter's distaff	Arial parts	Antimicrobial

Aantioxidants present in wheatgrass -

Beta-carotene -

Excited-state derivatives such as singlet oxygen and the excited triplet (diradical) states of other molecules may be quenched by interactions with conjugated diene systems such as those found in carotenes (Huber 1980), tocopherols or the melanins (Forrest et al., 1974). Vitamin A, itself, can pose a hazard to human health and even the risk of birth defects when taken in excess. Its precursor, β-carotene, can be taken in virtually any quantity without harmful effect. At the same time it provides the body with a store of the raw material from which it produces vitamin A naturally according to needs. β-carotene has been positively linked to increased protection against many forms of cancer, including lung, bladder, rectal, oral and dermal (skin) cancers.

Vitamin C -

Vitamin C acts synergistically with vitamin E and assists not only in the prevention of the formation of arterial cholesterol plaque but also, in sufficient quantities, has been shown to actually assist in the chelation ("dissolving") of existing cholesterol plaque thereby helping clear occluded (blocked) arteries, particularly coronary. Vitamin C is also specifically known to assist in the prevention of many forms of cancer, including pancreatic, rectal, cervical, esophageal and oral cancer. It is also a powerful free radical scavenger and thereby helps clean up the residues of cigarette smoke and other forms of air pollution.

Vitamin E -

As well as the primary defenses (scavenger enzymes and metal-ion sequestration), secondary defenses are also present. The cell membranes and plasma lipoproteins contain α-tocopherol, a lipid soluble molecule that functions as a chain-breaking antioxidant. Attached to the hydrophobic structure of α-tocopherol is an -OH group whose hydrogen atom is easily removed. Hence, peroxyl and alkoxyl radicals generated during lipid peroxidation preferentially combine with the antioxidant instead of with an adjacent fatty acid side chain. This therefore terminates the chain reaction, whence the term chain-breaking antioxidant. It also converts the α-tocopherol into a new radical, tocopherol-α, which is poorly reactive and unable to attack adjacent fatty acid side chains, consequently stopping the chain reaction. Evidence exists (Esterbauer et al., 1989, Wefers and Sies 1988) that the tocopherol radical can migrate to the membrane surface and reconvert to α-tocopherol by reaction with ascorbic acid (vitamin C). Both vitamin C and α-tocopherol seem to minimize the consequences of lipid peroxidation in lipoproteins and in membranes, should this process begin (Wefers and Sies 1988). α-tocopherol is the most effective lipid-soluble chain breaking antioxidant in-vivo in humans (Ingold et al., 1986). The content of α-tocopherol in circulating low-density lipoproteins helps to determine their resistance to lipid peroxidation and thus may affect the development of atherosclerosis, a disease in which lipid peroxidation is involved (Gey et al., 1987). Low plasma levels of α-tocopherol and vitamin C correlate with an increased incidence of myocardial infarction and of some forms of cancer (Gey et al., 1987). Vitamin E is present in small quantities in many foods but its uptake is all too often inhibited by low-fat or no-fat diets as fats or oils are the essential carriers of vitamin E without which absorption or uptake of this, the most powerful of the antioxidant vitamins, is severely restricted. Vitamin E is known to fight infection, promote healing and to assist in the prevention of lung and gastro-intestinal (including bowel) cancer. It achieves this by again preventing free radicals from entering cells where nuclear and DNA damage would otherwise ensue.

Wheatgrass is rich in chlorophyll, magnesium, Iron, selenium, zinc, chromium, and antioxidant vitamins like vitamins A, E, C, B_{12}, folic acid, pyridoxine, host of other minerals and amino acids that have significant nutritious and medicinal value. Since deformation of RBC, caused by oxidation of excess α-chains, is the

main causative factor for hemolysis and therefore, increased frequency of repeated blood transfusions in thalassemia; antioxidants and magnesium present in wheatgrass may reduce the need of such repeated transfusions.

In the light of foregoing discussion the present study is planned to investigate beneficial effects of *Tritium aestivum* for management of iron overload and other blood disorder like thrombocytopenia, hemophilia and immunomodulatory effects. In order to narrow down work we are planned to find out mechanism of action and possible active ingredients from *Triticum aestivum* responsible for these beneficial effects.

4. OBJECTIVES

Objectives of the present project were –

1. To carry out pharmacognostic study of *Triticum aestivum*.
2. To carry out phytochemical studies of *Triticum aestivum* (Wheatgrass) and its various extracts.
3. To evaluate iron chelating activity of various extracts of *Triticum aestivum.*
4. To isolate iron chelating constituent from extract of *Triticum aestivum*, using column chromatography.
5. To evaluate iron chelating activity of isolated constituent of *Triticum aestivum*, using pre-clinical study.
6. Investigation of anti-oxidant property of *Triticum aestivum* in iron overload condition.
7. Evaluation of therapeutic benefits of *Triticum aestivum* in thrombocytopenia.
8. Assessment of immunomodulatory activity of *Triticum aestivum*.

5. Materials and Methods

5.1 Pharmacognostic studies of *Triticum aestivum* (wheatgrass).

5.2 Preparation of various extracts of wheatgrass.

5.3 Phytochemical studies of various extracts of wheatgrass.

5.4 In-vitro evaluation of iron chelating activity in various extracts of wheatgrass.

5.5 In-vivo evaluation of iron chelating activity in various extracts of wheatgrass.

5.6 Isolation and characterization of iron chelating active constituent of wheatgrass.

5.7 In-vitro evaluation of iron chelating activity in active constituent of wheatgrass.

5.8 In-vivo evaluation of iron chelating activity of active constituents of wheatgrass.

5.9 Investigation into anti-oxidant property of *Triticum aestivum* in iron overload condition.

5.10 Evaluation of therapeutic benefit of *Triticum aestivum* in thrombocytopenia.

5.11 Assessment of immunomodulatory activity of *Triticum aestivum.*

5.1 Pharmacognostic studies of *Triticum aestivum* (wheatgrass)

Identification and growing of wheatgrass

Certified sample of species of wheat *Triticum aestivum* was acquired from Wheat Research Center, Gujarat Krushi University, Junagadh, Gujarat. The authenticity of this certified sample was also confirmed by comparing their morphological characters with description mentioned in different standard texts and floras (Percival J 1974). These wheat varieties were grown in plastic trays as per standard procedure described below (Wigmore 1985).

Cultivation and collection procedure for wheat grass (Wigmore 1985)

Procedure for growing wheatgrass -

1. Adequate quantity of unpolished wheat grain was soaked overnight in water in a container.
2. On the next day, the soaked wheat-grain were spread on the surface of the soil filled in plastic trays. Care was taken so that the grains did not touch one another.
3. A thin layer of soil was sprinkled on the wheat grains.
4. The tray was covered with a newspaper to provide darkness, which helps the sprouting.
5. The tray was kept in a covered balcony.
6. Next day the tray was uncovered to spray on some water and was covered again with the newspaper.
7. Step 6 was repeated every day until sprouting took place, after which the tray was left uncovered and watered everyday for 8 days.
8. On 9th day the wheatgrass was harvested by cutting it with a clean pair of scissors about 1/2" above the surface of the soil.

To characterize this variety of *T. aestivum,* the grass were subjected to microscopic study, which included transverse section, surface preparation and powder study. For powder study grass was cleaned and dried in a dark place for four days. It was powdered, passed through 40# and stored in airtight bottles.

Pharmacognostic studies

a. Macroscopic study:

Both fresh and dried samples of *T. aestivum* were subjected to study for their morphological characters.

b. Microscopic study:

Free hand transverse sections of fresh aerial part of *T. aestivum* were studied under microscope and compared with literature. Powder study of wheatgrass was performed for sample and compared with literature.

5.2 Preparation of various extracts of wheatgrass

Various extracts of wheatgrass were prepared by different solvents using soxhlet apparatus. Dried powder (60#) of *Triticum aestivum* was defatted with petroleum ether (60˚-80˚) (3 X 500ml) for 1 hour; allowing macerating for 24h each time. The combined extracts, upon concentrating in vacuum, yielded greenish yellow semisolid residue. (Ext P, 2.8 % w/w).

After defatting, powder was subjected to air dried and for extraction (60˚-80˚) with chloroform, exhaustively. The chloroform extract (Ext C) was concentrated to yield 3.4 % w/w. Afterwards the chloroform extract marc was air dried and extracted with acetone. The acetone extract (Ext A) was concentrated to yield 4.3 % w/w. After acetone extract, marc was air dried and extracted with methanol. The methanol extract (Ext M) was concentrated to yield 4.1 % w/w. Marc of methanol extract was successively extracted with water. Water extract (Ext W) was separately concentrated to yield 5.3 % w/w.

Solid powder obtained from these successive extractions of acetone, methanol and water were subjected for evaluation of pharmacological activities like iron over load induce thalassemia model, thrombocytopenia model, immunomodulatory activities.

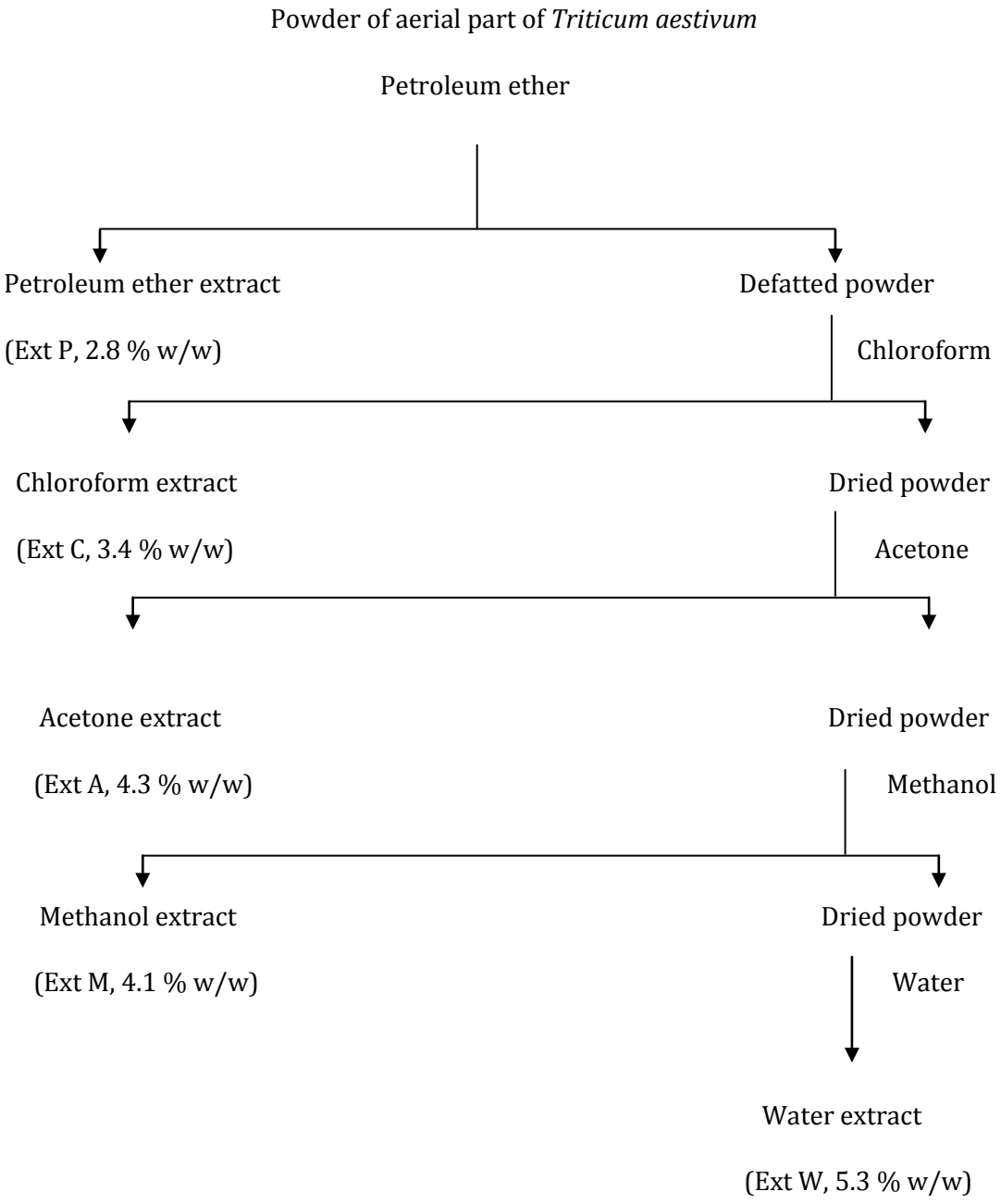


Figure 5.1: Scheme for successive extraction of aerial part of *Triticum aestivum* using soxhlet apparatus.

5.3 Phytochemical studies of various extracts of wheatgrass

Petroleum ether, chloroform, acetone, methanol and water extracts of wheatgrass were subjected to various tests to determine presence of phytoconstituents specifically, flavonoids and phenolic compounds.

'Controls': Controls were prepared for each test. This includes similar treatment/procedure as the 'test' sample, but no tests samples i.e. powder/extract were added. All inferences/conclusions were based on comparison of 'test' with 'control', and not with 'test' alone.

Test for flavonoids:

Shinoda test (Geissman A 1955)

1 gm of powder of different extracts of wheatgrass were extracted with 10 ml of ethanol (95%) for 15minutes, on a boiling water bath and filtered. To the filtrate a small piece of magnesium ribbon and 3 to 4 drops of concentrated sulphuric acid were added. Formation of red color indicated presence of flavonoids in extracts (Flavanone).

Tests for phenolic compounds:

a. *Test with $FeCl_3$*: (Clerk J et al, 1947)
   Freshly prepared $FeCl_3$ solution was added to each extracts of wheatgrass. Development of brownish green color indicated presence of phenolic content in extracts.
b. *Test with Folin ciocalteu reagent:* (Harborne JB , 1973)
   Folin ciocalteu reagent was added to each extracts of wheatgrass. Development of bluish green color in extracts indicated presence of phenolic contents with catechol or hydroquinone nuclei.

Quantitative determination of total phenolic in various extracts and isolation compound using Folin-Ciocalteau method

(Singleton VL and Rossi JA 1965, Somers TC 1980)

Folin-Ciocalteau (FC) colorimetry 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 color 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.

Materials

Sample: methanol and water extracts of *T. aestivum*

Standard: Gallic acid

Reagent: Folin-Ciocalteau (FC) reagent, stored in dark and discarded if reagent becomes visibly green, freshly prepared Sodium carbonate solution.

Apparatus: Spectrophotometer set to 765 nm, with 2-ml plastic or glass cuvettes.

Reagents and Solutions

- Deionized or distilled water were used in all recipes and protocol steps.
- Gallic acid calibration standards

  0.5 gm gallic acid was dissolved in 10 ml ethanol and then dilute to 100 ml with water (5 g/liter final). Different concentrations of gallic acid solution were prepared using water (50, 100, 250, and 500 mg/liter concentrations).
- Standards will retain 98% of their potency for 2 weeks if kept closed under refrigeration (4°C), but this potency is retained for only 5 days at room temperature.
- Sodium carbonate solution

  A fresh sodium carbonate solution was prepared by using dissolved 200 gm anhydrous sodium carbonate in 800 ml water and boiled. After cooling, solution was filtered through Whatman no. 1 filter paper.

Procedure:

1. 50 µl sample of gallic acid calibration standard, or blank (deionized or distilled water) was added to a test tube.

2. 1.58 ml water followed by 100 µl FC reagent was added to this sample. These solutions were inverted and incubated for 1 to 8 minutes. The incubation period was not more than 8 minutes.
3. 300 µl sodium carbonate solution was added, mixed and incubated again for 2 hr at room temperature. A final volume of 2 ml was made using distilled water.
4. Absorbance of samples was measured at 765 nm and analyzed in UV-visible spectrophotometer.

5.4 In-vitro evaluation of iron chelating activity in various extracts and isolated compound of wheatgrass

The chelation of ferrous ions by extracts was estimated by method of Dinis et al., 1994. Briefly, 50 µl of 2 mM $FeCl_2$ was added to 1 ml of different concentrations of the methanol extract (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) of wheatgrass. 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 $[(A_0 - A_S)/ A_S] \times 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of extract/standard. Na_2EDTA was used as positive control.

BIOLOGICAL EVALUATION / PHARMACOLOGICAL STUDIES OF VARIOUS EXTRACTS OF *Triticum aestivum*

Various extracts and isolated compounds of *T. aestivum* were subjected to evaluation of **iron overload induced thalassemia model, thrombocytopenia and immunomodulatory activity** in various animal models.

SELECTION OF ANIMALS

All animals were housed at ambient temperature (22±1^{0}C), in 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).

5.5. In-vivo valuation of iron chelating activity in various extracts of wheatgrass

Induction of Iron overload

Male Sprague-Dawley rats initially weighing 150-200 gm were used for present study. The rats were given six Intraperitoneal injections of iron-dextran (12.5 mg/l00 g body wt.) evenly distributed over a 30 days of period which results in condition of chronic iron overload, which was very resemble to iron overload in thalassemia. Control rats were injected with an equal volume of dextran at the same time. (Hachiro et al 2002, British journal of hematology, 1990, Addison GM et al., 1972)

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

Group 1: Normal control received dextrose solution

Group 2: Disease control treated with iron dextran (12.5mg/l00g body wt.)

Group 3: Disease control treated with desferoxamine (40 mg/kg, p.o., per day)

Group 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day)

Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day)

Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day)

All the studies were carried for a period of 30 days. During the study, blood samples, urine samples and fecal sample were collected on 15 and 30 days under fasting conditions. Histopathology of heart, liver and kidney were performed at the end of 30 days to study iron overload complications.

Blood sample collection and analysis for biochemical parameters

At the end of 15 days and 30 days of treatment period, blood samples were collected from rats under fasting conditions, from retro orbital plexuses using light ether anesthesia, in clean dry centrifuge tubes. Blood sample were subjected for estimation of -

A. Hematological parameters

a. Hemoglobin content
b. Total RBC, PCV, MCV, MCH, MCHC
c. Total WBC count
d. Differential WBC count
   Neutrophil, Lymphocyte, Eosinophil, Basophil counts and Total lymphocyte count and
e. Platelet count
   using fully automated hematoly analyzer - Model XS-800i – Sysmex.

Collected blood samples were allowed to clot for 30 min at room temperature and serum were separated by cooling centrifugation at 5000 rpm (REMI CM-12 cooling centrifuge) for 20 min and stored at –20°C until the analysis was carried out.

Serum samples were subjected to estimation of -

B. Serum Iron: Sampling, reagent delivery, mixing, processing and printing of results are automatically performed by fully automated biochemistry Dimension-Rx L Max-Dade Behring Analyzer.
C. Serum Ferritin: Sampling, reagent delivery, mixing, processing and printing of results are automatically performed by fully automated immunology Analyzer- sysmex-XS-800i ® system.
D. SGPT
E. SGOT
F. Creatinine

G. Creatine kinase (CKMB)

Urine and fecal sample collection and analysis for iron content

Animals were placed in metabolic cages after treatment with extracts for a period of 12 hours. At the end of day 15 and day 30, urine and fecal samples were collected using metabolic cage. Total volume of urine and weight of faces were recorded and subjected for determination of iron in sample using fully automated biochemistry Dimension-Rx L Max-Dade Behring Analyzer.

Analysis of iron in urine

All urine samples were centrifuged at 14000 rpm for 5 min to remove mucus and epithelial cells. After approximately dilutions with water, the supernatant was directly injected into fully automated biochemistry Dimension-Rx L Max-Dade Behring Analyzer.

Analysis of iron in faces

The fecal samples were subjected to dry at a constant weight in 20 minutes at medium power in a microwave. An accurately weighed amount of dried feces (100-300 mg) was mixed with a known volume of water in a glass mortar and grounded well to form a uniform homogenate. Typically concentrations of fecal in homogenate were prepared in range of 20–25 % w/v. An accurately weighed amount of homogenate was then placed in a crucible and dried for 10 min at medium power in microwave oven. To the dried residue 250 micro liter of concentrated nitric acid was added and subjected for digestion at medium power for 10 min. Digestion was repeated with three of four more additions of nitric acid to obtain residue which was reconstituted with 500 micro liters of 5 mM HCl by bath sonicator. These prepared samples were subjected to estimation of iron using fully automated biochemistry Dimension-Rx L Max-Dade Behring Analyzer. (British journal of hematology, 1990)

All above mentioned parameters were estimated to find out in-vivo iron chelating property of various extracts of *Triticum aestivum* and to find out its beneficial effects in prevention of complication in heart, liver and kidney due to iron overload.

A. Estimation of hematological parameters

Various hematological parameters like

f. Hemoglobin content
g. Total RBC, PCV, MCV, MCH, MCHC
h. Total WBC count
i. Differential WBC count
   Neutrophil, Lymphocyte, Eosinophil, Basophil counts and Total lymphocyte count and
j. Platelet count

were estimated using fully automated hematoly analyzer - Model XS-800i – Sysmex.

B. Estimation of serum iron (Hachiro et al., 2002)

Principle

Under acidic conditions (pH 4.5), iron (Fe^{+3}) bound to protein transferrin is released in presence of reducing agent, ascorbic acid, (Fe^{+3}) is reduced to (Fe^{+2}). The resulting product, Fe^{+2} forms a blue complex with 3-(2-pyridyl)-5, 6-bis-2-(5-furyl sulfonic acid)-1,2,4- triazine, disodium salt (Ferene®). The absorbance of the complex, measured using a bichromatic (600, 700 nm) endpoint technique, is directly proportional to concentration of transferrin-bound iron in serum.

Fe^{+3}- transferrin —› Fe^{+3} + Transferrin

2 Fe^{+3} + Ascorbic Acid —› 2 Fe^{+2} + Dehydroascorbic Acid + 2 H^{+}

Fe^{+2} + 3 Ferene® —› Fe^{+2}- Ferene®$_3$ complex

(absorbs at 600 nm)

Procedure:

Test conditions:

- Sample size - 50 µl
- Reagent 1 volume - 100 µl
- Reagent 2 volume - 25 µl
- Diluents size - 225 µl
- Test temperature - 37^{o} C
- Wavelength - 600 and 700 nm
- Type of measurement - bichromatic endpoint

Sampling, reagent delivery, mixing, processing and printing of results were automatically performed by fully automated biochemistry Dimension-Rx L Max-Dade Behring Analyzer.

C. Serum ferritin (Addison GM et al, 1972)

Ferritin is major soluble iron storage protein from which iron may be mobilized for synthesis of hemoglobin, myoglobin and other iron containing proteins. Ferritin is present in high concentrations in the cytoplasm of reticuloendothelial cells, liver cells, spleen cells and developing red precursors in bone marrow. Studies on patients with iron-deficiency and iron overload abnormalities confirmed that measurement of serum ferritin concentration reflect amount of storage iron in body.

Principle

Ferritin present in test sample is bound with monoclonal antibody immobilized on a magnetic solid phase and enzyme-labeled monocolonal antibody in the reagent. The magnetic beads are washed to remove unbound enzyme-labeled monoclonal antibody and are then incubated with a fluorogenic substrate, 4-methyl-umbelliferyl phosphate (4MUP). The amount of enzyme-labeled monoclonal antibody that binds to the beads is directly proportional to the ferritin concentration in test sample. A standard curve is constructed and unknown sample concentrations are calculated using this curve.

Sampling, reagent delivery, mixing, processing and printing of results were automatically performed by fully automated immunology Analyzer- sysmex-XS-800i ® system.

D. Serum Glutamic Pyruvic Transaminase (SGPT /ALT/GPT)

(Span Diagnostics Ltd., India)

Principle

Glutamic Pyruvic Transaminase catalyse the reaction between α–ketoglutaric acid and alanine giving L- Glutamic acid and Pyruvic acid. Pyruvic acid in the presence of lactate dehydrogenase reacts with NADH giving lactic acid and NAD.

The rate of NADH consumption is determined photometrically and is directly proportional to GPT activity in the sample.

Reagents

Reagent I: Buffer reagent
Reagent II: Enzyme reagent

Sample

Serum or Plasma

Reagent preparation

4 ml of buffer reagent was mixed with 1 ml of enzyme reagent.

Procedure

Pipette into test tubes

Sample	100 πl
Reagent	1000 πl

Reactive samples were subjected to fully automated biochemistry Dimension-Rx L Max-Dade Behring Analyzer which gives direct reading of SGPT.

E. Serum Glutamate Oxaloacetate Transaminase (SGOT/AST/GOT)

(Span Diagnostics Ltd., India)

Principle

Aspartate transaminase catalyses reaction between alpha- ketoglutaric acid and L- aspartame giving glutamate and oxaloacetate. Oxaloacetate in presence of malate dehydrogenase reacts with NADH giving malate and NAD rate of NADH decrease is determined photometrically and is directly proportional to GOT activity in the sample.

Reagents

Reagent I: Buffer reagent
Reagent II: Enzyme reagent

Sample

Serum or plasma

Reagent preparation

4 ml of buffer reagent was mixed with 1 ml of enzyme reagent.

Procedure

Pipette into test tubes

Sample	100 πl
Reagent	1000 πl

Reactive sample subjected to fully Semi-auto analyzer RA-50 Bayer co. which gives direct reading of SGOT.

F. Estimation of serum creatinine (Picrate method, Henry et al., 1974)
(Span Diagnostics Ltd., India)

Principle

Creatinine in a protein free solution reacts with alkaline picrate and produces a red colored complex, which is measured colorimetrically at 520nm.

Procedure:

Deproteinization of test sample

0.5 ml of serum sample was mixed well with 0.5 ml distilled water and 3 ml picric acid (Reagent 1). It was kept in boiling water bath exactly for one minute and cooled immediately under running tap water and centrifuged.

2.0 ml of supernatant from the above step was mixed with 1.0 ml sodium hydroxide solution (Reagent 2). 0.5 ml of distilled water and working creatinine standard mixed with 1.5 ml picric acid and 0.5 ml sodium hydroxide solution served as blank and standard respectively. All the tubes were allowed to stand at room temperature after thorough mixing for 20 min. The absorbance of blank, standard and samples were measured immediately against distilled water at 520nm.

G. Creatine kinase (CKMB)
(Span Diagnostics Ltd., India)

Principle

This procedure involves measurement of creatine kinase (CK) activity in the presence of an antibody to CK-M monomer. This antibody completely inhibits the activity of CK-MM and half of CK-MB while not affecting the B subunit of CK-MB and CK-BB. Then the CK method is used to quantitatively determine CK-BB

activity. CK catalysis the reaction between creatine phosphate and ADP, giving creatine and ATP. ATP and glucose in the presence of G6PDH oxidizes, and reduces NAD to NADH. The rate of NADH formation is determined photometrically at 340 nm and is directly proportional to CK-BB activity. The CK-MB activity is calculated by multiplying CK-BB X 2.

Reagents

Reagent I: Enzyme Reagent I

Reagent II: Enzyme Reagent II

Sample

Serum free of haemolysis, heparinised plasma, protected from light.

Reagent preparation

4 ml of enzyme reagent I was mixed with 1 ml of enzyme reagent II.

Procedure

Pipette into test tubes

Sample	50µl
Reagent	1ml

Solution was mixed well and change in absorbance was measure after 10 min at 37^{o} C. procedure was repeated and reading was taken at every min for next 5 min and ΔA/ min was calculated.

Calculation

ΔA/ min × 3376 = U/ I CKBB

CKMB= CKBB X 2

HISTOPATHOLOGICAL STUDY

Histopathological study of major organs included heart, liver and kidney were carried out to study protective effects of different extracts of *T. aestivum* on iron overload induce complications on animals.

Fixation of the tissues

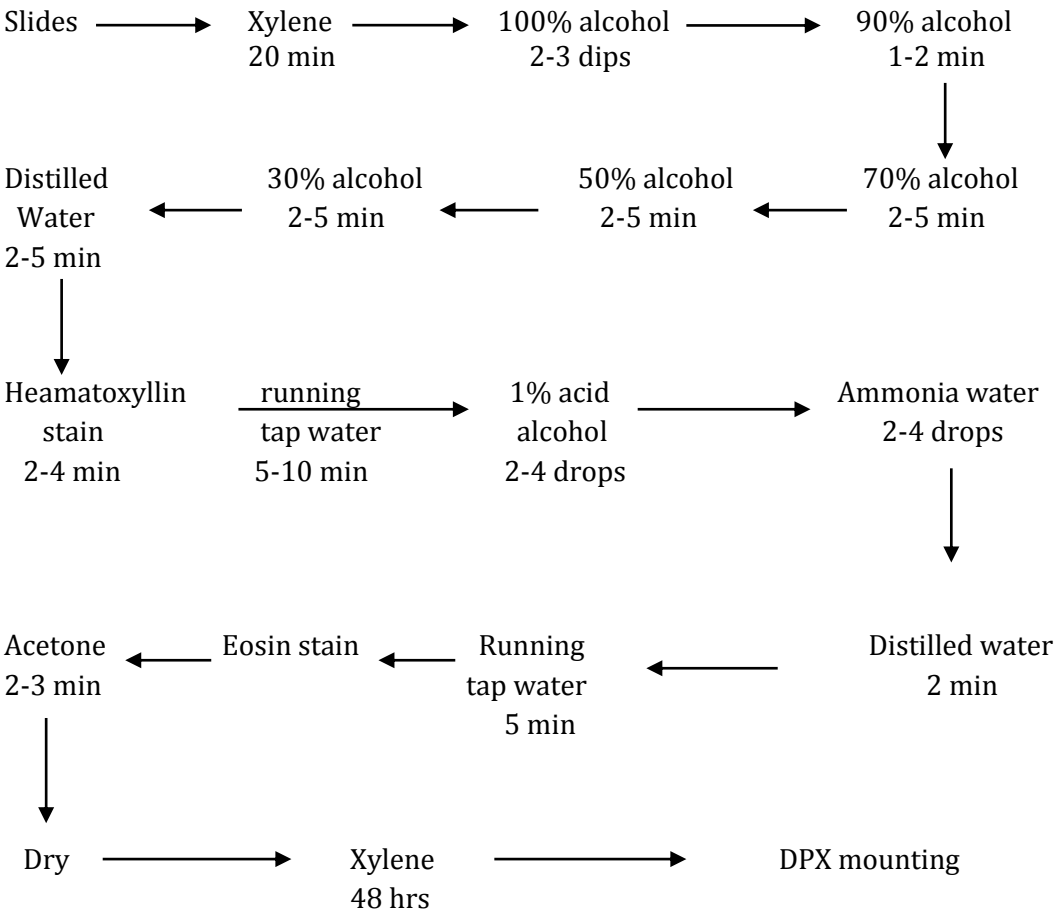
Dissected tissues were washed with normal saline and then kept in 10% formal saline. The tissues were then kept in Bouin's fixative for 18 to 24h. Tissues were then washed twice with distilled water and kept in 70% alcohol. A pinch of lithium carbonate was added to remove excessive stain. The tissues were washed and kept in 70% alcohol again. After that tissues were transferred to 90% alcohol and kept in it overnight. Next morning all tissues were transferred into 100% alcohol and kept for 3h. Then the tissues were transferred to xylene and kept till they become transparent.

Microtomy

Tissues were fixed on melted paraffin in wooden blocks, so that sectioning can be performed. Several sections of 3 μm thickness were taken from each tissue and sections with uniform shape and size were selected for histology. Selected sections were fixed on the clear glass slide with the help of egg albumin.

Staining

Tissues were stained using Heamotoxyllin and Eosin (H & E) stain.



5.6 Isolation and characterization of iron chelating active constituent of wheatgrass

Preparation of column chromatography for isolation of compound

Dried power of methanol extract (Extract M) 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 of eluent were collected.

Collected factions 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.

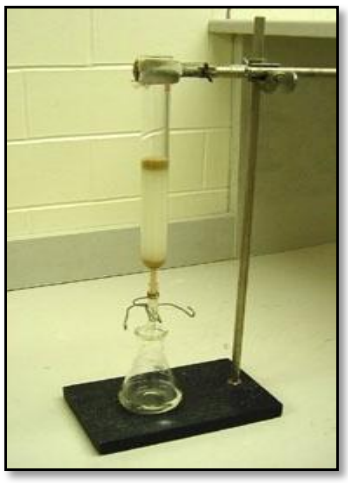


Figure 5.2: Assembly set up for column chromatography

Isolation and identification of compound PI_1:

Fraction 71-76 eluted using methanol: water: acetone: glacial acetic acid (1:0-80:0.5:0.1) was subjected to concentrate which yielded brown crystals. On recrystallization from methanol gave needles (8.2mg) having melting point 215-218 ^{0}C. An IR spectrum was recorded on FT-IR-8400S, Shimadzu instrument. Mass spectra was obtained using 2010EV LCMS Shimadzu instrument.

The identity of isolated compound was confirmed by TLC plate. These isolated compound gave black colour spot after spraying of 5 % ferric chloride solution on TLC that suggest nature of these compound was belonged to phenolic group.

Isolated compound was subjected to determine in-vitro quantitative analysis of phenolic content, in-vitro and in-vivo iron chelating activity.

5.7 In-vitro evaluation of iron chelating activity in active constituent of wheatgrass

The chelation of ferrous ions by extracts was estimated by method of Dinis et al., 1994. Briefly, 50 µl of 2 mM $FeCl_2$ was added to 1 ml of different concentrations of extract (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml). 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 solution was thereafter measured at 562 nm. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated as $[(A_0 - A_S)/ A_S] \times 100$, where A_0 was the

absorbance of control, and A_s was absorbance of extract/standard. Na_2EDTA was used as positive control.

5.8 In-vivo evaluation of iron chelating activity in active constituent of wheatgrass

Male Sprague-Dawley rats initially weighed 150-200 gm were used for these experiments. The rats were given single i.p. injections of iron-dextran (25 mg/l00 gm body wt.) which resulted in condition of acute iron overload. Control rats were injected with an equal volume of dextran at same time. (British journal of hematology, 1990)

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 PI_1
(40 mg/kg, p.o. for 2 days)

At the end of day two, 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.

5.9 Investigation of anti-oxidant property of *T. aestivum* in iron overload condition

At the end of 30 days period of iron overloaded conditions, animal were sacrificed by spinal cord dislocation technique and liver homogenates were prepared. The prepared homogenates were used to estimate tissue protein levels (of Lowry et al., 1951), SOD by Misra et al (1984), catalase by Aeibi et al., 1974, reduced Glutathione (GSH) by Beutler et al (1963) and tissue lipid peroxidation, malondialdehyde-MDA by Ohkawa et al (1979).

Preparation of tissue homogenate:

Reagents

1. 0.25M sucrose solution: 85.57 gm of sucrose was dissolved in 1000 ml of distilled water.
2. Tris HCl buffer (10mM, pH 7.4) solution: 1.21 gm of Tris was dissolved in 900 ml of distilled water; pH was adjusted to 7.4 with 1 M HCl and diluted up to 1000ml with distilled water.

Procedure:

Animal were sacrificed by spinal dislocation technique and liver was dissected out, rinsed with ice cold distilled water followed by sucrose solution (0.25 M). It was blotted free of blood and tissue fluid then weighed on analytical balance. Then it was cross-chopped with surgical scalpel into fine slices and was placed in chilled sucrose solution, quickly blotted on a filter paper. The tissue was minced and homogenized in ice-cold 10 mM Tris HCl buffer at a concentration of 10% (w/v) with 25 strokes of tight Teflon pestle of glass homogenizer at a speed of 2500 RPM.

The prepared homogenates were centrifuged in cooling centrifuge at 6000 RPM for 20 minutes; temperature was maintained at -5 to 1^{o}C during centrifugation. Clear supernatant was separated and used to estimate tissue protein levels, SOD, Catalase, Reduced Glutathione (GSH) and tissue lipid peroxidation (Malondialdehyde-MDA).

A. Estimation of tissue protein levels

The liver tissue protein was estimated by the methods of Lowry et al., 1951.

Reagent A: 2%Na_2CO_3 in 0.1N NaOH solution

Reagent B: 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate

Reagent C: alkaline copper sulphate solution (mixture of 50ml of Reagent A and 1ml of Reagent B)

Reagent D: dilute Folin Phenol Reagent (1N)

The Folin Phenol Reagent was titrated with NaOH solution to a phenolphthalein end point. On the basis of this titration, the Folin Phenol Reagent was diluted (about 2 fold) to make it 1N in acid.

Working standard: It was prepared from human serum diluted 100 to 1000 fold (approximate 700-70 λ per ml). This in turn was titrated with standard solution of crystalline Bovine Serum Albumin. The 1 gm is equivalent to 0.97 λ of serum protein.

Procedure:

To a sample of 5-100 λ of protein in 0.2 ml in a test tube, 1 ml of reagent C was added. Mixed well and allowed to stand for 10 min or longer at room temperature. About 0.1 ml of reagent D was added very rapidly and mixed within a second or two. After 30 min or longer the samples were read in a spectrophotometer. For the range 5-25 λ of protein per ml of final solution, it was described to make reading at or near λ=750 n, the absorbance peak. For stronger solution, the reading might be kept in a workable range by reading near λ=500 nm. Final calculation was made from the standard curve.

B. Measurement of Malondialdehyde (MDA) level

Lipid peroxidation product MDA was estimated as per the method described by Ohkawa et al (1979).

Principle

One molecule of MDA reacts with two moles of Thio barbituric acid under mildly acidic conditions to from a pink colored chromogen optically measured at 532 nm.

Procedure:

Supernatant (0.2 ml) was mixed with 0.2 ml of 8% W/V sodium dodecyl sulfate, 1.5 ml of 20% acetic acid in 0.27 M hydrochloric acid, 1.5ml freshly prepared of thiobarbituric acid (TBA) (1%W/V) and 0.6 ml of distilled water. The mixture was heated in a water bath at 95 ^{o}C for 45 minute, cooled and 2 ml of the mixture was mixed with 2 ml of 10% trichloro acetic acid. The resulting mixture was centrifuged at 1000 rpm for 5 min. The intensity of pink color developed was read against blank at 532 nm. The amount of (MDA) (thiobarbituric acid reactive material) was calculated using molar extinction coefficient 1.56 X 10 ^{5} M^{-1} Cm^{-1} and reported as nmoles of MDA/mg protein.

C. GSH level (Reduced Glutathione)

Reduced GSH levels in tissue homogenates were estimated as per the method described by Beutler et al (1963).

Principle

All of the non protein sulfhydryll groups in form of GSH-DTNB (5, 5'-dithiobis 2-nitro benzoic acid) is a disulfide compound which is readily reduced by sulfhydryll compound, forming a highly colored yellow anion, optically measured at 412 nm.

Procedure:

To 1 ml of sample (tissue homogenate), 1 ml of 10%TCA was added. The precipitated fraction was centrifuged and to 0.5 ml supernatant, 2 ml of DTNB reagent was added. The final volume was made up to 3ml with phosphate buffer. The color developed was read at 412 nm. Standard curve for GSH was prepared using glutathione. Results were expressed as µmole of GSH/mg tissue.

D. Superoxide dismustase (SOD)

SOD was estimated as per the method described by Misra et al (1984).

Principle

Rate of auto oxidation of epinephrine and the sensitivity of this auto oxidation to inhibition by SOD were augmented as pH was raised from 7.8-10.2, O_2 generated

by xanthine oxidase reaction, caused by the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O_2 introduced. The auto oxidation of epinephrine proceeds by at least two distinct pathways only one of which is free radical chain reaction involving O_2 and hence inhabitable by SOD.

Procedure:

Supernatant (0.1ml) of sample was mixed with 0.1 ml EDTA ($1x10^{-4}$ M), 0.5 ml of carbonate buffer (pH 9.7) and 1 ml of Epinephrine ($3x\ 10^{-3}$M). The optical density of formed adrenochrome was read at 480 nm for 3 min at an interval of 30 sec. the enzyme activity has been expressed in terms of U/min/mg protein. One unit of enzyme activity is defined as the concentration required for the inhibition of the chromogen production by 50% in one minute under the defined assay conditions.

E. Catalase

Decomposition of H_2O_2 in presence of catalase was estimated by Aeibi et al., 1974.

Principle

In U.V. range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of catalase activity.

Procedure:

A 50μl supernatent was added to buffered substrate (50 mM phosphate buffer, pH 7 containing 10 mM H_2O_2) to make total volume 3 ml. The decrease in the absorbance was read at 240 nm for 2.5 min at an interval of 15 sec. The activity was calculated using extinction co-efficient of H_2O_2 0.041/μmoles/cm^2. Results were expressed as μmoles of H_2O_2 utilized/min/mg protein.

5.10 Evaluation of therapeutic benefit of *Triticum aestivum* in thrombocytopenia

Induction of thrombocytopenia

Healthy male wistar rats weighed 150-200 gm were used for present study. Thrombocytopenia was induced in rats by busulfan according to methods of Evensen et al. 1968 and Deeg HJ 1999. Busulfan at a final concentration of 10 mg/ml in polyethylene glycol was prepared and was infused doses of 25 mg busulfan/kg body weight each at 1, 5, 10 and 15 days of treatment period which produced significant reduction in platelet counts in rats, which showed severe bleeding conditions as found in thrombocytopenia. Busulfan is an alkylating agent with myeloablative properties and activity against non-dividing marrow cells and, possibly, non-dividing malignant cells.

Treatment protocol

The experimental animals were divided into five groups, six animals in each group.

Group I : Normal healthy control

Group II : Diseases control, busulfan induced thrombocytopenic rats (25 mg busulfan/kg)

Group III : Busulfan induced thrombocytopenic rats treated with wheat grass fresh juice (5 ml/kg, p.o., day)

Group IV : Busulfan induced thrombocytopenic rats treated with methanol extract (50 mg/kg, p.o., day)

Group V : Busulfan induced thrombocytopenic rats treated with wheat grass acetone extracts (50 mg/kg, p.o., day)

All the studies were carried for a period of three weeks. After 21 days of treatment period blood samples were collected under fasting conditions and were subjected to estimations.

Blood sample collection and blood analysis

Blood samples were collected in clean dry centrifuge tubes at the end of three weeks of treatment period by retro orbital plexuses under light ether anesthesia and were collected in EDTA tube to prevent clot formation at room temperature.

A. Estimation of hematological parameters

Various hematological parameters like

a. Hemoglobin content
b. Total RBC, PCV, MCV, MCH, MCHC
c. Total WBC count
d. Differential WBC count
   Neutrophil, Lymphocyte, Eosinophil, Basophil counts and Total lymphocyte count and
e. Platelet count

were estimated using fully automated hematoly analyzer - Model XS-800i – Sysmex.

B. Determination of clotting time [Lee and white method]

Principle: During coagulation sol form of the blood is changed to gel from. The time elapsed between the moment of escape of blood outside the vessel and the observation of physical change is taken as clotting time.

Apparatus:

1. Sterile disposable pricking needle or lancet
2. Stop watch
3. Dry glass capillary tube (narrow diameter 1 top 2 mm, minimum 10 cm long.)
4. Cotton Swab of absorbent cotton
5. Spirit wetted, cotton swab.

Chemicals:

70 % v/v ethyl alcohol or 70 % v/v denatured spirit

Procedure:

1. Blood was collected from animal by retro orbital plexus method under light anesthetic conditions.
2. Immediately stop watch was started.
3. After every 30 seconds, a small piece of capillary was breaked.
4. Step 3 was repeated till fibrin thread appeared at broken end of capillary tube.
5. Clotting time was recorded as interval between pricking finger and first appearance of fibrin thread at broken ends of capillary tube.

C. Determination of bleeding time

Principle

The time elapsed between the moment blood escapes from vessel and the cessation of its flow is defined as the bleeding time the usual bleeding time is 1-3 minutes and clotting time is 4-10 minutes. Bleeding time is prolonged during thrombocytopenia.

Procedure:

1. The tip of finger of rats was sterilized with spirit and bold prick was given so that blood flows freely. The stopwatch was immediately started.
2. The blood was soaked on a filter paper (while soaking, the filter should not touch the skin of finger). This was repeated every 10 seconds till no blot appears on the papers.
3. The time from the first appearance of the blood to the cessation of bleeding was the bleeding time.

5.11 Assessment of immunomodulatory activity of *Triticum aestivum*

Experimental animals

Experimental animals Albino Wistar rats weighed between 180–220 gram and Swiss albino mice weighed between 25–35 gram were used for present study.

Treatment protocol: The animals were distributed into 4 groups (n=6). The first group served as control, second group received standard drug – *Ocimum sanctum* extract (OSE) at a dose of 100 mg/kg orally, third and fourth group received fresh juice (5 ml/kg/p.o/day) and methanolic extracts (100 mg/kg/p.o./day) of *Triticum aestivum* respectively.

Experimental models

A. Carbon clearance test

(Das M et al., 1998, Gokhale AB et al., 2003, Jayathirtha MG et al., 2004)

The four groups of swiss albino mice were administered drug or vehicle for 5 days orally. After 48 hours of last dose of drug, mice were injected with 0.1 ml of indian ink via tail vein. Blood samples were withdrawn at 0 min and 15 min. A 50 µl blood sample was mixed with 4 ml of 0.1% sodium carbonate solution and absorbance of this solution was determined at 660 nm. The phagocytic index K was calculated using following equation:

$$K = (\text{Loge } OD_1 - \text{Loge } OD_2)/15$$

Where OD_1 and OD_2 were the optical densities at 0 and 15 min respectively.

B. Cyclophosphamide induced Neutropenia:

(Thatte UM et al., 1987, Heppner GH et al., 1976)

Swiss albino mice received the drug or vehicle orally for 10 days. On 10th day, a neutropenic dose of cyclophosphamide (200 mg/kg, sc) was administered and this day was labeled as day zero. Blood samples were collected through retro-orbital vein. The total leucocyte count (TLC) and DLC were performed prior to and on day 3 after injection of cyclophosphamide. The TLC and DLC in treated groups were compared with the values of the control group.

C. Neutrophil adhesion test

Fulzele SV et al., 2003, Shinde UA et al., 1999.

Albino Wistar rats were divided into different groups and were treated orally with drug or vehicle for 14 days. On day 14, blood samples were collected from retro orbital plexus into heparinized vials and differential leukocyte count was determined. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 10 min at 37°C. The incubated blood samples were again analyzed for DLC. The difference in the neutrophils count before and after incubation of blood sample with nylon fibers was determined.

Statistical analysis

Results are presented as mean ± SEM. Statistical differences between the means of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test. Data were considered statistically significant at $P \leq 0.05$ and highly significant at $P \leq 0.001$. Statistical analysis was performed using Sigma stat statistical software.

6. RESULTS

6.1 Pharmacognostic studies of *Triticum aestivum* (wheatgrass).

6.2 Phytochemical studies of various extracts of wheatgrass.

6.3 In-vitro evaluation of iron chelating activity in various extracts of wheatgrass.

6.4 In-vivo evaluation of iron chelating activity in various extracts of wheatgrass.

6.5 Isolation and characterization of iron chelating active constituent of wheatgrass.

6.6 In-vitro evaluation of iron chelating activity in active constituent of wheatgrass.

6.7 In-vivo evaluation of iron chelating activity of active constituents of wheatgrass.

6.8 Investigation of anti-oxidant property of *Triticum aestivum* in iron overload condition.

6.9 Evaluation of therapeutic benefit of *Triticum aestivum* in thrombocytopenia.

6.10 Assessment of immunomodulatory activity of *Triticum aestivum.*

6.1 Pharmacognostic studies of *Triticum aestivum* (wheatgrass)

Macroscopic and microscopic studies

Macroscopical features -

In our investigation, certified samples of specie of wheat viz. *Triticum aestivum* was used to grow in plastic trays as per the standard procedure mentioned in material and method chapter. In conformation with description in literature; the leaves were near glabrous, auriculate, with blades narrow to broadly linear, 2–20 mm wide, flat and without cross venation. (Percival, J. 1974) The leaf blade was linear and parallel–veined with mid rib projecting on the back, continuing someway along the sheath.

Fig 6.1.1: *Triticum aestivum* (Wheat grass) and wheat grass juice.

Microscopical studies -

Microscopic studies of transverse sections, surface preparations and powder studies of wheatgrass viz *Triticum aestivum,* were conducted using high-resolution microscope. The structure of wheatgrass leaf showed elaborate epidermis with characteristic stomata and trichomes, green assimilating parenchyma, conducting vascular bundles and longitudinal strands of fibrous stereome or supporting tissue.

Surface preparations of *Triticum aestivum* -

The epidermis of the blade of the leaf was found to be composed of a number of diverse elements arranged in parallel rows along the long axis. Some of the individual rows consisted entirely of elongated cells placed end to end, each cell, with convex cutinized outer wall 4-5 μm thick, appearing in longitudinal section as a narrow rectangle 150-300 μm long and 15-20 μm wide. In other rows characteristic lines of long cells alternating with stomata were also present, trichomes or hairs of various lengths were found scattered along the rows at more or less at regular intervals. The trichomes or hairs were always unicellular. To the right and left of the central line were rows of long cells interspersed with hairs. Parallel to these, at the base of the ridge, there were single or double lines of stomata. In the furrow between two ridges was a band of three to seven rows of elongated cells, whose walls were thinner and not so distinctly parallel to each other are bulliform cells or motor cells (Figure 6.1.2).

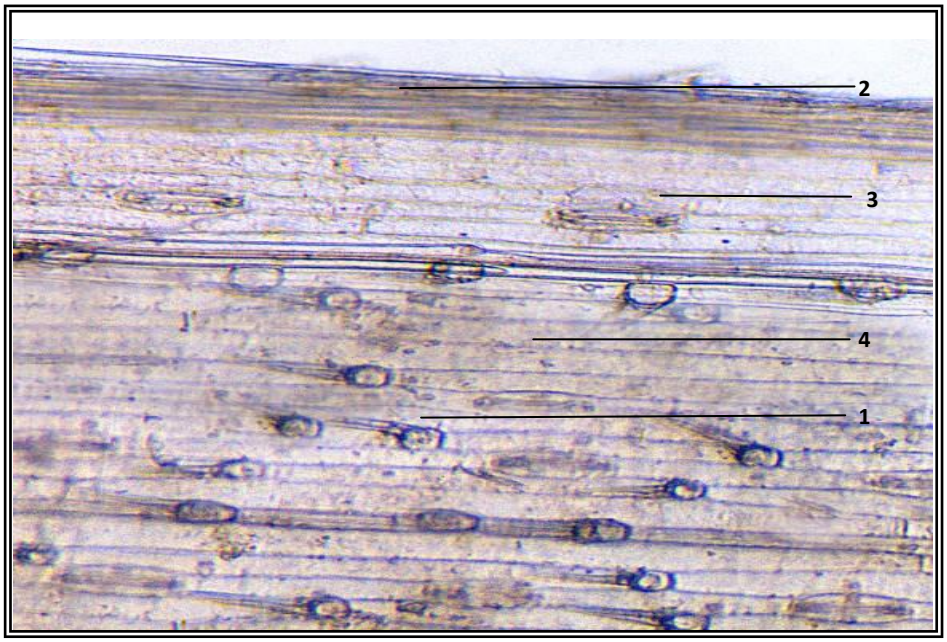


Figure 6.1.2 Surface preparation of *T. aestivum*

1. Unicellular trichome,
2. Stereome fiber
3. Double raw of stomata
4. Rows of mot

Transverse section of *Triticum aestivum* leaf (General description) -

On the upper surface of the leaf there was a series of longitudinal ridges or ribs, the lower surface being almost flat. The epidermal cells covering ridges differed in form and arrangement from those over the furrows and along the edge of the leaf. Running along the summit of each ridge there was a single row of elongated thick-walled and pitted cells alternating with hairs. On the flank of the ridge, right and left of the central line, there were three to five rows of long cells interspersed with short one and hairs. Parallel to these, at the base of the ridge, were single or double lines of stomata. In the furrow between two ridges there was a band of three to seven rows of elongated cells, whose walls were thinner. They were not distinctly parallel to each other are bulliform cells or motor cells.

The trichomes or hairs were always unicellular, and varied in length and stoutness. Some of them were blunt on the edges of older leaves where as others were short and stout, 20-30 πm long, with fine curved points rendering the surface scabrid. On the leaves of *T. aestivum*, ample numbers of hairs were present, while in *T. dicoccum* and *T. durum* they were sparsely distributed on the surface of the leaf. These were usually more on the upper epidermis than the lower epidermis. Each stoma on the leaf consisted of four cells, the two guard cells being narrow, with specially thickened walls round the stomatal pore and thin-walled widely dilated ends: the pore when closed appears as a narrow slit 30-40 πm long. The ratio of the number of stomata on the upper and lower epidermis respectively was about 10:7. In the transverse section the pores of the stomata were seen to be in communication with large intracellular cavities in the mesophyll, called lacune. The parenchyma of the leaf consists chiefly of thin-walled assimilating tissue, containing lenticular chloroplasts 4.5-6 πm in diameter. The cells of the chlorophyll-containing tissue in the central part of the leaf were much more irregular in shape and are loosely packed, with large intracellular spaces between them. Chloroplasts were present especially present in the subepidermal layers. In each cell on the outside of stereome, and between the vascular bundles, there was a single crystal or cluster of crystals of calcium oxalate. Vascular bundles were somewhat nearer to the lower surface than the upper surface of the leaf. All vascular bundles were collateral, with the xylem towards the upper surface of the leaf and the phloem bellow. In the xylem there

were one or two vessels 20 πm in diameter with annular or spiral thickening with narrow elliptical pits. Each bundle was surrounded by an inner and outer sheath; the former (the 'mestome') enclosed the vascular strand, and was composed of elongated thick-walled cells; the outer or 'parenchyma sheath' was more conspicuous and consisted of thin-walled cells, almost circular in transverse section. Above and below the bundles, and arranged parallel with them along the leaf were strands of stereome or supporting tissue consisting of sclerenchymatous fibers.

Transverse section of *Triticum aestivum* leaf -

It had more number of trichomes than other species, mostly in lower epidermis. Motor cells were clearly seen, almost equal in size on both the sides. The parenchymatous cells of outer bundle sheath were larger and also more in number than other verities. The cells of epidermis were nearly circular in shape. The medial vein was broader than other species. Xylem vessels were 4-5 in number. Outer sheath of vascular bundle was complete (Figure 6.1.3).

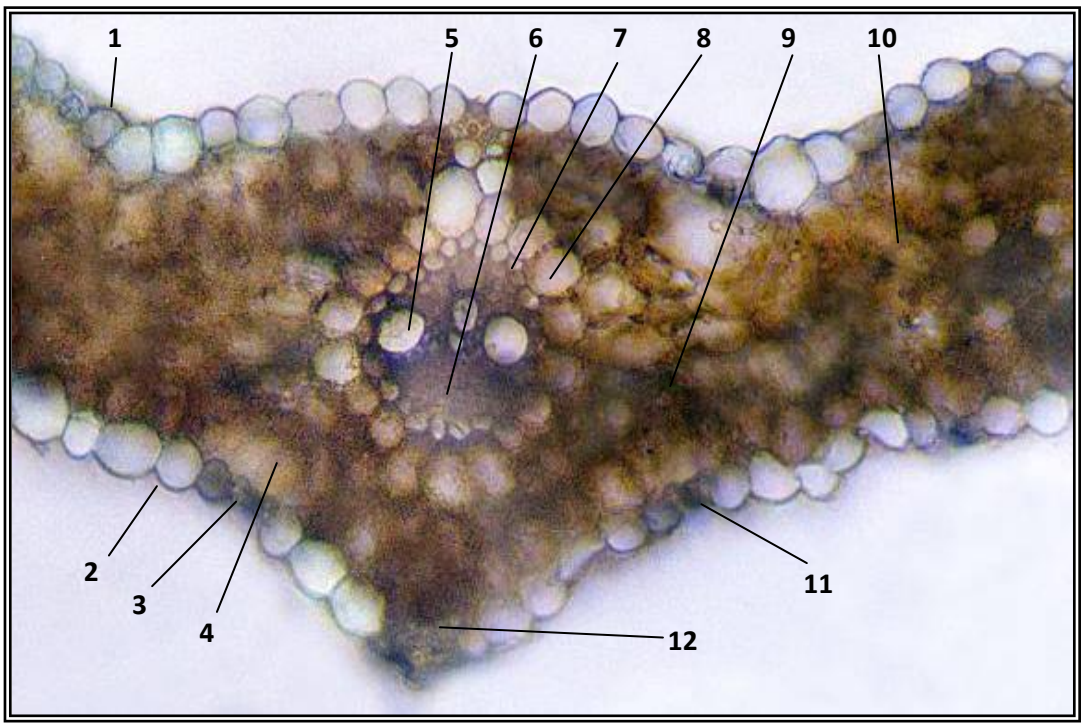


Figure 6.1.3 Transverse section (Cellular) of *Triticum aestivum*

1. Epidermis 2. Motor cells 3. Stomata 4. Lacuna 5. Xylem 6. Phloem
7. Inner sheath of vascular bundle 8. Outer sheath of vascular bundle
9. Mesophyll 10. Parenchyma 11. Guard cell 12. Stereome

Characteristics of various species of *Triticum* powder -

Powder characters of *Triticum aestivum* –

Epidermal cells in surface view were elongated and rectangular having few numbers of stomata. Trichomes were simple, uniseriate, unicellular and long with pointed end and swollen bases. Smaller ones were hook-shaped with broad base while longer trichomes were more in number than smaller ones. Fibers were scattered here and there, found as single or in groups. They were thin-walled and lignified. Vessels were single or together in groups of 2-3, pitted, reticulated and annular type. Pitted vessels were more in number (Figure 6.1.4).

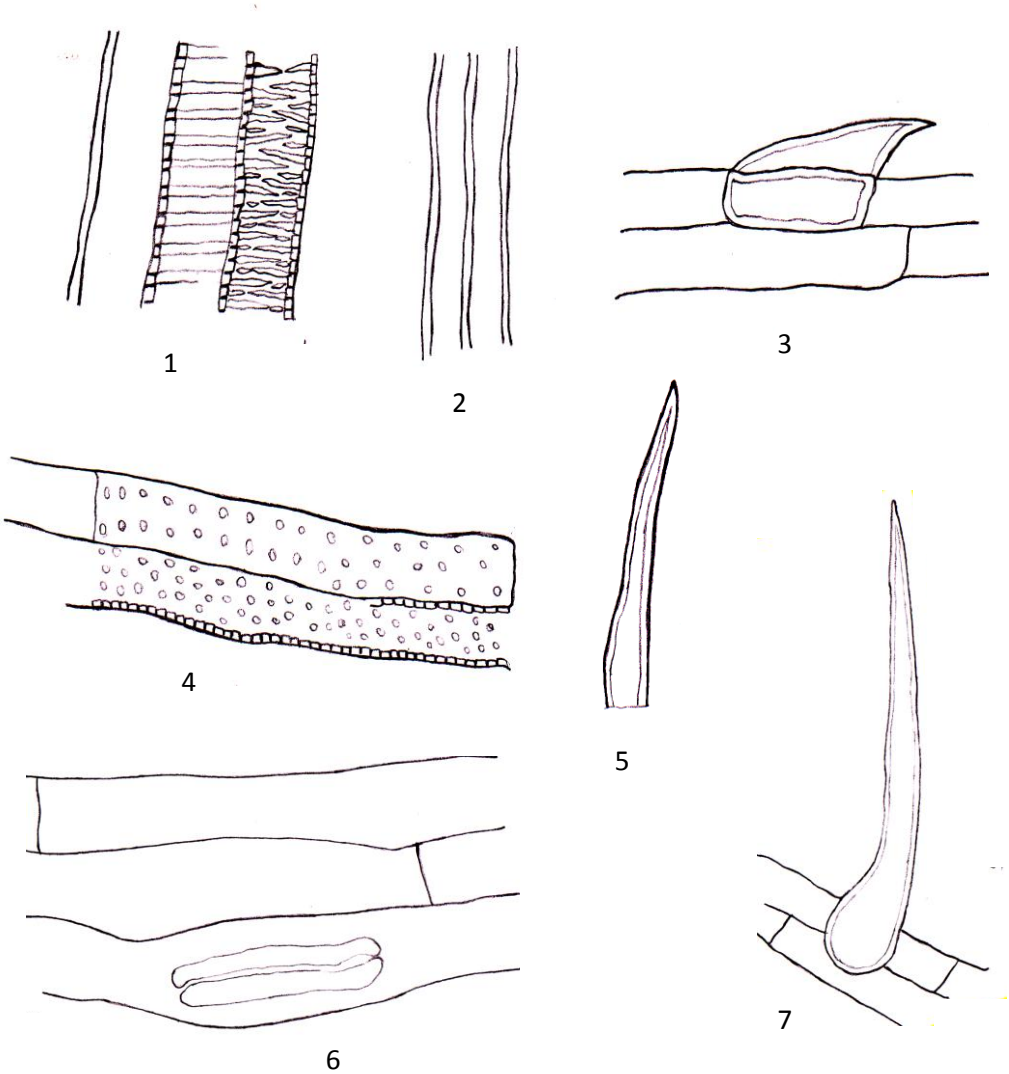


Figure 6.1.4 Characters of *T. aestivum*

1. Reticulated vessels
2. Group of fibers
3. Hook-shaped trichome
4. Pitted vessels with pitted parenchyma
5. Broken trichome
6. Epidermis in surface view with stomata
7. Uniseriate, unicellular simple trichome

6.2 Phytochemical screening of various extracts of *Triticum aestivum*

Preliminary phytoprofile

Powder leaves were subjected to successive solvent extraction. The different extracts were examined for their % yield (table 6.2.1).

The extracts obtained from successive solvent extraction process were subjected to various chemical tests to determine the presence of phytochemical constitutes like phenolics, tannins, and flavanoids. The resulted are reported in table 6.2.2.

Table 6.2.1: % yield of various extracts of *Triticum aestivum* using soxhlet apparatus.

Sr. No.	Extracts of *T. aestivum*	Colour of extracts	Consistency of extracts	% yield W/W
1.	Petroleum ether	Light green	Sticky	2.8
2.	Chloroform	Green	Semi solid Sticky	3.4
3.	Acetone	Dark green	Semi solid Sticky	4.3
4.	Methanol	Brownish green	Semi solid Sticky	4.1
5.	Water	Pale yellow	Sticky	5.3

Successive solvent extracts of wheatgrass with petroleum ether, chloroform, acetone, methanol and water were prepared, employing soxhlet apparatus. Highest extractive value was that of water extract (5.3 %), followed by acetone (4.3 %), methanol (4.1 %), chloroform (3.4 %) and petroleum ether with lowest extractive value of 2.8 %.

There is a direct relation beteween iron chelatory activity and the contents of phenolic and flavonoids in some extracts as reported by Ebrahimzadeh et al., 2008. Hence, the extracts obtained from successive solvent extraction process were subjected to shinoda and $FeCl_3$ tests to determine the presence of phenolics, tannins and flavanoids. Methanol and water extracts showed presence

of phenolics, tannins and flavanoids, while petroleum ether, chloroform and acetone extracts had no phenolic and flavanoids components. (Table 6.2.2)

Table 6.2.2: Qualitative chemical tests of different extracts of *Triticum aestivum.*

Class of compounds	Petroleum ether extract	Chloroform extract	Acetone extract	Methanol extract	Water extract
Phenolics/ tannins	-	-	-	+	+
Flavanoids	-	-	-	+	+

Quantitative determination of total phenolic in extracts using Folin Ciocalteau (FC) method

Phenolic compounds are known as powerful chain breaking antioxidants (Shahidi F et al., 1992) and also possess chelating property (Ebrahimzadeh et al., 2008). Phenols are very important plant constitute because of their scavenging ability due to their hydroxyl groups (Hatano T et al., 1989). In addition, it has been reported that phenolic compounds are associated with anti-oxidant activity and play a crucial role in stabilizing lipid peroxidation (Yen GC, et al., 1993).

The total phenolic content present in methanol and water extracts of *Triticum aestivum* were shown in table 6.1.3. In methanol and water extracts 506.92 $\pm$ 16.36 and 198.5 $\pm$ 10.61 µg Gallic acid equivalent of phenol content were detected. Phenolic content of methanol extract was found to be higher compared to water extract.

Table 6.2.3: Estimation of total phenolic content in methanol and water extract of *T. aestivum.*

Sample	Total Phenolics (µg/mg Gallic acid equivalent)			Mean ± SEM
	I	II	III	
Methanol extract	492.58	503.45	524.75	506.92 ± 16.36
Water extract	210.21	189.51	195.78	198.5 ± 10.61

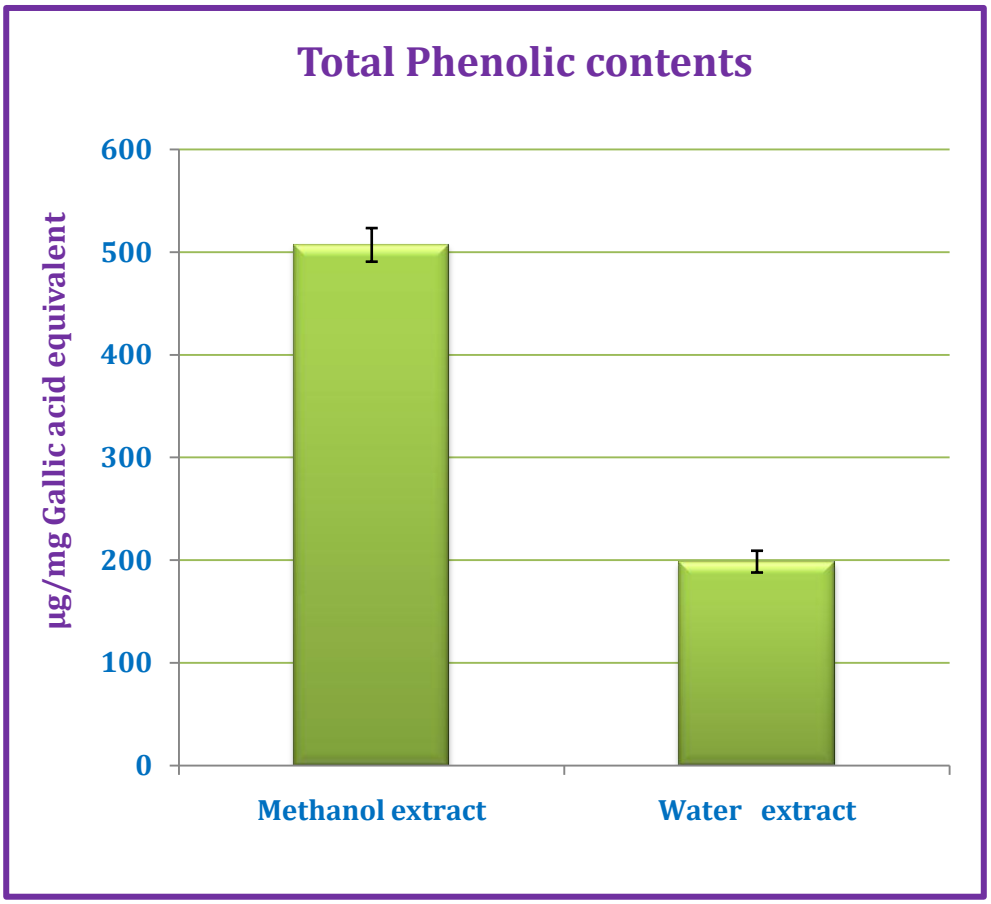


Fig 6.2.1: Total phenolic content in methanol and water extracts of wheatgrass.

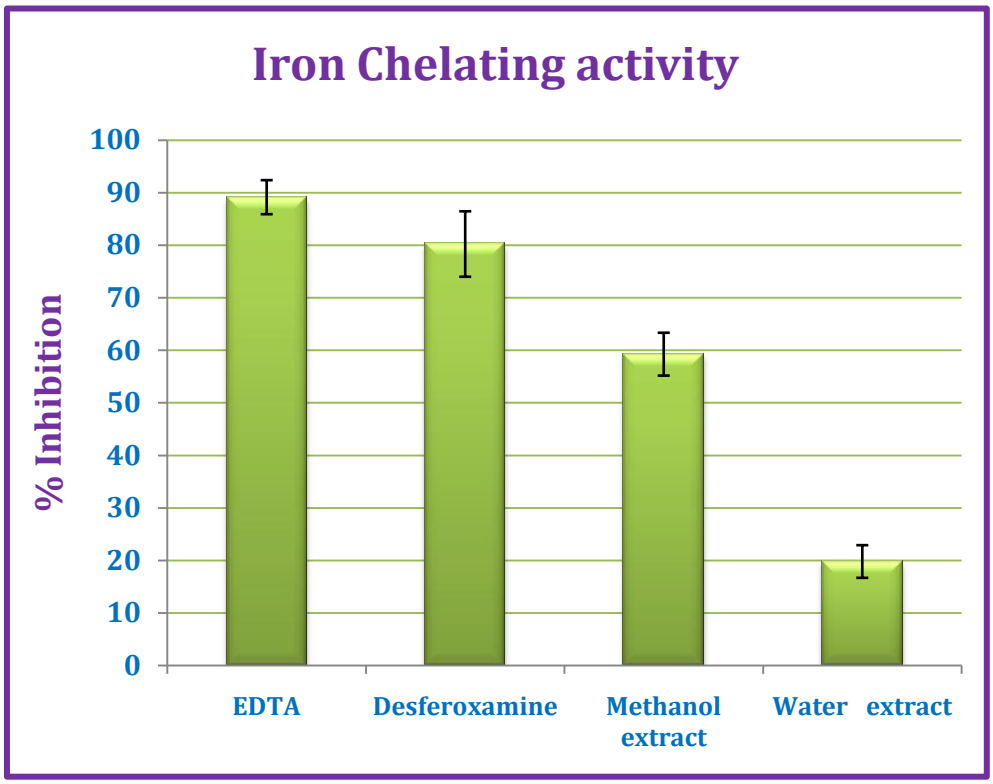


Fig 6.2.2: Iron chelating activity of various extracts of wheatgrass and its comparison with desferoxamine and EDTA.

6.3 In-vitro evaluation of iron chelating activity in various extracts of wheatgrass

The chelating of Fe^{2+} by extracts was estimated by the method of Dinis et al. 1994. 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+} possess 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 (Aboul-Enein AM et al., 2003). The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions.

Methanol and water extracts of *T. aestivum*, interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. % inhibition of chelating effect of various

extracts of *Triticum aestivum,* disodium EDTA and desferoxamine on Fe^{2+} and ferrozine complex formation is shown in table 6.3.1.

Table 6.3.1: Iron chelating property (% inhibition of complex formation between Fe^{2+} -ferrozine) of methanol and water extracts of *T. aestivum*, EDTA and Desferoxamine.

Concentration of Sample	% inhibition of complex formation			
	EDTA	Desferoxamine	Methanol extract	Water extract
0.2 mg/ml	6.70 ± 0.21	9.14 ± 0.83	4.37 ± 0.13	0.79 ± 0.02
0.5 mg/ml	15.06 ± 0.35	17.18 ± 2.01	12.68 ± 1.53	7.61 ± 1.40
1.0 mg/ml	49.80 ± 1.85	61.18 ± 5.37	32.64 ± 3.18	14.71 ± 1.86
2.0 mg/ml	89.11 ± 3.24	80.19 ± 6.23	59.22 ± 4.08	19.75 ± 3.11

Values are expressed as Mean ± S.E.M

Iron chelating property of methanolic and water extracts of *T. aestivum* were compared to standard iron chelator drug desferoxamine which is use in treatment of iron overload patients of thalassemia and EDTA at different concentration level (table 6.3.1). % inhibition of complex formation between Fe^{2+} -ferrozine were found in desferoxamine, EDTA, methanolic and water extracts of *T. aestivum* in acceding order. In-vitro iron chelating activities of EDTA, desferoxamine and both extracts were found to be increased with increase in their concentration with highest activity at concentration of 2 mg/ml. The chelating activity of methanol extract (59.22 ± 4.08) was found to be significantly higher compared to water extract (19.75 ± 3.11).

BIOLOGICAL EVALUATION / PHARMACOLOGICAL STUDIES OF VARIOUS EXTRACTS OF *Triticum aestivum* (Wheatgrass)

Various extracts *T. aestivum* were subjected for beneficial effects in **iron overload induced thalassemia model, antioxidant activities, thrombocytopenia and immunomodulatory** in various animal models.

6.4 In-vivo evaluation of iron chelating activity in various extracts of wheatgrass

Intraperitoneal injections of iron-dextran (12.5 mg/l00 g body wt.) evenly distributed over a 30 days period that results in condition of chronic iron overload (serum iron - 6099 ± 252 µg/dl) which is found as same as iron overload conditions as in thalassemia. Control rats were injected with an equal volume of dextran at the same time showed normal level of iron (serum iron - 203 ± 17 µg/dl) in rats.

All the studies were carried out for a period of 30 days. During the blood sample, urine sample and facial sample were collected on 15 and 30 days under fasting conditions.

Hematological parameters

Beneficial effects of wheatgrass on hemoglobin and RBC parameters on iron overloaded rats

Iron overloaded group rats exhibited significant decrease in Hb count (10.78 ± 0.84 gm/dl) and RBC count (6.77 ± 0.29 m/cmm) as compared to normal control group rats (Hb-14.57 ± 0.61 gm/dl, RBC- 8.65 ± 0.49 m/cmm) suggesting toxic effect of iron excess on Hb and RBC synthesis. 15 days treatment with desferoxamine (Hb-13.5 ± 0.51 gm/dl, RBC- 7.86 ± 0.58 m/cmm), water extract (Hb-13.83 ± 0.31 gm/dl, RBC- 7.69 ± 0.52 m/cmm) and methanol extract (Hb-14.03 ± 0.68 gm/dl, RBC- 8.13 ± 0.87 m/cmm) in iron over loaded rats produced significant increase in Hb levels and RBC counts. The increase in RBC count indicates that wheatgrass may have stimulated haemopoietic process while rise in hemoglobin content indicates stimulation of hemoglobin synthesis in individual RBC. Treatment with acetone extract in iron overload rats did not produce any significant increase in Hb level (10.67 ± 0.68 gm/dl) and RBC count (7.13 ± 0.44 m/cmm) as compared to iron overloaded rats (Figure 6.2.1). Over

all, there was improvement in Hb level and RBC count after treatment with wheatgrass extracts after 15 and 30 days in iron overload rats. (Table 6.4.1).

Table 6.4.1: Erythropoetic effects of various extracts of wheatgrass on day 15 and 30 on iron overload induced thalassemic rats.

Hematological Parameters	NC (n=6)		DC (n=6)		DCD (n=6)		DCWT (n=6)		DCMT (n=6)		DCAT (n=6)	
	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days
Hb gm/dl	14.57 ± 0.61	13.93 ± 0.98	10.78 ± 0.84*	9.92 ± 0.87*	13.53 ± 0.51#	14.5 ± 0.77#	13.83 ± 0.31#	14.15 ± 0.39#	14.03 ± 0.68#	15.08 ± 0.47#	10.67 ± 0.68	11.95 ± 0.72
RBC m/cmm	8.65 ± 0.49	8.21 ± 0.48	6.77 ± 0.29*	6.38 ± 0.37*	7.86 ± 0.58#	8.33 ± 0.18#	7.69 ± 0.52#	8.71 ± 0.71#	8.13 ± 0.87#	8.30 ± 0.25#	7.13 ± 0.44	7.48 ± 0.42

Values are expressed as Mean ± S.E.M
*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

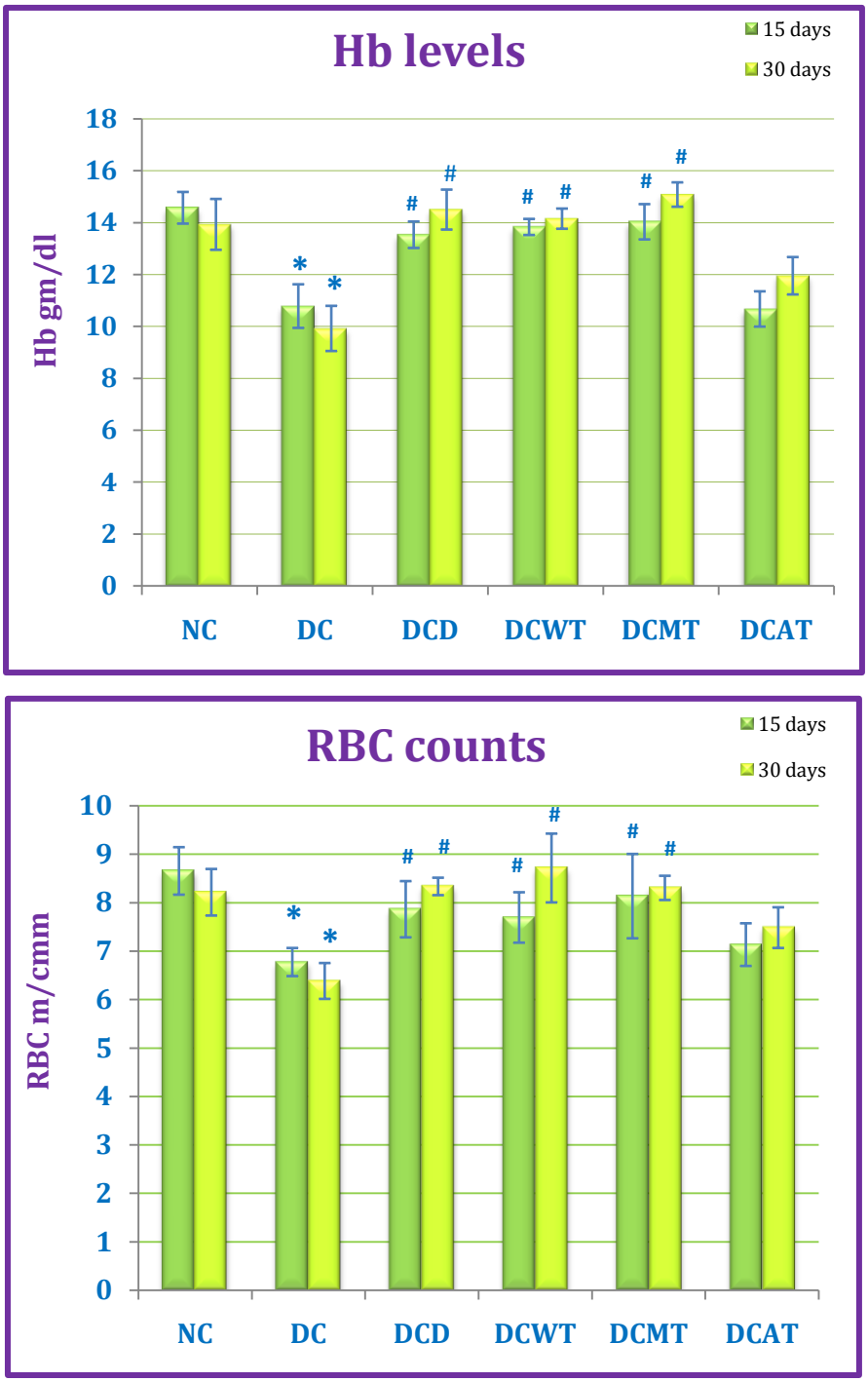


Fig 6.4.1: Increase erythropoetic activity of activity of various extracts of wheatgrass in iron overloaded animals.

Values are expressed as Mean ± S.E.M
*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

Beneficial effects of wheatgrass on defense system and platelet count on iron overloaded rats

Accumulation of iron in body leads to suppression of bone marrow resulting in reduction of total and differential leucocytes counts. This was observed in iron overloaded rats of disease control group (3.89 ± 0.24 10^3/πl) as compared to placebo group (5.98 ± 0.41 10^3/πl). After 15 and 30 days treatment with desferoxamine (4.01 ± 0.23 10^3/πl), water extract (4.93 ± 0.27 10^3/πl) and methanol extract (4.53 ± 0.32 10^3/πl) of wheatgrass in iron over loaded rats total WBC count was significant increased. (Table 6.4.2) The increase in leukocyte count indicates that wheatgrass may have stimulating effect on bone marrow and also on synthesis of all types of leucocytes. Treatment with acetone extract in iron overload rats did not produce any significant increase in total and differential leucocytes counts as compared to iron overloaded group rats. (Fig 6.4.2) These results indicate beneficial effect of wheatgrass on immune system.

Similarly, treatment with methanol extract (699.6 ± 38.6 10^3/πl) and water (793.6 ± 43.5 10^3/πl) extract of wheatgrass, significantly increased platelet counts in iron over loaded group rats as compared to diseases control group rats (530.2 ± 32.6 10^3/πl). Acetone extract did not produce any significant increase in platelet count (590.5 ± 59.5 10^3/πl) as compared to disease control group. These data indicate beneficial effect of wheatgrass in platelet deficiency disorders. (Table 6.4.2)

Tale 6.4.2: Effect of Wheatgrass on patients with β-thalassemia (major) with respect to changes in parameters related to platelet and natural defense mechanism of the body.

Hematological Parameters	NC (n=6)		DC (n=6)		DCD (n=6)		DCWT (n=6)		DCMT (n=6)		DCAT (n=6)	
	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days
Platalet (10^3/πl)	933.6 ± 66.7	976.9 ± 54.9	530.2 ± 32.6*	412.6 ± 51.2*	601.4 ± 48.5#	598.3 ± 51.2#	793.6 ± 43.5#	839.5 ± 35.7#	699.6 ± 38.6#	748.4 ± 56.3#	590.5 ± 59.5	521.3 ± 62.1
Total WBC counts												
WBC (10^3/πl)	5.98 ± 0.41	5.84 ± 0.51	3.89 ± 0.24*	3.26 ± 0.31*	4.01 ± 0.23#	4.09 ± 0.41#	4.93 ± 0.27#	5.13 ± 0.41#	4.53 ± 0.32#	4.72 ± 0.42#	3.99 ± 0.26	3.44 ± 0.39
Differential WBC counts												
Neutrophil (10^3/πl)	2.63 ± 0.13	2.87 ± 0.16	1.86 ± 0.09*	1.72 ± 0.12*	2.07 ± 0.15#	2.16 ± 0.20#	2.43 ± 0.21#	2.67 ± 0.31#	2.23 ± 0.24#	2.32 ± 0.20#	1.98 ± 0.16	2.01 ± 0.19
Lymphocytes (10^3/πl)	1.52 ± 0.11	1.62 ± 0.13	1.02 ± 0.07*	0.98 ± 0.08*	1.13 ± 0.12#	1.31 ± 0.09#	1.34 ± 0.12#	1.54 ± 0.17#	1.25 ± 0.08#	1.42 ± 0.14#	1.06 ± 0.09	1.21 ± 0.15
Monocytes (10^3/πl)	0.44 ± 0.03	0.48 ± 0.03	0.18 ± 0.02*	0.14 ± 0.01*	0.26 ± 0.02#	0.31 ± 0.03#	0.39 ± 0.03#	0.44 ± 0.04#	0.36 ± 0.03#	0.43 ± 0.05#	0.22 ± 0.03	0.23 ± 0.02
Eosinophil (10^3/πl)	0.13 ± 0.01	0.14 ± 0.01	0.04 ± 0.005 *	0.02 ± 0.005 *	0.06 ± 0.01#	0.08 ± 0.01#	0.12 ± 0.02#	0.13 ± 0.02#	0.07 ± 0.01#	0.09 ± 0.01#	0.04 ± 0.01	0.05 ± 0.01
Basophil (10^3/πl)	0.11 ± 0.01	0.12 ± 0.01	0.03 ± 0.005 *	0.04 ± 0.005 *	0.05 ± 0.01#	0.07 ± 0.01#	0.11 ± 0.01#	0.12 ± 0.01#	0.08 ± 0.02#	0.10 ± 0.02#	0.03 ± 0.01	0.06 ± 0.01

Values are expressed as Mean ± S.E.M
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- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

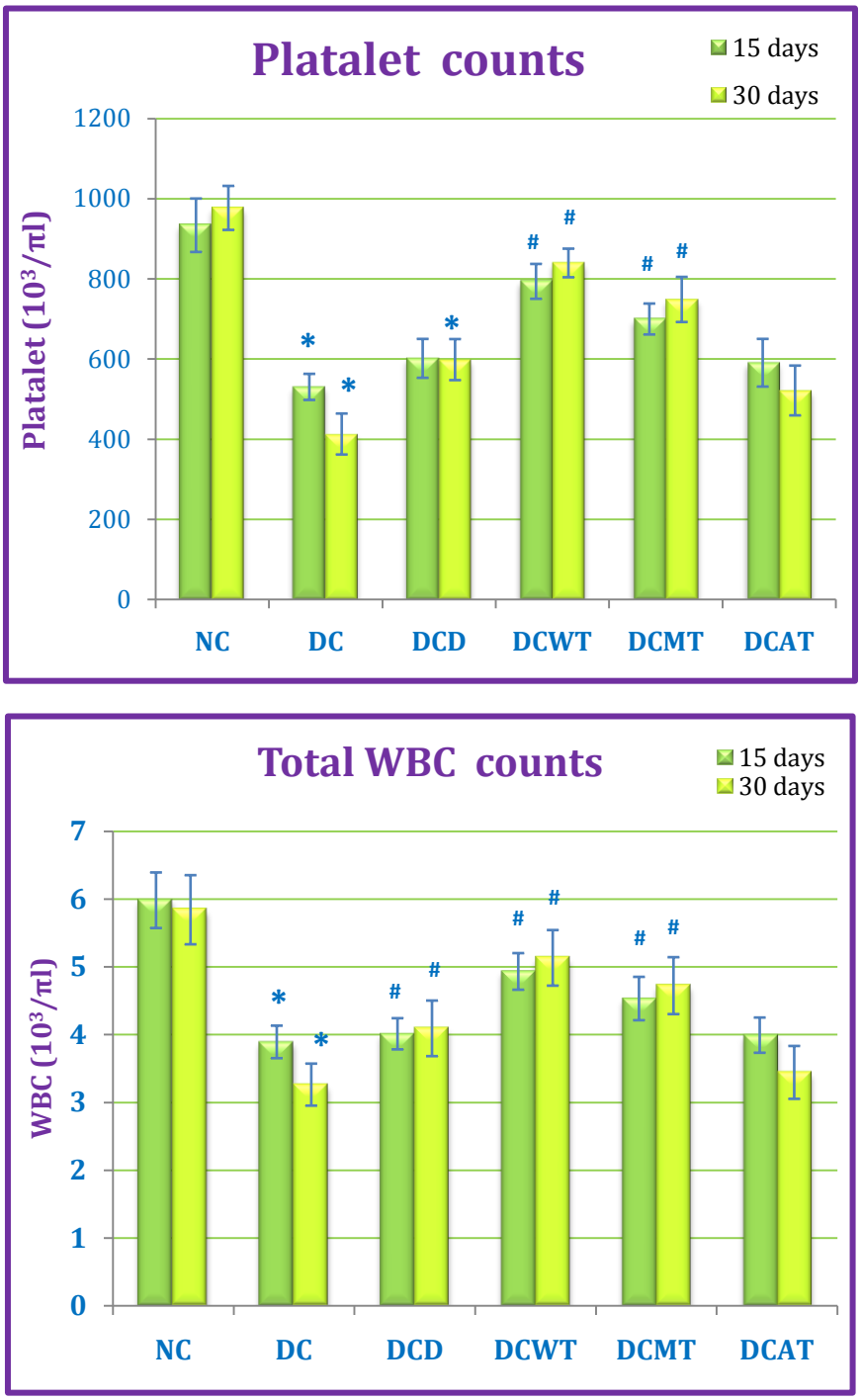

Fig 6.4.2: Beneficial effects of various extracts of wheatgrass on platelet counts, total and differential WBC counts.

Values are expressed as Mean ± S.E.M
*- significantly different from normal control ($p < 0.05$)
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Group 1: Normal control received dextrose solution (NC)
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Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

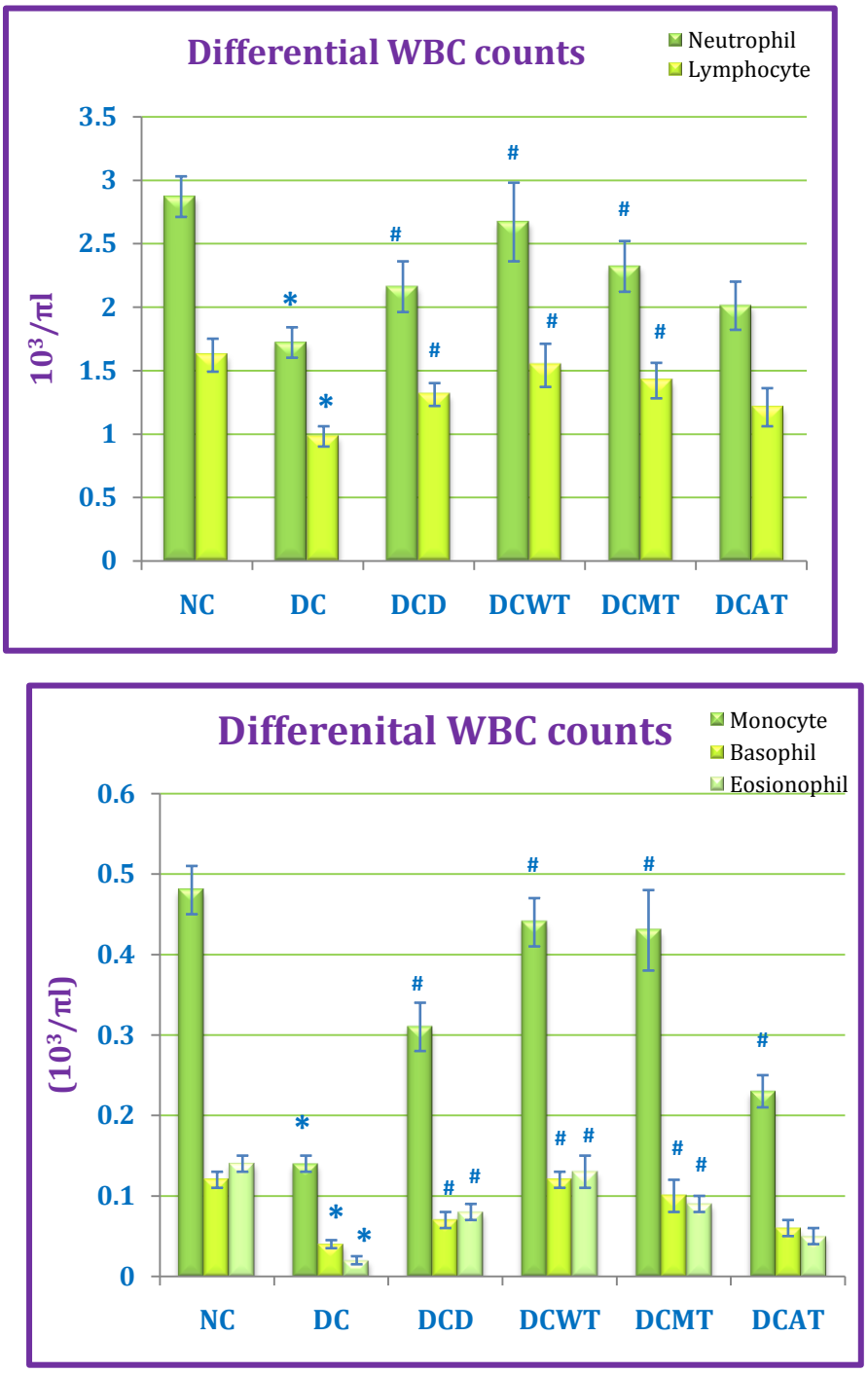

Fig 6.4.2: Beneficial effects of various extracts of wheatgrass on platelet counts, total and differential WBC counts.

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Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

Beneficial effects of wheatgrass on different hematological parameters on iron overload rats

Iron overloaded rats showed significant reduction in HCT (%), MCV(fL), MCH(pg), MCHC(g/dl), RDW-SD(fL), RDW-CV(%), PDW(fL), MPV (fL), P-LCR(%) and PCT(%) counts as compared to normal control rats which indicates toxic effects of iron on blood (Table 6.4.3). After 15 and 30 days treatment with water and methanol extracts of wheatgrass showed increase in these parameters which indicates beneficial effects of wheatgrass on various blood disorders. Acetone extracts was not produced significant effects as water and methanol extracts.

Table 6.4.3: Beneficial effects of wheatgrass on different hematological parameters on iron overloaded rats.

Hematological Parameters	NC (n=6)		DC (n=6)		DC (n=6)		DCWT (n=6)		DCMT (n=6)		DCAT (n=6)	
	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days
HCT (%)	47.2 ± 3.9	46.1 ± 4.5	28.9 ± 1.99	28.5 ± 3.76	32.8 ± 4.8	35.7 ± 5.2	36.8 ± 4.9	42.7 ± 6.4	30.8 ± 2.9	38.4 ± 4.8	25.8 ± 2.8	32.3 ± 3.6
MCV(fL)	55.4 ± 6.1	53.9 ± 3.8	44.0 ± 3.7	36.9 ± 3.1	46.0 ± 4.8	46.9 ± 5.2	53.6 ± 4.0	55.6 ± 3.2	45.7 ± 5.6	55.9 ± 4,7	43.8 ± 4.9	44.9 ± 5.7
MCH(pg)	22.1 ± 1.9	19.8 ± 2.3	14.2 ± 0.9	13.7 ± 2.1	15.7 ± 0.9	18.4 ± 2.3	18.9 ± 1.6	19.4 ± 2.7	16.3 ± 0.9	18.9 ± 2.5	15.3 ± 1.8	16.3 ± 2.8
MCHC(g/dl)	31.8 ± 4.1	30.2 ± 3.6	24.3 ± 2.7	23.9 ± 3.5	26.8 ± 2.6	27.8 ± 3.9	30.6 ± 3.4	32.7 ± 5.8	28.1 ± 2.6	27.9 ± 3.7	25.0 ± 4.2	27.4 ± 3.7
RDW-SD(fL)	27.8 ± 4.1	28.4 ± 3.8	23.7 ± 3.9	22.5 ± 2.4	24.7 ± 4.1	23.6 ± 3.6	26.3 ± 2.8	27.8 ± 4.1	24.8 ± 1.9	25.9 ± 3.3	23.9 ± 2.2	24.9 ± 4.0
RDW-CV(%)	16.9 ± 1.7	18.9 ± 1.8	13.6 ± 2.6	12.7 ± 1.8	14.8 ± 2.6	15.3 ± 2.6	15.2 ± 3.8	17.4 ± 4.2	13.8 ± 2.6	16.6 ± 2.6	13.9 ± 3.1	14.7 ± 2.0
PDW(fL)	8.9 ± 1.1	9.3 ± 2.1	5.9 ± 1.3	5.4 ± 0.98	6.4 ± 1.3	6.8 ± 0.95	7.8 ± 1.9	8.4 ± 1.1	6.9 ± 2.0	7.1 ± 1.4	6.2 ± 0.87	6.1 ± 0.79
MPV (fL)	8.2 ± 1.2	8.4 ± 1.6	5.7 ± 0.57	5.1 ± 0.6	6.1 ± 0.82	6.9 ± 0.47	7.7 ± 1.4	7.9 ± 1.1	6.3 ± 0.73	7.1 ± 0.6	5.9 ± 0.7	6.8 ± 0.78
P-LCR(%)	10.4 ± 1.3	11.2 ± 1.6	8.1 ± 1.5	7.9 ± 0.98	8.7 ± 0.98	9.5 ± 1.2	10.1 ± 2.1	11.3 ± 0.99	9.4 ± 1.3	10.5 ± 1.7	8.3 ± 1.1	8.5 ± 0,92
PCT(%)	78 ± 4	79 ± 7	67 ± 6	63 ± 5	71 ± 6	72 ± 8	76 ± 8	77 ± 8	73 ± 7	75 ± 6	70 ± 8	69 ± 6

Values are expressed as Mean ± S.E.M
*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

Beneficial effects of wheatgrass on iron overload

A. Serum iron and serum ferritin levels

Intraperitoneal injections of iron-dextran (12.5 mg/l00 g body wt.) evenly distributed over a 30 days period on Sprague dwaley rats resulted in condition of chronic iron overload (serum iron - 6099 ± 252 µg/dl). Control group rats injected with an equal volume of dextran showed normal level of iron (serum iron - 203 ± 17 µg/dl). There was significant increase in serum ferritin level in iron overloaded group rats (1.13 ± 0.07 mg/dl) compared to normal control group rats (4.83 ± 0.51 mg/dl). All the studies were carried out for a period of 30 days. Blood, urine and feacal samples were collected on 15th and 30th days under fasting conditions and were subjected for various biochemical parameters. (Fig 6.4.3)

After 15 days of treatment, there was significant reduction in serum iron and ferritin levels in desferoxamine group (serum iron - 2876 ± 281 µg/dl, serum ferritin - 2.74 ± 0.42 mg/dl). There were significant reduction in serum iron and ferritin levels after treatment with water extract group (serum iron - 3510 ± 264 µg/dl, serum ferritin - 3.32 ± 0.19 mg/dl) and methanol extract group (serum iron - 4636 ± 142 µg/dl, serum ferritin - 3.97 ± 0.29 mg/dl) of wheatgrass compared to disease group. Treatment with acetone extract did not significantly reduce serum iron or ferritin levels (serum iron - 5222 ± 314 µg/dl, serum ferritin – 4.64 ± 0.38 mg/dl) compared to disease control. (Fig 6.4.3)

These data suggest water and methanol extracts have effectiveness in reduction of iron overload which may be benefits in iron overload disorders as desferoxamine. (Table 6.4.4)

Table 6.4.4: Beneficial effects of various extracts of wheatgrass on iron over load rats with respect to changes in parameters related to its mechanism of action.

Parameters	NC (n=6)		DC (n=6)		DCD (n=6)		DCWT (n=6)		DCMT (n=6)		DCAT (n=6)	
	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days
Serum Iron µg/dl	203 ± 17	221 ± 31	6099 ± 252*	6640 ± 291*	2876 ± 281#	2193 ± 128#	4636 ± 142#	4293 ± 132#	3510 ± 264#	3173 ± 202#	5222 ± 314	5130 ± 216
Serum Ferritin mg/dl	1.13 ± 0.07	1.19 ± 0.08	4.83 ± 0.51*	6.45 ± 0.68*	2.74 ± 0.42#	2.29 ± 0.39#	3.97 ± 0.29#	4.24 ± 0.58#	3.32 ± 0.19#	3.60 ± 0.60#	4.64 ± 0.38	5.89 ± 0.45
Urine Iron µg/dl	26.2 ± 6.4	21.9 ± 7.4	69.2 ± 7.7*	80.8 ± 11.0*	422.5 ± 79.1#	488.9 ± 73.7#	256.0 ± 32.6#	293.4 ± 39.4#	296.5 ± 33.8#	322.8 ± 11.6#	94.0 ± 11.9	105.3 ± 15.7
Faces Iron µg/dl	2.83 ± 0.3	2.75 ± 0.25	9.17 ± 2.5*	11.0 ± 1.2*	31.0 ± 5.3#	40.0 ± 3.0#	17.67 ± 2.1#	23.0 ± 2.6#	30.33 ± 2.5#	32.5 ± 1.5#	12.0 ± 1.01	12.67 ± 1.2

Values are expressed as Mean ± S.E.M
*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

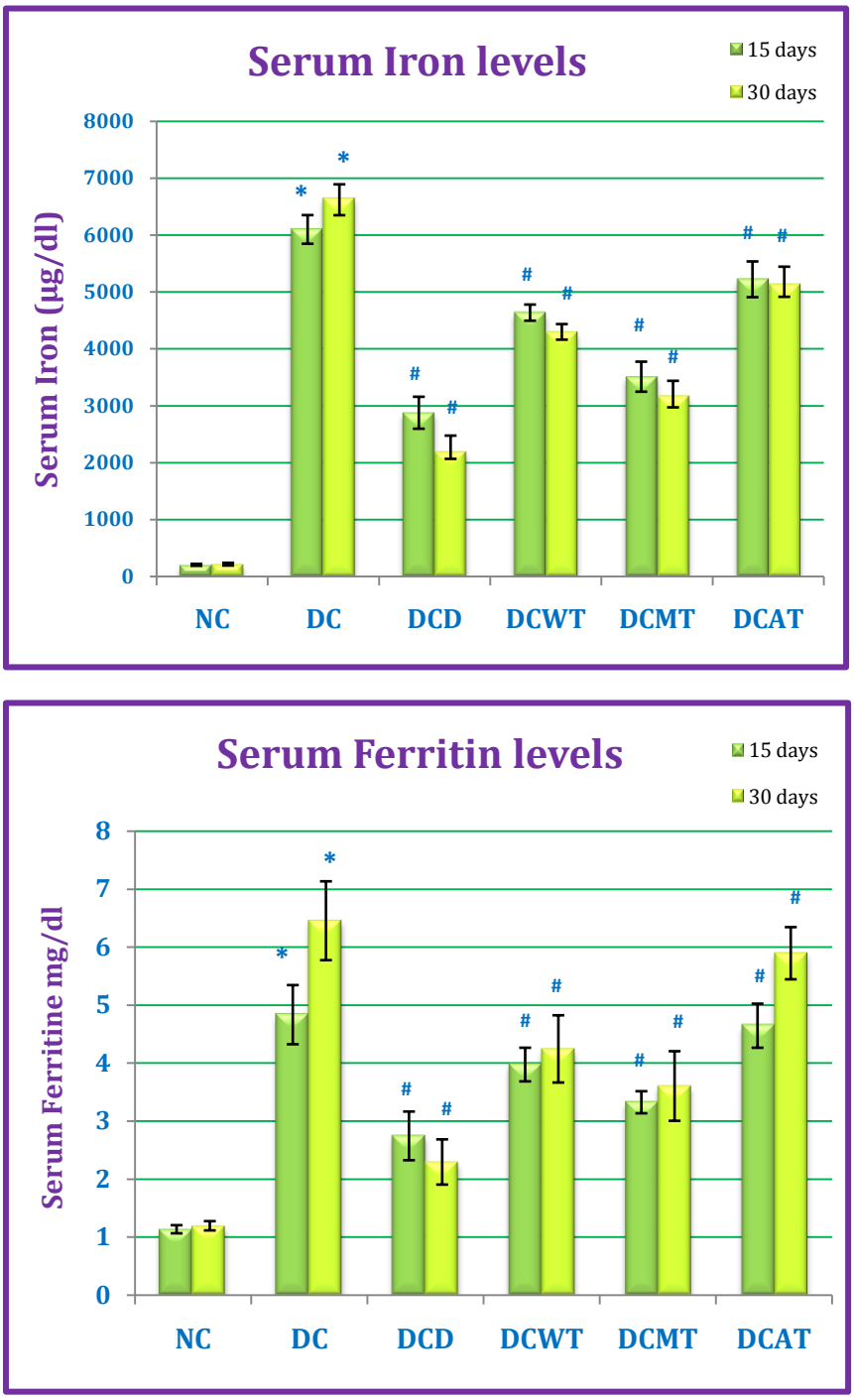


Fig 6.4.3: Beneficial effects of various extracts of wheatgrass on iron over load rats with respect to changes in parameters related to its mechanism of action.

Values are expressed as Mean ± S.E.M
*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

B. Urine iron and faces iron levels

No changes were observed in urine and fecal iron in iron overloaded group rats (urine iron – 69.2 ± 7.7 µg/dl, faces iron – 9.17 ± 2.5 µg /dl) and placebo group (urine iron – 26.2 ± 6.4 µg/dl, faces iron – 2.83 ± 0.3 µg /dl), because iron does not excrete from body. There was significant increase in urine iron and faces iron levels in desferoxamine group (urine iron - 422.5 ± 79.1 µg/dl, faces iron - 31.0 ± 5.3 µg /dl), water extract group (urine iron - 256.0 ± 32.6 µg/dl, faces iron - 17.67 ± 2.1 µg /dl) and methanol extract group (urine iron - 296.5± 33.8 µg/dl, faces iron - 30.33 ± 2.5 µg /dl) compared to iron overloaded group rats (urine iron - 69.2 ± 7.7 µg/dl, faces iron - 9.17 ± 2.5 µg /dl). (Fig 6.4.4) Increase in urine and faecal excretion of iron in rats treated with water and methanol extracts of wheatgrass indicate iron chelating property of wheatgrass that was comparable to desferoxamine group. Similarly there were beneficial effects observed after 30 days treatment period with wheatgrass in iron overloaded rats. (Table 6.4.4) Treatment of acetone extracts did not produce any significant increase in urine iron (94.0 ± 11.9 µg /dl) and faces iron (12.0 ± 1.01 µg /dl) levels compared to diseases control group. These data suggest effectiveness of water and methanol extracts in reduction of iron overload in rats by increase iron excretion in urine and faeces.

These data suggest water and methanol extracts have effectiveness in reduction of iron overload which may be benefits in iron overload disorders as desferoxamine. (Table 6.4.4)

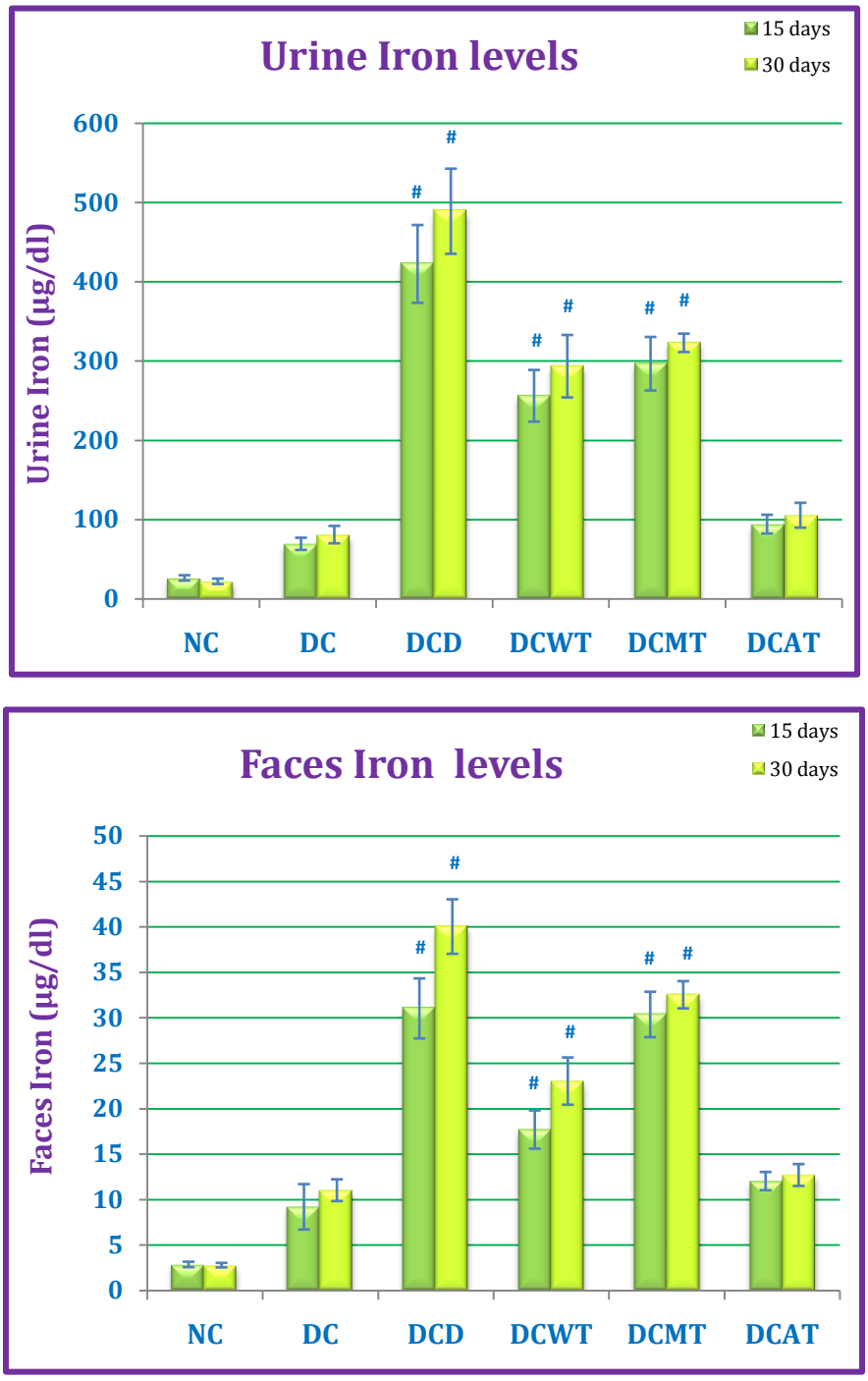


Fig 6.4.4: Beneficial effects of various extracts of wheatgrass on iron over load rats with respect to changes in parameters related to its mechanism of action.

Values are expressed as Mean ± S.E.M
*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

C. Protective effects of wheatgrass on iron overload complications on vital organs

Excess iron in vital organs, even in mild cases of iron overload, increases the risk for liver disease (cirrhosis, cancer), kidney diseases, heart attack or heart failure, diabetes mellitus etc. and in some cases premature death.

SGPT and SGOT levels

There were significant increases in SGPT (101.9 ± 8.7 µg/l) and SGOT (170.9 ± 11.3 µg/l) levels in iron overloaded group as compared to normal control group (SGPT – 12.4 ± 1.9 µg/l, SGOT - 46.28 ± 5.2 µg/l). After treatment with water and methanol extracts of wheatgrass there was significant reduction in these enzyme levels (water extract SGPT – 95.65 ± 6.9 µg/l, SGOT - 148.0 ± 6.5 µg/l; methanol extract SGPT – 81.9 ± 5.8 µg/l, SGOT - 132.4 ± 8.8 µg/l) indicating protective effects of extracts in liver complications due to iron overload. (Table 6.4.5)

Serum Creatinine and Creatinine Kinase Levels

Serum creatinine and creatinine kinase levels were significant increased in iron overloaded rats group (serum creatinine- 1.76 ± 0.08 mg/dl and creatinine kinase- 398.2 ± 23.7 µg/l) as compared to placebo group (serum creatinine- 0.67 ± 0.08 mg/dl and creatinine kinase- 91.8 ± 8.76 µg/l). Methanol and water extracts treated animals showed reduction in levels of these enzymes (water extract, serum creatinine- 1.59 ± 0.05 mg/dl and creatinine kinase- 335.7 ± 17.9 µg/l; methanol extract, serum creatinine- 1.45 ± 0.07 mg/dl and creatinine kinase- 316.5 ± 11.5 µg/l) indicating that these extracts prevent damage to vital organs like kidney and heart in iron overload complications. (Table 6.4.5)

Table 6.4.5: Beneficial effect of various extracts of *Triticum aestivum* on iron over complications on major organ heart, liver and kidney.

Parameters	NC (n=6)		DC (n=6)		DCD (n=6)		DCWT (n=6)		DCMT (n=6)		DCAT (n=6)	
	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days
SGOT µg/l	46.28 ± 5.2	48.03 ± 3.4	170.9 ± 11.3*	191.6 ± 12.0*	117.3 ± 5.7#	105.9 ± 5.6#	148.0 ± 6.5#	144.5 ± 3.8#	132.4 ± 8.8#	126.4 ± 5.7#	170.3 ± 7.8	168.8 ± 6.9
SGPT µg/l	12.8 ± 2.5	12.4 ± 1.9	101.9 ± 8.7*	160.4 ± 14.6*	77.20 ± 11.0#	63.8 ± 5.2#	95.65 ± 6.9#	128.1 ± 5.7#	81.9 ± 5.8#	89.1 ± 10.3#	97.8 ± 3.7	155.3 ± 5.7
Serum creatinine mg/dl	0.67 ± 0.08	0.63 ± 0.63	1.76 ± 0.08*	1.90 ± 0.06*	1.31 ± 0.11#	1.12 ± 0.09#	1.59 ± 0.05#	1.49 ± 0.04#	1.45 ± 0.07#	1.33 ± 0.09#	1.70 ± 0.11	1.63 ± 0.11
Creatine kinase (CKMB) µg/l	91.8 ± 8.76	80.7 ± 10.3	398.2 ± 23.7*	446.0 ± 12.1*	219.0 ± 23.8#	189.2 ± 15.4#	335.7 ± 17.9#	325.7 ± 14.4#	316.5 ± 11.5#	271.4 ± 16.0#	360.1 ± 15.6	378.1 ± 9.1

Values are expressed as Mean ± S.E.M
*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

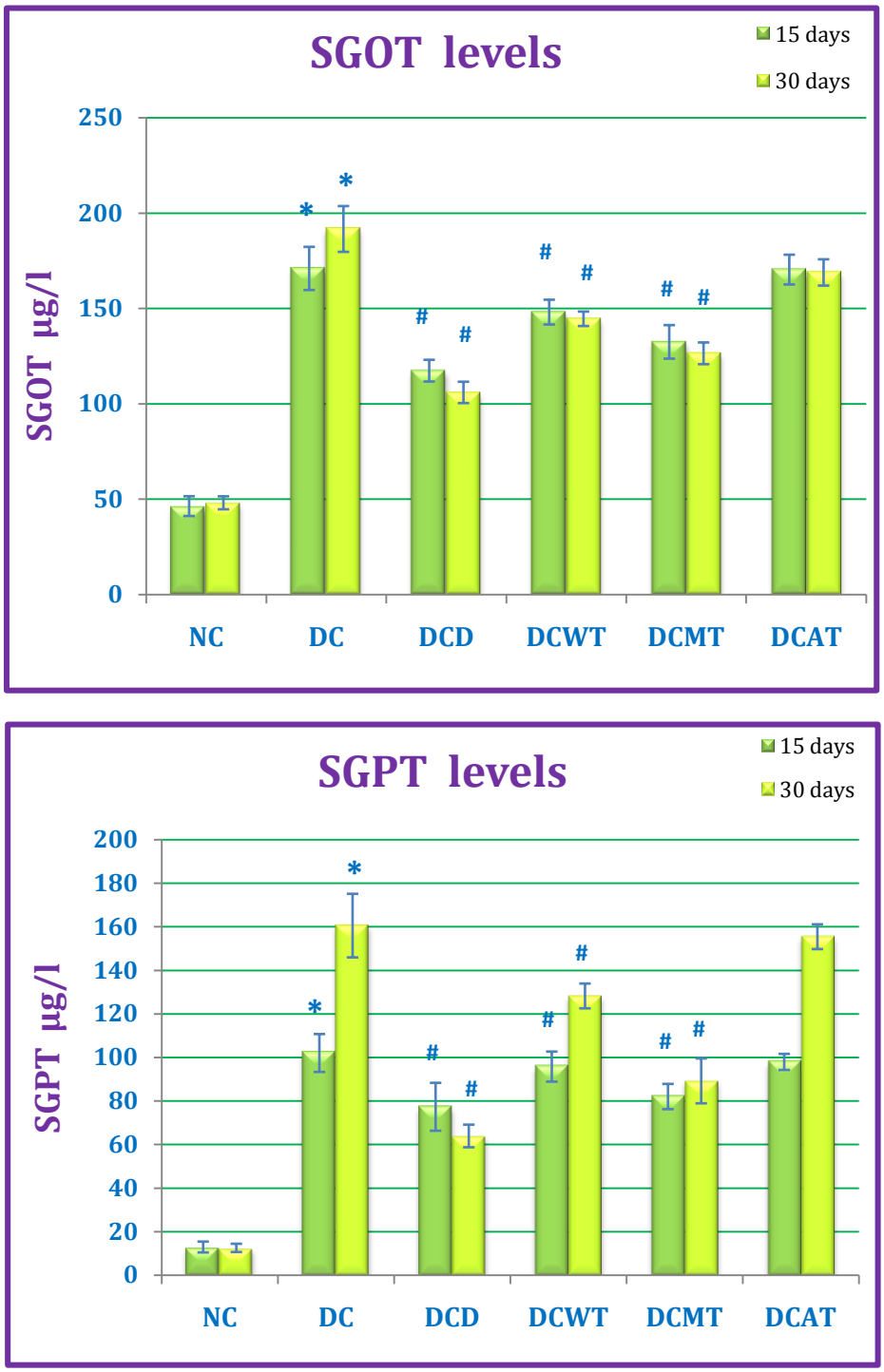


Fig: 6.4.6 Beneficial effect of various extracts of *Triticum aestivum* on iron overload induce liver complication.

Values are expressed as Mean ± S.E.M
*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

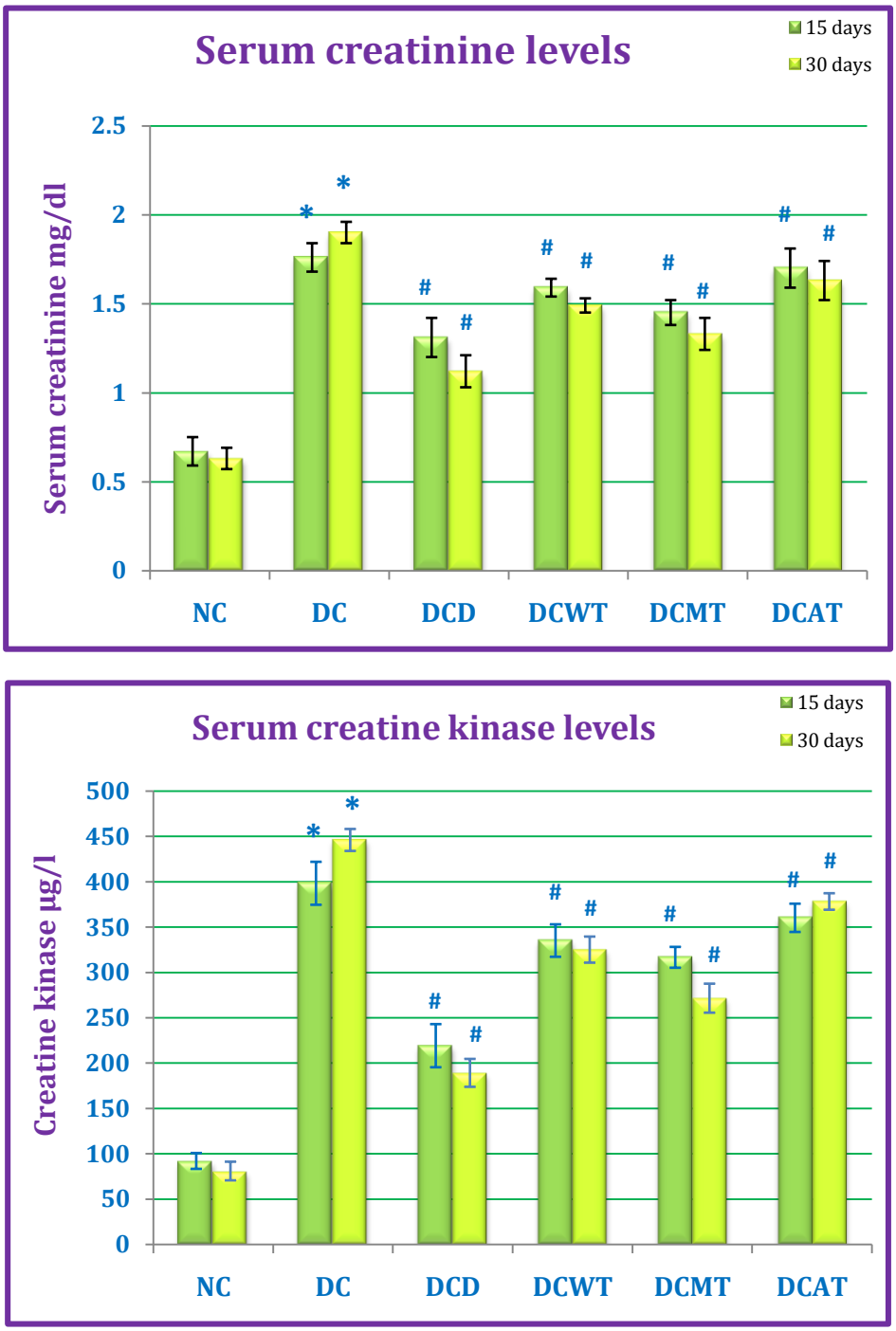


Fig: 6.4.7 Beneficial effects of various extracts of *Triticum aestivum* on iron overload induce kidney and cardiac complications.

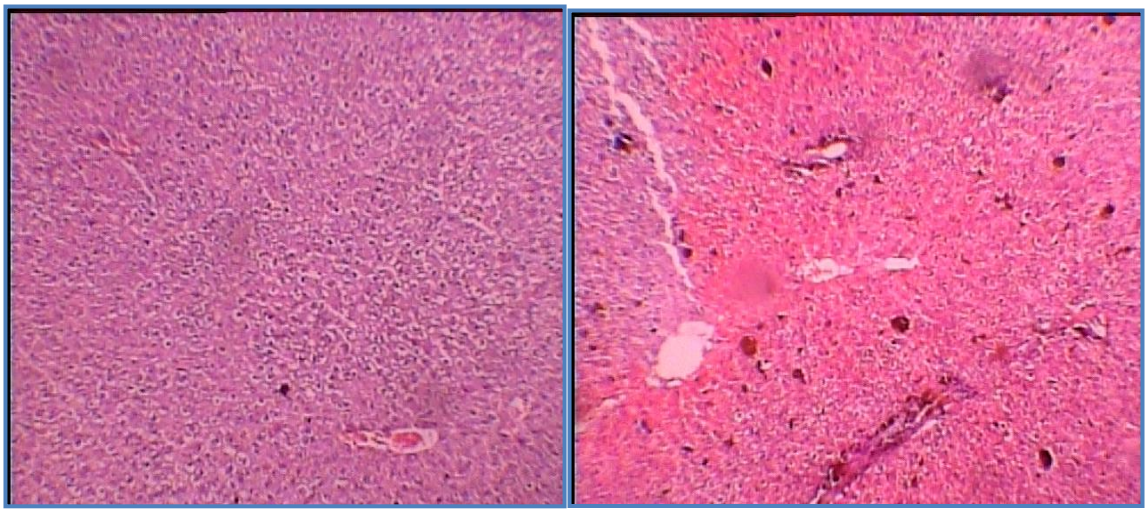
Values are expressed as Mean ± S.E.M
*- significantly different from normal control ($p < 0.05$)
- significantly different from diseases control ($p < 0.05$)
Group 1: Normal control received dextrose solution (NC)
Group 2: Disease control treated with iron dextran (12.5mg/l00g body wt.) (DC)
Group 3: Disease control treated with desferoxamine (40 mg/kg, p.o., per day) (DCD)
Group 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

D. Histopathological study of liver, kidney and heart

Beneficial effects of wheatgrass on Iron overloaded Liver complications

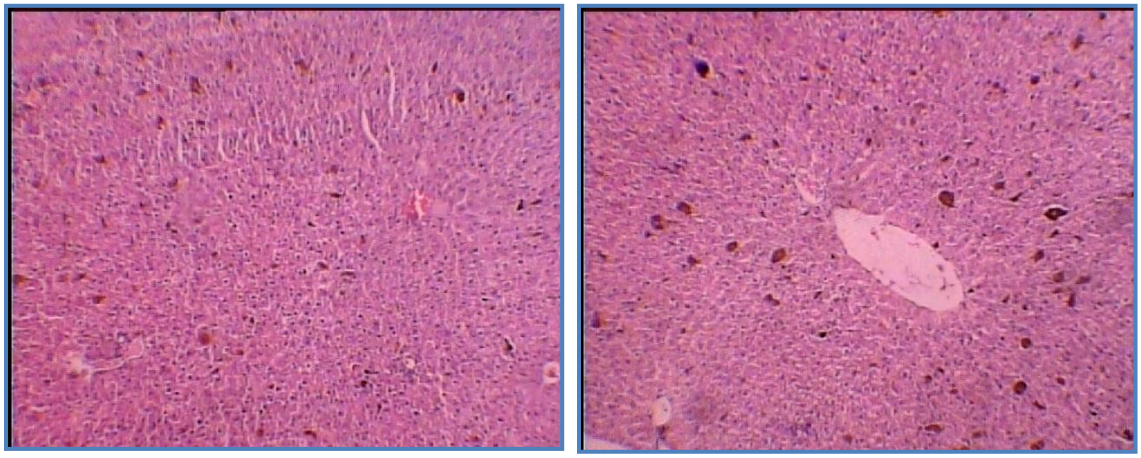
Hepatotoxicity is the most common finding in iron over-load because liver is the main recipient of the excess iron. (Zurlo MG et al., 1989)

Results of histopathological study of liver suggested that chronic treatment with desferoxamine and water, methanol and acetone extracts of wheatgrass reduce iron pigmentation, pleomorphism, vaculation, fibrosis, disarrangement and degeneration of hepatocytes as compared to iron overloaded group animals. The degree of protection was found to be minimal with acetone extracts group. (Fig. 6.4.8)



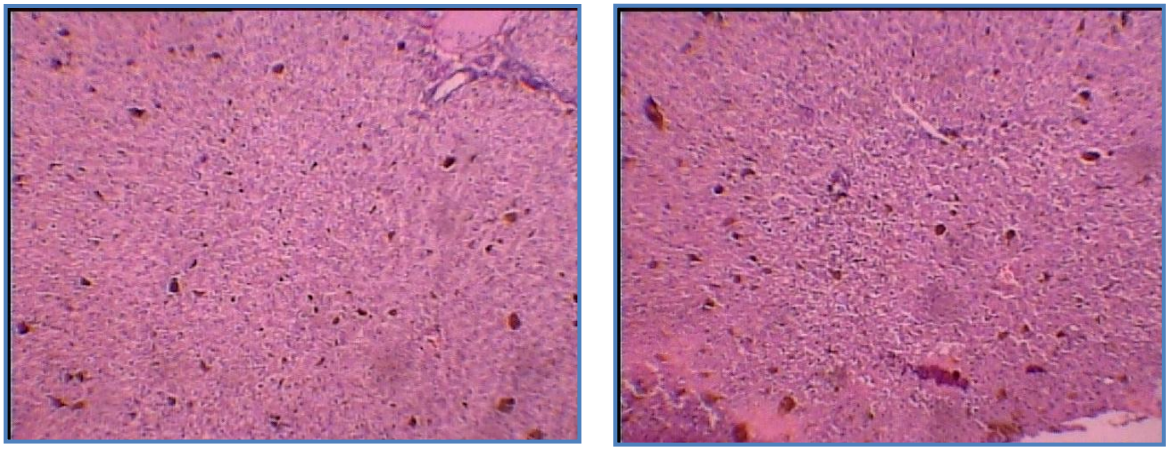
a. Normal control

b. Iron overloaded Diseases control



c. iron overload rats treated with desferoxamine

d. iron overload rats treated with water extract



e. iron overload rats treated with methanol extract

f. iron overload rats treated with acetone extract

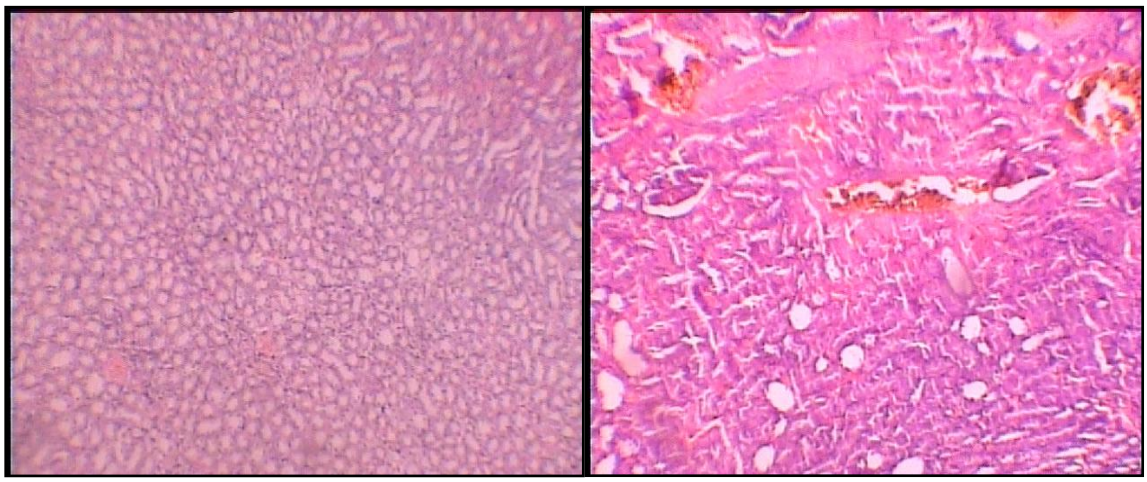
Fig 6.4.8: Protective effects of various extracts of wheatgrass on iron overloaded liver complications.

Beneficial effects of wheatgrass on Iron overloaded Kidney complications

It was reported that an iron-deficient diet or iron chelators prevent the development of tubulointerstitial disease and renal functional deterioration in nephrotoxic serum nephritis. Baliga et al. have demonstrated that cytochrome $P450_{12}$ and, more specifically, cytochrome $P450_{2B1}$, an isozyme present in the glomerulus, are sources of catalytic iron that participate in glomerular injury. The evidence reviewed suggests the possibility of using iron chelators to halt the progression of kidney disease. Lin et al. have shown that chelation therapy with ethylenediaminetetraacetic acid (EDTA) in patients with chronic renal insufficiency results in a reduced rate of decline in the glomerular filtration rate (Lin JL et al., 2003).

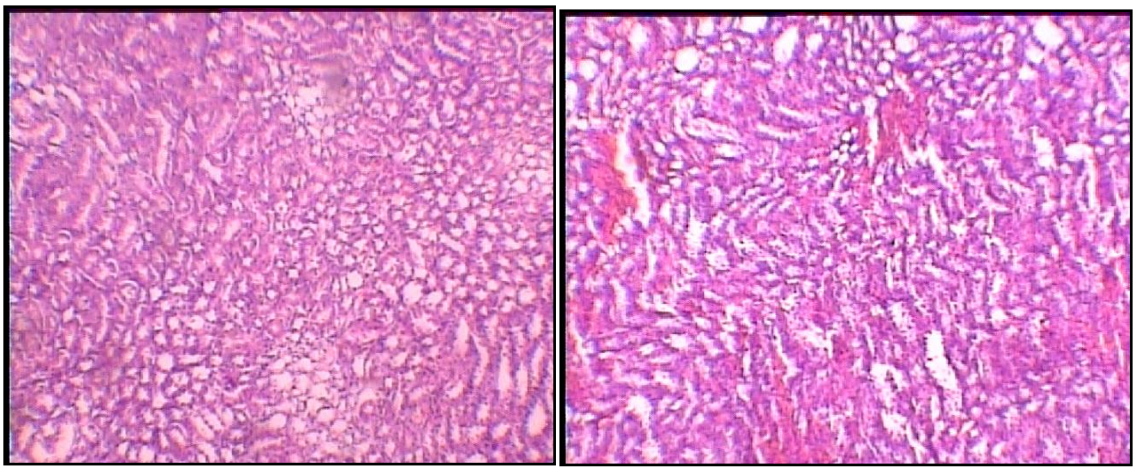
Normal structure of the cortex and medulla was observed in the kidney of normal control rats (Figs 6.4.9). The animals exposed to chronic iron dextran showed damage of renal tubules and glomeruli. Hypertrophy of epithelial cells and degeneration of epithelia of renal tubules with infiltration of mononuclear cells, dilation of glomerul and mononuclear cell infiltrates were evident in all diseases control rats. (Fig 6.4.9). Pathological changes in kidney ultra structure (injured brush-border microvilli and swollen proximal convoluted tubular cells) were observed when iron dextran.

Histology of kidney in iron overload group rat treated with desferoxamine, a iron chelator, showed reduced damage of renal tubules and glomeruli. Pathological changes was also prevented by desferoxamine. Our result suggest protective effects of methanol, water and acetone extracts of wheatgrass in iron overload kidney complications as it reduced damage of kidney ultra structure (injured brush-border microvilli and swollen proximal convoluted tubular cells). Protective effects found much better in methanol extract and less in acetone extract. (Fig 6.4.9)



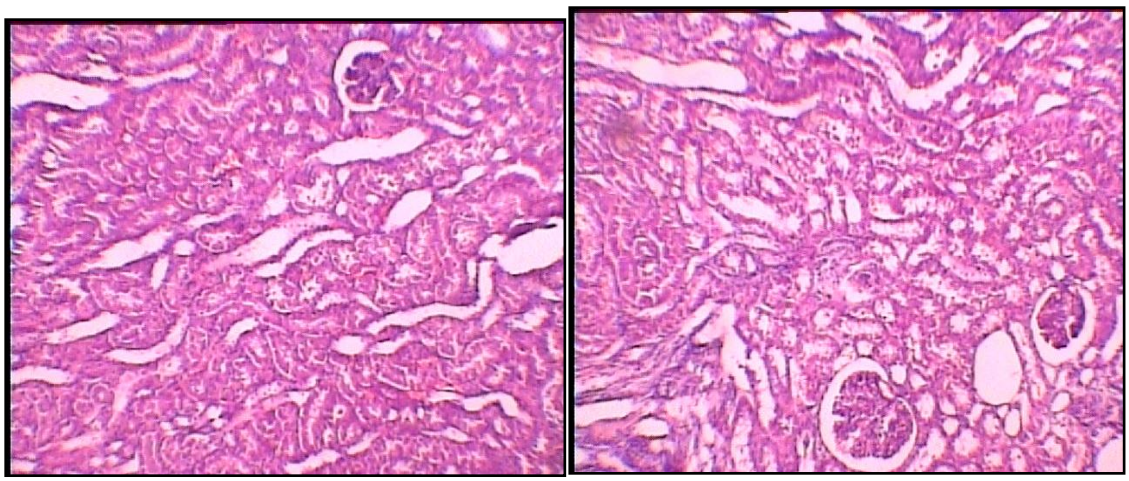
a. Normal control - kidney

b. Diseases control – kidney



c. Diseases control - with desferoxamine

d. Diseases control – with methanol extract



e. Diseases control – with water extract

f. Diseases control– with acetone extract

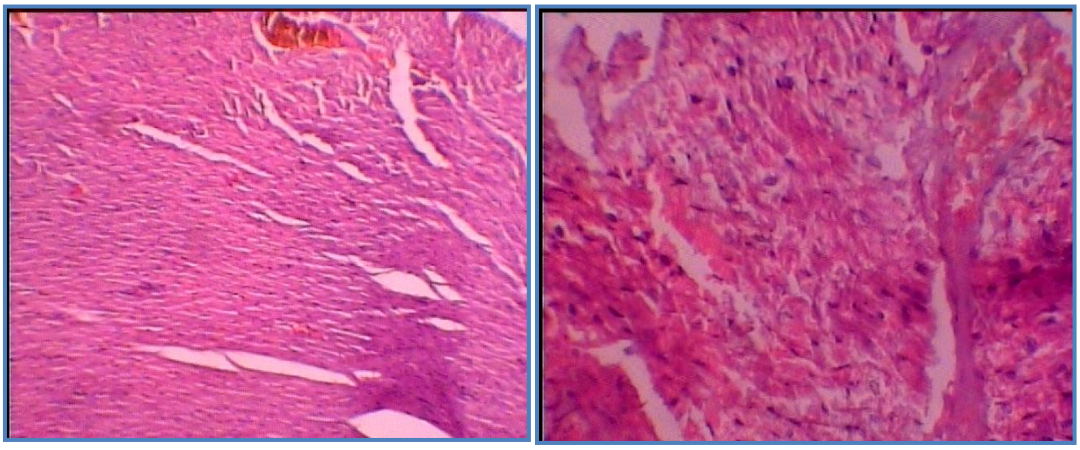
Fig 6.4.9: Protective effects of various extracts of wheatgrass on iron overloaded kidney complications

Beneficial effects of wheatgrass on Iron overloaded cardiac complications

Iron-overload cardiomyopathy is a common cause of CV death worldwide in subjects in their second and third decades of life. (Weatherall DJ, Clegg JB et al., 1996, Olivieri NF, 1999) Indeed, iron-overload cardiomyopathy is the most important determinant of survival in European, (Olivieri NF, 1999) North American, (Weatherall DJ, 2001) and Chinese (Li CK, et al., 2002) patients with thalassemia major. Long-term follow-up studies in beta-thalassemia patients have established that the level of cardiac iron accumulation correlates directly with both the occurrence of heart disease and mortality, (Brittenham GM et al., 1994) while in patients with primary haemochromatosis, CV disease also contributes significantly to their mortality and morbidity. (Niederau C, 1996)

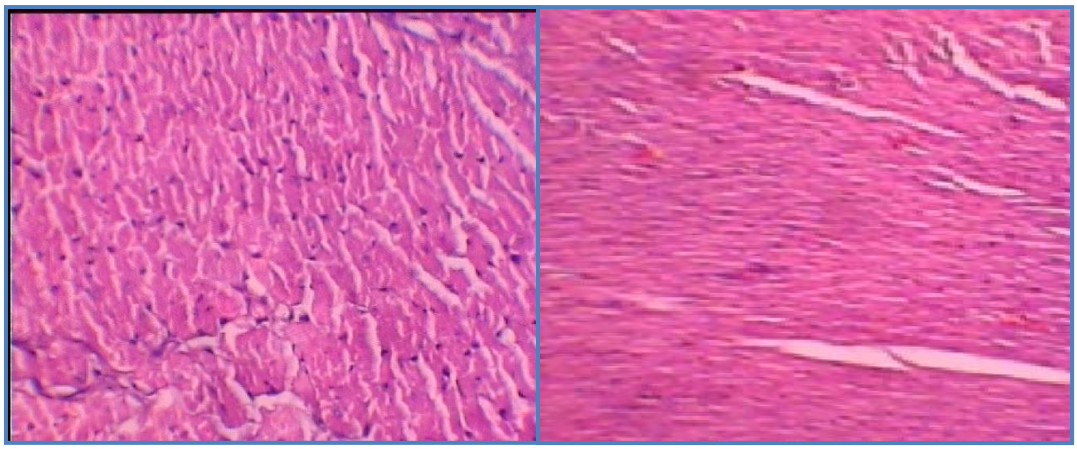
Hearts from rat injected chronically with iron displayed extensive interstitial fibrosis and myocyte vacuolar degeneration with mild inflammatory infiltrate compared to placebo (Figure 6.4.10) there was vascular hemorrhage and hypertrophy observed in iron overload rats compared to placebo.

Treatment with desferoxamine and extracts of methanol and water of wheatgrass showed protective effects on myocytes as well as reduces fibrosis and hypertrophy of myocytes. Vascular hemorrhages were also found to be reducing in iron overloaded group rats treated with methnoal and water extracts. Treatment with acetone extracts was produce less protective effects. (Figure 6.4.10)



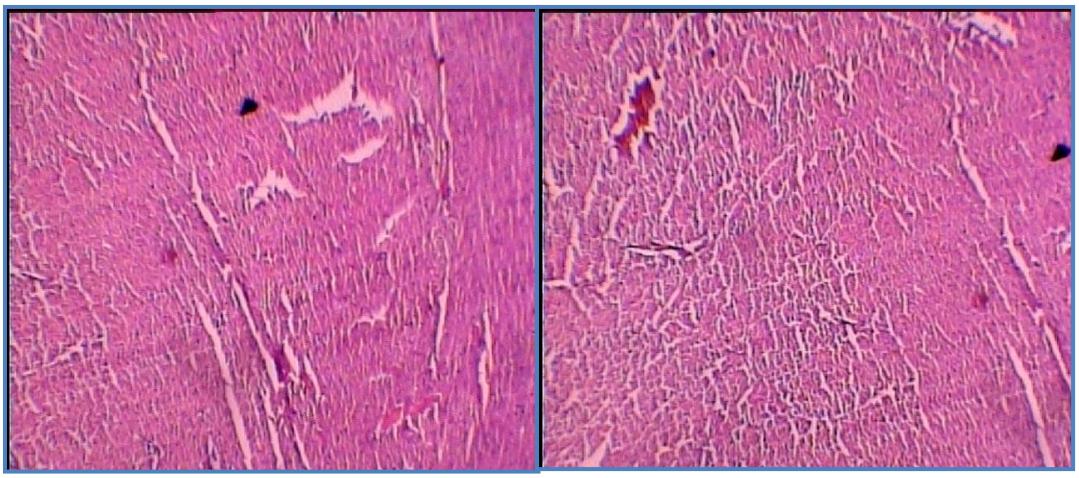
a. Normal control

b. Diseases control



c. Diseases control with desferoxamine

d. Diseases control with methanol extract



e. Diseases with water extract

f. Diseases with acetone extract

Fig 6.4.10: Protective effects of various extracts of wheatgrass on iron overloaded cardiac complications.

6.5 Isolation and characterization of iron chelating active constituent of wheatgrass

Since, the results, obtained so far, revealed maximum chelating activity in methanol extract group, we decided to process the methanol extract further, for the purpose of isolation of active iron chelator constituent form *Triticum aestivum.*

Column chromatography fraction 71-76 eluted using methanol: water: acetone: glacial acetic acid (1:0-80:0.5:0.1) solvent system followed by concentration yielded brown crystals. On recrystallization with methanol produced needle shaped crystals of the active compound (PI_1), having melting point of 215-218 ^{0}C.

TLC Study

The identity of the isolated compound PI_1, was confirmed by comparing the R_f value 0.682 on TLC plate. The isolated compound gave black colored spot on spraying 5 % ferric chloride solution on TLC plate suggesting phenolic nature.

Spectroscopic analysis

The isolated iron chelator compound was subjected to LCMS and IR spectroscopic analysis, for its molecular characterization. The compound was found to be aromatic in nature containing phenolic group. The data of Spectral analysis, IR and GC-MS of isolated pure compound mentioned in table 6.5.1.

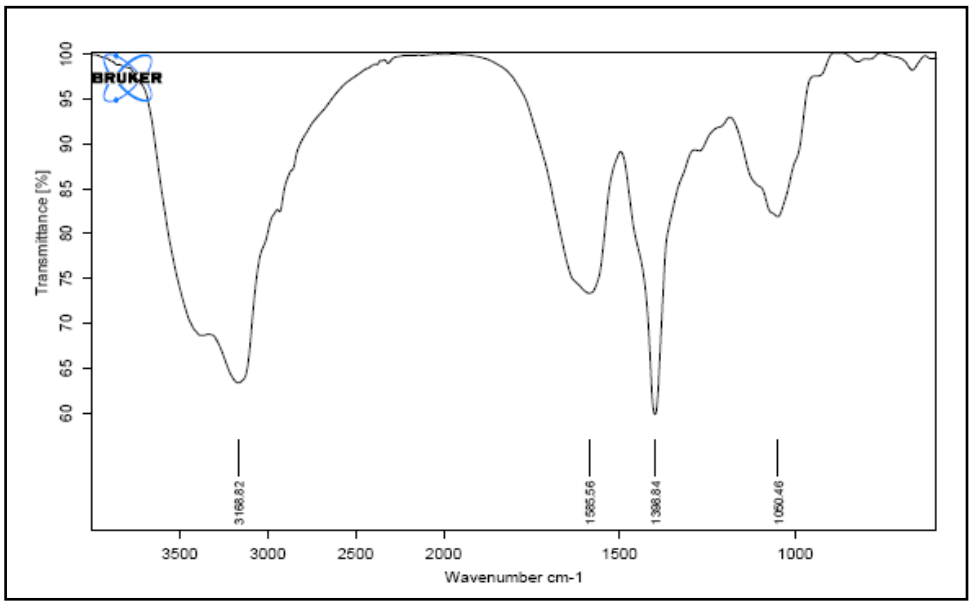


Fig 6.5.1: IR spectroscopy of isolated compound PI_1 from *Triticum aestivum.*

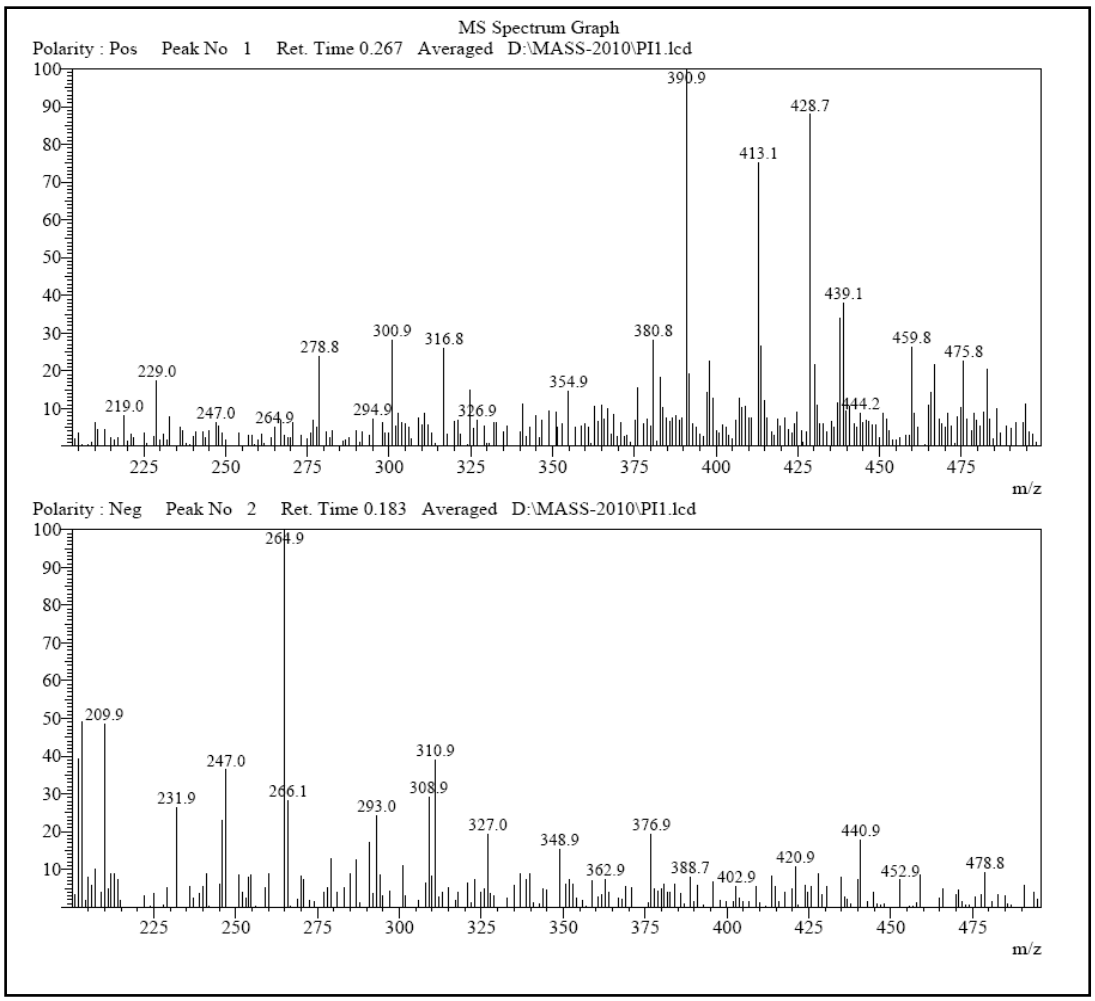


Fig 6.5.2: Mass spectroscopy of isolated compound PI_1 from *Triticum aestivum.*

Table 6.5.1: IR and MASS spectral value of isolated pure compound PI_1.

Spectra	Spectral value	Inference
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

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

The total phenolic content in isolated compound from methanol fraction of *Triticum aestivum,* using Folin-Ciocalteau (FC) method, was found to be 434.14 ± 28.02 µg Gallic acid equivalent of phenol.

6.6 In-vitro evaluation of iron chelating activity in active constituent of wheatgrass

In-vitro iron chelating activity of isolated compound was compared with standard iron chelator drug, desferoxamine 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 PI_1.

6.7 In-vivo evaluation of iron chelating activity of active constituent of wheatgrass

Intraperitoneal injections of iron-dextran (12.5 mg/l00g of body wt.), evenly distributed over a 2 days period, resulted in condition of acute iron overload, in SD rats. Control group rats injected with an equal volume of dextran, showed normal serum level of iron. At the end of day 2, urine samples were collected and analyzed for iron content. No significant changes in urine iron levels were observed in iron overloaded rats (34.25 ± 3.8 µg/dl) and normal control rats

(26.2±6.4 µg/dl). 2 day's treatment with desferoxamine (urine iron- 108.75±7.4 µg/dl) and isolated compound (urine iron- 62.21±9.4 µg/dl) in iron overloaded rats produced significant increase in urine iron levels compared to diseases control rats. The chelating power or efficacy of the isolated compound was 34.5% compared to that of desferoxamine.

Table 6.7.1: Comparison of excretion of iron in urine between desferoxamine and isolated compound 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#

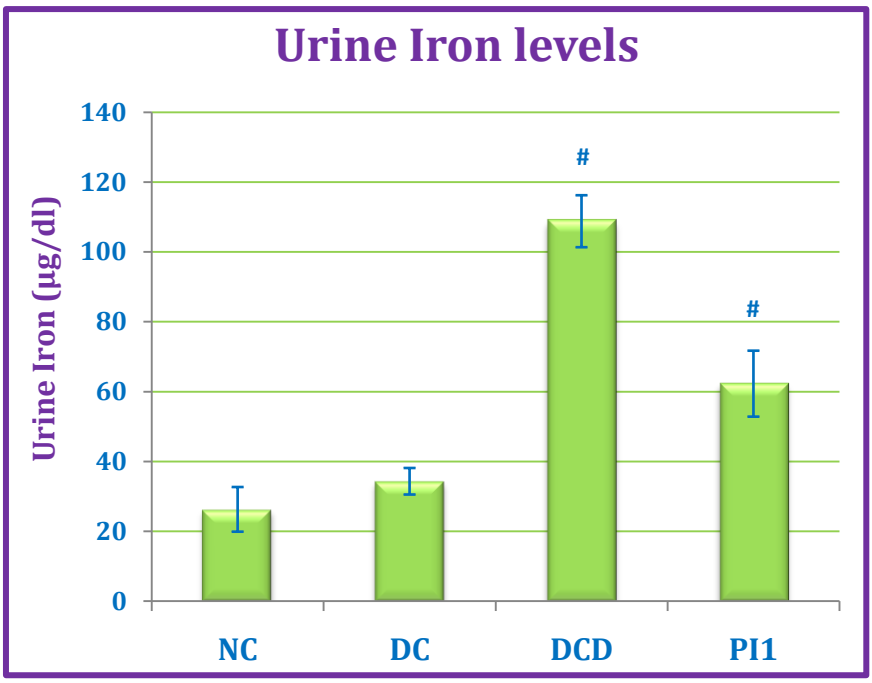


Fig 6.7.1: Iron chelating property of isolated compound PI_1 compared to desferoxamine.

- 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)

6.8 Investigation of anti-oxidant property of *Triticum aestivum* in iron overload condition

At the end of 30 day period of iron overloaded conditions, animal were sacrificed by spinal dislocation technique and liver homogenate was prepared. The prepared homogenates were used to estimate tissue protein levels (of Lowry et al., 1951), SOD by Misra et al (1984), Catalase by Aeibi et al., 1974, Reduced Glutathione (GSH) by Beutler et al (1963) and tissue lipid peroxidation, Malondialdehyde-MDA by Ohkawa et al (1979) to role of anti-oxidant and beneficial effects of wheatgrass on oxidative stress induce toxicities.

In our study, at the end of 30 days treatment periods, liver homogenates of iron overloaded group rats showed significant increase in OFRs, MDA level (2.56 ± 0.18 nmoles/mg protein) and decrease in SOD (0.73 ± 0.07 units/min/mg protein), catalase (0.98 ± 0.14 units/min/mg protein) and glutathione levels (7.22 ± 1.75 µgm/mg protein) compared to normal group rats (MDA- 0.74 ± 0.11 nmoles/mg protein, SOD- 1.42 ± 0.1 units/min/mg protein, catalase- 4.59 ± 1.11 units/min/mg protein , GSH- 12.69 ± 1.04 µgm/mg protein). Treatment with methanol and water extracts of wheatgrass significantly reduced MDA level (1.24 ± 0.06 and 1.16 ± 0.08 nmoles/mg protein) in the rat liver homogenates. There were significant improvements in GSH, SOD and catalase levels in iron overloaded group rats treated with methanol (SOD- 1.29 ± 0.13 units/min/mg protein, Catalase- 3.47 ± 0.17 units/min/mg protein, GSH- 10.8 ± 0.7 µgm/mg protein) and water extracts (SOD- 1.35 ± 0.11 units/min/mg protein, Catalase- 3.78 ± 0.69 units/min/mg protein, GSH- 11.82 ± 0.51 µgm/mg protein). No significant changes were observed in MDA, SOD, catalase and glutathione levels in acetone extract treated group. These data indicate strengthening of antioxidant defense by wheatgrass in iron overload condition. (Table 6.8.1)

Table 6.8.1: Effect of *T. aestivum* on antioxidant parameters in iron over load induced thalassemic rats.

Anti-oxidant Parameters	NC (n=6)	DC (n=6)	DCD (n=6)	DCWT (n=6)	DCMT (n=6)	DCAT (n=6)
SOD (units/min/mg protein)	1.42 ± 0.10	0.73* ± 0.07	1.20# ± 0.11	1.35# ± 0.11	1.29# ± 0.13	0.95 ± 0.09
Catalase (units/min/mg protein)	4.59 ± 1.11	0.98* ± 0.24	2.08# ± 0.37	3.78# ± 0.69	3.47# ± 0.17	1.89# ± 0.31
MDA (nmoles/mg protein)	0.74 ± 0.11	2.56* ± 0.18	1.79# ± 0.18	1.16# ± 0.08	1.24# ± 0.06	2.33# ± 0.10
GSH (µgm/mg protein)	12.69 ± 1.04	7.72* ± 1.75	9.21# ± 1.12	11.82# ± 0.51	10.8# ± 0.70	8.65 ± 1.85

*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

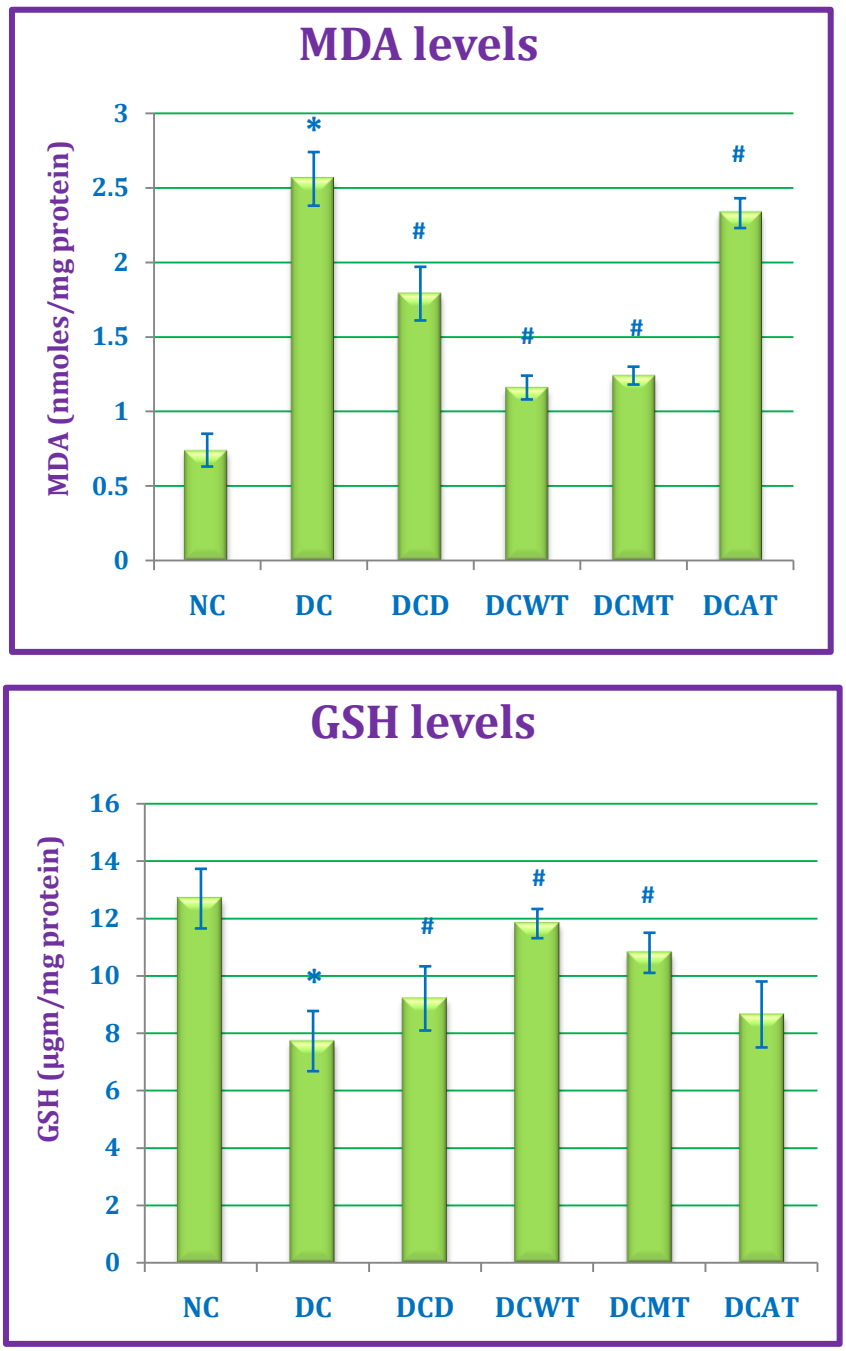
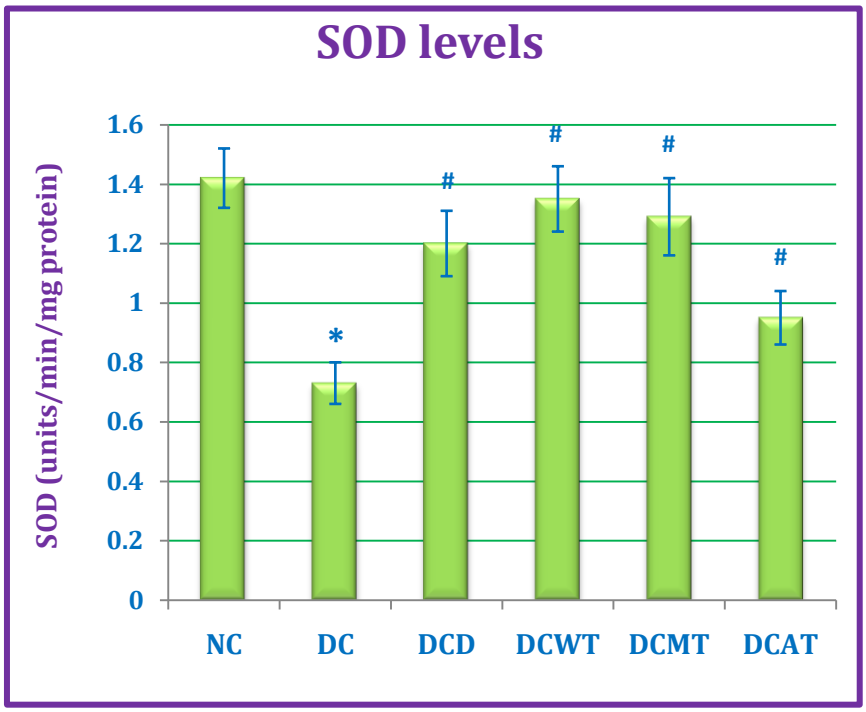


Fig 6.8.1: Beneficial effects of wheatgrass on anti-oxidant parameters in iron overload animals.

*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)



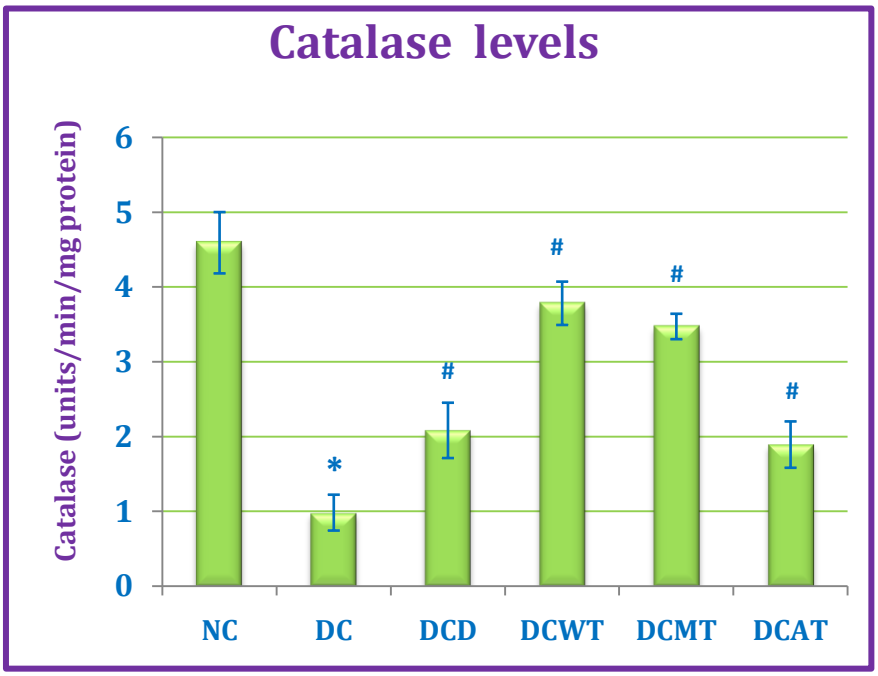


Fig 6.8.1: Beneficial effects of wheatgrass on anti-oxidant parameters in iron overload animals.

*- significantly different from normal control ($p < 0.05$)
- 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 4: Disease control treated with water extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCWT)
Group 5: Disease control treated with methanol extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCMT)
Group 6: Disease control treated with acetone extract of *T. aestivum* (100 mg/kg, p.o., per day) (DCAT)

6.9 Evaluation of therapeutic benefit of *Triticum aestivum* in thrombocytopenia

For evaluation of effects of wheatgrass in thrombocytopenia, busulfan was used to induce experimental thrombocytopenia. Busulfan is an alkylating agent with myeloablative properties and activity against non-dividing marrow cells and possibly, non-dividing malignant cells. Busulfan solution, at concentration of 10 mg/ml in polyethylene glycol, was prepared and infused in wistar rats at doses of 25 mg busulfan/kg body weight each, at 1, 5, 10 and 15 days of interval produced pancytopenia with significant reduction in platelet count mimicking severe bleeding conditions as found in thrombocytopenia.

Hemoglobin level and RBC count

Disease control group rats which, received busulfan showed significant reduction in Hb (8.1 ± 0.75 gm/dl) and RBC count (5.4 ± 0.5 m/cmm) compared to normal healthy group rats (Hb- 10.9 ± 0.98 gm/dl, RBC- 6.8 ± 0.89 m/cmm) indicating anemia in iron overloaded rats. Treatment with fresh wheatgrass juice (Hb- 10.5±1.1 gm/dl, RBC- 6.7±0.59 m/cmm), methanol extract (Hb- 10.2 ± 1.2 gm/dl, RBC- 6.1 ± 0.47 m/cmm) and acetone extract (Hb- 9.7 ± 0.89 gm/dl, RBC- 6.3 ± 0.7 m/cmm) of wheatgrass, produced significant increase in Hb levels and RBC counts in diseased rats. Decrease in blood Hb level and RBC count in rats was significantly prevented by treatment with fresh juice, methanol and acetone extracts of wheat grass. Thus, wheatgrass seems to help improve blood purification and also, to increase hemoglobin level and RBC count near to normal. (Table 6.9.1)

Table 6.9.1: Beneficial effects of *Triticum aestivum* fresh juice, methanol and acetone extracts on Hb level and RBC count.

Blood parameters	Normal Control	Diseases Control	DWFJ	DWM	DWA
Hemoglobin (gm/dl)	10.9±0.98	8.1±0.75*	10.5±1.1#	10.2±1.2#	9.7±0.89
RBC (10^6/πl)	6.8±0.89	5.4±0.50*	6.7±0.59#	6.1±0.47#	6.3±0.7

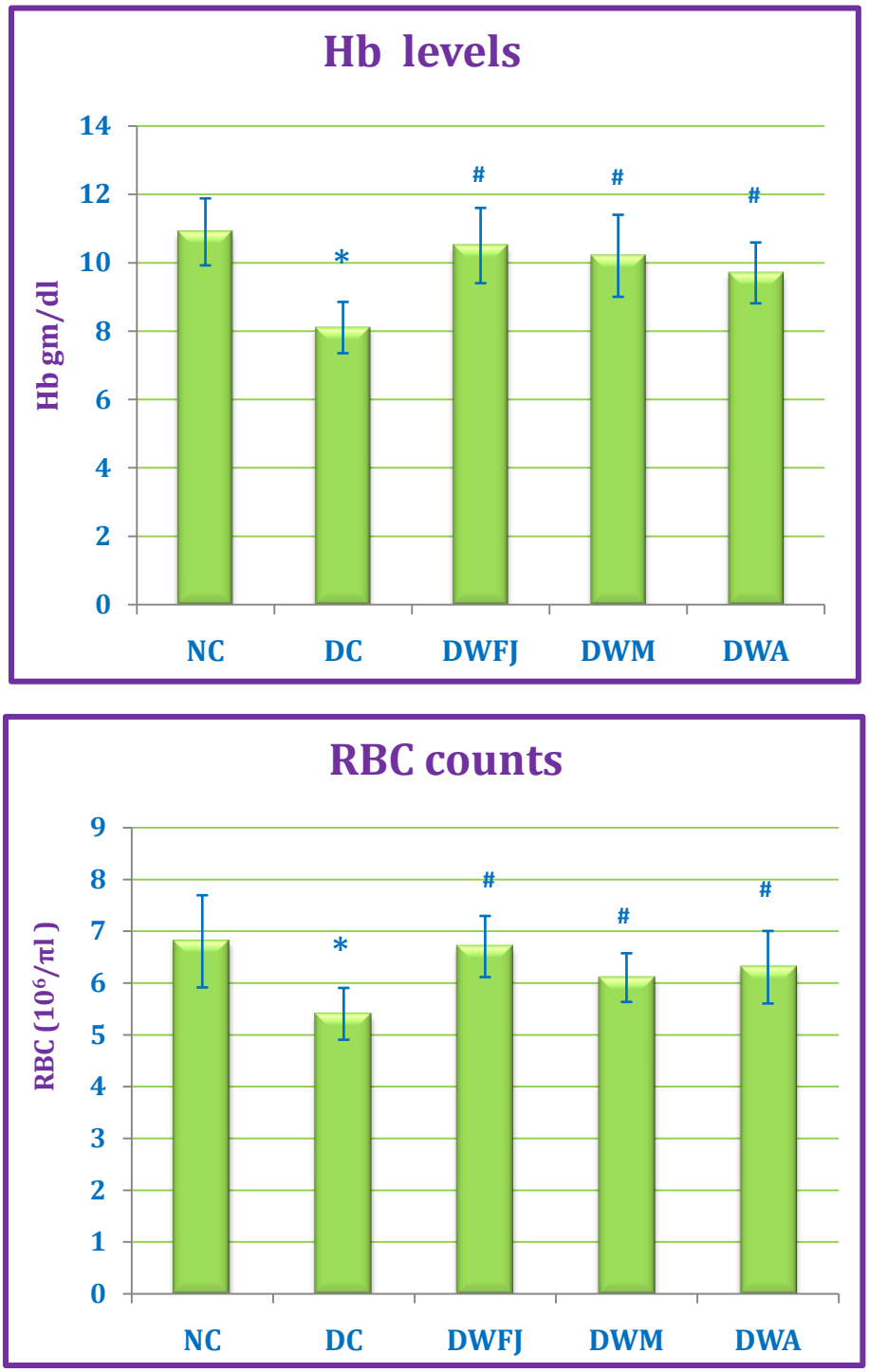


Fig 6.9.1: Beneficial effects of *Triticum aestivum* fresh juice, methanol and acetone extracts on Hb level and RBC counts.

Values are expressed as Mean ± S.E.M, *- significantly different from normal control ($p < 0.05$), # - significantly different from diseases control ($p < 0.05$), DWFJ- **D**iseases rats treated with **W**heatgrass **F**resh **J**uice, **D**WM- **D**iseases rats treated with **W**heatgrass **M**ethanol extract, **D**WA- **D**iseases rats treated with **W**heatgrass **A**cetone extract

Total WBC count

Disease control group rats which received busulfan showed significant reduction in total WBC and differential WBC counts compared to normal healthy group rats. Treatment with fresh wheatgrass juice, methanol extract produced significant increase in total WBC counts and differential WBC counts, in busulfan induced pancytopenic rats. Treatment with acetone extract did not produce significant increase in total WBC counts. Disease control group rats showed pancytopenia (reduction in all blood cells count) compared to normal healthy control group rats. Treatment with fresh wheatgrass juice and different extracts showed increase in WBC counts compare to disease control group. (Table 6.9.2)

Table 6.9.2: Effect of *Triticum aestivum* fresh juice, methanol and acetone extracts treatment on differential WBC count and other blood parameters on control and disease rats.

Blood parameters	Normal Control	Diseases Control	DWFJ	DWM	DWA
WBC (10^3/πl)	4.98±0.49	3.26±0.31*	4.78±0.52#	4.58±0.43#	3.89±0.26
Differential WBC					
Neutrophil (10^3/πl)	2.63±0.35	1.89± 0.28*	2.45± 0.09#	2.01± 0.11#	2.32± 0.12#
Lymphocytes (10^3/πl)	1.52±0.08	1.02±0.07*	1.63±0.08#	1.26±0.07#	1.48±0.09#
Monocytes (10^3/πl)	0.42±0.07	0.11±0.08*	0.43±0.06#	0.28±0.03#	0.37±0.03#
Eosinophil (10^3/πl)	0.12±0.01	0.02±0.003*	0.09±0.002#	0.03±0.0001#	0.11±0.01#
Basophil (10^3/πl)	0.11±0.002	0.04±0.003*	0.12±0.01#	0.07±0.004#	0.09±0.002#
OTHER BLOOD PARAMETERS					
HCT (%)	44.2	33.5	43.9	42.5	38.2
MCV(fL)	58.3	47.0	56.3	54.2	51.0
MCH(pg)	19.2	15.2	17.2	16.1	16.3
MCHC(g/dl)	32.7	25.6	31.8	30.0	27.8
RDW-SD(fL)	32.1	27.1	30.2	29.3	28.9
RDW-CV(%)	18.6	13.6	15.3	16.2	14.9
PDW(fL)	8.5	5.8	7.2	7.7	7.2
MPV (fL)	8.1	7.2	7.9	7.6	7.4
P-LCR(%)	10.5	8.6	9.7	9.4	9.1
PCT(%)	0.78	0.71	0.76	0.75	0.73

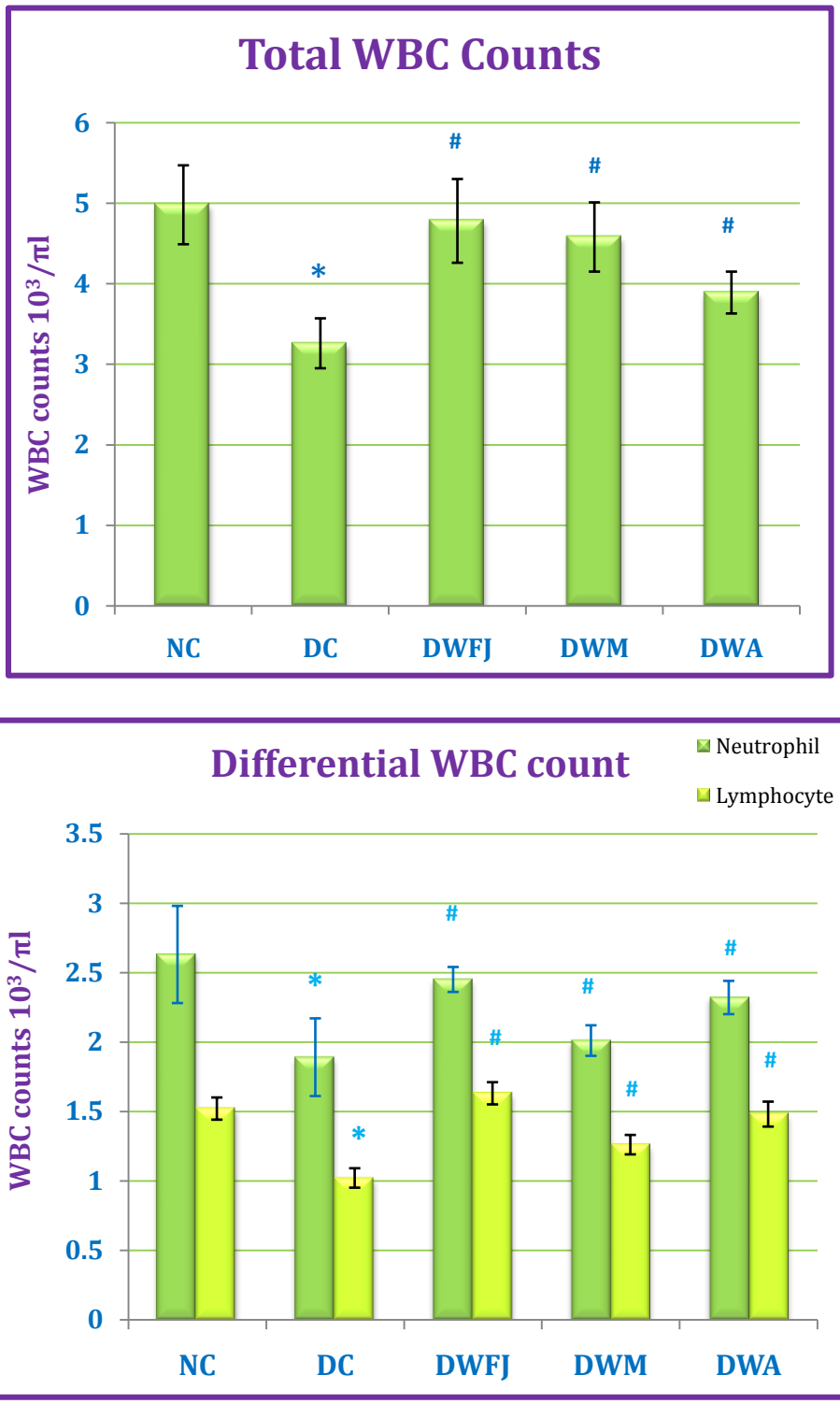


Fig 6.9.2: Beneficial effects of *Triticum aestivum* fresh juice, methanol and acetone extract on Total and differential WBC counts.

Values are expressed as Mean ± S.E.M, *- significantly different from normal control ($p < 0.05$), # - significantly different from diseases control ($p < 0.05$), DWFJ- **D**iseases rats treated with **W**heatgrass **F**resh **J**uice, **D**WM- **D**iseases rats treated with **W**heatgrass **M**ethanol extract, **D**WA- **D**iseases rats treated with **W**heatgrass **A**cetone extract

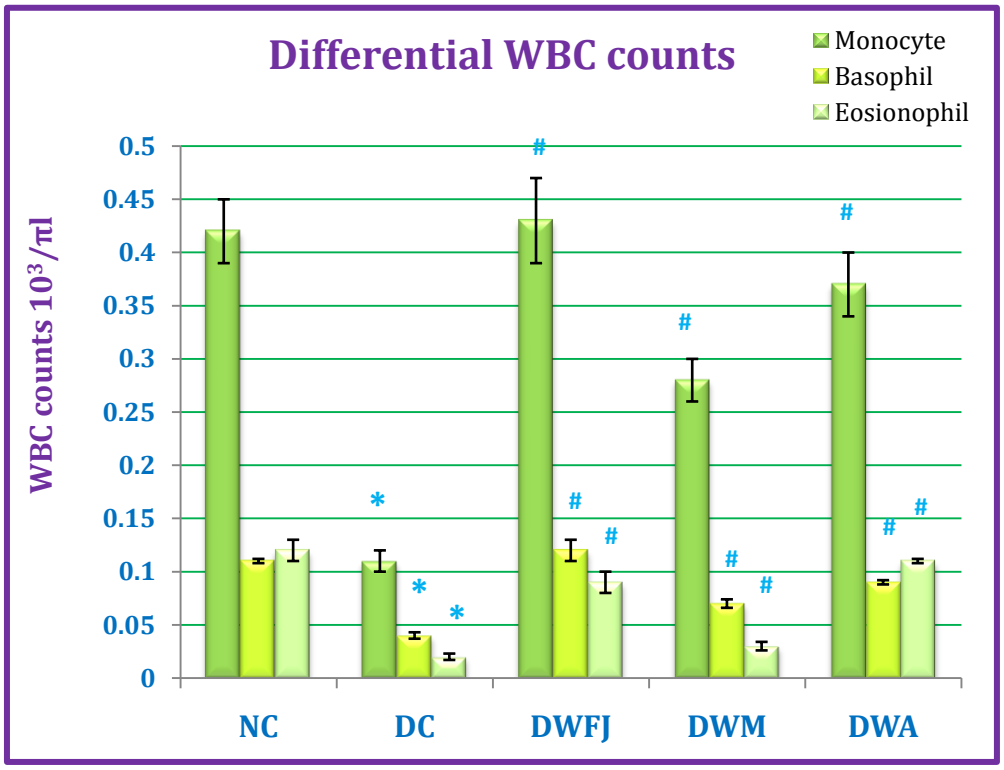


Fig 6.9.2: Beneficial effects of *Triticum aestivum* fresh juice, methanol and acetone extract on Total and differential WBC counts.

Values are expressed as Mean ± S.E.M, *- significantly different from normal control ($p < 0.05$), # - significantly different from diseases control ($p < 0.05$), DWFJ- **D**iseases rats treated with **W**heatgrass **F**resh **J**uice, **D**WM- **D**iseases rats treated with **W**heatgrass **M**ethanol extract, **D**WA- **D**iseases rats treated with **W**heatgrass **A**cetone extract

Platelet count

In disease control group rats which received busulfan, there was significant reduction in platelet count (523 ± 46 10^3/πl) compared to normal healthy group rats (905 ± 82 10^3/πl) indicating thrombocytopenia. Treatment with fresh wheatgrass juice (804 ± 72 10^3/πl), methanol extract (761 ± 58 10^3/πl) and acetone extract (708 ± 63 10^3/πl) produced significant increase in platelet count as compared to disease control group rats. Decrease in platelet count in these rats was significantly prevented by treatment with fresh wheatgrass juice, methanol and acetone extracts of wheatgrass. (Table 6.9.3)

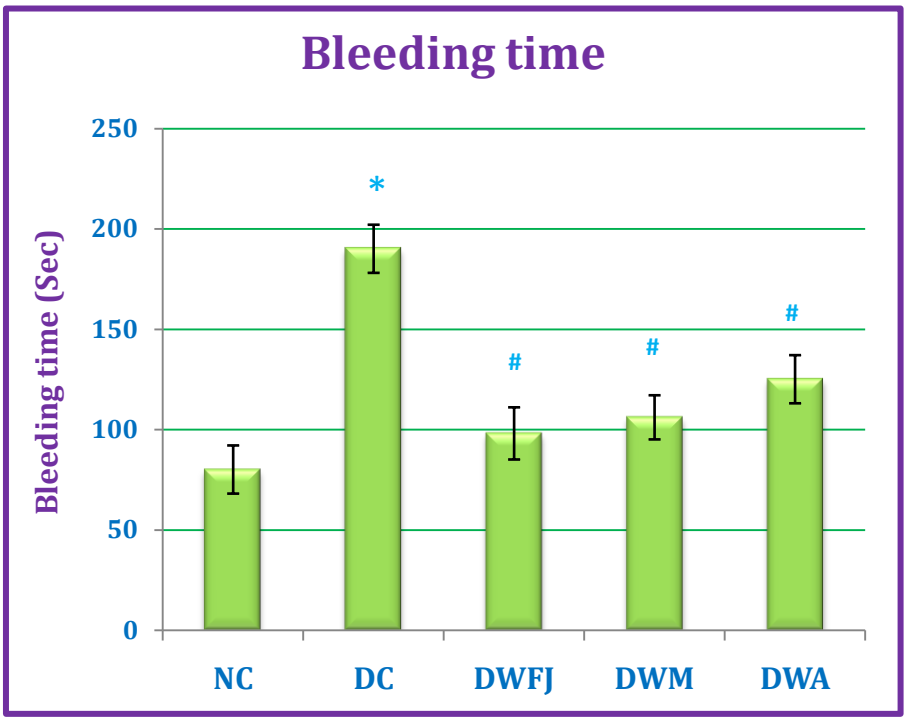
Bleeding time and clotting time

Disease control group rats which received busulfan showed significant increase in bleeding (190 ± 18 sec) and clotting time (390 ± 35 sec) as a result of reduction in platelet counts compared to normal healthy group rats (bleeding time- 80 ± 12 sec, clotting time- 130 ± 22 sec) indicating hemophilia and thrombocytopenia in animals. Treatment with fresh wheatgrass juice (bleeding time- 98 ± 13 sec, clotting time- 150 ± 23 sec), methanol extract (bleeding time- 106 ± 17 sec, clotting time- 196 ± 24 sec) and acetone extract (bleeding time- 125 ± 15 sec, clotting time- 214 ± 30 sec) produced significant reduction in bleeding and clotting time in disease suffering rats. Increases in bleeding and clotting time in rats were significantly prevented by treatment with fresh juice, methanol and acetone extract of wheatgrass. Thus wheatgrass seems to help in reducing bleeding and clotting time, near to normal. (Table 6.9.3)

Table 6.9.3: Effect of *Triticum aestivum* fresh juice, methanol and acetone extracts treatment on bleeding and clotting times on control and disease rats.

Blood parameters	Normal Control	Diseases Control	DWFJ	DWM	DWA
Platelet (10^3/πl)	905±102	523± 46*	804± 72#	761± 58#	708± 163#
Bleeding time(sec)	80 ± 12	190 ± 18*	98 ± 13#	106 ± 17#	125 ± 15#
Clotting Time (sec)	130 ± 22	390 ± 35*	150 ± 23#	196 ± 24#	214 ± 30#

Values are expressed as Mean ± S.E.M, *- significantly different from normal control ($p < 0.05$), # - significantly different from diseases control ($p < 0.05$), DWFJ- **D**iseases rats treated with **W**heatgrass **F**resh **J**uice, **D**WM- **D**iseases rats treated with **W**heatgrass **M**ethanol extract, **D**WA- **D**iseases rats treated with **W**heatgrass **A**cetone extract



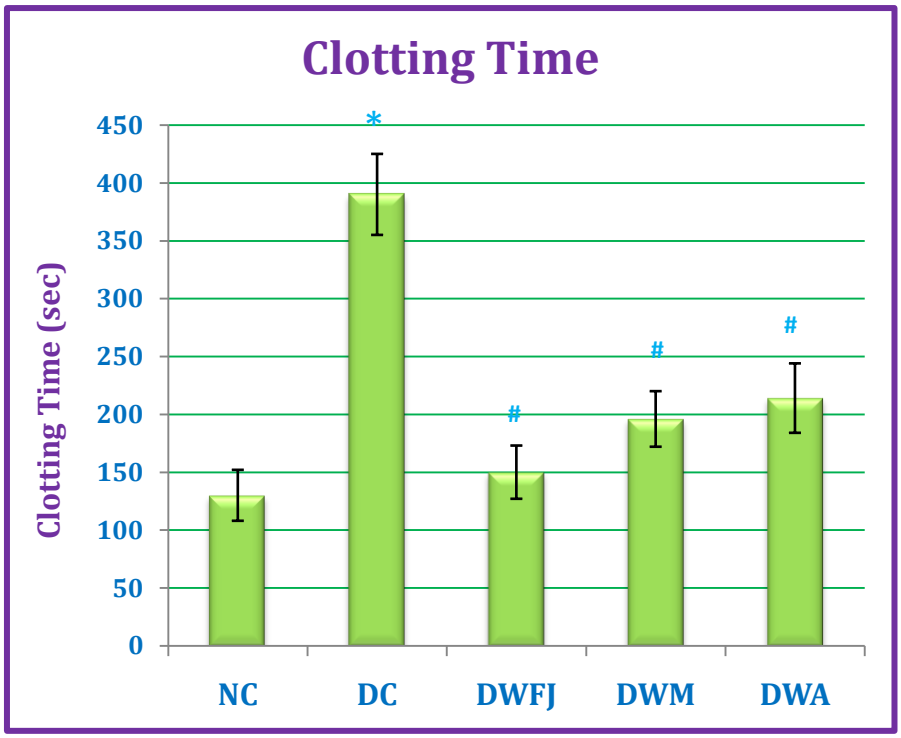


Figure 6.9.3: Effect of *Triticum aestivum* fresh juice, methanol and acetone extracts treatment on platelet count, bleeding and clotting times on control and disease rats.

Values are expressed as Mean ± S.E.M, *- significantly different from normal control ($p < 0.05$), # - significantly different from diseases control ($p < 0.05$), DWFJ- **D**iseases rats treated with **W**heatgrass **F**resh **J**uice, **D**WM- **D**iseases rats treated with **W**heatgrass **M**ethanol extract, **D**WA- **D**iseases rats treated with **W**heatgrass **A**cetone extract

6.10 Assessment of immunomodulatory activity of *Triticum aestivum*

For investigation of beneficial effects of wheatgrass on immune system, reduction in cyclophosphamide-induced neutropenia, Neutrophil adhesion test and carbon clearance test, were used in our study.

Carbon clearance assay

Carbon clearance test was carried out to evaluate effect of drugs on the reticulo-endothelial system (RES). It is a diffuse system of phagocytic cells, comprising of fixed tissue macrophages and mobile macrophages. The phagocytic cells in this system comprise of mononuclear phagocyte system (MPS). Macrophages are the major differentiated cell in MPS. Cells of the RES and MPS are known to be important in the clearance of particles from bloodstream. When colloidal ink containing carbon particles is injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation (Das M et al., 1998, Gokhale et al., 2003). Water and methanol extracts of wheatgrass showed significant increase in phagocytic index. Hence, we infer that wheatgrass may stimulate the reticuloendothelial system. (Table 6.10.1).

Table 6.10.1: Effect on phagocytic index in carbon clearance assay.

Treatment	Phagocytic index
Vehicle (1ml/kg, po)	0.0121 ± 0.0019
Water extract	0.0345 ± 0.0038*
Methanol extract	0.0685 ± 0.061*

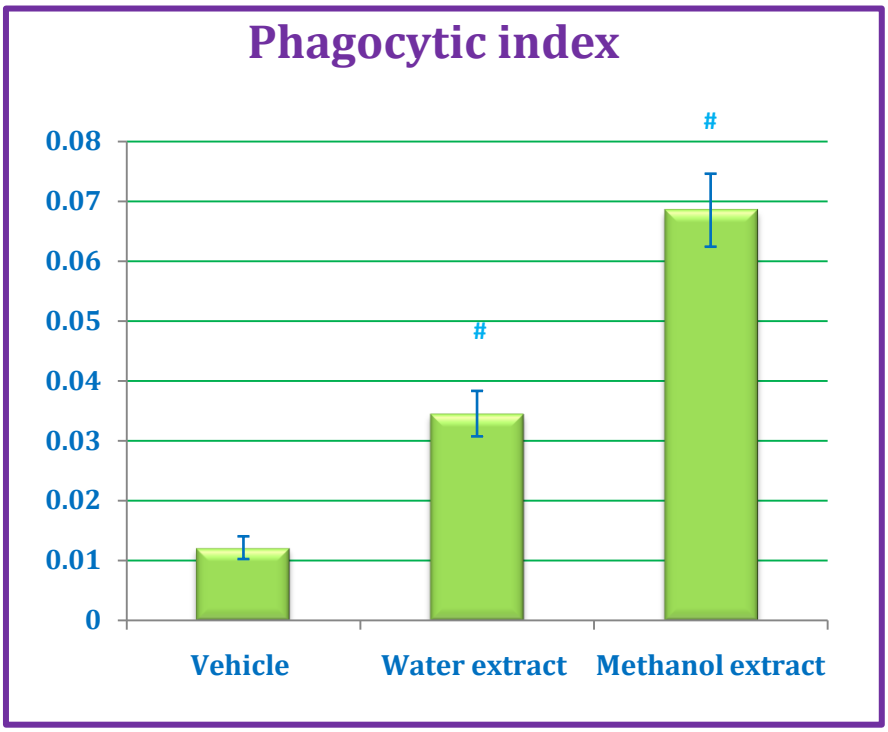


Figure 6.10.1: Effect on phagocytic index in carbon clearance assay.

All values are mean±SEM, n=5-6, *P<0.01 when compared to control group

Cyclophosphamide induced neutropenia

Cyclophosphamide belongs to nitrogen mustard subclass of alkylating agents and acts as an immunosuppressive agent by causing alkylation of DNA, in turn by interfering in DNA synthesis and function. It is also used extensively as an immunosuppressant (Thatte UM et al., 1987). Administration of cyclophosphamide (200 mg/kg, sc) produced a decrease in neutrophil count in all groups. Water extract of *Triticum aestivum* decreased neutrophil count significantly compared to control group. (Table 6.10.2)

Table 6.10.2: Effects of cyclophosphamide induced neutropenia.

Treatment	Total leukocyte count (cell/mm³)		Reduction in cell number	% reduction	% neutrophils	
	Before	After			Before	After
Vehicle (1 ml/kg, p.o)	5500 ± 365.95	2600 ± 223.23*	2900.00 ± 227.65	58.00	13.16 ± 1.01	7.50 ± 0.76*
OSE (100 mg/kg, po)	5041.65 ± 296.20	2783.32 ± 259.70*	2258.32 ± 315.80	44.80	12.65 ± 1.30	8.83 ± 1.35*
Water extract	5862.12 ± 156.20	3122.20 ± 215.41*	2739.08 ± 245.5	46.65	12.83 ± 0.98	9.33 ± 1.11*
Methanol extract	6332.30 ± 468.80	3332.20 ± 388.52*	3000.10 ± 358.52	52.64	16.16 ± 0.87	9.00 ± 1.00*

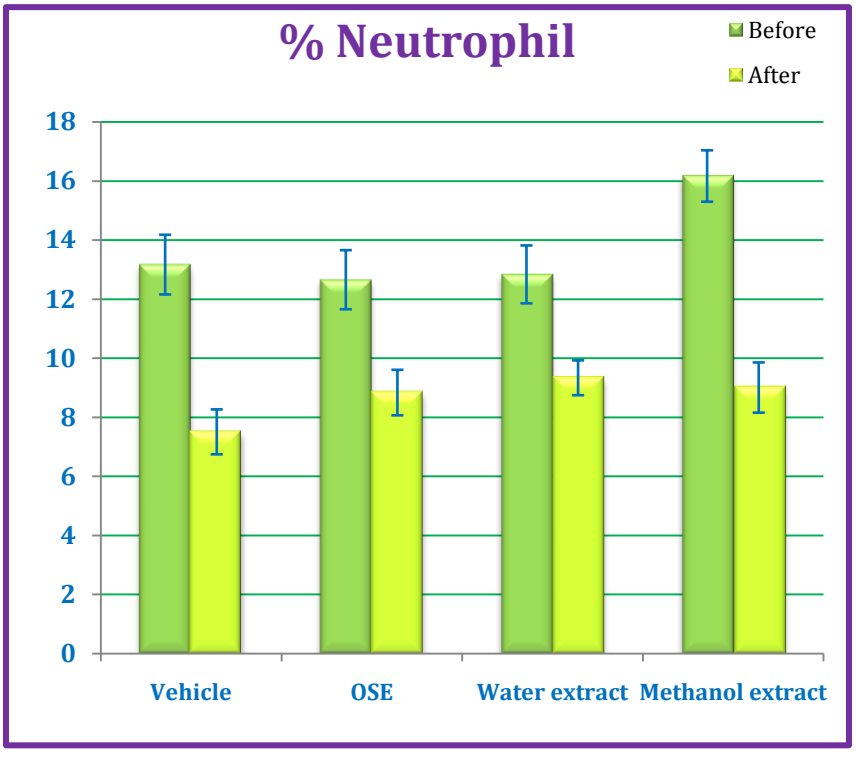


Figure 6.10.2: Effects of cyclophosphamide induced neutropenia.

All values are mean±SEM, n=5-6, *P<0.01 when compared to control group

Neutrophil adhesion test

When blood samples were incubated with nylon fibers, a reduction in neutrophil percentage due to the adhesion of neutrophils to the nylon fibers was observed. There was significant increase in neutrophil adhesion to nylon fibres and increase in macrophage induced phagocytosis in carbon clearance test along with reduction in cyclophosphamide induced neutropenia. The methanol extract of wheatgrass was more effective than the water extract. The increase in adhesion of neutrophil to nylon fibres indicates migration of cells from blood vessels and the number of neutrophils reaching the site of inflammation (Shinde UA et al., 1999). Increase in neutrophil adhesion to nylon fibres may be due to up regulation of β_2 integrins that are present on surface of neutrophils through which; they adhere firmly to nylon fibres. Hence, it can be inferred that wheatgrass causes stimulation of neutrophil migration towards the site of inflammation. Results of the present study also suggest that wheatgrass may stimulate cell mediated immunity. (Table 6.10.3)

Table 6.10.3: Effect on neutrophil adhesion in rats.

Treatment	Neutrophil (%)		Difference A-B
	UB (A)	NFTB (B)	
Vehicle (1ml/kg,po)	24.50 ± 0.85	20.65 ± 1.08	3.85 ± 0.47
OSE (100 mg/kg,po)	20.00 ± 1.86	10.32 ± 0.55	9.68 ± 1.58*
Water extract	22.32 ± 1.05	11.50 ± 1.40	10.80 ± 1.16*
Methanol extract	22.66 ± 1.05	10.66 ± 1.05	12.00 ± 0.78*

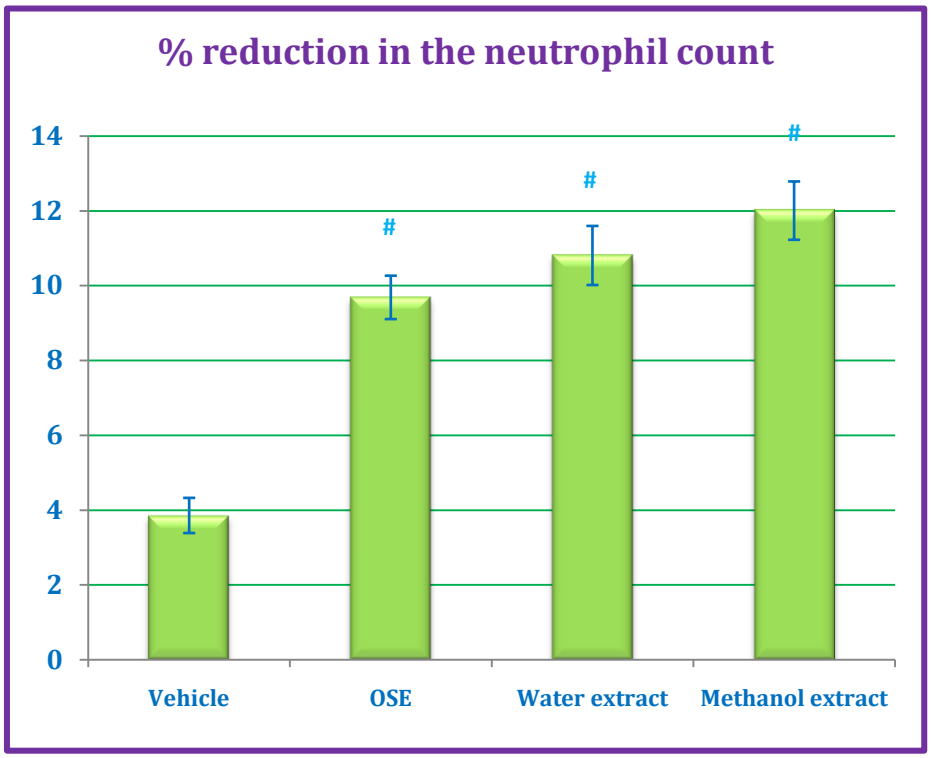


Figure 6.10.3: Effect on neutrophil adhesion in rats.

All values are mean±SEM, n=5-6, *P<0.01 when compared to control group

Thus, the results indicate beneficial effects of wheatgrass in iron overload condition, immnocompromised condition and thrombocytopenia. The active constituent isolated from wheatgrass also shows significant iron chelating potential.

7. DISCUSSION

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 the iron released from the breakdown of transfused red blood cells and the excess iron is deposited as hemosiderin and ferritin in the liver, spleen, endocrine organs and myocardium. 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 (Taher et al., 2006; Rund and Rachmilewitz, 2005; Loukopoulos, 2005).

Synthetic agents like desferrioxamine and deferiprone, used as chelators for the treatment of iron overload in thalassemia, are accompanied by serious side effects and certain limitaions including the need for parenteral administration, arthralgia, nausea, gastrointestinal symptoms, leucopenia, agranulocytosis and obviously the heavy cost. In addition, they are not suitable for use during pregnancy. Therefore the search for more effective and safer chelator for treatment of thalassemia and other blood disorders has become the need of the hour.

Hence, the major objective of the present project was to evaluate beneficial effect of wheatgrass on iron overload and to isolate, characterize and evaluate the active phytochemical constituent in wheatgrass, responsible for its iron chelating activity. For this aqueous, acetone and methanol extracts of wheatgrass were prepared. Among all the extracts, water extract produced maximum yield while, petroleum extract produced least yield. Flavanoid, phenoics and tannins were found to be present in water and methanol extracts, while acetone extract did not contain these constitutes. This was confirmed using shinoda and $FeCl_3$ in vitro qualitative tests. Phenolic compounds are known to possess chelating property (Ebrahimzadeh et al., 2008). Phenols are very important plant

constituents because of their scavenging ability due to their hydroxyl groups (Hatano T et al., 1989). Since, phenolic nature of a phytochemical constituent is closely related to its chelating activity, we decided to use detection method for phenolic compound as the method for detection and isolation of chelating compound in wheatgrass extracts.

In our investigation, water and methanol extracts of wheatgrass were subjected to in vitro quantitative determination of total phenolic contents, using FC method. There was higher amount of total phenolic content in methanol extract compared to water extract. Thus, methanol extract showed promise of maximum concentration of iron chelating compound among all three extracts.

In vitro quantitative estimation of iron chelating activity of various extracts of wheatgrass was carried out using Dinis et al. method.[8] Iron chelating activity of methanol extract was higher than that of water extract and significantly closer to desferoxamine, a standard drug used as a chelator in treatment of iron overload in thalassemia patients. This iron chelating property was found to be reversible, as confirmed by discoloration of red colored Fe^{2+}-extract complex on heating at above 60^{o} C. Thus, our in vitro investigations confirmed that methanol extract of wheatgrass has highest phenolic content and also highest concentration of iron chelating constituent among the three extracts.

To confirm in vitro findings about presence of iron chelator constituent, the water extract, methanol extract and acetone extracts of wheatgrass were subjected to in vivo testing on rats and their iron chelating activities were compared with that of desferoxamine. Iron overload was induced in rats by intra peritoneal injections of iron-dextran (12.5 mg/l00 g body wt.) evenly distributed over 30 days period, which resulted in condition of chronic iron overload that resembled thalassemia. [9] [10]

There was significant decrease in serum iron and serum ferritin levels in methanol and water extract treated animals, compared to untreated disease control group while, in acetone extract group there was no significant reduction in serum iron and serum ferritin levels, compared to untreated disease control group. However, rate of reduction was found to be higher in methanol extract

compared to water extract. Rate of reduction of serum iron and serum ferritin by methanol extract was near to desferoxamine treated group. Water and methanol extracts increased excretion of iron in urine and feaces which was found to be near to desferoxamine treated group. While in acetone group there was no significant increase in iron excretion. Thus, in vivo findings in our study suggest that possibly the same iron chelator constituent is present in both methanol and water extracts (probably in higher concentration in methanol extract) and that this constituent has comparable iron chelating activity to that of desferoxamine. The mechanism of this iron chelating activity seems to be through increase in excretion of iron in urine as well as in faeces and that the chelate complexation process with iron is reversible.

Isolation of active chelating constituent from wheatgrass was a crucial step in our project. Since, the results, obtained so far, revealed that maximum chelating activity was exhibited by methanol extract, we decided to process the methanol extract further for the purpose. A solvent system suitable for phenolic compounds was designed (methanol: water: acetone: glacial acetic acid (1:6:0.5:0.1), using which the methanol extract was subjected to TLC. All spots on the TLC plate were sprayed with 5 % ferric chloride solution. Since, phenolic compound gives black color with FeCl3, the spot of phenolic compound could be easily identified on the TLC plate. The compound was later, isolated in larger quantity, using the same solvent system and same color technique, by column chromatography. During purification process, on recrystallization, the compound gave needle-shaped crystals having melting point 215-218 ^{0}C. The purified isolated compound PI_1 was found to have R_f value 0.682. The total phenolic content present in this isolated compound was found to be 434.14 $\pm$ 28.02 µg Gallic acid equivalent phenol. Thus, a potential chelating compound was isolated from wheatgrass in sufficient quantity for estimation of in vivo activity through animal studies and for spectral characterization.

In vitro iron chelating power of the isolated compound was found to be 50% compared to standard iron chelator drug, desferoxamine. When subjected to in vivo study, the isolated compound significantly increased iron excretion in urine as compared to disease control group. The chelating power or efficacy of the compound was found to be 34.5% to that of desferoxamine.

The isolated iron chelator compound was subjected to LCMS and IR spcectroscopic analyses, for its molecular characterization. The compound was found to be aromatic in nature containing phenolic group.

Further, during estimation of iron chelating activity of wheatgrass extracts, blood samples collected from iron overloaded animals revealed some other beneficial effects of wheatgrass on blood composition, unrelated to its iron chelating activity.

While, there was significant decrease in hemoglobin content and RBC count in iron overloaded rats compared to normal control rats, these parameters were significantly increased in water extract group and methanol extract group, compared to disease control group at the end of 15 days and 30 days treatment.

Further, there was significant increase in total and differential WBC count in water and methanol extract groups compared to diseases control group. While in acetone extract these counts were not changed significantly. These data indicate that wheatgrass improves defense mechanism of body in iron overloaded patients.

Also, there was significant increase in thrombocyte count in water and methanol extract groups compared to diseases control group. While in acetone extract these counts were not changed significantly. Thus, data indicate that wheatgrass improves over all blood picture in iron overloaded patients.

There were significant increases in SGOT, SGPT, Serum creatinine and creatine kinase levels in disease control group indicating damage to various vital organs. In water extract treated group and methanol extract treated group all these parameters were significantly decreased as compared to diseases control group while in acetone extract treated group there was no significant change in these parameters compared to disease control group. These data suggest protective effect of wheatgrass on vital organs like liver, kidney and heart in iron over load condition.

In iron overload condition, oxidative stress is ultimately involved in dysfunction of vital organs including cardiovascular system (Shinar and Rachmilewitz, 1990; Hebbel et al., 1990; Grinberg et al., 1995). Antioxidant and other supportive therapies protect RBC against oxidant damage (Kukongviriyapan et al., 2008; Filburn et al., 2007). Also, a higher rate of LDL oxidation in thalassemia patients is due to a lower concentration of vitamin E and C in the LDL particles. Enrichment with vitamins E and C was effective in preventing LDL oxidation in patients with thalassemia (Rachmilewitz et al., 1979; Livrea et al., 1996). Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications and thereby improves quality of life and overall survival (Shinar and Rachmilewitz, 1990; Hebbel et al., 1990).

In our study, iron overloaded rats showed significant increase in OFRs, MDA level and decrease in SOD, catalase and glutathione levels in rat liver homogenates compared to normal rats. Treatment with methanol and water extracts of wheatgrass significantly reduced MDA level in the rat liver homogenates. There was significant improvement in GSH, SOD and catalase levels in iron overloaded rats treated with methanol and water extracts. There were no significant change in MDA or SOD, catalase and glutathione levels in acetone extract treated group. These data indicate strengthening of antioxidant defense by wheatgrass in iron overload condition.

For evaluation of effects of wheatgrass in thrombocytopenia, busulfan was used to induce experimental thrombocytopenia. Busulfan is an alkylating agent with myeloablative properties and activity against non-dividing marrow cells and possibly, non-dividing malignant cells. Intraperitoneal injection of busulfan produced, significant reduction of platelet count in wistar rats, resulting in severe bleeding tendency as found in thrombocytopenia. A significant loss of blood cells was evident in diseases control rats over the period of three weeks. Three weeks treatment with fresh wheatgrass juice (5 ml/kg, p.o.), methanol extract and acetone extract (100 mg/kg/p.o./day each) produced a significant increase in all blood cell counts. Treatment with fresh juice, methanol and acetone extracts showed significant increase in hemoglobin, RBC, total and differential WBC and platelet counts in pancytopenic rats as compared to disease

control group. Disease control rats showed significant increase in bleeding and clotting time indicating hemophilia and thrombocytopenia. Treatment with fresh juice, methanol and acetone extracts showed decrease in bleeding and clotting time period. Thus, data indicate beneficial effect of wheatgrass in thrombocytopenia.

Chronic treatment with fresh juice, methanol and acetone extracts increased hemoglobin and RBC count indicating therapeutic usefulness of wheatgrass in anemia and other hemoglobin and RBC related disorders.

For investigation of beneficial effects of wheatgrass on immune system, reduction in cyclophosphamide-induced neutropenia and carbon clearance test were used, in our study. Cyclophosphamide induces myelosuppression in the experimental animals. It belongs to nitrogen mustard subclass of alkylating agents and acts as an immunosuppressive agent by causing alkylation of DNA, in turn by interfering in DNA synthesis and function. It is also used extensively as immunosuppressant (Thatte UM et al., 1987). There was an increase in neutrophil adhesion to nylon fibres, increase in macrophage induced phagocytosis in carbon clearance test and reduction in cyclophosphamide induced neutropenia. The methanol extract of wheatgrass was more effective than water extract. The adhesion of neutrophil to nylon fibres indicates the migration of cells in the blood vessels and the number of neutrophils reaching the site of inflammation (Shinde UA et al., 1999). Increase in neutrophil adhesion to nylon fibres may be due to upregulation of β_2 integrins that are present on surface of neutrophils through which, they adhere firmly to nylon fibres. Hence, it can be inferred that wheatgrass causes stimulation of neutrophils towards the site of inflammation. The results of the present study also suggest that wheatgrass may stimulate cell mediated immunity.

Carbon clearance test is carried out to evaluate effect of drugs on the reticulo-endothelial system (RES). This is a diffuse system comprising of phagocytic cells, comprising of fixed tissue macrophages and mobile macrophages. The phagocytic cells in this system comprise of mononuclear phagocyte system (MPS). Macrophages are the major differentiated cell in MPS. Cells of the RES and MPS are known to be important in the clearance of particles from bloodstream.

When colloidal ink containing carbon particles are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation (Das M et al., 1998, Gokhale et al., 2003). Water and methnol extracts of wheatgrass showed significant increase in phagocytic index. Hence, we infer that wheatgrass may stimulate the reticuloendothelial system.

Thus, we conclude that regular intake of wheatgrass can be useful in management of iron overload diseases and can possibly, replace the current regime of painful, expensive and harmful drugs like desferoxamine or deferiprone. We have also, isolated a new iron chelator compound from wheatgrass. Further characterization as well as detailed toxicological and clinical studies of the iron chelator molecule, may provide a new chemical entity for better management of iron overload diseases like thalassemia. Our study also confirms, scientifically, the traditional use of wheatgrass in immnocompromised conditions and thrombocytopenia.

8. CONCLUSION

In nutshell, our investigation reveals following important inferences –

1. We have confirmed iron chelating activity of wheatgrass and its various extracts.
2. We have isolated, purified and characterized a potential iron chelating compound from wheatgrass. The chelating power of the compound was found to be 34.5% compared to desferroxamine.
3. The mechanism of iron chelating activity of wheatgrass seems to be the increase in excretion of iron through urine as well as faeces and that the chelate complexation process with iron is reversible.
4. Data of our study indicate that wheatgrass improves overall blood picture in iron overloaded patients by increasing Hb, RBC, WBC and platelet counts.
5. Our data suggest that wheatgrass protects vital organs like liver, kidney and heart in iron over load conditions like thalassemia.
6. The results of our study indicate strengthening of antioxidant defense of body by wheatgrass in iron overload condition.
7. Our data indicate potential beneficial effect of wheatgrass in thrombocytopenia.
8. Our results indicate that wheatgrass possesses immunomodulatory activity through stimulation of reticuloendothelial system and cell mediated immunity.

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10. ANNEXURE-I

CPCSEA approval certificate for conduction of project

R. K. COLLEGE OF PHARMACY
Kasturbadham, Rajkot - 360 020

CERTIFICATE

This is certify that the research project no. **RKCP/COL/RP/09/02** entitled **"Investigation into mechanism of action and pharmacological evaluation of various extracts of *Triticum aestivum* (Wheat) grass with special reference to its beneficial effects on iron overload and blood disorders"** has been approved by IAEC committee during meeting on 7th March 2009.

Dr. T. R. Desai
Principal and Chairperson - IAEC
R. K. College of Pharmacy

7/3/09
Dr. K. B. Patel
CPCSEA nominee

11. ANNEXURE-II

Research paper publications in various international journals

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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.

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Immunomodulatory Effect of Hydroalcoholic Extract of *Triticum aestivum* on Laboratory Animals

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ABSTRACT:

The immunomodulatory effect of hydroalcoholic extract of *Triticum aestivum* (Gramineae), commonly known as wheat grass was studied at two doses - 5 mg/kg and 50 mg/kg. The effect was studied in different animal models including neutrophil adhesion test, carbon clearance assay and cyclophosphamide induced neutropenia. Wheat grass hydroalcoholic powder extract showed neutrophil adhesion to nylon fibers, produced a significant increase in phagocytic index and a significant protection against cyclophosphamide induced neutropenia indicating its effect on cell mediated immunity and humoral immunity. From the above results, it was concluded that hydroalcoholic extract of wheat grass powder has a significant effect on immune system.

Keywords: *Triticum aestivum,* cyclophosphamide, phagocytic index, cell mediated and humoral immunity.

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RESEARCH ARTICLE

Pharmaceutical Sciences

INVESTIGATION INTO THERAPEUTIC ROLE OF *TRITICUM AESTIVUM* (WHEAT) GRASS IN BUSULFAN INDUCE THROMBOCYTOPENIA

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ABSTRACT

Triticum aestivum, wheatgrass is a cereal grass of Gramineae (Poaceae) family has been traditionally used, to treat various diseases like cancers, diabetes, gastritis, ulcers, pancreas and liver problems, anemia, skin problems and constipation etc. 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 B12, iron, folic acid, pyridoxine and many other minerals, amino acids and enzymes, which have significant nutritious and medicinal value. Wheat grass is a rich source of phenolic and flavanoid content. Thrombocytopenia is a lower than normal number of platelets in the blood. Platelets play an important role in clotting and bleeding. The present study was planned to evaluate beneficial effects *T. aestivum* on busulfan induced thrombocytopenic animals. We have made the unexpected observation that fresh juice, methanol and acetone extracts of *T. aestivum* significantly increase Hb levels, RBC, total WBC and differential WBC counts in pancytopenic rats. Wheatgrass significantly increase platelet counts and reduce bleeding and clotting time in thrombocytopenic rats. In conclusion, our data suggest *Triticum aestivum* possess beneficial effect in thrombocytopenia and pancytopenia conditions by increase platelet counts. In addition it also produces immunostimulant effects.

KEY WORDS *Triticum aestivum,* thrombocytopenia, pancytopenia

List of research papers excepted in various internatational journals

Sr. No.	Title of research paper	Name of Journal	Remarks
1.	Investigation into iron chelating activity of Triticum aestivum (wheat grass) in iron-dextran induce iron overload model of thalassemia	Journal of Pharmacy Research ISSN No. 0974-6943	Impact factor - 1.09
2.	Investigation into beneficial effects of *Triticum aestivum* (wheat grass) in iron overload complications	Pharmacology online ISSN No. 1827-8620	-