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(CGPA 2.93)

Desai, Tusharbindu R., 2005, *Invetigation into the Mechanism of Action and Effects of Triticum Aestivum (Wheat) Grass,* thesis PhD, Saurashtra University

http://etheses.saurashtrauniversity.edu/id/eprint/188

INVESTIGATION INTO
THE MECHANISM OF ACTION
AND EFFECTS OF
TRITICUM AESTIVUM (WHEAT) GRASS

A Thesis Submitted To
SAURASHTRA UNIVERSITY
For The Award Degree Of
DOCTOR OF PHILOSOPHY
IN PHARMACOLOGY
(Faculty Of Medicine)

By

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April 2005

Statement under ordinance Ph. D. 7 of Saurashtra University

The contents of this thesis are my own work carried out under the supervision of Prof. Ramesh K. Goyal and leads to some contribution in pharmacy, supported by necessary references.

(Tusharbindu R. Desai)

C E R T I F I C A T E

This is to certify that the thesis entitled "**Investigation Into The Mechanism Of Action And Effects Of *Triticum Aestivum* (Wheat) Grass**" represents the bonafide work of **MR. TUSHARBINDU RAMESHCHANDRA DESAI,** carried out under my guidance and supervision. The above-mentioned work was carried out in **B. K. MODY GOVT. PHARMACY COLLEGE, RAJKOT** during the period of years 2001-2005. The work is up to my satisfaction.

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ACKNOWLEDGEMENTS

Date: April, 2005   **(Tusharbindu R. Desai)**

Dedicated To
My Illustrious Father
(Shri R. A. Desai)

CONTENTS

1. ABSTRACT

Over one-third of the population in developing countries lack access to essential medicines. According to WHO report, over 80% of the world population relies on traditional medicine largely plant based for their primary healthcare needs, especially in the under developed and developing countries, because of better cultural acceptability, better compatibility with the human body and lesser side effects. The provision of safe and effective herbal therapies could become a critical tool to increase access to health care. However, in the last few years there has been a major increase in their use in the developed world. In Germany and France, many herbs and herbal extracts are used as prescription drugs. About 25% of modern medicines are descended from plants first used traditionally. The Indian systems of medicine have identified 1500 medicinal plants, of which 500 species are mostly used therapeutically. The Ayurved drug market alone is to the order of Rs. 3500 crores. Besides this, there is also a growing demand for natural products including items of medicinal value/pharmaceuticals, food supplements and cosmetics in both domestic and international markets. Presently, India's export from medicinal and herbal plants is Rs.3000 crores. India, with its diversified biodiversity has a tremendous potential and advantage in this emerging area. India is sitting on a gold mine of well-recorded and well-practiced knowledge of traditional herbal medicine. But, unlike China, India has not been able to capitalize on this herbal wealth by promoting its use in the developed world despite their renewed interest in herbal medicines. This can be achieved by judicious product identification based on diseases found in the developed world for which no medicine or only palliative therapy is available; such herbal medicines will find speedy access into those countries. Backward integration from market demands will pay rich dividends. Strategically, India should enter through those plant-based medicines, which are already well accepted in Europe, USA and Japan. Simultaneously, it should identify those herbs (medicinal plants), which are time-tested and dispensed all over in India.

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. 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. 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 ''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. 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, which have significant nutritious and medicinal value.

Anemia is a hematological condition in which there is quantitative deficiency of circulating hemoglobin, often accompanied by a reduced number of red blood cells. Further, causes of anemia are blood loss, impaired erythropoiesis and abnormal erythrocyte destruction. Nutritional deficiencies (iron, vitamin B_{12} and folic acid) are the most common cause of anemia through out the world (Brown 1991).

Thalassemia is one of the most common groups of genetic blood disorder. Countries like Italy, Greece and Cyprus have the highest frequency of Thalassemia cases in the world. There exists a Thalassemic "belt" that includes countries like Turkey, Iran, Afghanistan onto Pakistan and India. 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 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%). Very high rates have been found in certain communities such as Sindhis 12.4% and in Lohana Gujratis 13.6 % (Ambekar et al., 2001).

The thalassemias ($\propto$ and β) 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. The fact that there are only two genes for the beta chain of hemoglobin makes ß-thalassemia simpler to understand. The β-globin gene is present, but produces little β-globin protein. The degree of suppression varies. 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. 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). Management of ß-thalassemia major (*Cooley's Anemia)* requires patients to have life-long regimen of regular blood transfusions coupled with iron chelation therapy. 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.

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Generally, regular blood transfusions and iron chelation treatment with desferrioxamine are initiated early in life; therefore, the patients and their families have to sustain regular treatment throughout their childhood, adolescent, and adult years. The release of hemoglobin, during hemolysis, and the subsequent therapeutic transfusion lead to systemic iron overloading that further potentiates the generation of Reactive Oxygen Species (ROS) and consequent oxidative stress, in thalassemia. The iron-induced liver damage in thalassemia may play a major role in the depletion of lipid-soluble antioxidants. Data indicate that dietary magnesium supplementation improves some of the characteristic cellular function abnormalities of β-thalassemia intermedia.

Thus, in thalassemia, there is a vicious cycle of iron overload leading to oxidative stress with consequent increase in hemolysis and increase in blood transfusion requirement causing further iron overload and its toxicities on other organs. Replenishing magnesium and antioxidants by administration of wheatgrass can break this vicious cycle.

Some research workers have studied chlorophyll, one of the major ingredients present in wheatgrass. The chemical similarity between hemoglobin and chlorophyll was first suggested by Verdel (1855). 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. The deficiency of magnesium in serum or erythrocytes has also been reported in human β-thalassemia. These deficiencies may play a significant role in various cellular abnormalities characteristic of this disorder. The iron-induced liver damage in thalassemia may play a major role in the depletion of lipid-soluble antioxidants like vitamin A and vitamin E. Considering the above facts, we hypothesized that major ingredients of wheatgrass like chlorophyll, beta-carotene, vitamin A, vitamin E, vitamin C, selenium, zinc, magnesium, iron, folic acid and vitamin B_{12} may be useful in treatment of anemia and ß-

thalassemia. Hence, we have undertaken clinical studies to evaluate the effects of wheatgrass in patients with anemia and thalassemia.

Traditionally, wheatgrass has been used as an adjunct in treatment of cancer. It has also been suspected that wheatgrass is useful as anticancer preparation by virtue of its several components like chlorophyll, P_4D_1 compound, abscisic acid and laetrile (vitamin B_{17}). Chlorophyllin, which is obtained by hydrolysis of chlorophyll to remove phytyl alcohol, is an efficient antimutagenic agent and has been used as a dietary supplement or to diminish the intensity of the discomforting side effects of chemopreventive therapy. More recently, the cancer chemopreventive properties of chlorophylls have come to be recognized. Chlorophyll has been reported to exhibit anti-mutagenic activity in short-term genotoxicity assays.

Apart from the use of wheatgrass in cancer, it has been recommended that the topical application of wheatgrass juice is useful for treatment of skin infections. It has also been claimed that wheatgrass juice may have antibacterial activity and that chlorophyll limits the growth of many types of germs not by directly killing them, but by providing an environment, which interferes with their growth especially against anaerobic bacteria, those that do not require oxygen.

As mentioned above, fresh wheatgrass has been proposed to be used as a juice, which is prepared in a mixer/blender with addition of little water followed by filtration through a cloth. In a chronic disease like thalassemia, the drug treatment is of long duration, may even be for years. In such a circumstances the factor of patient compliance becomes very important. Outcome of the therapy will largely depend upon regular supply (round the year and in all seasons) and acceptability of the drug by patient. As a pharmaceutical scientist, preparation of a suitable dosage form is prime area of research in the development of new drug formulations. Preparation of wheatgrass tablets was a challenge for us. We attempted to use different drying techniques such as freeze drying, spray drying and shade drying to prepare dried wheatgrass powder. It is well known that the stability of components present in

wheatgrass, like chlorophyll, beta-carotene, vitamin A, vitamin E, vitamin C etc, are adversely affected upon exposure to changes in air, light, humidity and temperature. Since, all of the above mentioned drying processes involve exposure of wheatgrass to some of these variables, potency of wheatgrass powder obtained at the end of each drying process might be different, consequently affecting the therapeutic effectiveness and out come of the clinical trial. Hence, we also evaluated the potency of dried wheatgrass powder, using UV spectra and HPTLC methods. It is well known that the contents of an herbal drug can be affected by changes in species variety and choice of soil or fertilizer used. In order to ascertain the most therapeutically potent verity of Wheat and best type of fertilizer to grow wheatgrass, we carried out UV spectral and HPTLC analysis of different verities of wheat grown in similar soil/fertilizer and same variety of wheat grown in different soils/fertilizers.

In nutshell the objectives of the present project were -
1. *To carry out pharmacognostic study and phytochemical standardization of different verities of Triticum grass (wheatgrass) and crude extract using HPTLC fingerprinting.*
2. *To evaluate clinical effects of wheatgrass in the treatment of anemia, β - thalassemia (major), cancer and bacterial diseases.*
3. *To prepare tablets of wheatgrass and to evaluate the most suitable method of drying the wheatgrass for the same.*
4. *To standardize various formulations of wheatgrass by HPTLC.*
5. *To evaluate effect of wheat variety and fertilizer on potentially therapeutic contents in wheatgrass.*

In our investigation, certified samples of three major species of wheat viz. *Triticum aestivum*, *Triticum durum* and *Triticum dicoccum* were acquired form the Wheat Research Center, Gujarat Krushi University, Junagadh, Gujarat. Adequate quantity of unpolished wheat grain of these varieties were soaked overnight in water and were grown in plastic trays filled with soil, on the next day. Trays were watered adequately everyday, for 8 days. On 9th day the

wheatgrass was harvested. To characterize and differentiate among the three varieties of wheatgrass, all three varieties of the grass were subjected to microscopic study, which included transverse sections, surface preparations and powder study. These wheat varieties were grown in plastic trays as per the standard procedure. Microscopic studies of transverse sections, surface preparations and powder studies of the three species of wheatgrass were conducted 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). The leaf blade was linear and parallel –veined with mid rib projecting on the back continuing someway along the sheath. In *T. Dicoccum* the hairs on the swollen base of leaf were longer than those of other species. The longest leaves were possessed by *T. durum*. 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 vary much in length and stoutness. On the leaves of *T. aestivum,* ample numbers of hair were present, while in *T. dicoccum* and *T. durum* they were sparsely distributed on the surface of the leaf. 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 stomatal 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 except in *T. durum*. The pharmacognostic characteristics observed in our study were in confirmation with that reported in the literature.

Phytochemical analyses of dried wheatgrass powder revealed that wheatgrass contains protein (25.2 %), carbohydrates (52.4 %), chlorophyll (5.12 %), iron (43.5 mg/100 gm), magnesium (1455 mg/100 gm), zinc (97.8 mg/100 gm), beta-carotene (10.9075 I. U./100 gm), insoluble dietary fiber (64.54 %) and fat (1.75 %). Our quantitative analyses revealed that wheatgrass is a rich source of chlorophyll, various minerals like iron, magnesium, calcium, phosphorus, antioxidants like beta carotene and insoluble dietary fibers while being low in fat content.

Three different techniques were adapted to prepare dried wheatgrass powder including spray drying, freeze drying and shade drying. In freeze-drying technique, fresh wheatgrass was frozen to sub-zero temperature and subsequently subjected to low-temperature heating (10° C -15° C) in vacuum to evaporate crystallized water content. Dried wheatgrass was then milled to obtain powder. In spray drying technique, fresh wheatgrass was pressed in a hydraulic press to obtain juice. The juice was then sprayed in aerosol form through a nebulizing nozzle in a conical vessel from top. A hot air counter current (55° C) was passed from bottom of the vessel. The nebulized juice settled on the bottom of the vessel in the form of powder. In shade drying technique, fresh wheatgrass was dried at room temperature in a well-ventilated dark room. The dried wheatgrass after 3-4 days of drying period was powdered in a mill.

Samples of different methanolic extracts of wheatgrass and methanolic solutions of reference standard beta-carotene were used for TLC. Suitably diluted sample solutions were spotted on pre-coated silica gel 60 F_{254} TLC plates (E. Merck) using CAMAG Linomat IV Automatic Sample Spotter. The plates were developed in the solvent system consisting of Hexane: Acetone

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(65:35). The plates were dried at room temperature and scanned using CAMAG TLC scanner 3 at UV 254 and 354 nm and Rf values, absorption spectra of resolved bands were recorded. In all, 13 spots were observed at different R_f values viz. (1) 0.07(Light green), (2) 0.21(Yellow), (3) 0.27(Grey), (4) 0.35(Yellow), (5) 0.40(Yellow), (6) 0.47(Light green), (7) 0.54(Yellow), (8) 0.57(Green), (9) 0.61(Blue green), (10) 0.64(Light gray), (11) 0.69(Light gray), (12) 0.74(Grey), (13) 0.92(Orange). By comparing standard Rf values and colors reported in literature, out of 13 spots representing 13 different components of wheatgrass, seven components at spot number 4, 7, 8, 9, 11, 12 and 13 were identified as xanthophyll c, xanthophyll a, chlorophyll b, chlorophyll a, pheophytin b, pheophytin a and beta-carotene respectively. In comparative TLC study, chlorophyll a was found to be present in significant quantity in fresh wheatgrass and was absent in rest of the formulations. Intense grey-colored spots of pheophytin compensated the absence of chlorophyll in these formulations, as pheophytin is the degradation product of chlorophyll. Except chlorophyll a, 12 components were found present in shade-dried powder. Spot no. 4, 5, 6, 9 and 10 were absent in freeze-dried and spray-dried powders. Beta-carotene was present in all formulations. *Thus shade drying, the natural method of drying, was found to preserve all components of wheatgrass and hence, the most suitable one.*

In qualitative HPTLC chromatograms of methanolic extracts of Standard β Carotene, fresh wheatgrass, shade-dried wheatgrass powder, freeze-dried wheatgrass powder and spray dried wheatgrass powders were subjected to HPTLC study. The chromatograms were scanned in combined as well as individual modes at 254 nm and 354 nm wavelengths. Findings of qualitative HPTLC confirmed the patterns obtained in TLC study. Shade drying, the natural method of drying, was found to preserve all components of wheatgrass. Individual spots (components), in chromatogram of shade-dried powder, were scanned at 254 nm wavelength to obtain their spectra for the purpose of identification of these components of wheatgrass in further investigations.

While, qualitative HPTLC was carried out on extracts of wheatgrass formulations at maximum concentration (for detecting presence of various constituents), for quantitative evaluation the methanolic extracts of equivalent amounts of these formulations were subjected to quantitative HPTLC analyses along with those of different species of wheatgrass. Chromatograms of the formulations or species were recorded. Area under curve (AUC) of different components were measured in chromatogram of each formulation or species and analyzed for comparison with that of fresh wheatgrass. Since all the constituents of wheatgrass could not be identified or were not present in all chromatograms, the AUCs of only chlorophyll, pheophytin and beta-carotene were used for the purpose of comparison of contents among formulations of wheatgrass.

The quantitative HPTLC analyses of wheatgrass grown from three major wheat species revealed that _Triticum aestivum_ had lowest contents (76.8%) as compared to the contents of _Triticum durum_ (86.92%) and _Triticum dicoccum_ (100 %). Thus, it seems that wheatgrass grown from tetraploid species of wheat, especially _Triticum dicoccum_ (local variety called as DDK) and _T. durum_, is more suitable for medicinal use as compared to the hexaploid species i.e. _T. aestivum_ or the common wheat.

To determine the effect of fertilizer on quality of wheatgrass, same species of wheat i.e. _T. durum_ was grown separately in plain soil, in compost fertilizer and in organic fertilizer. When subjected to quantitative HPTLC analyses, contents of wheatgrass grown in organic fertilizer (91.36%) and that of wheatgrass grown in compost fertilizer (92.05%) were nearly similar while the contents of wheatgrass grown in plain soil (100%) were highest among the three. Thus, presence of a fertilizer does not seem to hamper or interfere much with the sensitive phytochemical processes being carried out in the young growing grass in early stages of growth.

It is well known that the stability of components present in wheatgrass, like chlorophyll, beta-carotene, vitamin A, vitamin E, vitamin C etc, are likely to be adversely affected upon exposure to changes in air, light, humidity and

temperature. This fact was confirmed by the results of our analyses. When subjected to quantitative HPTLC analyses, wheatgrass tablets of spray-dried powder was found to have lowest contents (21.66%) as compared to the contents of tablets of freeze-dried powder (46.68%) and shade-dried powder (87.75%) considering the contents of methanolic extract of fresh wheatgrass as 100%. *Hence, it may be concluded that the most suitable method of preparing dry wheatgrass powder, among the above three techniques, is shade drying technique.*

Traditionally, wheatgrass juice is prepared by grinding fresh wheatgrass in mixer/grinder adding little water that is subsequently filtered through a cloth. When subjected to quantitative HPTLC analyses the fresh wheatgrass juice, prepared by traditional method, was found to have 96.7% contents considering the contents of methanolic extract of fresh wheatgrass as 100%. *Thus, the traditional method i.e. fresh wheatgrass juice seems to be the best method for wheatgrass therapy compared to any formulations of wheatgrass that were made in our study.*

The *in-vitro* antibacterial activity profiles of undiluted wheatgrass juice as well as acetone and methanolic extracts of wheatgrass were studied by agar–well diffusion method. Acetone and methanolic extracts did not show any kind of antibacterial activity while, fresh and undiluted wheatgrass juice exhibited mild antibacterial activity against *Staphylococcus* aureus, *Bacillus cereus, Salmonella typhimurium* and *Kleibsella pneumoneae* at 2.7 gm/ml concentration. In terms of zone of inhibition, the diameter of the zone was found to be 12.1, 12.4, 13.2 and 12.2 % respectively. It is known that availability of iron increases host susceptibility to *K. pneumoniae* infection and that thalassemic patients are chronic sufferers of iron overload. Thus, the antibacterial activity of wheatgrass could be beneficial for thalassemic patients, in addition to its clinical effects on blood picture. Investigation in to antibacterial profile of wheatgrass revealed two important features. The antibacterial activity was exibited only by undiluted extract of wheatgrass. Further, this antibacterial activity was lost within 2 hours of preparing the

extract. This leads us to conclude that topical application of only fresh and undiluted wheatgrass extract could be beficial in treatment of skin infections.

The antiproliferative activity of wheatgrass was studied by comparing seed germination rate of green grams, groundnuts and wheat in presence of distilled water (control) and wheatgrass juice. Presence of wheatgrass juice reduced rate of wheat germination by 50% while those of green gram and groundnut were reduced by 70% and 90% respectively. The strong antiproliferative activity exibited by wheatgrass could be a clue to its potential anticancer activity. Chlorophyll, abscisic acid and laetrile present in wheatgrass may contribute to this activity.

To assess clinical effects of wheatgrass on anemia an open clinical trial of wheatgrass on patients with anemia was carried out at Ayurved Hospital, working under Civil Hospital, Rajkot. Necessary permission for conducting the clinical trial was obtained from the concerned ethical committee. Twenty anemic patients (age 20 to 50 years) were enrolled for the trial after taking informed consent. The patients were given wheatgrass tablets with dosage regimen of 2 tablets (shade-dried wheatgrass powder 250 mg.) 3 times in a day for 1 month. Blood samples were collected before and at the end of period of clinical trial (i.e. 1-month period). To assess effect of wheatgrass on haemopoietic activity, hemoglobin gm%, RBC count, WBC count were recorded while, to assess effect of wheatgrass on defense mechanism neutrophil count, lymphocyte count and total lymphocyte count were recorded. After 1-month treatment with wheatgrass, hemoglobin content (Hb gm%) was found to be significantly ($P<0.05$) increased (before treatment: 11.75 ± 0.245, after treatment: 12.63 ± 0.307). RBC count was significantly ($P<0.05$) decreased (before treatment: 4.72 ± 0.132, after treatment: 4.37 ± 0.087). The decrease in RBC count indicated that wheatgrass might have suppressed haemopoietic process. Rise in hemoglobin content indicated stimulation of hemoglobin synthesis in individual RBC, probably by replenishment of deficient nutritional factors like iron. It is known that the major cause of anemia, in absence of any primary disease, is malnutrition. WBC count

(before treatment: 8615 ± 593, after treatment: 7725 ± 440), neutrophil count (before treatment: 62.2 ± 1.51, after treatment: 60.8 ± 1.7), lymphocyte count (before treatment: 32.25 ± 1.62, after treatment: 33.7 ± 1.86) and total lymphocyte count (before treatment: 2661.5 ± 83, after treatment: 2483 ± 120) were not affected significantly (P<0.05). This showed that either wheatgrass had no effect on body's defense mechanism or the duration of treatment i.e.1 month, was insufficient for such an effect.

To assess clinical effects of wheatgrass on β-thalassemia (major) a randomized clinical trial of wheatgrass on β-thalassemia was carried out at K. T. Children Hospital working under Civil Hospital, Rajkot. Necessary permission for conducting the clinical trial was obtained from the concerned ethical committee. Thalassemic patients, visiting K. T. Children Hospital regularly for blood transfusion and registered at the hospital were enrolled for the trial, after taking informed consent. The patients were given wheatgrass tablets with dosage regimen of 2 tablets (wheatgrass powder 250 mg.) 3 times in a day for 9 months. Blood samples were collected at the start, after 6 months and at the end of period of clinical trial. The parameters recorded were Hemoglobin content, Total RBC count, PCV, MCV, MCH, MCHC, Total WBC count, Neutrophil count, Eosinophil count, Basophil count, Lymphocyte count, Serum iron, Serum ferritin, Serum magnesium and TBARS.

After 9 months treatment with wheatgrass, Hemoglobin gm% in normal-range subgroup (i.e. Male-13.5-18 gm%, F–12-16 gm%) was significantly decreased (before treatment: 12.87 ± 0.10, after treatment: 10.77 ± 0.36). RBC count in normal-range subgroup (Male-4.5-6.5/cmm, Female-4.2-5.4/cmm) was also significantly decreased (before treatment: 4.55 ± 0.13, after treatment: 3.74 ± 0.20). This may be an indication of haemopoietic suppression effect of wheatgrass.

Reticulocyte count in abnormal-range subgroup was significantly decreased (before treatment: 10.8 ± 2.55, after treatment: 2.8 ± 1.21). The chronic hypoxic condition in thalassemia, which may occur due to persistent low

hemoglobin content of blood, stimulates secretion of erythropoietin and is reported to increase the ineffective erythropoiesis and reticulocyte count. Decrease in reticulocyte count observed in our study, after wheatgrass treatment, indicates an increase in the tissue oxygenation and decrease in ineffective erythropoiesis.

Mean corpuscular volume (MCV) in normal-range subgroup was significantly increased (before treatment: 86.26 ± 1.06, after treatment: 77.02 ± 2.58) and that in abnormal-range subgroup was also significantly increased (before treatment: 77.63 ± 0.94, after treatment: 82.99 ± 1.74). Mean corpuscular hemoglobin concentration (MCHC) in abnormal-range subgroup was significantly increased (before treatment: 28.85 ± 1.06, after treatment: 32.63 ± 1.06). Positive changes in both these parameters reflect correction of both hypochromia and microcytosis, thus exhibiting reversal of abnormal condition in thalassemia. In our investigation, the increase in MCHC i.e. increase in hemoglobin content of individual RBC was possibly offset by haemopoietic suppression effect of wheatgrass i.e. decrease in number of RBC. Hence, the reduction in overall transfusion requirement was not observed after 9 months treatment with wheatgrass. This further supports a positive effect of wheatgrass in thalassemia.

After 9 months treatment with wheatgrass, various parameters in abnormal-range subgroup, including platelet count (before treatment: 199000 ± 126572.1, after treatment: 258000 ± 154149.3), WBC count (before treatment: 13875.0 ± 2902.8, after treatment: 16057.1 ± 2033.11), neutrophil % (before treatment: 51.69 ± 2.04, after treatment: 54.23 ± 3.39), lymphocyte % (before treatment: 40.00 ± 1.83, after treatment: 40.53 ± 2.00) and total lymphocyte count (before treatment: 5274.00 ± 916.94, after treatment: 6968.29 ± 1364.91) were not affected to any significant level, while eosinophil count (Eosin %) was significantly decreased (before treatment: 3.22 ± 1.14, after treatment: 0.33 ± 0.22). Eosinophilia is seen in allergic disorders. Thalassemic patients on regular blood transfusion therapy are subject to various allergens hence, eosinophilia is commonly found in thalassemic patients. Thus, reversal

of eosinophilia may be an indicator of much acclaimed detoxifying or antiallergic property of wheatgrass.

Treatment with wheatgrass did not produce any effect on serum iron, serum magnesium and TBARS. These parameters were included to investigate probable mechanism of action of wheatgrass. Thus, our data indicate that although wheatgrass treatment exhibits beneficial therapeutic effects in thalassemia, the mechanism of action is neither iron or magnesium supplementation nor replenishment of antioxidants.

As wheatgrass contains substantial quantity of iron that is contraindicated for thalassemic patients, the major apprehension expressed by patients and doctors, at the outset of this study, was the possibility of increase in iron load after wheatgrass treatment. Since, the plasma or serum ferritin is the most commonly used indirect estimate of body iron stores, we measured serum ferritin levels in thalassemic patients. In our investigation serum ferritin level, against all predictions, was significantly decreased at the end of 9 months treatment with wheatgrass. In absence of substantial rise in hemoglobin content, the only possible explanation for decreased iron load is increased excretion of iron. Thus, the most notable outcome of our investigation, apart from ascertaining the ability of wheatgrass to increase hemoglobin content, is the detection of its probable ability to decrease iron load in thalassemia. Wheatgrass may well be the long sought-after safe, oral and economical iron-chelating agent for thalassemic patients.

Comparison of effects of wheatgrass treatment in different groups of patients reveals that patients above 12 years age had more beneficial effects of wheatgrass treatment as compared to the patients below 12 years age. This was probably due to the lower dose of wheatgrass (two tablets twice in a day) administered to patients bellow 12 years age as compared to higher dose (two tablets three time in a day) to patients above 12 years age. Significant difference in the clinical effectiveness between these two dosage regimen indicate that the minimum dose of wheatgrass should be two tablets (250 mg tablets) three times in a day, for thalassemic patients of all age group.

Reversal of ineffective erythropoiesis and consequent reversal of splenomegaly with subsequent release of trapped lymphocytes may be the cause of significantly increased total lymphocyte count (before treatment: 1768.5 $\pm$ 151.04, after treatment: 2351.8 $\pm$ 157.97) in patients of above 12 years age grouping our investigation. Reversal of splenomegaly after wheatgrass treatment is further supported by the fact that lymphocyte count was significantly increased (before treatment: 26.5 $\pm$ 2.47, after treatment: 41.5 $\pm$ 1.06) in patients with spleen, while it remained unaffected in spleenectomized group.

In nutshell, our studies support, scientifically, some of the therapeutic claims for wheatgrass. The potential benefit of wheatgrass in thalassemia appears to be convincing and opens new vista of investigation.

2. INTRODUCTION

Herbal medicine is still the mainstay of about 75% of the world population, especially in the under developed and developing countries, for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side effects. However, in the last few years there has been a major increase in their use in the developed world. In Germany and France, many herbs and herbal extracts are used as prescription drugs. Their sales in the countries of European Union were around $ 6 billion in 1991 and may be over $ 20 billion now. About 25% of modern medicines are descended from plants first used traditionally.

According to The National Medicinal Plants Board, Ministry of Health and Family Welfare, Govt. of India ' India has 15 agro climatic zones, with 47000 different plant species and 15000 medicinal plants The Indian Systems of Medicine have identified 1500 medicinal plants, of which 500 species are mostly used therapeutically. The medicinal plants contribute to cater 80% of the raw materials used in the preparation of drugs. The effectiveness of these drugs mainly depends upon the proper use and sustained availability of genuine raw materials. The domestic market of Indian Systems of Medicine and Homoeopathy is about Rs. 4000 crores (2000), which is expanding day by day. The Ayurved drug market alone is to the order of Rs. 3500 crores. Besides this, there is also a growing demand for natural products including items of medicinal value/pharmaceuticals, food supplements and cosmetics in both domestic and international markets. Presently, India's export, from medicinal and herbal plants, is Rs. 3000 crores. India, with its diversified biodiversity has a tremendous potential and advantage in this emerging area.

According to WHO report, over 80% of the world population relies on traditional medicine largely plant based for their primary healthcare needs. The EXIM bank of India, in its report (1997) has reported the value of medicinal plants related trade in India of the order of 5.5 billion US dollars and is growing rapidly. According to WHO, the International market of herbal products is estimated to be US $ 62 billion which is poised to grow to US $ 5

trillion by the year 2050. India's share in the global export market of medicinal plants related trade is just 0.5%. In India, the herbal drug market is about $ one billion and the export of plant-based crude drugs is around $ 80 million (Esau 1974). Herbal medicines also find market as nutraceuticals (health foods) whose current market is estimated at about $ 80–250 billion in USA (Cutter 1978). India is sitting on a gold mine of well-recorded and well-practiced knowledge of traditional herbal medicine. But, unlike China, India has not been able to capitalize on this herbal wealth by promoting its use in the developed world despite their renewed interest in herbal medicines. This can be achieved by judicious product identification based on diseases found in the developed world for which no medicine or only palliative therapy is available; such herbal medicines will find speedy access into those countries. Backward integration from market demands will pay rich dividends. Strategically, India should enter through those plant-based medicines, which are already well accepted in Europe, USA and Japan. Simultaneously, it should identify those herbs (medicinal plants), which are time-tested and dispensed all over in India.

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. The wheat plant is an annual grass. In early growth stages the wheat plant consists of a much-compressed stem or crown and numerous narrowly linear or linear-lanceolate leaves. For over fifty years, researchers have known that the cereal plant, at this young green stage, is many times richer in 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. The jointing stage is that point at which the internodal tissue in the grass leaf begins to elongate, forming a

stem. This stage represents the peak of the cereal plant's vegetative development (Kohler 1944).

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 – (I) *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. (II) *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*. (III) *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.

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

Dr. Ann Wigmore, U. S. A. founder director of the Hippocrates Health Institute, Boston, U.S.A. was one of the proponents of the 'Wheatgrass Therapy'. Dr. Wigmore utilized the chlorophyll present in wheatgrass as body cleanser, rebuilder and neutralizer of toxins. She 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 (Wigmore 1985).

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, which have significant nutritious and medicinal value.

Anemia is a hematological condition in which there is quantitative deficiency of circulating hemoglobin, often accompanied by a reduced number of red blood cells. Further, causes of anemia are blood loss, impaired erythropoiesis and abnormal erythrocyte destruction. Nutritional deficiencies (iron, vitamin B_{12} and folic acid) are the most common cause of anemia through out the world (Brown 1991).

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. In terms of cost of ideal maintenance of these children a staggering Rs. 150 crores is required to be spent every year (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, βγ,) 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). α-thalassemia occurs when one or more of the four α chain genes fails to function. With α-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.*

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. 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. 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).

Management of ß-thalassemia major (*Cooley's Anemia)* 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 desferrioxamine 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).

The release of hemoglobin, during hemolysis, and the subsequent therapeutic transfusion lead to systemic iron overloading that further potentiates the generation of Reactive Oxygen Species (ROS) in thalassemia. A number of major cellular defense mechanisms exist to neutralize and combat the damaging effects of these reactive substances. The enzymic system functions by direct or sequential removal of ROS (superoxide dismutase, catalase, and glutathione peroxidase), thereby terminating their activities. Nonenzymic defense consists of scavenging molecules that are endogenously produced (GSH, ubiquinols, uric acid) or those derived from the diet (vitamins C and E, lipoic acid, selenium, riboflavin, zinc, and the carotenoids or vitamin A) (Chan et al., 1999). The iron-induced liver damage in thalassemia may play a major role in the depletion of lipid-soluble antioxidants (Livrea et al., 1996). Data indicate that dietary magnesium supplementation improves some of the characteristic cellular function abnormalities of β-thalassemia intermedia (De Franceschi et al., 1998).

Patients receiving regular blood transfusions, if given less than two-thirds of the recommended desferrioxamine dose, are known to have increased risk of developing complications later in life as a result of accumulation of iron (Brittenham et al., 1994, Gabutti and Piga 1996). Desferioxamine is required to be given at least five times a week through an injection under the skin very slowly with the help of an electrical or mechanical gadget tied to the child. The

cost of the gadget is around Rs 10,000 to 15,000. Thus, in thalassemia, there is a vicious cycle of iron overload leading to oxidative stress with consequent increase in hemolysis and increase in blood transfusion requirement causing further iron overload and its toxicities on other organs. Replenishing magnesium and antioxidants by administration of wheatgrass can break this vicious cycle.

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). 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.

The deficiency of magnesium in serum or erythrocytes has also been reported in human β-thalassemia. These deficiencies may play a significant role in

various cellular abnormalities characteristic of this disorder (De Franceschi et al., 1998). The iron-induced liver damage in thalassemia may play a major role in the depletion of lipid-soluble antioxidants like vitamin A and vitamin E (Livrea et al., 1996). Degradation of chlorophyll following ingestion by humans produces several chlorophyll derivatives, of which pheophytin, pyro-pheophytin, and pheophorbide have been under study for their potential medical benefits (Chernomorsky 1999). Pheophorbide-a showed antioxidant activity against lipid auto-oxidation. The extent of activity was comparable to that of ∞-tocopherol, a powerful and well-known antioxidant (Gentile 1991, Nakamura 1996, Lee 1999). Considering the above facts, we hypothesized that major ingredients of wheatgrass like chlorophyll, beta-carotene, vitamin A, vitamin E, vitamin C, selenium, zinc, magnesium, iron, folic acid and vitamin B_{12} may be useful in treatment of anemia and ß-thalassemia. A recent report by Marvaha et al (2004) has provided an evidence to support this hypothesis wherein in a pilot study it was reported that the administration of wheatgrass significantly reduces blood transfusion requirement in thalassemic patients. *Therefore, one of the major objectives of the present project was to evaluate clinical effects of wheatgrass in anemia and β-thalassemia (major).*

Traditionally, wheatgrass has been used as an adjunct in treatment of cancers. Wigmore (1985) suspected that wheatgrass is also useful as anticancer preparation by virtue of its several components like chlorophyll, P_4D_1 compound, abscisic acid and laetrile (vitamin B_{17}). According to Te et al., (1997) chlorophyllin, which is obtained by hydrolysis of chlorophyll to remove phytyl alcohol, is an efficient antimutagenic agent and has been used as a dietary supplement or to diminish the intensity of the discomforting side effects of cancer preventive therapy. It is possible that chlorophyllin may have beneficial effects when used in combination with cancer preventive therapy. More recently, the cancer chemopreventive properties of chlorophylls have come to be recognized. Chlorophyll has been reported to exhibit anti-mutagenic activity in short-term genotoxicity assays (Dashwood 1997). Chlorophyll-rich plant extracts, as well as water solutions of a chlorophyll derivative (chlorophyllin), dramatically inhibit the carcinogenic effects of

common dietary and environmental chemicals (Kimm et al., 1982, Ong et al., 1986). Using the standard Ames test, it has been shown that an extract of wheat grass, when applied to known chemical mutagens (which cause cells to become cancerous), decreased their cancer-causing ability by up to 99 percent (Li et al., 1978). Later studies by the same investigators showed that several green vegetables provide anti-mutagenic protection from a number of cancer causing chemicals (Li et al., 1980). This activity was found to be proportional to the amount of chlorophyll in the vegetables (Lai 1978).

Apart from the use of wheatgrass juice in anemia and cancer, it has been recommended that the topical application of wheatgrass juice is useful for treatment of skin infections (Wigmore 1985). It has also been claimed that wheatgrass juice may have antibacterial activity. Chlorophyll limits the growth of many types of germs not by directly killing them, but by providing an environment, which interferes with their growth especially against anaerobic bacteria, those that do not require oxygen (Smith 1955). Chlorophyll solutions provide significant relief of pain, reduction of inflammation, and the control of odor for patients with serious mouth diseases (Gruskin 1940). Chlorophyll has also been used successfully to treat chronic and acute sinusitis, vaginal infections, and chronic rectal lesions (Gruskin 1940). *In the light of these reports we also made an attempt to evaluate anticancer and antibacterial activity of wheatgrass.*

As mentioned above, fresh wheatgrass has been proposed to be used as a juice, which is prepared in a mixer/blender with addition of little water followed by filtration through a cloth (Marwaha et al., 2004). In a chronic disease like thalassemia, the drug treatment is of long duration, may even be for years. In such a circumstances the factor of patient compliance becomes very important. Outcome of the therapy will largely depend upon regular supply (round the year and in all seasons) and acceptability of the drug by patient. As a pharmaceutical scientist, preparation of a suitable dosage form is prime area of research in the development of new drug formulations. In the present investigation we decided to prepare suitable formulation that is as effective as juice per se.

Preparation of wheatgrass tablets was a challenge for us. We attempted to use different drying techniques such as freeze drying, spray drying and shade drying to prepare dried wheatgrass powder. In freeze-drying technique, fresh wheatgrass was frozen to sub-zero temperature and subsequently subjected to low-temperature heating (5° C -10° C) in vacuum to evaporate crystallized water content. Dried wheatgrass was then milled to obtain powder. In spray-drying technique, fresh wheatgrass was pressed in a hydraulic press to obtain juice. The juice was then sprayed in aerosol form through a nebulizing nozzle in a conical vessel from top. A hot air counter current (55° C) was passed from bottom of the vessel. The nebulized juice settled on the bottom of the vessel in the form of powder. In shade-drying technique, fresh wheatgrass was dried at room temperature in a dark room. The dried wheatgrass after 3-4 days of drying period was powdered in a mill.

It is well known that the stability of components present in wheatgrass, like chlorophyll, beta-carotene, vitamin A, vitamin E, vitamin C etc. are adversely affected upon exposure to changes in air, light, humidity and temperature. Since, all of the above mentioned drying processes involve exposure of wheatgrass to some of these variables, potency of wheatgrass powder obtained at the end of each drying process might be different, consequently affecting the therapeutic effectiveness and out come of the clinical trial. *Hence, we also evaluated the potency of dried wheatgrass powder, using UV spectra and HPTLC methods.*

It is well known that the contents of an herbal drug can be affected by changes in species variety and choice of soil or fertilizer used. In order to ascertain the most therapeutically potent verity of Wheat and best type of fertilizer to grow wheatgrass, *we carried out UV spectral and HPTLC analysis of different verities of wheat grown in similar soil/fertilizer and same variety of wheat grown in different soils/fertilizers.*

In nutshell the objectives of the present project were -

1. To carry out pharmacognostic study and phytochemical standardization of different verities of *Triticum* grass (wheatgrass) and crude extract using HPTLC fingerprinting.

2. To prepare tablets of wheatgrass and to evaluate the most suitable method of drying the wheatgrass for the same.

3. To standardize various formulations of wheatgrass by HPTLC.

4. To evaluate effect of wheat variety and fertilizer on potentially therapeutic contents in wheatgrass.

5. To evaluate effects and mechanism of action of wheatgrass in treatment of anemia, β-thalassemia (major), cancer and bacterial diseases.

3. REVIEW OF LITERATURE

3.1 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 ha).

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.

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.

Different species of wheat -

Wheat species differ from one another both morphologically and genetically. Triticum species can be placed in three groups, according to whether their

body cells contain 14, 28 or 42 chromosomes. The basic haploid number being 7, these groups are described as Diploid, Tetraploid and Hexaploid respectively. Diploid species include *T. boeoticum* (Wild Eincorn - most ancient variety of wheat) and *T. monococcum* (Eincorn). *T. boeoticum* was growing in Southwestern Asia before the advent of agriculture. *T. monococcum* is now grown to a limited extent in the mountainous region of Yugoslavia, Asia Minor and North Africa. Diploid species can be readily crossed to yield Tetraploid group. Tetraploid species include *T. dicoccum* (Emmer wheat, local variety -DDK) and *T. durum* (Durum wheat or Macaroni wheat, local varieties - Bansi, Kathia, Khandwa, Raj 1555). *T. dicoccum* is one of the most ancient of cultivated cereals. It was formerly grown in the United States for feed on a limited acreage but now has substantially disappeared from cultivation. It is grown to a limited extent in the nilgiri hills and the neighboring areas and is preferred for the preparation of suji or rawa. *T. durum*, next important species to *T. aestivum*, is used mainly for the manufacture of semolina which is made into macaroni, spaghetti and related products. Although high in gluten, *T. durum* is not good for baking. Instead, it is often ground into semolina, the basis for excellent pasta, such as spaghetti and macaroni. It is grown to a considerable extent in parts of Gujarat and central peninsular India. It is preferred for preparation of vermicelli or sewian. When crossed with Diploid species, Tetraploid species yield Hexaploid group. Hexaploid species include *T. aestivum* (common wheat, bread wheat, local varieties - Sharbati, Lal kanak, Lok1, GW 273). *T. aestivum* is the most evolved and widely cultivated of all wheat species. It is high in protein (10-17 %) and yields flour rich in gluten, making it particularly suitable for yeast breads. It is also preferred for the preparation of biscuits, cake and pastry manufacture. In India, *T. aestivum* is the most widely grown wheat species.

The principal cultivated grasses are the cereal grains —wheat, rice, corn, barley, oats, rye and millet (Baker 1978). Various researchers have known that the cereal plant, at this young green stage, contains many times the level of vitamins, minerals and proteins found in the seed kernel, or grain product of the mature cereal plant. The nutrient content of these grasses varies with their stage of growth and growing conditions, rather than with the species of cereal

grass (Kahn 1985). 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, the nutritional level in the leaves drops rapidly while the fiber content increases rapidly. The jointing stage is that point at which the internodal tissue in the grass leaf begins to elongate, forming a stem. This stage represents the peak of the cereal plant's vegetative development (Kohler 1944); factors involved in photosynthesis and plant metabolism would be expected to increase up to this stage. Sucrose, the simple carbohydrate found in table sugar, is the primary molecule from which all organic (carbon containing) molecules are formed in the plant (Duffus and Duffus 1984). At the appropriate times and rates, sucrose is converted into amino acids (which make up all proteins), complex carbohydrates, lipids (fats), and nucleic acids (DNA and RNA). The degree of conversion of sugars to specific complex nutrients is dependent on the activity levels of specific enzymes in the plant. Enzyme activity levels are dependent on the plant's growth stage. Chlorophyll, protein, and most of the vitamins found in cereal grasses reach their peak concentrations in the period just prior to the jointing stage of the green plant. Although this period lasts for only a few days, cereal grasses which are consumed, as food supplements should be harvested precisely during this stage of the wheat or barley plant's development. After the jointing stage, the stem forms branches and continues to elongate. The chlorophyll, protein, and vitamin contents of the plant decline sharply as the level of cellulose increases. Cellulose, the indigestible plant fiber, provides structural stability for the growing stem.

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 3.1 and table 3.2, 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.

Optimum Health Institute

Table – 3.1 Nutritional analysis of wheatgrass

Protein,	1.959	Calories,	21.0 Cal
Carbohydrates total	2.09	Moisture,	959
Ash,	.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,	061 mg;	Vitamin A	427 ILI 9
Vitamin B1	08 mg,	Vitamin B2,	0.13 mg;
Vitamin B3,	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,food	0.03 g;
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

Sweet Wheat

Table 3.2 Major ingredients in sweet wheatgrass juice powder

Main Ingredients	Content %		
Carbohydrates	23.5	Fat	3.7
Moisture	0	Protein	46.7
		Ash (Minerals)	26.1
Mineral & trace minerals mgs/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 3.3 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 Wheatgrass	100	25	17	515	66,080	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)

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.

Medicinal uses of wheatgrass –

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.

In the following chapters, we have discussed the possible therapeutic effectiveness and mechanisms of action of wheatgrass juice, in various diseases.

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.

3.2 Significance of antioxidants in wheatgrass -

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.
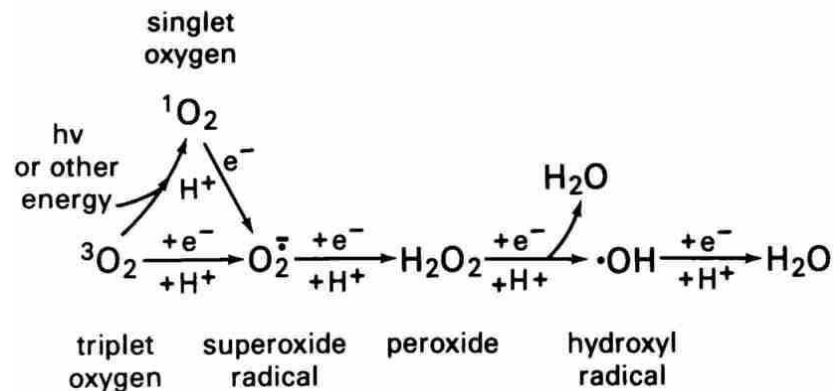

Figure 3.1 - Formation of reactive oxygen species -

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 02 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 H2O and O2 (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).

Exogenous antioxidants – antioxidants 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 $\propto$-tocopherol, a lipidsoluble molecule that functions as a chain-breaking antioxidant. Attached to the hydrophobic structure of $\propto$-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 $\propto$-tocopherol into a new radical, tocopherol-O, 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 $\propto$-tocopherol by reaction with ascorbic acid (vitamin C). Both vitamin C and $\propto$-tocopherol seem to minimize the consequences of lipid peroxidation in lipoproteins and in membranes, should this process begin (Wefers and Sies 1988). $\propto$-tocopherol is t1he most effective lipid-soluble chainbreaking antioxidant in vivo in humans (Ingold et al., 1986). The content of $\propto$-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 $\propto$-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.

3.3 Significance of chlorophyll in health and disease -

The most important light-absorbing pigments, in the thyllakoid membrane of a plant leaf, are the chlorophylls, green pigments with polycyclic, planar structures resembling the protoporphyrin of hemoglobin, except that Mg^{+2}, not Fe^{+2}, occupies the central position. The four inward oriented nitrogen atoms of chlorophyll are coordinated with the Mg^{+2}. The heterocyclic five-ring system that surrounds the Mg^{+2} has an extended polyene structure, with alternating single and double bonds. Such polyenes, characteristically show, strong absorption in the visible region of spectrum; the chlorophylls exhibit high molar absorption coefficients and are therefore well suited for absorbing visible light during photosynthesis. Chloroplasts of higher plants always contain two types of chlorophyll. One is invariably chlorophyll a, and the second in many species is chlorophyll b, which has an aldehyde group instead of a methyl group attached to ring II. Although both are green, their absorption spectra are slightly different, allowing the two pigments to complement each other's range of light absorption in visible region. Most higher plants contain about twice as much chlorophyll a as chlorophyll b. Chlorophyll itself is not a single molecule but a family of related molecules, designated chlorophyll a, b, c, and d. Chlorophyll a is the molecule found in all plant cells and therefore its concentration is what is reported during chlorophyll analysis. Chlorophyll d is found only in marine red algae, but chlorophylls b and c are common in fresh water. Chlorophyll a and chlorophyll b can be separated by shaking a light petroleum solution of chlorophyll with aqueous methanol: chlorophyll-a remains in the light petroleum but chlorophyll b is transferred into the aqueous methanol. Cholorophyll a is a bluish-green solid and cholorophyll b is a dark green solid, both giving a green solution in organic solutions. In natural chlorophyll there is a ratio of 3 to 1 (of a to b) of the two components. In addition to chlorophylls, the thyllakoid membranes secondary light absorbing pigments, together called the accessory pigments, the carotenoids and phycobilins. Carotenoids may be yellow, red or purple. The most important are beta-carotene, a red-orange isoprenoid compound that is the precursor of vitamin A in animals, and the yellow carotenoid, xanthophyll. The carotenoid pigments absorb light at wavelengths other than

those absorbed by chlorophylls and thus are supplementary light receptors. Phycobilins are linear tetrapyrroles that have the extended polyenes system found in chlorophylls, but not their cyclic structure or central Mg^{+2}. Examples are phycoerythrin and phycocynin.

All the above-mentioned pigments allow plants to absorb energy from visible light. In addition to this, chlorophyll has been reported to be useful in several clinical conditions. Possibly, the presence of magnesium offers wide range of therapeutically useful effects.

Degradation of chlorophyll following ingestion by humans produces several chlorophyll derivatives, of which, pheophytin, pyro-pheophytin, and pheophorbide have been under study for their potential medical benefits (Chernomorsky 1999). Magnesium is rather easily displaced from the molecule of chlorophyll, when it is heated in the presence of organic acids. Treatment of chlorophyll-a, with acid removes the magnesium ion replacing it with two hydrogen atoms giving an olive-brown solid, pheophytin-a. Pheophytins are much more stable than naturally occurring chlorophyll. Hydrolysis of pheophytin-a splits off phytol and gives pheophorbide-a. Similar compounds are obtained if chlorophyll b is used. Removal of the phytyl group from the molecule of chlorophyll is catalyzed by the enzyme chlorophyllase, found in some vegetables. Hydrolysis of the ester linkage yields a compound, known as a chlorophyllide, which is water-soluble. Further degradation of chlorophyllide produces pheophorbide. Thus, when a chlorophyll molecule breaks down, a number of distinct pheophytins, chlorophyllides, and pheophorbides will be produced, depending on the parent molecule. Chlorophyll can also be reacted with a base, which yields a series of phyllins, magnesium porphyrin compounds. Treatment of phyllins with acid gives porphyrins. Numerous studies have shown that chlorophyll and Cu-chlorophyllin, a water-soluble chlorophyll derivative, possess antimutagenic and antigentoxic activities. Little information about the absorption and metabolism of these compounds by humans is available. In vitro digestion has been used to access the relative bioavailability of numerous minerals and nutrients from different food matrices. The general consensus among

researchers was that chlorophyll molecules were too large to be absorbed by the body. But in recent studies, chlorophylls potential as a health promoter has taken a leap forward (Chernomorsky 1999). In the study, artificial digestion system, known as the Coupled In Vitro Digestion and Caco-2 Human Cell Model, showed that absorptive cells lining the small intestine actually do uptake pheophytins chlorophyll derivatives such as pheophytins, pyropheophytins and chlorophyllins formed during digestion of spinach puree.

Medicinal uses of chlorophyll –

Chlorophyll and anemia -

Chlorophyll is not so unique in its chemical make-up. 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. The similarities between chlorophyll and heme are not limited to appearance and function (Milgrom 1985). Chemists report that the synthesis of heme by animals can occur in much the same way as the synthesis of chlorophyll in plants (Battersby 1988). 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. This opinion was reinforced by the observation that animals, which ate only leafy green plants, had ample amounts of hemoglobin in their red blood cells (Patek 1936). 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). Drs. Hughes and Latner fed several doses and forms of chlorophyll to anemic rabbits in 1936. Extremely 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 (Hughes and Latner 1936). Although green foods have long been considered useful for their "blood-building" qualities, the chlorophyll found in green foods is itself valued for many other therapeutic purposes. The number of surface conditions in which chlorophyll has been successfully used would be unbelievable were they not so well documented (Smith 1944).

Chlorophyll and cancer -

There is scientific evidence that chlorophyll and the nutrients found in green foods offer protection against toxic chemicals and radiation. In 1980, Dr. Chiu Nan Lai at the University of Texas Medical Center reported that extracts of wheat grass and other green vegetables inhibit the cancer-causing effects of two mutagens (benzopyrene and methylcholanthrene) (Lai et al, 1980). The more chlorophyll in the vegetable, the greater the protection from the carcinogen. Several laboratories have verified that chlorophyll can reduce the ability of carcinogens to cause gene mutations in the last decade. Chlorophyll-rich plant extracts, as well as water solutions of a chlorophyll derivative (chlorophyllin), dramatically inhibit the carcinogenic effects of common dietary and environmental chemicals (Kimm et al, 1982, Ong et al, 1986). Ames testing shows that chlorophyllin neutralizes the cancer-causing action of mixtures of coal dust, tobacco, fried beef, red wine, and other compounds. In this capacity, chlorophyllin is more effective than vitamin A, vitamin C, or vitamin E against mutations induced by the same mixtures (Ong et al, 1989, Ong et al, 1986). Chlorophyll and its related chemicals are being investigated for potential protection against carcinogens, including the dietary carcinogens found in cooked muscle meats (heterocyclic amines), smoked or barbecued foods (polycyclic hydrocarbons), and peanut mold (aflatoxin) (Zitgler 1995). Epidemiological evidence has suggested that diets containing yellow-green vegetables are associated with protection against carcinogenic effects and related mutagenic, clastogenic, and genotoxic activities (Sarkar 1994, Waladkhani 1998, Sarkar 1996). Unlike other chemoprotective phytochemicals, which are present naturally in small quantities in plants,

chlorophyll is especially abundant in green leafy plants. For example, spinach can contain up to 5.7% chlorophyll on a dry-weight basis (Harttig 1998, Gentile 1991). Chlorophyll, ubiquitous to all green plants, and having been shown to have medical value, has received tremendous interest as a nutritional supplement. However, some evidence suggests that the observed medical effects of chlorophyll in plants are actually the result of the total effect of the interaction between different components of the whole plant, in addition to the sole effects of chlorophyll (Lea 1999, Waladkhani 1998). Other phytochemicals that may work in therapeutic combination with chlorophylls include carotenoids, flavonoids, indole, isothiocyanate, poyphenolic compounds, protease inhibitors, sulfides, and terpens (Waladkhani 1998). Epidemiological surveys have demonstrated that the frequent consumption of fresh vegetables and fruit is associated with reduced risk of some cancers, including gastrointestinal cancer in humans (Sarkar 1994, Nakamura 1996, Sarkar 1996). Chlorophylls and chlorophyll-related products, both natural and man-made, have been shown to support normal cellular function, as demonstrated experimentally through in vitro, in vivo, and animal studies (Higashi Okai 1998). Studies demonstrate that chlorophyll and chlorophyll-related products support normal cellular health through anti-mutagenic activity. Studies have found that chlorophyll binds carcinogens and reduces their uptake (Harttig 1998, Nakamura 1996). Chlorophylls have shown a dose-related protective effect against carcinogens found in the human diet. Chlorophylls form complexes with carcinogens while they are still in the digestive tract, thereby limiting their bioavailability. Chlorophylls also reduce the binding of carcinogens to DNA in the liver (Zitgler 1995). Terwell and van der Hoeven have shown through in vivo studies that chlorophyll and chlorophyllin are inhibitors for the mutagenicity of cigarette smoke condensate (Negishi 1989). Chlorophyllin has been used as a dietary supplement or to diminish the intensity of the discomforting side effects of anticancer therapy. The increase in micronuclei in bone marrow polychromatic erythrocytes in response to anticancer therapy was reduced by concomitant chlorophyllin treatment. We conclude that chlorophyllin may have beneficial effects when used in combination with anticancer therapy (Te et al., 1997).

Chlorophyll and its derivatives are also being studied in relation to photodynamic therapy, a procedure that harnesses the energy of light to treat certain diseases. In photodynamic therapy, chlorophyll and its derivatives are used as photosensitizers that kill cancer cells and act as antiviral agents (Lee 1990). Chlorophyll-related compounds have been effective in photodynamic treatment of pancreatic cancer cells, gross leukemia virus and malignant melanoma, in various *in vitro*, *in vivo* and animal studies (Fiedor 1993, Rosenbach and Belkin 1996, Lee 1999).

Chlorophyll and inflammation -

In this century medical scientists have found chlorophyll to be effective in the general fields of detoxification, deodorization, and the healing of wounds. The power of chlorophyll as an effective deodorizing agent was first scientifically demonstrated in the 1940s. In the decade that followed, toilet paper, diapers, chewing gum, bed sheets, toothpastes, shoe liners, and a number of other products containing various amounts of green coloring and crude chlorophyll extracts began appearing on store shelves. In the wake of this "chlorophyll hysteria", a number of researchers began serious investigation of the therapeutic uses of chlorophyll (Smith 1955). The leaves and green parts of plants have been used for centuries to accelerate wound healing. Among the ancients, the greenest plants were chosen for health remedies (Smith 1944). Chlorophyll has been recognized for its antinflammatory, antioxidant, wound healing, and deodorant properties (Zitgler 1995, Nakamura 1996). The ideal wound treatment stimulates repair of damaged tissues and inhibits the growth of bacteria (Carpenter 1949). Chlorophyll does both! Even crude preparations of chlorophyll are effective in stimulating the growth of healthy granuloma tissue and fibroblasts—both on actual wounds and in laboratory cultures (Smith 1955). Nakamura found that pheophorbide a, a naturally occurring major degradation product of chlorophyll, had anti-inflammatory effects when tested in animal studies. The anti-inflammatory effects have been linked to inhibition of leukocyte activation, which is important to maintaining normal tissue and cellular health (Nakamura 1996). Nenonen and others have found that lactobacilli-rich and chlorophyll-rich drinks were associated with decreased disease activity in arthritis (Nenonen 1998). In addition, the foul

odors associated with surface wounds and ulcers rapidly disappear following chlorophyll application (Gruskin 1940). The medical literature is replete with reports demonstrating these effects. Surface wounds and sores due to surgery, compound fractures, osteomyelitis (bone inflammation), decubitus (bed sores), and routine cuts and scrapes all show fast and dramatic improvement with the topical use of chlorophyll. Chlorophyll therapy has saved limbs from amputation. Chlorophyll is also known to reduce the itching, pain and local irritation of surface wounds (Smith 1955). Burns caused by heat, chemicals, and radiation also heal faster with chlorophyll therapy, whether or not they are infected. Chlorophyll was used to prolong the survival of skin grafts before the development of the immune-suppressing drugs, which are now used. The action of chlorophyll on wounds has a unique feature. Most medicines become less effective with repeated use. In contrast, an initial application of chlorophyll makes a wound more sensitive to its healing benefits with repeated use (Smith 1955). Chlorophyll is considered to have the most constant and marked effect of all agents for stimulating cell proliferation and tissue repair (Smith 1955). Collings (1945) demonstrated that the healing time of wounds is shorter with chlorophyll therapy than with penicillin, vitamin D, sulfanilamide, or no treatment. Chlorophyll also accelerates wound healing by reducing hemagglutination and inflammation. When a tissue is injured, foreign substances in the blood generally cause blood cells to clump together. This limits the amount of nutrients available for repair of the injured tissue. When chlorophyll is administered to a wound, this clumping is reduced, so the lag time associated with tissue repair is shortened (Sack and Barnard 1955). Chlorophyll decreases swelling by reducing the synthesis of fibrin (the protein associated with blood clot formation) (Miller et al., 1960, Miller et al., 1958). This gives chlorophyll a mild blood thinning, or heparin-like property, which can enhance the effectiveness of local immune defenses. Chlorophyll has also been shown to be extremely effective in speeding the healing of peptic ulcers and wounds, which develop internally in the gastro-intestinal tract. Several studies document the use of chlorophyll in the treatment of ulcers resistant to more conventional therapies. The results are quite impressive. In the Offenkrantz study, 20 of the 27 patients with chronic ulcers were relieved of pain and other symptoms in 24 to 72 hours

(Offenkrantz 1950). Complete healing of the damaged tissues, as demonstrated by X-ray examination, occurred in 20 of 24 cases within two to seven weeks. These reports include case descriptions of dramatic recoveries from severe, long standing problems. Chlorophyll has been cited as therapeutic for inflammation of the ear, the mucous membrane of the nose, and the sinuses (Duke 1992). Chlorophyll has also been found to be supportive of normal kidney function, acceleration of wound healing, treatment of ulcers, and reduction of fecal, urinary, and body odor in geriatric patients (Duke 1992, Zitgler 1995).

European investigators report preliminary favorable results in the use of chlorophyll in the treatment of pancreatitis. The chlorophyll is thought to influence several enzymatic reactions, which complicate this disease (Chernomorsky and Segelman 1988). Other intestinal diseases have also been effectively treated with chlorophyll. Rafsky and Krieger (Rafsky and Krieger 1948) report positive results obtained with the use of rectal implants of chlorophyll solutions for the treatment of a variety of diseases of the colon including spastic colitis, sigmoiditis, and ulcerative colitis. The majority of the patients in the study showed definite improvement. Chlorophyll appears to alter the metabolism of colonic bacteria. Its use is associated with reduced formation of skatole, a substance formed by the bacterial breakdown of proteins (Smith 1955).

Chlorophyll in gastrointestinal disorders -

Researchers observed a side benefit when chlorophyll was used to treat peptic ulcers. Chlorophyll tended to "promote regularity" in the patients studied (Offenkrantz 1950). According to several investigators, chlorophyll did not act simply to stimulate bowel activity, as does a laxative. Rather, it promoted bowel regularity, stimulating bowel action only when that action was sluggish. The same effect was noted in a 1980 study of the use of chlorophyllin (a water soluble chlorophyll derivative) to reduce body and fecal odors in a geriatric nursing care facility (Young and Beregi 1980). It was found that chlorophyll did reduce offensive odors, as anticipated, but also that it promoted regular bowel movements in these patients. Chlorophyll use also

reduced the amount of intestinal gas experienced by the patients. And, as chlorophyll has no toxic side effects, the "gratifyingly good results" obtained made it preferable to the use of "drastic laxatives". Today, patients to deodorize the surfaces and contents of colostomies routinely use chlorophyll tablets. Chlorophyll is also administered to incontinent patients to reduce odors in health care facilities (Young and Beregi, 1980). Topical chlorophyll ointments and solutions for healing and deodorizing wounds are still available, as are chlorophyll-containing toothpastes and chewing gums.

Chlorophyll and oxidative stress -

Antioxidant activity of chlorophyll-related compounds, including chlorophyllin, has been reported in several studies. Nakamura (1996) found that the naturally occurring chlorophyll derivative, pheophorbide a, showed antioxidant activity against lipid auto-oxidation. The extent of activity was comparable to alpha-tocopherol, a powerful and well-known antioxidant (Gentile 1991, Nakamura 1996, Lee 1999).

Chlorophyll and bacterial infections -

During the 1950s, many laboratories tested chlorophyll's power to kill germs. The consensus of these reports was that, for the most part, chlorophyll is bacteriostatic, and only slightly bacteriocidal. This means that chlorophyll limits the growth of many types of germs not by directly killing them, but by providing an environment, which interferes with their growth. It is particularly effective against anaerobic bacteria, those, which do not require oxygen (Smith 1955). Dentists and physicians have successfully used chlorophyll to control mouth infections such as pyorrhea and Vincent's angina. Chlorophyll solutions provide significant relief of pain, reduction of inflammation, and the control of odor for patients with serious mouth diseases (Gruskin 1940). There are several reported cases of the successful use of chlorophyll for bacterial endocarditis, an infection of the tissue surrounding the heart (Smith 1944). Chlorophyll has also been used successfully to treat chronic and acute sinusitis, vaginal infections, and chronic rectal lesions (Gruskin 1940).

Green plants are still used as healing agents by traditional health practitioners throughout the world. But chlorophyll is used in a rather limited way in our modern medical system. The enthusiasm with which chlorophyll was once studied subsided with the development of antibiotics and steroid drugs. Dr. S. A. Chernomosky, in a 1988 review article in the New Jersey Medical Journal, states that the treatment of patients with slow-healing wounds is still problematic, and that the increased use of chlorophyll compounds may offer a useful alternative in this area (Chernomorsky and Segelman 1988).

Chlorophyll and radiation -

Green vegetables provide protection from radiation damage in test animals. This information has been reported in the scientific literature since the early 1950s. Early reports showed that certain vegetables significantly reduced mortality in rats exposed to lethal doses of X-rays (Spector and Calloway, 1959). Dark green broccoli offered more protection than the lighter green cabbage. In a later study, the same vegetables were shown to reduce the damage caused by radiation (Calloway et al., 1962). These protective effects were more pronounced when even darker green vegetables such as mustard greens and alfalfa leaves were used. When two or more of the green vegetables were fed together, the positive resistance to radiation was greatest.

Safety of chlorophyll –

Chlorophyll has found diverse applications in both medicine and industry (Duke 1992). In response to recent concerns over the safety of chemical additives used as food colorants, the food industry has witnessed a renewed interest in using plant pigments as natural colorants. Chlorophyll, produced mainly from grasses, is one of the top four pigments used in the food industry for the coloring of food and drink ((Duke 1992, Lea et al., 1999). Chlorophyllin is the man-made sodium, iron, or copper salt of chlorophyll in which the central magnesium ion of chlorophyll is replaced by the metal ion. In Japan, chlorophyllin is used as a food additive for coloration (Negishi 1989). Chlorophyll is of special interest to the food industry because of its recognized safety. Several in vivo experiments and animal experiments show chlorophyll

to be safe (Sarkar 1996, Negishi 1997). Experiments report the absence of adverse events and obvious toxicity at up to 5% chlorophyll in animal diets (Furukawa 1998). In hundreds of experiments and trials on humans and test animals, chlorophyll therapy has always been shown to have no toxic side effects. Not just low toxicity, NO toxicity--whether ingested, injected or rubbed onto a surface (Smith 1944). This fact alone makes chlorophyll one of the most unique therapeutic substances known to medical science. In medicine, chlorophyll has attracted great interest as a fundamental component of a healthy diet and as a potential therapeutic agent (Sarkar 1996, Negishi 1989). Several chlorophyll-related chemicals are also receiving attention: chlorophyllin, the metal solution of chlorophyll, and several natural and synthetic chlorophyll derivatives (Chernomorsky 1999, Hajri 1999, Gentile 1991).

3.4 Significance of magnesium in health and disease -

The major electrolytes in the body are sodium, potassium, calcium, magnesium, chloride, bicarbonate and phosphate. Magnesium is the most abundant divalent cation in living cells and it plays a vital role in many cellular processes (Wacker 1968, Flataian 1993, Fry and Proctor 1993). Average adult body contains approximately 1000 mmol (2000 mEq/L) magnesium, 99% of which is in bone and the intracellular compartment (Abbott et al., 1993, Rude 1993). Of the 1% remaining in the vascular space, 25% is bound to proteins; it is the ionized 75% that exerts the physiological effect. Serum magnesium is maintained in the normal range of 0.8-1.2 mmol/L (1.6 to 2.4 meq/L) by efficient renal conservation and the ability to draw on the intracellular space for replacement ions, when intake falls. Consuming foods high in oxalic acid, like spinach, cocoa, and tea, can inhibit the body's processing of magnesium. Hypomagnesaemia is characterized by serum magnesium level < 0.8 mmol/l. It may be caused by amphotericin B, cis-platinum, diuretics, diarrhea, hypervitaminosis D, vitamin D-deficiency, vomiting, hyperaldosteronism and aminoglycosides. Symptoms of magnesium deficiency include tremor, hyperreactive reflexes, confusion and seizures. Clinical deficiency of magnesium can be treated by administering $MgSO_4$ (i v 0.15-0.25 mmol/kg/day) or Mg_2O (300-600 mg PO BID-TID). The physiologic function of magnesium (along with calcium and phosphate) is to maintain membrane potentials for nerve conduction and muscle contraction. Magnesium is an essential nutrient, meaning that body needs it for healthy functioning. It is found in significant quantities throughout the body and used for numerous purposes, including muscle relaxation, blood clotting, and the manufacture of ATP. Cytosol contains Mg^{+2}, which binds to ATP and ADP. In most enzymatic reactions that involve ATP as phosphoryl donor, the true substrate is $MgATP^{-2}$. Transfer of phosphate group in any biochemical reaction in a cell requires presence of Mg^{+2}. The phosphate groups of ATP, ADP and glycolytic intermediates form complexes with Mg^{+2}, and the substrate binding sites of many of the glycolytic enzymes are specific for these Mg-phosphate complexes. Thus, phosphated intermediates bind to Mg^{+2} and form Mg-Phosphate complex. This complex binds to kinase or

phosphorylase enzyme for further processing. Nearly all the glycolytic enzymes require Mg^{+2} for activity (Lehninger 1993).

Several hundreds of enzymes, directly or indirectly, are (via the need of Mg-ATP) dependent on Mg^{+2} (Wacker 1980). Thus, magnesium is a cofactor required for activity of many important enzymes and other biomolecules (Holoenzyme = Apoenzyme + Coenzyme + Metal ion (Mg^{+2})). Enzymes and physiological processes, which require Mg^{+2} for their activity, are listed bellow:-

Enzyme	Function
• Hexokinase-	- Glycolysis
• Glucokinase-	-Glycogenesis, to lower BGL (faulty in diabetes)
• Glycolytic enzymes-	-affected in diabetes
• Phosphorylases-	-Muscle Phosphorylase, Liver Phosphorylase
• ATPases-	-Na-K ATPase (excitability of muscles and nerves)
• Ca ATPase	- in sarcoplasmic reticulum -contraction and relaxation of muscles
• Creatine Kinase-	-Muscle contraction (Creatine phosphate + ADP = ATP + Creatine)
• Phosphodiastrase	-breakdown of cAMP and termination of cAMP action

- Synthesis of LDL receptors -lowering of serum cholesterol
- Neurotransmitter release from nerve terminals (Ca^{+2} + ATP)
- Hormone release from exocrine glands, exocytosis (Insulin release)
- Synthesis of mRNA, DNA dependant- RNA polymerase (Insulin synthesis)
- Synthesis of protein, Activation of amino acid, initiation and elongation require Mg^{+2}, (Insulin synthesis)
- DNA replication (immune response, Wound healing)

- Phosphorylation of G-proteins (signal transduction)
- Phosphorylation of tyrosine kinase (Insulin signal transduction)
- Synthesis of Glut-4 (Insulin action- sensitivity)
- AMPA receptors (for glutamate) are coupled to Ca^{+2} channel, which is normally blocked by Mg^{+2}. Deficiency of Mg^{+2} would remove this block, resulting in excitotoxicity.

Recently it has been reported that magnesium plays in the transmission of hormones (such as insulin, thyroid, estrogen, testosterone, DHEA, etc.), neurotransmitters (such as dopamine, catecholamines, serotonin, GABA, etc.), and minerals and mineral electrolytes. It is concluded that it is magnesium status that controls cell membrane potential and through this means controls uptake and release of many hormones, nutrients and neurotransmitters. It is magnesium that controls the fate of potassium and calcium in the body. If magnesium is insufficient potassium and calcium will be lost in the urine and calcium will be deposited in the soft tissues (kidneys, arteries, joints, brain, etc.). Magnesium protects the cell from aluminum, mercury, lead, cadmium, beryllium and nickel. Evidence is mounting that low levels of magnesium contribute to the heavy metal deposition in the brain that precedes parkinson's, multiple sclerosis and alzheimer's disease. It is probable that low total body magnesium contributes to heavy metal toxicity in children and is a participant in the etiology of learning disorders. Magnesium is also an important component of plants. The most important light-absorbing pigments in the thyllakoid membrane of a plant leaf are the chlorophylls, green pigments with polycyclic, planar structures resembling the protoporphyrin of hemoglobin, except that Mg^{+2}, not Fe^{+2}, occupies the central position. The four inward oriented nitrogen atoms of chlorophyll are coordinated with the Mg^{+2} (Lehninger 1993).

Medicinal uses of magnesium -
Magnesium has been called "nature's calcium channel–blocker." The idea refers to magnesium's ability to block calcium from entering muscle and heart cells. This may be the basis for magnesium's effects on migraine headaches

and high blood pressure. Studies indicate that there may be a link between low magnesium levels and thymus gland-cancer and diabetes. Diabetes appears more frequently in areas of the country where the drinking water is lower in magnesium. These two theories are more controversial and less clear than the others. Some physicians feel that more premature babies are born to women with low magnesium levels. Persons with kidney failure must be very careful when taking any food or medication with magnesium because the kidney clears excess magnesium from the body.

Magnesium and oxidative stress -

It is well known that magnesium plays a key role in oxidative metabolism in the mitochondria (Jung et al., 1990). There is overproduction of NO in magnesium deficiency. NO endogenously activates neutrophils to generate Superoxide ions, which combine with NO to produce peroxynitrite radicals (oxidative stress) to damage myocardium. Magnesium deficiency resulted in 70% decrease in plasma glutathione and 220% increase in TBARS levels in rats (Tong et al., 1997). Part of the increased oxidative stress could be contributed by increased NO production; simultaneous increases in production of both Superoxide anions and NO would likely lead to the formation of peroxynitrite which per se is highly reactive and might contribute directly to depletion of glutathione (Beckman et al., 1990, Radi et al., 1994). Increased oxidative stress is involved in cardiovascular injury during Magnesium deficiency in the rat (Weglicki et al., 1992, Weglicki et al., 1994). Increased free radical generation is involved in cardiovascular injury during Magnesium deficiency and vitamin E provides protection against this injury (Feedman et al, 1990). Several investigators have reported decrease in RBC glutathione, during magnesium deficiency (Weglicki et al., 1994, Mak et al., 1994, Mills et al., 1986).

Magnesium and inflammation -

Circulating levels of neutrophils increased 3-4 fold in Mg-deficient rats as compared to magnesium-sufficient rats from 3rd week (Kurantsin et al., 1994). Magnesium deficiency results in early elevation of neuropeptides (substance P, CGRP) followed by later increases in inflammatory cytokines including

gamma-interferon, IL-1, IL-6 and TNF-alpha in both rat and mice (Weglicki et al., 1992, Weglicki et al., 1994, Weglicki and Phillips 1992, Weglicki et al., 1992(a), Weglicki 1994(a)). As part of their proinflammatory activities, these neuropeptides and cytokines might be able to activate a number of cells including macrophages, neutrophils and endothelial cells to generate oxyradicals and NO (Billingham 1987, Whicher et al., 1990, Mak et al., 1995, Rock et al., 1995). Significant tissue inflammatory lesions occur only after 2 weeks of magnesium deficient diet (Weglicki et al., 1992, Weglicki et al., 1994, Weglicki and Phillips 1992, Weglicki et al., 1992(a), Weglicki 1994(a)).

Magnesium and cardiovascular diseases -

Magnesium works with calcium and potassium to regulate blood pressure. Several studies suggest that magnesium supplements can reduce blood pressure in people with hypertension (Sanjuliani et al., 1996, Witteman et al., 1994, Dyckner and Wester 1983, Henderson et al., 1986). A double-blind placebo-controlled trial of 50 individuals with blockage in the arteries to the heart (coronary artery disease) found that supplementation with magnesium at 730 mg daily significantly improved exercise tolerance (Shechter et al., 2000). In addition, a double-blind study of 42 individuals with heart disease found that magnesium supplements reduced the tendency of the body to form blood clots (Shechter 2000). Results indicate that hypomagnesaemia is associated with decreased levels of Mg^{+2} and elevated levels of Ca^{+2} in the rat heart and moreover internal Mg^{+2} is able to modulate the Ca^{+2} current through L-type Ca^{+2} channel, which in turn may be involved in regulation of contractile force in the heart. Mg^{+2} seems to play an important second messenger-signalling role in the control of myocardial contractility possibly by regulating cellular Ca^{+2} mobilization (Jaipaul et al., 1997). Magnesium deficiency or a reduction of dietary intake of magnesium plays an important role in aeitiology of diabetes and numerous cardiovascular diseases including thrombosis, atherosclerosis, ischemic heart disease, myocardial infarction, hypertension, arrhythmias and congestive heart failure in humans (Wallach 1987, Altura and Altura 1986, Altura and Altura 1995, Maguire 1990, Woods 1991, Durlach and Mareschi 1991, Elin 1987). Magnesium supplementation can bring about a significant decrease in blood pressure and a stabilization of cardiac

arrhythmias and acute myocardial infarction (Altura and Altura 1995, Woods 1991, Woods et al., 1992, Abraham 1987). The precise cellular mechanism(s) by whereby magnesium can exert its useful effect on cardiac excitability and contractility as well as on high blood pressure is not yet fully understood. However, it is believed to act by a number of cellular pathways including activation of enzymes and regulation of ion channel activities and transport processes (Flataian 1993, Fry and Proctor 1993, Agus et al., 1989, Romani et al., 1993, Murphy et al., 1991). Catecholamines can evoke marked Mg^{+2} efflux, which is associated with a concomitant increase in force of contraction the heart (Howarth et al., 1994, Hustler et al., 1995). Magnesium may also be useful for improving blood pressure (Shechter et al., 2000, Sanjuliani et al., 1996, Witteman et al., 1994) and preventing or treating coronary artery disease (Dyckner and Wester 1983, Shechter et al., 2000). There is also some evidence that magnesium may decrease the atherosclerosis risk caused by hydrogenated oils, margarine-like fats found in many "junk" foods (Kummerow et al., 1999). Hypomagnesaemia may lead to an impairment of Ca^{+2} transporting processes in the heart resulting in an increase in heart Ca^{+2} content (Wacker 1968, Flataian 1993, Murphy et al., 1991). In human subject hypomagnesaemia is often associated with electrolyte imbalace and severe cardiovascular disorders including arrhythmias (Altura and Altura 1986, Altura and Altura 1995, Woods 1991, Whang 1993). Reduction in either external or internal ionized Mg^{+2} can result in a marked increase in Ca^{+2} influx through L-type Ca^{+2} channels (Fry and Proctor 1993, Agus et al., 1989). Reduction in ionised magnesium can result in sub-optimal performance of the Na-K-ATPase pump (Skou 1979, Fischer and Giroux 1984) and Ca-ATPase pump (Vormann et al., 1995). (Increase in $(Na^{+})_I$, Na-Ca exchange in reverse, increase in $(Ca^{+2})_I$).

Magnesium and growth retardation -

Rats on low magnesium diet ate less food and grew more slowly than the control rats on control magnesium diet. This reduction in growth has been attributed to changes in insulin-like growth factor and growth hormone accompanying magnesium deficiency (Dorup et al., 1991).

Magnesium and migraine -

A recent double-blind study found that regular use of magnesium helps prevent migraine headaches. In this 12-week trial, 81 people with recurrent migraines were given either 600 mg of magnesium daily or placebo (Peikert et al., 1996). By the last 3 weeks of the study, the treated group's migraines had been reduced by 41.6%, compared to a reduction of 15.8% in the placebo group. The only side effects observed were diarrhea (in about one-fifth of the participants) and, less often, digestive irritation. Similar results have been seen in other smaller double-blind studies (Taubert 1994, Facchinetti et al., 1991). Several preliminary studies suggest that regular use of magnesium can help prevent migraine headaches (Peikert et al., 1996, Taubert 1994, Facchinetti et al., 1991).

Magnesium and kidney function -

Magnesium may also be useful for reducing the incidence of kidney stones (Johansson et al., 1980). Magnesium inhibits the growth of calcium oxalate stones in the test tube (Li et al., 1985) and decreases stone formation in rats (Parivar et al., 1996). However, human studies have had mixed results. In one 2-year open study, 56 people taking magnesium hydroxide had fewer recurrences of kidney stones than 34 people not given magnesium (Johansson et al., 1980). In contrast, a double-blind (and, hence, more reliable) study of 124 individuals found that magnesium hydroxide was essentially no more effective than placebo (Ettinger et al., 1988).

Magnesium and premenstrual syndrome -

A 6-month double-blind placebo-controlled study of 50 women with menstrual pain found that treatment with magnesium significantly improved symptoms (Seifert et al., 1989). The researchers reported evidence of reduced levels of prostaglandin $F_2\propto$, a hormone-like substance involved in pain and inflammation. A double-blind placebo-controlled study of 32 women found that magnesium taken from day 15 of the menstrual cycle to the onset of menstrual flow could significantly improve premenstrual mood changes (Facchinetti et al., 1991). Another small double blind preliminary study found

that regular use of magnesium could reduce symptoms of PMS-related fluid retention.73 In this study, 38 women were given magnesium or placebo for 2 months. The results showed no effect after one cycle, but by the end of two cycles, magnesium significantly reduced weight gain, swelling of extremities, breast tenderness and abdominal bloating (Facchinetti et al., 1991). Preliminary evidence suggests that the combination of magnesium and vitamin B_6 might be more effective than either treatment alone (De Souza et al., 2000). Preliminary double-blind trials suggest that magnesium may be useful for dysmenorrhea (menstrual cramps) (Fontana and Hogg 1990, Wagler et al., 1989) and symptoms of PMS (premenstrual syndrome), including menstrual migraines (Facchinetti et al., 1991, Facchinetti et al.,(a) 1991).

Magnesium and diabetes -

Open trials suggest that magnesium might decrease symptoms of restless legs syndrome (Hornyak et al., 1998, Popoviciu et al., 1993). Although there is no direct evidence that magnesium helps people with diabetes, such individuals are known to be deficient in magnesium (Elamin and Tuvemo 1990, Tosiello 1996, Eibl et al., 1995) and magnesium supplementation may be a good idea on general principle. However, individuals with severe kidney disease should take magnesium supplements only on their physician's advice.

Wheatgrass is rich in magnesium content hence, it may replenish body's depleted level of magnesium. From the forgoing discussion on benefits of magnesium supplementation in various pathological conditions, wheatgrass may offer potential therapeutic uses.

3.5 Pathophysiology and treatment of 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, βγ,) 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 ($\propto$). 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. ($\propto_2\gamma_2$). 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. ($\propto_2\beta_2$). 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 the functional form of hemoglobin. Complex biophysical characteristics of the hemoglobin tetramer permit the 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.

Human hemoglobins -

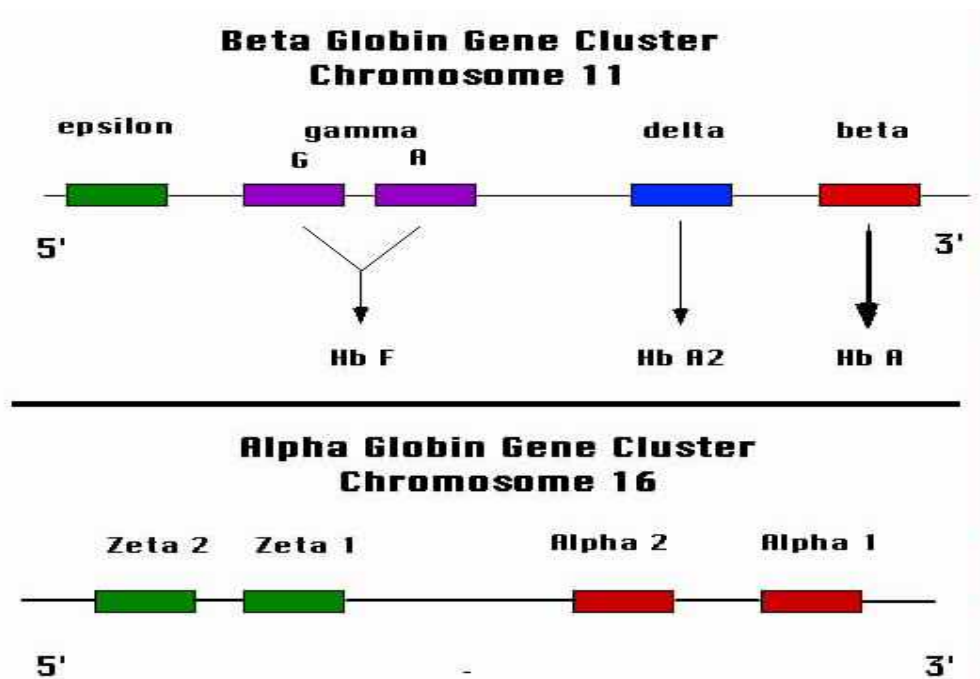| Embryonic hemoglobins – |
| --- |
| Gower 1- zeta(2), epsilon(2) |
| Gower 2- alpha(2), epsilon (2) |
| Portland- zeta(2), gamma (2) |
| |
| **Fetal hemoglobin -** |
| Hemoglobin F- alpha(2), gamma(2) |
| |
| **Adult hemoglobins -** |
| hemoglobin A_1- alpha(2), beta(2) - 97% |
| hemoglobin A_2- alpha(2), delta(2) – 2.5% |
| hemoglobin F- alpha(2), gamma(2) – 0.5% |

Genetics of thalassemia -

Figure – 3.2 Schematic representations of the globin gene loci

Alpha globin locus -

Each chromosome 16 has two alpha globin genes that are aligned one after the other on the chromosome. For practical purposes, the two alpha globin genes (termed $alpha_1$ and $alpha_2$) are identical. Since each cell has two chromosomes 16, a total of four alpha globin genes exist in each cell. Each of the four genes produces about one-quarter of the alpha globin chains needed for hemoglobin synthesis. The mechanism of this coordination is unknown. Promoter elements exist 5ʹ to each alpha globin gene. In addition, a powerful enhancer region called the locus control region (LCR) is required for optimal gene expression. The LCR is many kilobases upstream of the alpha globin locus. The mechanism by which DNA elements so distant from the genes control their expression is the source of intense investigation. The transiently expressed embryonic genes that substitute for alpha very early in development, designated zeta, are also in the alpha globin locus.

Beta globin locus -

The genes in the beta globin locus are arranged sequentially from 5' to 3' beginning with the gene expressed in embryonic development (the first 12 weeks after conception; called epsilon). The beta globin locus ends with the adult beta globin gene. The sequence of the genes is: epsilon, gamma, delta, and beta. There are two copies of the gamma gene on each chromosome 11. The others are present in single copies. Therefore, each cell has two beta globin genes, one on each of the two chromosomes 11 in the cell. These two beta globin genes express their globin protein in a quantity that precisely matches that of the four alpha globin genes. The mechanism of this balanced expression is unknown.

The lower panel shows the alpha globin locus that resides on chromosome 16. Each of the four alpha globin genes contributes to the synthesis of the alpha globin protein. The upper panel shows the beta globin locus. The two gamma globin genes are active during fetal growth and produce hemoglobin F. The "adult" gene, beta, takes over after birth.

Molecular biology -

Each globin gene consists of a string of nucleotide bases divided into 3 coding sequences termed exons and 2 noncoding regions known as introns or intervening sequences. Three other regions, known as regulatory regions, also exist in the 5' noncoding or flanking region of each globin gene.

The first is the promoter, which plays a major role in the transcription of the structural genes. The second region is the enhancer, which has an important role in promoting erythroid-specific gene expression, as well as in coordinating the changes in globin gene activity at different stages of development (embryonal, fetal, adult). Enhancers are able to influence gene expression, despite being located some distance away from the gene itself, and, unlike the promoter, they can stimulate transcription irrespective of their orientation relative to the transcription start site. Finally, master regulatory sequences, known as locus control regions (in the beta globin gene family) and HS40 (in the alpha gene complex), are responsible for activating the genes in erythroid cells.

Each of these regulatory sequences has a modular structure, consisting of short nucleotide motifs that act as binding sites for transcriptional activator or suppressor molecules. Such molecules activate or suppress gene expression in different cell types at different stages of development. Transcription of a certain gene is achieved by an initiation complex formed of certain proteins and a number of transcription factors, which interact with binding sites on the promoters and other regulatory sequences of the relevant genes.

When a gene is transcribed, mRNA is synthesized from one of the gene's DNA strands by the action of RNA polymerase. The initial product is a large mRNA precursor. Both exons and introns are initially present on this mRNA precursor; the introns ultimately are subsequently eliminated, and the exons are spliced together in the nucleus. At this stage, the mRNA, which also has been modified at both 5' and 3' ends, moves to the cytoplasm to act as a template for the production of globin chains.

Carrier molecules (transfer RNA [tRNA]) transport amino acids to the mRNA template. Each amino acid has a specific tRNA, which also contains 3 bases (anticodon), complimentary to the mRNA codons for that amino acid. The position of each amino acid in the globin chain thus is established by its corresponding triplet code (codon) in the globin gene. The CUG codon (cytidine/uridine/guanosine), for example, encodes the amino acid leucine, while the AAA codon (adenosine/adenosine/adenosine) encodes lysine. When a tRNA molecule carries the initial amino acid to the template, directed by codon-anticodon base pairing, globin chain synthesis begins.

Once the first tRNA is in place, a complex is formed between several protein initiation factors and the subunit of the ribosome that is to hold the growing peptide chains together on the mRNA as it is translated. A second tRNA moves in alongside, and a new amino acid is bound to the first with a peptide bond, resulting in a peptide chain 2 amino acids long. This process continues from left to right until a specific codon for termination is reached. At this point, the completed peptide chain drops off the ribosome-mRNA complex, and the ribosomal subunits are recycled. The globin chain is now ready to join a heme molecule and 3 other globin chains to form an Hb molecule.

The developmental switches from embryonic to fetal and then to adult Hb production are synchronized throughout the different organs of hematopoiesis (yolk sack, liver, bone marrow), which function at various stages of development. Even though the mechanism of such switches is not understood clearly, the globin gene promoter is known to contain information that specifies developmental stages of transcription.

Molecular pathology -
Mutations resulting in beta or alpha thalassemia are similar in principle but different in their patterns. More than 150 different mutations are known to result in various types of beta thalassemia. Major deletions are unusual (in contrast to alpha thalassemia), and most of the encountered mutations are either single base changes, small deletions, or insertions of 1-2 bases at a critical site along the gene. These mutations occur in both exons and introns.

For example, in a "nonsense" mutation, a single base change in the exon generates a "stop" codon in the coding region of the mRNA, resulting in premature termination of globin chain synthesis. This leads to the production of short, nonviable beta chains. Conversely, in the "frame shift" mutation, one or more bases on the exon are lost or inserted, resulting in a change in the reading frame of the genetic code or the production of a new stop codon.

RNA splicing mutations are fairly common and represent a large portion of all mutations resulting in beta thalassemia. These mutations corrupt the splicing process. The importance of precise splicing in the quantitative production of stable functional mRNA cannot be overemphasized. Slippage by even one nucleotide changes the reading frame of the mRNA. Specific consensus sequences exist at both ends of the RNA introns (at the junction with the exons); these motifs include GT in the 5′ (left end or donor site) consensus sequence and AG in the 3′ (right end or acceptor site) consensus sequence. Such sequences are obligatory for correct splicing, and a single substitution at the invariant GT or AG sequence prevents splicing altogether and results in beta-0 or alpha-0 thalassemia. Mutations in the other members of the consensus sequences, even though still highly conserved, result in variable degrees of ineffective beta globin production, causing milder types of beta thalassemia.

Mutations in exon sequences may activate a "cryptic" splice site. For example, in exon 1 of the beta globin gene, a consensus sequence that resembles a sequence in IVS-1 has been identified as the site for several distinct mutations, resulting in a gene that carries the features of both thalassemia and hemoglobinopathy at the same time (quantitatively and qualitatively abnormal Hb production). This type of mutation represents a clear link between the thalassemias and the hemoglobinopathies, and accordingly, these are labeled "thalassemic hemoglobinopathies." Thus, mutations at codon 19 (A to G), 26 (G to A), and 27 (G to T)—all in exon 1—result in reduced production of mRNA (thalassemia), due to inefficient splicing, and an amino acid substitution, encoded by the mRNA that is spliced and translated

(albeit inefficiently) into protein. The resulting abnormal Hbs are Malay, E and Knossos respectively.

The flanking regions of the beta globin gene are also sites for various mutations. A single base substitution involving the promoter element, for example, can down-regulate beta globin gene transcription, resulting in a mild form of beta thalassemia. Conversely, a mutation affecting the 3' end of the beta globin mRNA can interfere with it's processing, resulting in a severe form of beta thalassemia.

Clearly, many different beta thalassemia mutations exist, and compound heterozygosity frequently is encountered. The resulting laboratory findings may lead to confusion. An example is the patient who manifests symptoms of beta thalassemia major without an elevated HbA_2. The explanation for such a situation is often co-inheritance of beta and delta thalassemia. Delta/beta thalassemia further is divided into (delta/beta+) or (delta/beta-0). In the first type, a misalignment in the delta-beta genes during meiosis results in the production of fused delta/beta genes, a process responsible for the production of an Hb variant termed Hb Lepore. The fused delta/beta gene is under the control of a delta-globin gene promoter region (the beta gene promoter is deleted in the process). Since the delta gene promoter carries mutations that lead to ineffective transcription, the fused delta/beta chains are produced in limited amounts, resulting in thalassemia. This is in addition to the hemoglobinopathy.

Conversely, in delta/beta-0 thalassemia, a large deletion takes place in the beta globin gene cluster, removing both the delta and the beta genes, which also can extend to involve all globin genes on chromosome 11, thus producing (epsilon, gamma, delta, and beta-0) thalassemia.

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 beta^0 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 thalassemia intermedia 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. The factors that go into the diagnoses are: 1. The degree to which, the patient tolerates the anemia. 2. The threshold of the physician to transfuse patients with thalassemia. With regard to the tolerance of the anemia, most patients with thalassemia have substantial symptoms with a Hb of much below 7 or 8 gm/dl. With hemoglobins of this level, excess energy consumption due to the profound hemolysis can produce small stature, poor weight gain, poor energy levels, and susceptibility to infection. Further, the extreme activity of the bone marrow produces bone deformities of the face and other areas, along with enlargement of the spleen. The long bones of the arms and legs are weak and fracture easily. Patients with this clinical condition usually do better with regular transfusions. The need for regular transfusions would then place them under the heading of thalassemia major (see below). On the other hand, some patients with marked thalassemia can maintain hemoglobin of about 9 to 10 gm/dl. The exercise tolerance of these patients is significantly better. The question then becomes whether the accelerated bone marrow activity needed to maintain this level of hemoglobin causes unacceptable side effects such as bone abnormalities or enlarged spleen. This is a judgment decision. Some physicians to prevent these problems, even if they are slight, would transfuse a given patient at the critical borderline. The patient then would be *clinically* classified as having thalassemia major. Another physician might choose to avoid the complications of chronic transfusion. The same patient then would be *clinically* classified as thalassemia intermedia. The patient has thalassemia that is more severe than thalassemia trait, but not so severe as to require chronic transfusion as do the patients with thalassemia major. A patient can change status. The spleen is enlarged in these patients. The spleen plays a role in clearing damaged red cells from the blood stream. Since all of the red cells in patients with severe thalassemia have some degree of damage, clearance by the spleen accelerates the rate of cell loss. Therefore the bone marrow has to work harder to replace these cells. In some

patients, removal of the spleen slows the rate of red cell destruction just enough, that they can manage without transfusion, and still not have the unacceptable side-effects. In this case, the patient converts *clinically* from thalassemia major to thalassemia intermedia.

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, desferrioxamine (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.

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.

Management of ß-thalassemia major (*Cooley's Anemia)* 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 desferrioxamine 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).

Biochemistry of Iron –

Iron is the most important component of haemoglobin, the molecule of which contains four iron atoms. Approx. 2–5 g iron is normally present in the human body, depending on body weight. Some two-thirds of this quantity are incorporated into haemoglobin (100 ml blood contains approx. 15 g haemoglobin), about one-fifth is stored in the tissues, about one-tenth is contained in all types of cells as a component of various enzymes, and approx. 5% is bound in myoglobin; only about 2% circulates in the plasma. An average daily diet contains roughly 10–20 mg iron, but of this quantity the body normally absorbs only as much as is required to offset the amount of iron excreted each day, i.e. about O.5–1.O mg. Iron is absorbed from the gut by the cells of the intestinal mucosa in its non-poisonous bivalent form. It then either passes into the blood, where it becomes bound in its trivalent form to the iron-binding protein transferrin (siderophilin), or, alternatively, it is retained in the mucosal cells in the form of ferritin. Under normal circumstances, the iron in transferrin is made available to the erythropoietic cells for the synthesis of haemoglobin. Iron supplied to the body in excessive quantities becomes stored chiefly in the liver and in the reticulo-endothelial system, either as ferritin or as haemosiderin.

Iron overload -

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.

Clinical impact of iron overload in thalassemia -

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 (Witzleben and Wyatt 1961, 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 $\propto$ 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 thrive25. 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 thalassemia -
Blood transfusion therapy -

The combination of transfusion and iron chelation is now the reference treatment for thalassemia. This combination gives a marked improvement in survival, growth and sexual development if started at the right time and followed diligently. The standard policy is to maintain hemoglobin between 10 and 14 g/dl, if the blood supply permits. If adequate individual records are kept, the optimal transfusion therapy can be determined for each patient's clinical condition. One drawback of blood transfusion is that it exposes the patient to risk. Thus it is vital to improve blood safety continually and, find ways of reducing transfusion requirements.

Objectives of transfusion -

When the diagnosis of thalassemia major is clear-cut, regular transfusions should be started without delay. The transfusion scheme should keep the Hb level in the normal range, the current recommendation being to maintain a mean Hb = 10 -12 g/dl. Super-transfusion regimen, in which the mean Hb = 13g/dl, and Hyper-transfusion, maintaining a mean Hb = 12g/dl are also advocated. This scheme prevents 1. chronic hypoxemia 2. secondary bone change by reducing compensatory bone marrow hyperplasia 3. growth perturbation, whereas promotes normal physical activity 4. hypervolemia, which places a strain on the heart 5. early development of splenomegaly and hypersplenism, by reducing the number of abnormal RBC reaching the spleen and 6. excess gastro-intestinal iron absorption, which contributes to iron overload.

Amount of blood needed for transfusion -

The amount of blood needed for transfusion is estimated in relation with the patient's age and his clinical status. The total volume of blood fluctuates between 10-20ml / kg of body weight and the hematocrit of the RC (red cell) component may influence this calculation (60-75%). It is estimated that 3ml RCs / kg of body weight increase Hb per 1g/dl. A blood volume greater than 20ml / kg per transfusion must be avoided. However, the calculations are misleading for low-transfused patients (mean Hb=10g/dl or less) because they have a greatly expanded blood volume. To evaluate transfusion in such patients both the pre-transfusion and the post-transfusion Hb must be

measured routinely. Moreover, low-transfused patients are at risk for circulatory overload and therefore they have to be transfused with small volumes of RC but more frequently (8-12 days).

Transfusion rate -

The rate at which blood can be transfused without overloading the circulation depends on the patient's Hb level and cardiovascular status, and RC component used. When there is no cardiac problem: It is acceptable to give 5-7,5ml RC/Kg/ hour. If it is necessary to transfuse volumes > 20ml/Kg the transfusion must be given over 3-4 hours. When cardiac failure is present or Hb level < 5g/dl: No more than 5ml /Kg/ hour should be transfused on any one occasion, and infusion rate should not exceed 2ml /Kg / hour.

Transfusion interval -

The pre-transfusion Hb level required to maintain the recommended mean Hb = 12-12,5 g/dl, varies with the transfusion interval. In practice, the interval between transfusions varies from 2-4 weeks and it is well supported in well-being patients.

Personalizing the treatment -

1. The pre-transfusion Hb should be high enough (10-11g/dl) to inhibit marrow hypersensitivity. The exact level of pre-transfusion Hb can vary with Thalassaemia mutations involved, the patient's age, and the presence or absence of the spleen.

2. The post-transfusion Hb should not rise above 16g/dl. Higher levels increase blood viscosity, reduce tissue oxygenation and increase the risk of thrombosis. Moreover, blood consumption increases and iron overload is accelerated.

3. Transfusion interval is usually influenced by pragmatic considerations, such as the distance from a patient's home to the center, and the wish to minimize the social and psychological disturbance of repeated hospital visits.

4. To avoid waste of blood, the transfusion interval is also usually adjusted to allow the transfusion of a whole number of units (for small children, special pediatric blood bags are available).

Transfusion reactions in thalassemic patients -

<u>Acute</u> -

After infusion of only few millilitres of blood:

1. Hemolytic (AHTR) Intravascular: result from transfusion of ABO incompatible RCs (clerical error) and can be life-threatening.

2. Anaphylactic: Immediate hypersensitivity, occurs among patients who have a congenital deficiency in IgA. It begins mildly but can progress to shock and death.

3. Embolic: Air embolism is a concern, if air enters the system when containers or tubing are changed. Patient symptoms may include cough, dyspnea, chest pain and shock.

Steps to be followed:

1. Stop the transfusion immediately and maintain IV access with normal saline.

2. Perform clerical check of labels, forms and patient identification. If an error has been made, undertake then an immediate search to locate it and avert putting another patient at risk.

3. Report reaction to blood bank personnel.

4. Draw a post-transfusion sample and urine sample and send it along with the unused blood product and its administration set to the blood bank.

Towards the end or after the completion of the transfusion -

 1. *Circulatory Overload:* Transfusion that proceeds at too fast a rate may lead to congestive heart failure and pulmonary edema.

2. TRALI: should be considered whenever recipient experiences acute respiratory difficulties without evidence of cardiac involvement.

3. Metabolic: Hyperkalaemia may arise when RBC are transfused at the end of storage, when they lose their intracellular potassium.

4. FNHTR: occurs in about 1% of the transfusions. It is defined as an increase in $T^o > 1^oC$, without any other medical explanation. It may be caused by antileukocyte antibodies present in the recipient's plasma or to infusion of cytokines produced by leucocytes during component storage. It may be reduced by antipyretics and prevent by leucopoor RC components $< 1 \times 10^6$ WBC/unit.

5. Allergic (urticarial): Characterized by local redness, itching and hives. It is the result of either foreign allergen in the donor plasma, with which the recipient antibodies react, or transfused donor antibodies, which combine with recipient allergens. It may be reduced with antihistamines.

DELAYED:

After some days or weeks:

1. *Alloimmune Hemolytic Extravascular (DHTR):* an anamnestic response leads to antibody production and clinical symptoms in about 3-7 days post-transfusionally. The most common alloantibodies are in Rhesus (anti-D, anti-C^w, anti-E, anti-c, anti-C). Symptoms may be mild (fever, chills and moderate jaundice) to severe, depending on the hemolytic effect of antibody attachment to the surface of the RCs. Moreover, there is a failure of expected therapeutic response.

2. *Viral Contamination:* The risk varies between the different countries. It depends on transfusion screening programme, as well as the carrier rates of infection in the donor populations. The prevention of HIV and Hepatitis C are of great importance.

3. *Parasitic Contamination:* Malaria, Leishmaniose.

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).

Desferrioxamine (Desferal) –

Desferrioxamine 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 pilosusEttlinger et al.) 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:
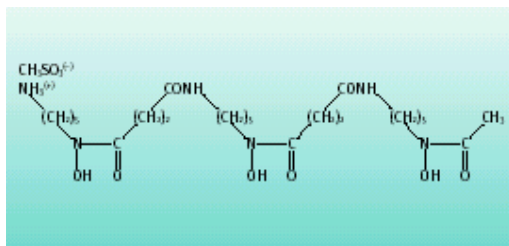
Desferrioxamine B
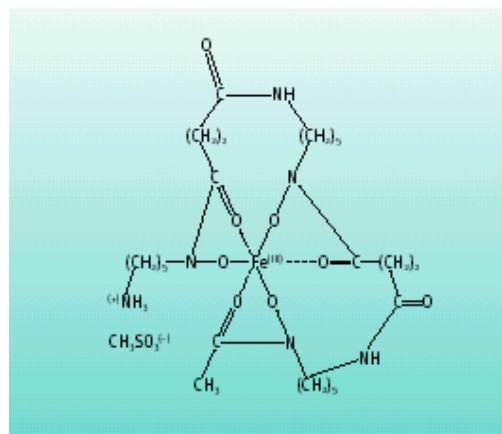
Ferrioxamine B

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 (Cmax) 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 (Cohen et al., 2000, 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).

Role of ascorbic acid in chelation therapy -
Patients on chelation therapy have been demonstrated to have some degree of vitamin C deficiency. This has been attributed in part to increased catabolism. Administration of vitamin C increases the urinary excretion of iron and raises both serum iron and ferritin levels. This probably is related to the fact that vitamin C slows down the conversion of ferritin to hemosiderin, leading to the availability of more chelatable iron. Conversely, vitamin C enhances iron-mediated peroxidation of membrane lipids, leading to significant toxicity, mostly cardiac dysfunction in patients who are receiving large doses of vitamin C supplementation in addition to chelation therapy. For this reason, only small doses should be administered to enhance chelation (3 mg/kg/d at the start of infusion of the chelator). Large doses should be avoided.

Vitamin E deficiency –
Vitamin E deficiencies have been reported in patients with severe thalassemia. Some of the hemolysis in this population even was attributed to peroxidation of the membrane lipids by an iron-mediated free radical effect. As an antioxidant, vitamin E is expected to decrease cell toxicity.

Folic acid deficiency –
This deficiency is a common complication in patients with thalassemia, mainly due to the extreme demand associated with the severe expansion of the marrow. Other causes, such as poor absorption and intake, also can contribute to folate deficiency. For this reason, folic acid (1 mg/d) has been recommended as a supplement for this patient population.

Hematopoietic stem cell transplantation –

HSCT only is recommended for selected patients. It is the only known curative treatment for thalassemia. Poor outcome after HSCT correlates with the presence of hepatomegaly and portal fibrosis and with ineffective chelation prior to transplant. Event-free survival for patients who have all 3 features is 59%, compared to 90% for those who lack all 3. Even though blood transfusion is not required after a successful transplant, chelation therapy continues to be needed in certain individuals to remove excessive iron. The optimal time to start such treatment is a year after the successful HSCT. Parents and/or caregivers of patients with severe thalassemia frequently are confronted with the question of choosing between standard therapy and HSCT. The 15-year cardiac disease-free survival for patients on standard therapy exceeds 90% and is similar for those without risk factors having undergone HSCT. Long-term outcome for transplant patients, including fertility, is not known. The cost of long-term standard therapy is known to be higher than the cost of transplant. The possibility of developing cancer after HSCT also should be considered. In many centers, the donor has to be a matched sibling with or without thalassemia trait.

Gene therapy –

This therapy is a very attractive therapeutic modality, the efficacy of which remains to be demonstrated.

Surgical Care –

The principal surgical procedure used for many patients with thalassemia is splenectomy. The spleen is known to contain a large amount of the labile nontoxic iron (i.e. storage function) derived from sequestration of the released iron. The spleen also increases RBC destruction and iron distribution (ie, scavenger function). These facts always should be considered before the decision is made to proceed with splenectomy. If the spleen acts as a store for nontoxic iron, thereby protecting the rest of the body from this iron, early removal of the spleen may be harmful (liver cirrhosis has been described in such individuals). Conversely, when the spleen becomes hyperactive, leading

to excessive destruction of RBCs and thus increasing the need for frequent blood transfusions, resulting in more iron accumulation, splenectomy is justified. Furthermore, if the labile iron pool in the spleen becomes the target for the action of the deferoxamine (ie, removing the nonharmful pool and leaving the toxic one), then splenectomy is justified even more. The goal in this confusing dilemma always should be to achieve a negative iron balance, which in many patients has been possible by continuous administration of subcutaneous deferoxamine. Several criteria are used to aid in the decision for splenectomy; a practical one suggests that splenectomy may be beneficial in patients who require more than 200-250 ml/kg of PRBC per year to maintain an Hb level at 10 g/dl. The risks associated with splenectomy are minimal, and many of the procedures now are performed by laparoscopy. Postsplenectomy risk of infections with encapsulated organisms and malaria in endemic areas is always a concern. The problem is minimal at the present time, since presplenectomy immunizations and postsurgical prophylactic antibiotics have decreased the rates of such complications significantly. Traditionally, the procedure is delayed whenever possible until the child is aged 4-5 years or older. Aggressive treatment with antibiotics always should be administered for any febrile illness while awaiting the results of cultures.

Prenatal DNA testing -

Successful prevention programs in different parts of the world have resulted in an impressive decline in the number of patients with severe forms of thalassemia. Ferrara, Cyprus, Sardinia, Greece, and the United Kingdom were among the first to report a significant decline in the birth rate of children with thalassemia major. Many other regions with more limited resources are following their steps with remarkable success. In addition to the effective prenatal diagnosis adopted in the countries mentioned, other measures such as premarital screening programs, genetic counseling, and restrictions on issuing marriage certificates and licenses also proved to be effective. Since many of the countries where thalassemia prevails are poor and cannot afford sophisticated preventive programs, more practical approaches clearly are needed.

Screening of school children, pregnant women, and individuals visiting public health facilities is effective in identifying individuals at risk who require further testing. A simple CBC, with emphasis on the red cell counts and indices, including the MCV, MCH, and RDW, is the main component of such screening processes. Those suspected to be positive are checked for elevated HbA_2, HbF, or both for confirmation. In some situations, this simple method is not adequate, and further testing; including analyses of globin chain synthesis must be performed to reach a final diagnosis.

Investigational agents known to increase HbF level -

In the past few years, several new approaches to the treatment of thalassemia have included marrow or stem cell transplantation, the use of young red blood cells ("neocytes" for transfusion), maintenance of a higher pre-transfusion hemoglobin level, new iron chelators and the use of drugs such as hydroxyurea, 5-AzaC and butyrate compounds to elevate fetal hemoglobin (HbF).

Fetal hemoglobin or HbF ($\propto_2\gamma_2$), which has a substantially higher affinity for oxygen than adult hemoglobin, develops in the fetus during the last six months of gestation. As both mother and fetus share the same blood supply, fetal hemoglobin essentially draws off oxygen from the mother's blood. This enables the fetus to survive in the uterus. After birth, fetal hemoglobin levels fall rapidly and in the adult represents less than one percent of total hemoglobin in the body. Thus, total adult hemoglobin comprises of HbA_1 - $\propto_2\beta_2$ (97%), HbA_2 - $\propto_2\delta_2$ (2.5%) and HbF - $\propto_2\gamma_2$ (0.5%). It has been found that stimulation or induction of fetal hemoglobin in thalassemia can improve the patient's clinical condition.

Increased transcription of fetal hemoglobin requires an open chromatin configuration dictated by –

1. DNA hypomethylation –

The γ-globin gene promoter has been shown to be nonmethylated at a CpG residue during fetal development; this dinucleotide sequence becomes

methylated (^{m}CpG) postnatally when γ-globin gene expression is silenced (Vander-Ploeg and Flavell 1980). Early studies demonstrated that treatment of tissue culture cells with the cytidine analog 5-azacytidine (5-azaC) led to cellular differentiation and DNA hypomethylation (Jones and Taylor 1980). Incorporation of 5-azaC into baboon DNA resulted in a 40- to 70-fold increase in γ-globin gene expression in the adult (DeSimone et al., 1982), and to hypomethylation of the γ-globin promoter (Lavelle et al., 1986). These early studies led to clinical trials of this analog in sickle cell anemia and ß-thalassemia (Ley et al., 1983, Ley and Anagnou 1983, Dover et al., 1983, Charache et al., 1983) that demonstrated significant increases in HbF levels (10%–25%), F cells (30%–40%), and total hemoglobin (1–3 g/dL) in patients receiving 5-azaC. When a 5-azaC analog incorporates into DNA as 5-aza-2-deoxycytidine, it covalently binds DNA methyltransferase (DNMT), thereby depleting DNMT concentration (Juttermann and Jaenisch 1994, Creusot et al., 1982). This decrease in DNMT results in DNA hypomethylation as DNMT is required for maintenance of the methylation pattern during replication. Patients treated with 5-azaC analogs show global hypomethylation.

2. Increased histone acetylation -

Histone proteins are found attached to DNA allowing long chromosomal strand to fold and stack on itself (i.e. formation of chromatin) when transcription is not in progress. Transcription requires an open chromatin configuration dictated by specific patterns of histone acetylation of amino acids in amino terminal histone. For example, histone acetylation is required for an active chromatin configuration, and acetylated histone does not associate with DNA having ^{m}CpG dinucleotides (Eden et al., 1998). The level of acetylation is, to a large extent, dependent on histone deacetylase (HDAC) activity. Butyrate exerts its effect by increasing histone acetylation and opening up the chromatin structure of the promoter of the γ-globin gene. Mechanism of induction of HbF by butyrate is that it Inhibits histone deacetylases and thereby increasing histone acetylation, which opens up the chromatin structure and makes DNA more accessible to transcription factors.

This results in increased transcription of the γ-globin gene by making its DNA more accessible to transcription factors.

3. Histone methylation -

There is a third level of regulation related to DNA methylation, and that is histone methylation. Histone methylation can have an activating or suppressing effect depending upon which amino acids are methylated, and the number of methyl groups added.

The current hypothesis is that histone modifications and DNA methylation may influence each other. It has also been shown that there is a direct connection between the enzymes that methylate DNA and methylate histones (Fuks et al., 2003). Thus DNA methylation appears to be central in transcriptional regulation. This cross talk suggests that inhibitors of DNMT and HDAC may collaborate to activate transcription (Bird 2001).

4. Materials and Methods

4.1 Pharmacognostic studies

Identification of wheat variety and growing of wheatgrass -

Certified samples of three major species of wheat viz. *Triticum aestivum*, *Triticum durum* and *Triticum dicoccum* were acquired form Wheat Research Center, Gujarat Krushi University, Junagadh, Gujarat. The authenticity of these certified samples was also confirmed by comparing their morphological characters with the description mentioned in different standard texts and floras (Percival 1974). Voucher specimens were deposited at B. K. Mody Government Pharmacy College, Rajkot, Gujarat. These wheat varieties were grown in plastic trays as per the standard procedure described below (Wigmore 1985).

Procedure for growing wheatgrass -

1. Adequate quantity of unpolished wheat grain were 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 everyday 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 and differentiate among the three varieties of wheatgrass, all three varieties of the grass were subjected to microscopic study, which included transverse sections, surface preparations and powder study. For

powder study the grass was cleaned and dried in a dark place for four days. It was powdered, passed through 40# and stored in airtight bottles.

4.2 Phytochemical Studies

Extraction Process -

For preparation of methanolic extract, 100 gram of fresh wheatgrass was crushed thoroughly, using mortar and pestle. The crushed wheatgrass was completely exhausted by adding small quantities of methanol and filtering off every time in a successive manner, to yield final volume of 1 liter. The methanolic extracts of different varieties of fresh wheatgrass, and also dried wheatgrass powders, obtained from different types of drying processes, were prepared by above-mentioned procedure. These extracts were subjected to thin layer chromatography.

Thin-Layer Chromatography and HPTLC finger printing -

Samples of different methanolic extracts and methanolic solutions of reference standards chlorophyll a, chlorophyll b and β carotene were used for TLC and HPTLC. Suitably diluted sample solutions were spotted on pre-coated silica gel 60 F_{254} TLC plates (E. Merck) using CAMAG Linomat IV Automatic Sample Spotter. The plates were developed in the solvent system consisting of Hexane: Acetone (65:35). The plates were dried at room temperature and scanned using CAMAG TLC scanner 3 at UV 254 and 354 nm and Rf values, absorption spectra of resolved bands were recorded.

4.3 Estimation of antibacterial activity -

Preparation of Extract -

Fresh Wheatgrass was crushed and the juice was filtered through filter paper (undiluted juice). Fresh Wheatgrass was also extracted in acetone and methanol separately. The extracts were concentrated and dried by evaporating the solvent. The anti-microbial activity of the undiluted juice and extracts was studied by agar-well diffusion method (Pelczar et al., 1993) at the concentration of 2.7 g/ml were tested against *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli,*

Pseudomonas aeruginosa, Kleibsella pneumoneae, Bacillus megaterium, Bacillus cereus and *Salmonella typhimurium*.

Activation of strain and preparation of plate -

A loop full of the test strain was inoculated in 25ml of nutrient broth and incubated for 24 hours on a rotary shaker so as to activate the given test bacterial strain. The Mueller Hinton Agar No. 2 (Hi-media) was prepared for the study of in-vitro antibacterial activity by agar diffusion method (Pharmacopoeia of India). Agar well diffusion method (Perez et al., 1990) was performed.22 ml of the Mueller Hinton Agar No. 2 media was poured into the 100mm diameter Petri-plate (Hi-media). The test bacterial culture (0.2 ml) was also added along with the media into the Petri plate.

Microbiological Assay -

Inoculation of the test strain was done by the pour-plate technique. The media is autoclaved at 121 lbs pressure.0.2ml of the activated strain was inoculated into the media with the help of an auto pipette when it reached 40-42 C temperatures. Shaking the sugar tube in between the palms will make the sugar tubes temperature reach somewhere between 40-42 C. Now the activated test bacterial strain (0.2 ml) is inoculated into the media. Again the tubes are rotated in between the palms for the proper homogenization of the bacterial in the media. The complete procedure of the plate preparation was done in the laminar airflow to maintain strict sterile and aseptic condition. After the inoculation the plates were kept for solidification. The solidification of the media took about 30 – 45 minutes. After the media got solidified, a ditch was made in the plates with the help of a sterile cup-borer (8.5mm). After introducing the cup-borer, with the help of a sterile forceps the agar beads were removed, form a well in the agar plate and 0.1ml of the test compound was introduced into the well. The inoculation of the crude drug was done with the help of auto pipette (Glaxo). The plates were kept for incubation for 24-36 hours depending upon the test bacterial strain used. The inhibitory activity of the compounds in various solvents was determined by comparing the sizes of inhibition zones of the different compounds in different solvents with those of the controls. The controls were those solvents in which the test compound is

dissolved. The positive result was determined by deducting the control (solvent) activity with that of the test (compound). The experiment was done in triplicates.

Media (1) Used:

N – Broth was used for activation of the test bacterial strains.

Media Composition:

Peptic digest of animal tissue	-	0.5 %
NaCl	-	0.5 %
Beef extract	-	0.15 %
Yeast extract	-	0.15 %
Distilled water	-	100 ml

Media (2) used:

Mueller Hinton Agar No. 2 was used for the antimicrobial susceptibility test.

Media Composition:

Casein acid hydrolysate	-	1.75 %
Beef heart infusion	-	0.2 %
Starch, soluble	-	0.15 %
Agar	-	1.7 %
Distilled water	-	100 ml

4.4 Estimation of antiproliferative activity –

Seeds of green grams, groundnuts and wheat were soaked in sufficient quantity of distilled water (control) in 5 petridishes each, overnight. Similarly, all three varieties of seeds were soaked in Wheatgrass juice prepared traditionally in water. Next day, the seeds were sown in small bowls filled with plain soil. The left over quantity of distilled water or Wheatgrass juice, used for soaking the seeds, was added to the soil of each bowl in corresponding manner. Seeds were allowed to germinate for 5 days with addition of sufficient quantity of distilled water to all the bowls, daily. On 6th day, the number of seeds germinated in each bowl was counted. Sprout length less than 2 mm

were not considered in counting. The experiment was repeated 5 times and average was reported.

4.5 Preparation of dried Wheatgrass powder -

For preparation of dried Wheatgrass powder (to be used for manufacturing of Wheatgrass tablets) different drying techniques were used such as freeze-drying, spray drying and shade drying. In freeze drying technique, fresh wheatgrass was frozen to sub-zero temperature and subsequently subjected to low-temperature heating (5 ^{o}C -10 ^{o}C) in vacuum to evaporate crystallized water content. Dried wheatgrass was then milled to obtain powder. In spray-drying technique, fresh wheatgrass was pressed in a hydraulic press to obtain juice. The juice was then sprayed in aerosol form through a nebulizing nozzle in a conical vessel from top. A hot air counter current (55 ^{o}C) was passed from bottom of the vessel. The nebulized juice settled on the bottom of the vessel in the form of powder. In shade-drying technique, fresh wheatgrass was dried at room temperature in a dark room. The dried wheatgrass after 3-4 days of drying period was powdered in a mill.

4.6 Preparation of wheatgrass tablets -

Wheat grains of *Triticum durum* variety were acquired and sown in plain soil without using any type of fertilizer. The wheatgrass was grown under specially constructed shades. The grass was harvested after 8 days from sprouting and shade-dried in well-ventilated dark rooms for 4 days. Dried wheatgrass was powdered in a mill. Tablets of wheatgrass were manufactured in a pharmaceutical tablet unit by adding suitable binders and excipients.

4.7 Clinical trial of wheatgrass on patients with anemia -

The clinical trial of wheatgrass on anemia was carried out at Ayurved Hospital working under Civil Hospital, Rajkot. Necessary permission for conducting the clinical trial was obtained from the concerned ethical committee. 20 anemic patients, visiting the hospital were enrolled for the trial, after taking informed consent.

Twenty patients suffering from anemia whose age ranged from 15 to 50 years were included in the trial. The patients were given wheatgrass tablets with dosage regimen of 2 tablets (wheatgrass powder 250 mg.) 3 times in a day for 1 month. Blood samples were collected at the start and at the end of period of clinical trial (i.e. 1-month period). The samples were analyzed for hemoglobin content by using standard method (Sahlǐs Hemoglobinometer) while, Total RBC count, Total WBC count, Neutrophil count, Lymphocyte count and Total lymphocyte count were recorded by using automatic cell counter – Model KX-21 – Sysmex.

4.8 Clinical trial of wheatgrass on patients with β-thalassemia (major) -

The clinical trial of wheatgrass on β-thalassemia was carried out at K. T. Children Hospital working under Civil Hospital, Rajkot. Necessary permission for conducting the clinical trial was obtained from the concerned ethical committee. Thalassemic patients, visiting K. T. Children Hospital regularly for blood transfusion and registered at the hospital were enrolled for the trial, after taking informed consent.

Twenty patients suffering from β-thalassemia (major) whose age ranged from 8 to 20 years were included in the trial. The patients of this group were given wheatgrass tablets with dosage regimen of 2 tablets (wheatgrass powder 250 mg.) 3 times in a day for 9 months. Blood samples were collected at the start, after 6 months and at the end of period of clinical trial (i.e. 9 months period).

The samples were analyzed for hemoglobin content by using standard method (Sahlǐs Hemoglobinometer) while, Total RBC count, PCV, MCV, MCH, MCHC, Total WBC count, Neutrophil count, Lymphocyte count, Eosinophil count, Basophil count and Total lymphocyte count were recorded by using automatic cell counter – Model KX-21 – Sysmex.

We also estimated Serum iron, Serum magnesium, Serum ferritin and Thiobarbituric acid reacting substances (TBARS).

4.9 Estimation of serum magnesium -

Serum magnesium was estimated by Calmagite Dye method.

Principle -

Magnesium ions react with Caimagite to produce a red complex, which is measured spectrophotometrically at 530 nm. Intensity of the color produced is directly proportional to magnesium concentration in the serum. To eliminate the interference of calcium during estimation, EGTA is included in the reagent. Heavy metal interference is prevented by the presence of cyanide and a surfactant system is included to remove protein interference.

Kit contents -

Reagent 1: Magnesium color reagent - Calmagite: 0.006°o w/v, Stabilizer: 1% w/v

Reagent 2: Ethylaminoethanol: 6%, EGTA: 1.18 mM, Potassium cyanide: 0.10%

Reagent 3: Magnesium Standard - Magnesium salt: 2 meq/l

Preparation of working reagent -

Ten volumes of Color reagent (Reagent 1) were mixed with one volume of Buffer reagent (Reagent 2). The mixture (working reagent) was prepared as per requirement for the day.

Procedure -

Pipette into Test Tubes	Blank	Standard	Test
Magnesium working reagent	1.0 ml	1.0 ml	1.0 ml
Standard	-	10 ml	-
Sample	-	-	10 ml
Distilled water	10 ml	-	-

All solutions were mixed and incubated at room temperature (22-28°C) for 10 min. The absorbance of the Test (A_T), Standard (A_S) and Blank (A_B) were read against distilled water at 530 nm (520 - 530 nm).

Calculations -

Magnesium conc. (meq/l)   = $(A_T- A_B)$ x 2 $(A_s – A_B)$

4.10 Estimation of serum iron–

Principle –

Transferrin-bound iron is released at an acid pH and reduced from ferric ion to ferrous ion. These ions react with frrozine to form a violate-colored complex, which is measured spectrophotometerically at 560 nm. The absorbance measured at this wavelength is proportional to serum iron concentration.

Reagents –

Iron buffer reagent – Hydroxylamine hydrochloride 220 mM in acetate buffer, pH 4.5 with surfactant.

UIBC buffer reagent – Tris 500 mM, pH 8.1 with surfactant, Sodium azide 0.05% (w/v) as preservative.

Iron color reagent – Ferrozine 16.7 mM in hydroxylamine hydrochloride

Iron standard (500 ug/dl) – Ferrous chloride in hydroxylamine hydrochloride.

Procedure –

Couvettes were labeled as blank, standard and sample. Table of setup instructions listed bellow, was followed.

Total iron -

Reagents	Test	Sample blank
Iron buffer	1ml	1 ml
Sample	200 ul	Ul
Color reagent	20 ul	-

Color was added and incubated for 10 minutes at 37 c.

Spectrophotometer was zeroed at 560 nm with reagent blank.

Absorbencies of all tubes were read and recorded.

Calculation –

A = Absorbance

STD = Standard

Total Iron (ug/dl) = $\underline{(A_{Test} - A_{blank})}$ X conc./ $A_{Std} - A_{blank}$

4.11 Estimation of Serum Ferritin–

Serum Ferritin was estimated using Access Immunoassay System a product of Beckman Coulter.

Principle –

Access Ferritin assay is a two-site immunoenzymatic (sandwich) assay. A sample is added to a reaction vessel with goat anti-ferritin-alkaline phosphatase conjugate and paramagnetic particles coated with goat anti-mouse: mouse anti-ferritin complexes. Serum or plasma (heparin) ferritin binds to the immobilized monoclonal anti-ferritin on the solid phase, while the goat anti-ferritin enzyme conjugate reacts with different antigenic sites on the ferritin molecules. After incubation in a reaction vessel, separation in a magnetic field and washing remove materials not bound to the solid phase. A chemiluminescent substrate, Lumi-phos* 530, is added to the reaction vessel and light generated by the reaction is measured with a luminometer. The light production is directly proportional to the concentration of ferritin in the sample. The amount of analyte in the sample is determined from a store multi-point calibration curve.

Procedure –

Ferritin assay was performed using automatic analyzer as per the Access Immunoassay System manual.

4.12 Estimation of free radicals (Thiobarbituric acid reacting substances)–

Lipid peroxidation product (MDA) was estimated by thiobarbituric acid reaction method (Ohkawa et al., 1979).

Procedure -

1.0 ml sample was mixed with 0.2 ml 4% (w/v) sodium dodecyl sulphate (SLS). 1.5 ml 20% acetic acid in 0.27M HCl (pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid (pH 7.4). Mixture was heated in hot water bath at $85^{\circ}C$ for 1 hr and after centrifugation at 1200 g for 10 minutes; intensity of pink color was read against reagent blank at 532 nm. The amount of MDA (TBA reactive material) was calculated using molar extinction coefficient 1.56×10^5 $M^{-1}.Cm^{-1}$ and has been reported as n moles of MDA per mg protein.

4.13 Statistical analysis–

Results are presented as mean ± SEM. Statistical differences between the means of the various groups were evaluated using student's paired 't' test. Data were considered as statistically significant at 'P' value of 5% (P   0.05).

5. RESULTS

5.1 Pharmacognostic Studies

Macroscopical features -

In our investigation, certified samples of three major species of wheat viz. *Triticum aestivum*, *Triticum durum* and *Triticum dicoccum* were used to grow wheatgrass in plastic trays as per the standard procedure. In conformation with the 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 1974). The leaf blade was linear and parallel–veined with mid rib projecting on the back, continuing someway along the sheath. In *T. Dicoccum* the hairs on the swollen base of leaf were longer than those of other species. The longest leaves were possessed by *T. durum*.

Microscopical studies -

Microscopic studies of transverse sections, surface preparations and powder studies of three varieties of wheatgrass viz *Triticum aestivum, Triticum durum* and *Triticum dicoccum* 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 different species of *Triticum* -

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 cutinised outer wall 4-5 μ thick, appearing in longitudinal section as a narrow rectangle 150-300 μ long and 15-20 μ wide. In other rows characteristic lines of long cells alternating with stomata were also present, and, except *T. durum,* 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 5.1 – 5.3).
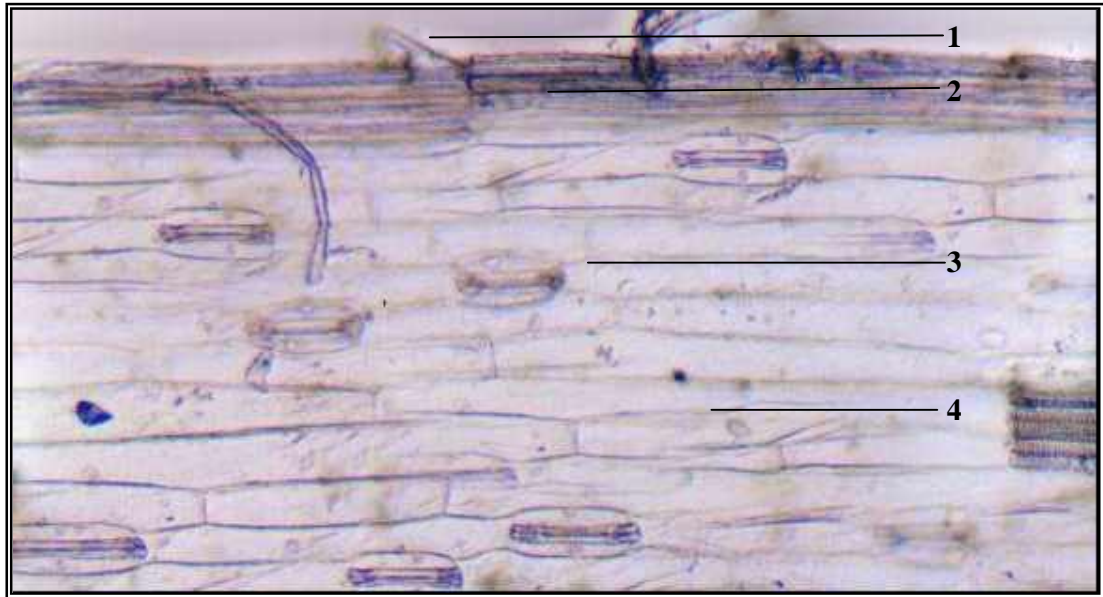
Figure 5.1 Surface preparation of *Triticum durum*
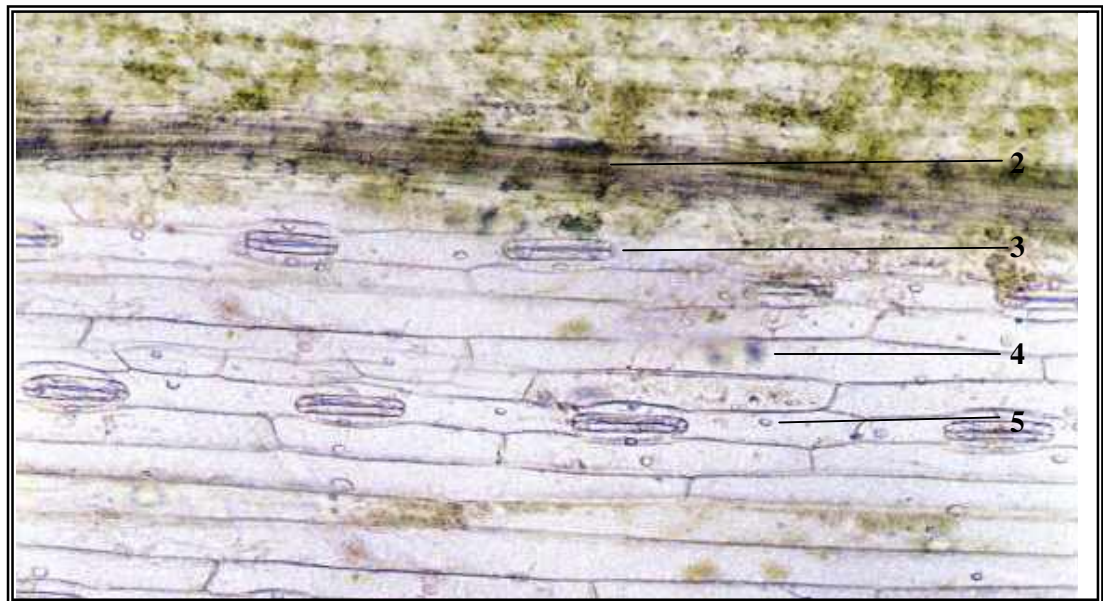
Figure 5.2 Surface preparation of *Triticum dicoccum*
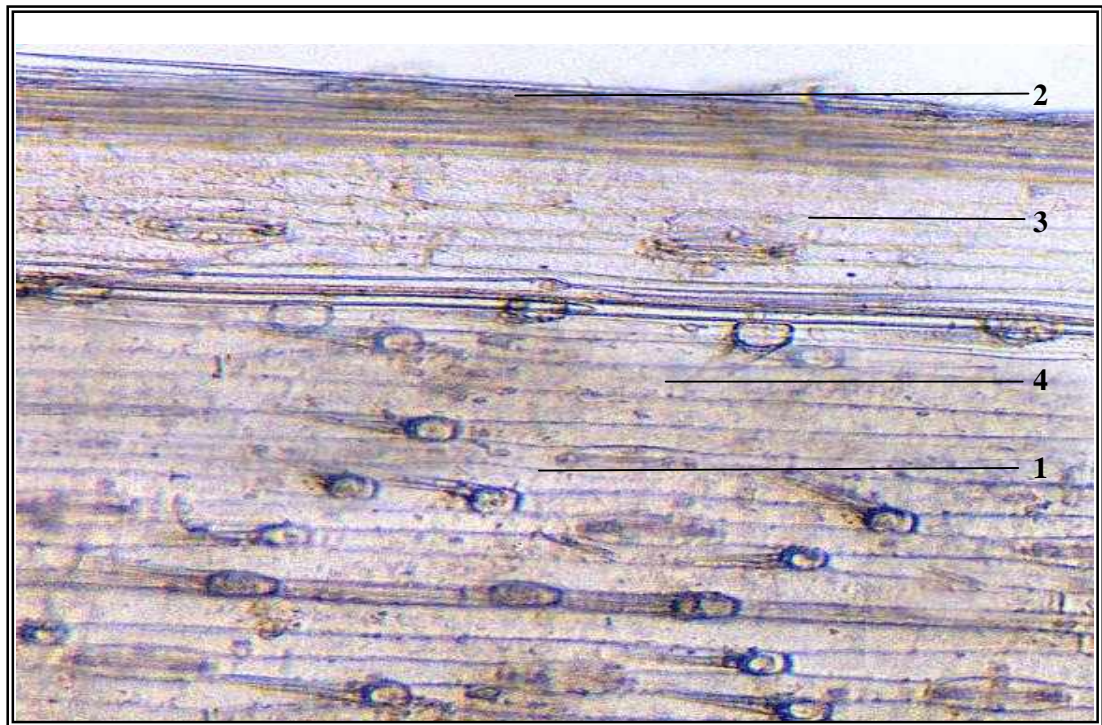
Figure 5.3 Surface preparation of *Triticum aestivum*

   1. Unicellular trichome
   2. Stereome fiber
   3. Double raw of stomata
   4. Rows of motor cells
   5. Calcium oxalate crystal

Transverse section of *Triticum* 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 the 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 µ 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 µ 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 4.5-6µ 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 µ 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 bellow the bundles, and arranged parallel with them along the leaf were strands of stereome or supporting tissue consisting of sclerenchymatous fibers.

Differentiating characters among transverse sections of leaf of various *Triticum* species -

Transverse section of *T. durum* leaf -

Trichomes were almost absent or very rare but stomata were more in number than other verities. Lower epidermal cells had more motor cells in comparison to upper one. Lower portion of median vein was more bulging than other species. Xylem vessels were more in number than in other species. Outer sheath of vascular bundles were incomplete (Figure 5.4 and 5.7).

Transverse section of *T. dicoccum* leaf -

Motor cells were not seen clearly. Outer sheath of vascular bundle was incomplete and parenchymatous cells of the sheath were smaller than other species. Xylem vessels were 2-3 in number. Each ridge had almost one row of simple pointed trichomes (Figure 5.5 and 5.8).

Transverse section of *T.* 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 5.6 and 5.9).

Transverse sections (Diagrammatic) of different species of Triticum -
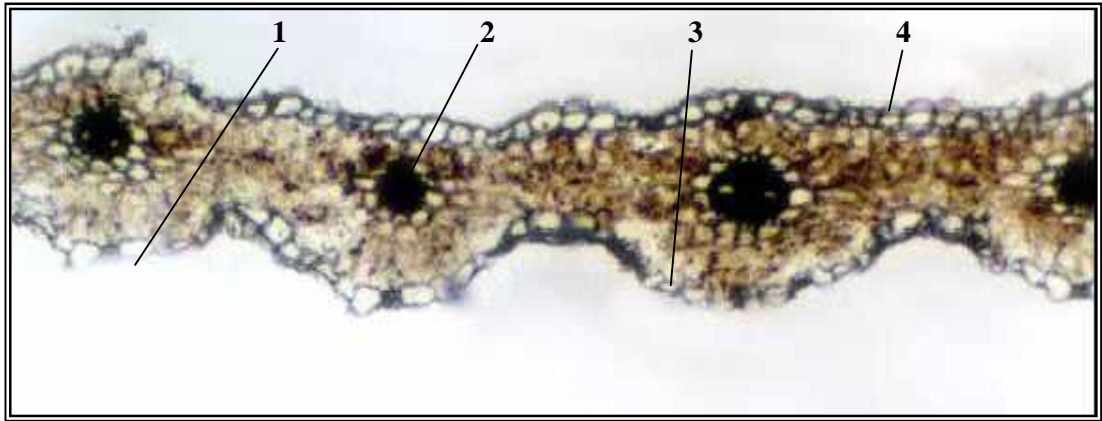
Figure 5.4 Transverse section (Diagrammatic) of *Triticum durum*
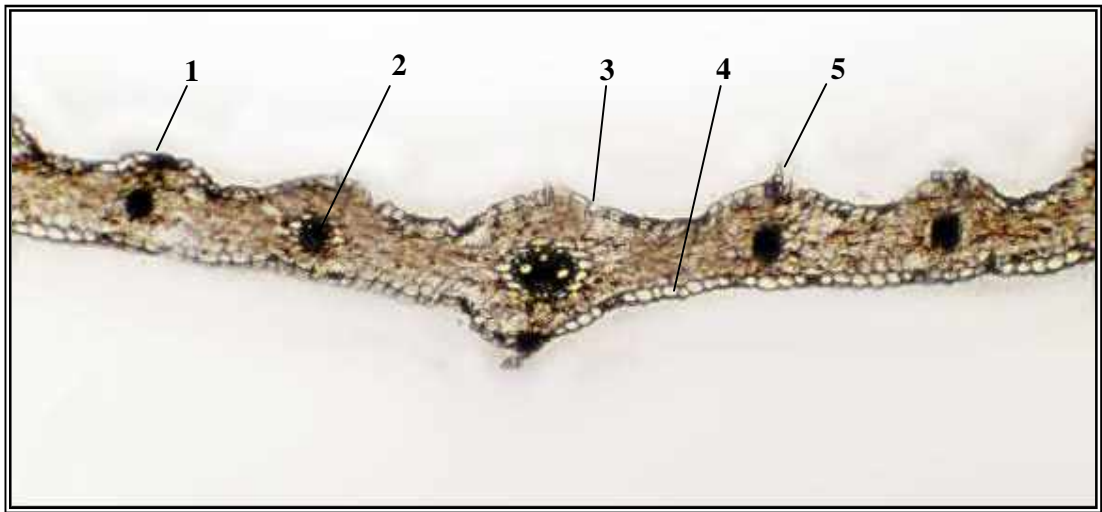
Figure 5.5 Transverse section (Diagrammatic) of *Triticum dicoccum*
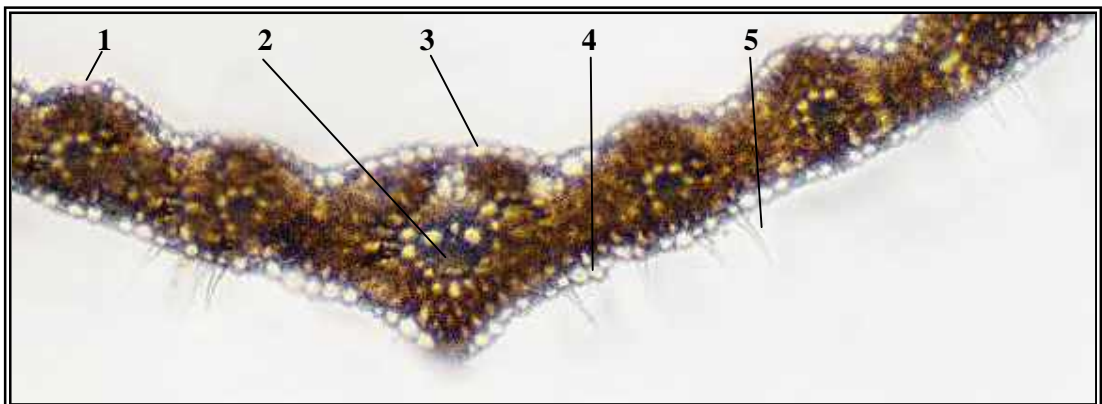
Figure 5.6 Transverse section (Diagrammatic) of *Triticum aestivum*

1. Ridge
2. Vascular Bundle
3. Outer epidermis
4. Inner epidermis
5. Trichome

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Transverse sections (Cellular) of different species of *Triticum* -
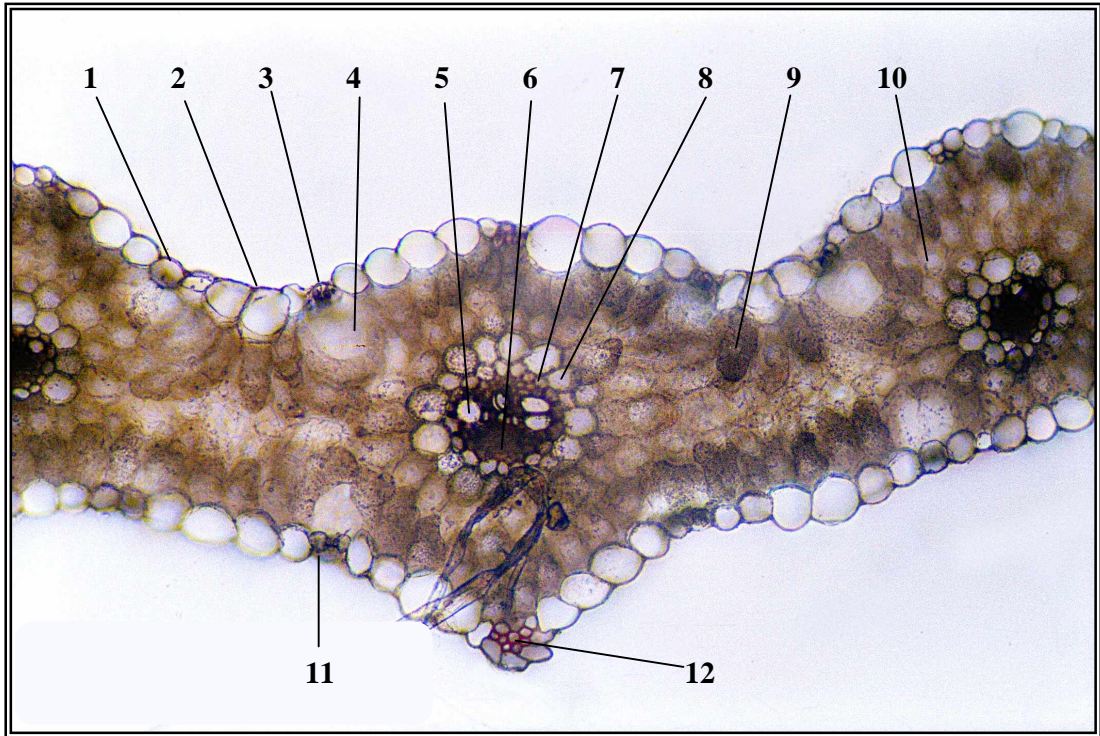
Figure 5.7 Transverse section (Cellular) of *Triticum durum*
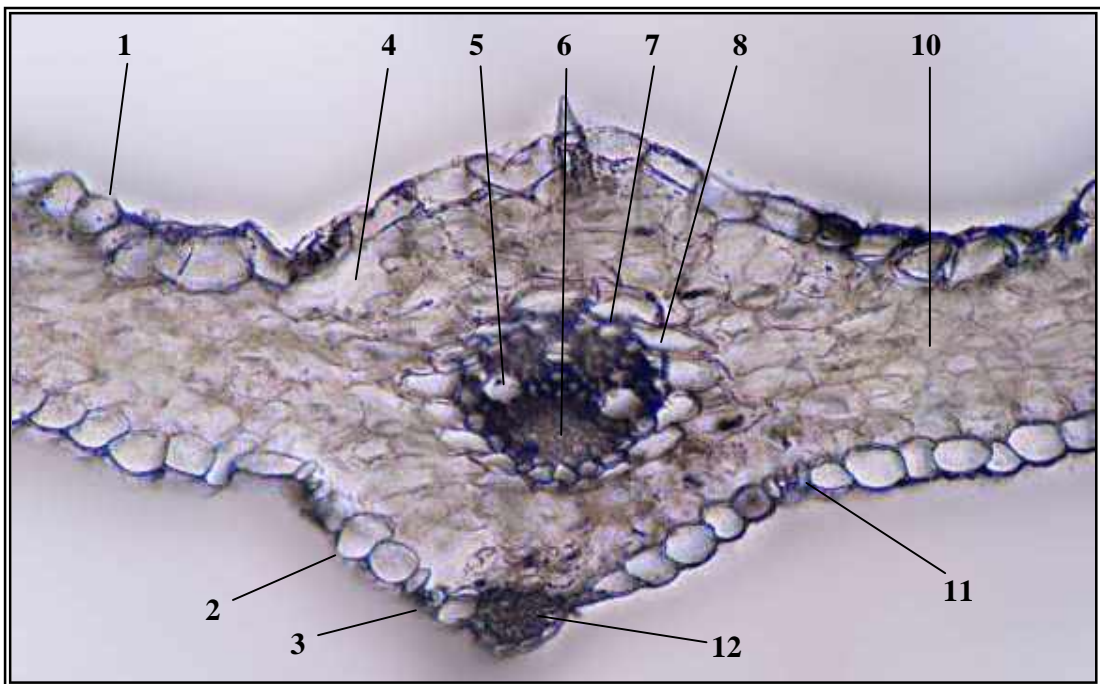
Figure 5.8 Transverse section (Cellular) of *Triticum dicoccum*
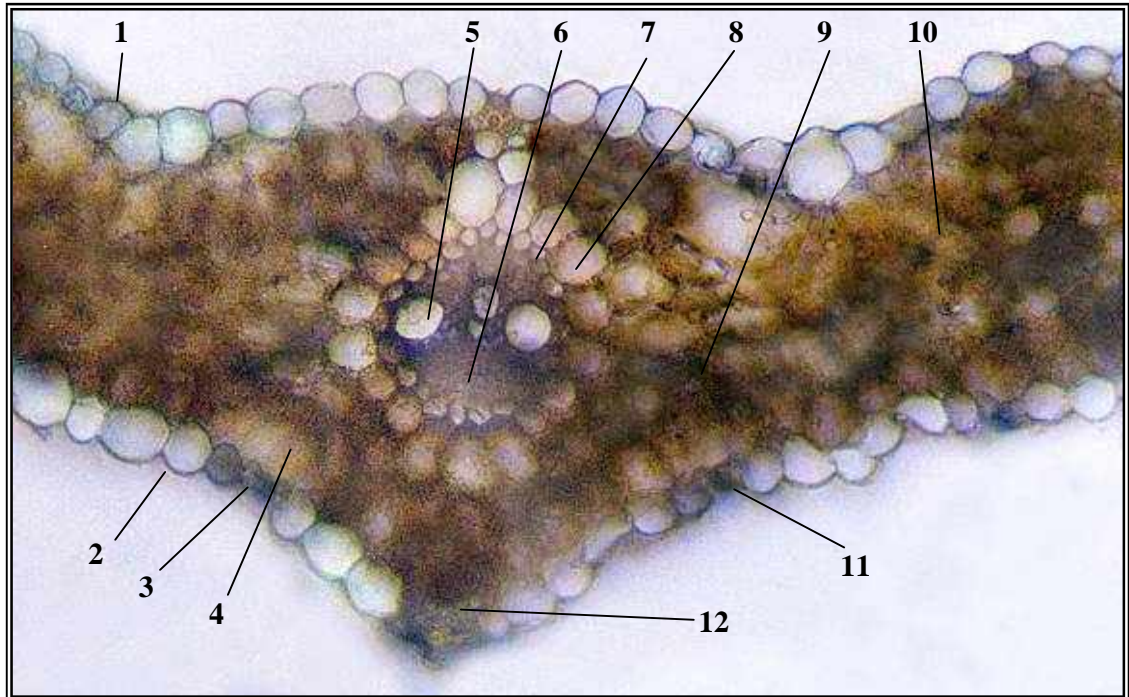
Figure 5.9 Transverse section (Cellular) of *Triticum aestivum*

**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 5.10).

Triticum durum differed in trichomes only, which were simple, unicellular and uniseriate but were smaller in size than *T. aestivum*. Fibers were more in number and reticulated vessels were almost absent (Figure 5.11).

Triticum dicoccum differed form both the samples in following characters –
Trichomes were simple, unicellular and uniseriate but of both the sizes i.e.
smaller and longer in length. Fibers were broader than other two samples.
Only one type of vessels i.e. annular was present (Figure 5.12).
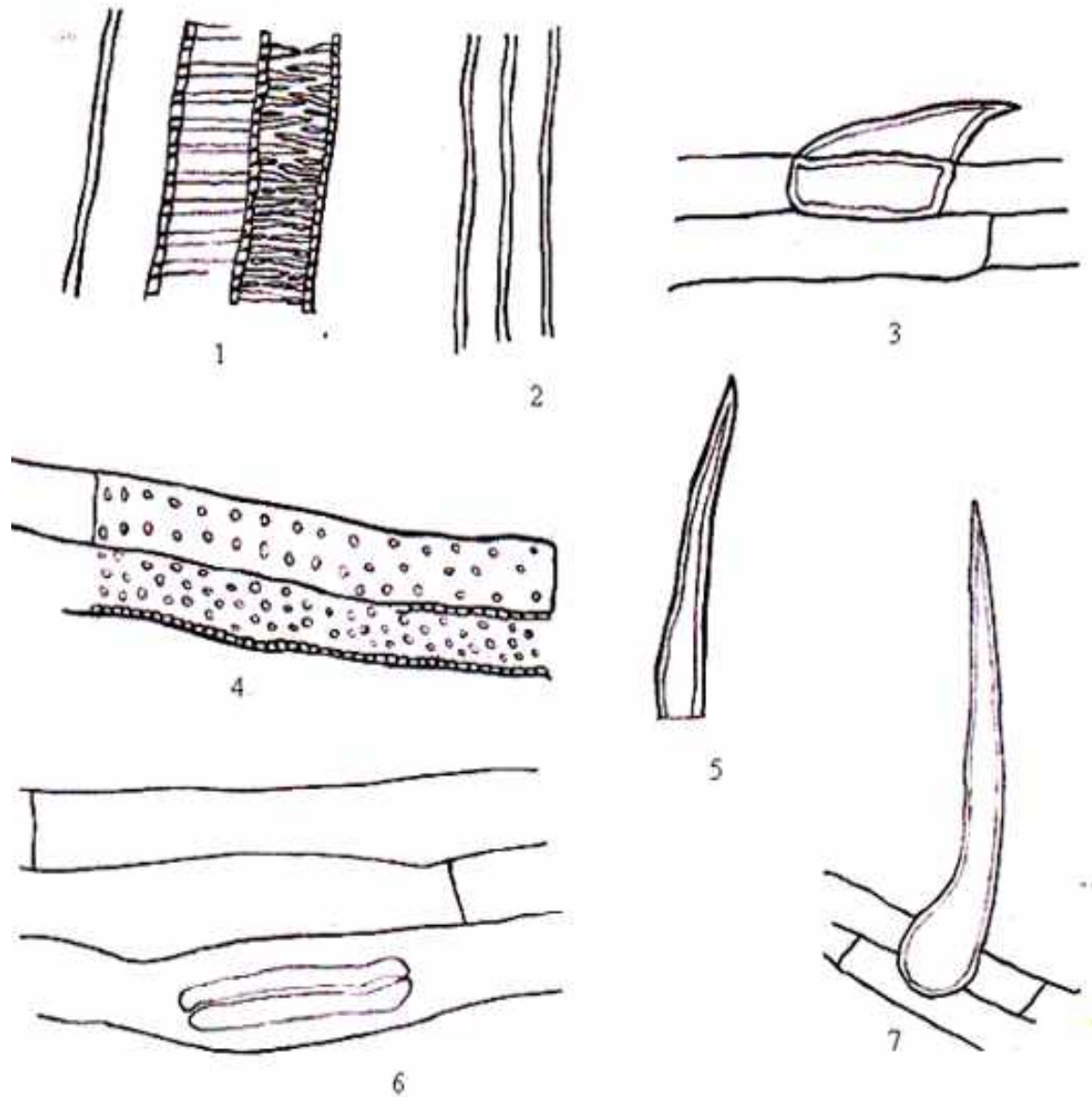
Figure 5.10 Characters of *T. aestivum* powder

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

98
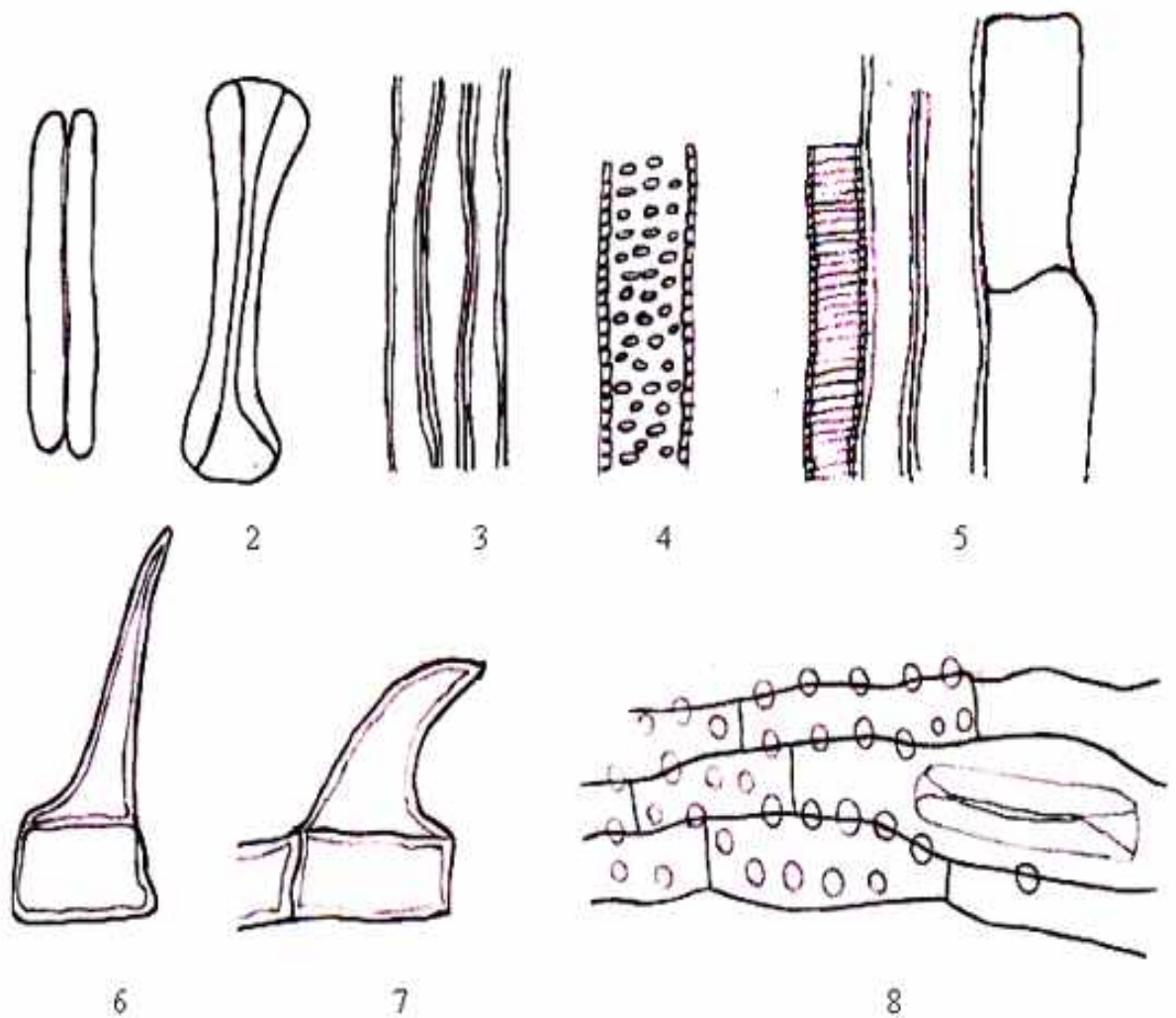
Figure 5.11 Characters of *T. durum powder*

1. and 2. Stomata in scattered form
3. Thin walled fibers
4. Pitted vessels
5. Xylem vessels associated with fibers and parenchyma
6. and 7. Uniseriate, unicellular simple trichomes
8. Epidermis of the leaf overlapped with palisade cells
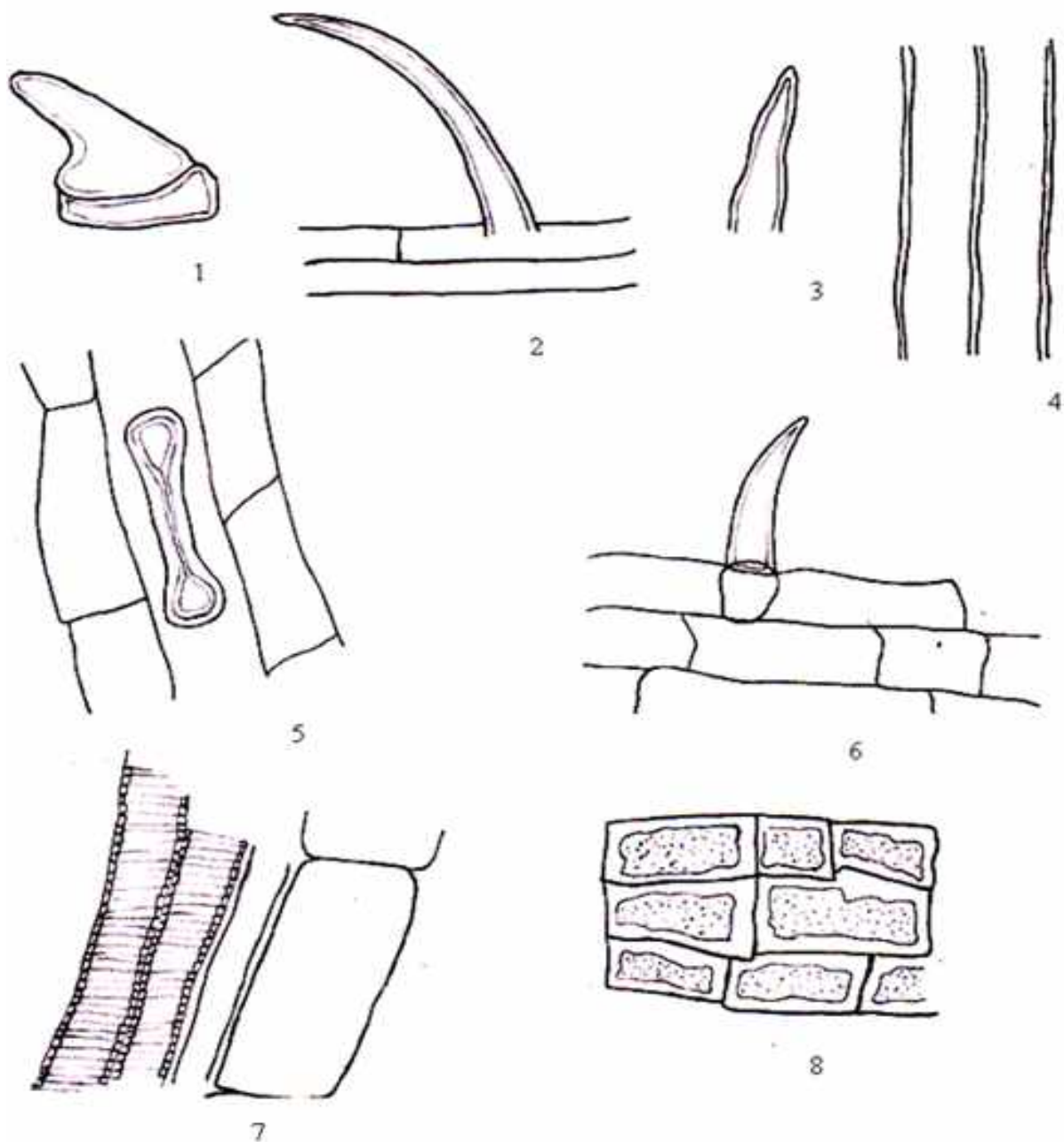
Figure 5.12 Characters of *T. dicoccum* powder

1. and 2. Trichomes
3. Broken trichome
4. Thin walled fibers
5. Stomata
6. Epidermal cells with trichome
7. Xylem vessels attached with thin walled fibers and parenchyma
8. Palisade cells

5.2 Phytochemical Studies

5.2.1 Phytochemical analysis -

The shade-dried powder of wheatgrass, used in our study, was analyzed at Sathyam Analytical Laboratory, Bombay. In the phytochemical analyses wheatgrass was found to be rich in chlorophyll, various minerals like iron, magnesium, calcium, phosphorus, antioxidants like beta-carotene and insoluble dietary fibers while, the fat content was very low (Table - 5.1).

Table – 5.1

Phytochemical Analysis of Wheatgrass Powder		
Sr. No.	**Constituent**	**Quantity**
1.	Moisture	4.4 %
2.	Ash	4.15 %
3.	Protein	25.2 %
4.	Fat	1.75 %
5.	Crude fiber	0.29 %
6.	Carbohydrates	52.4 %
7.	Calories	390 kcal/100 gm
8.	Calcium	32 mg/100 gm
9.	Phosphorus	850 mg/100 gm
10.	Chlorophyll	5.12 %
11.	Iron	43.5 mg/100 gm
12.	Magnesium	1455 mg/100 gm
13.	Zinc	97.8 mg/100 gm
14.	Beta Carotene	10.9075 I. U./100 gm
15.	Soluble dietary fibers	0.3545 %
16.	Insoluble dietary fiber	64.54 %
17.	Total dietary fiber	65.56 %

5.2.2 Thin layer chromatography -

TLC of methanolic extracts of Standard β Carotene, fresh wheatgrass, shade-dried wheatgrass powder, freeze-dried wheatgrass powder, spray-dried wheatgrass powder showed, in all, 13 spots at different R_f values (Fig 5.10) viz. (1) 0.07(Light green), (2) 0.21(Yellow), (3) 0.27(Grey), (4) 0.35(Yellow), (5) 0.40(Yellow), (6) 0.47(Light green), (7) 0.54(Yellow), (8) 0.57(Green), (9) 0.61(Blue green), (10) 0.64(Light grey), (11) 0.69(Light grey), (12) 0.74(Grey), (13) 0.92(Orange). By comparing standard Rf values and colors reported in literature, out of 13 spots representing 13 different components of wheatgrass, seven components at spot number 4, 7, 8, 9, 11, 12 and 13 were

identified as xanthophyll c, xanthophyll a, chlorophyll b, chlorophyll a, pheophytin b, pheophytin a and beta carotene respectively (Table - 5.2) (Figure 5.13).
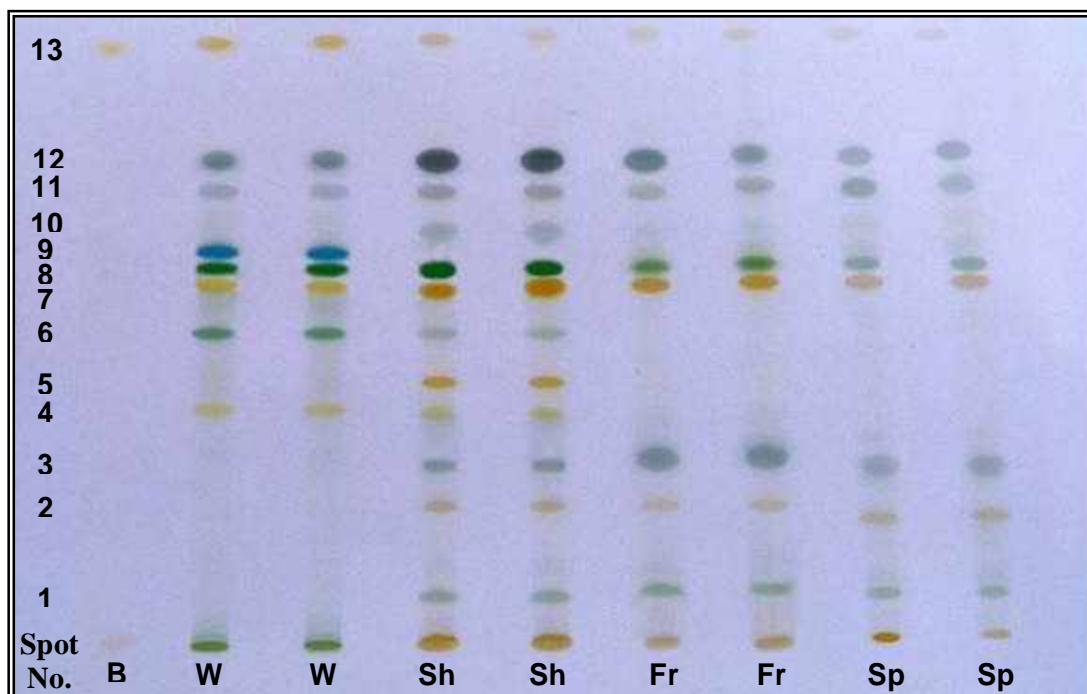

Figure 5.13 TLC of Methanolic extracts of B, W, Sh, Fr and Sp

B = Standard β Carotene   W = Fresh wheatgrass Juice

Sh = Shade-dried wheatgrass powder

Fr = Freeze-dried wheatgrass powder

Sp = Spray-dried wheatgrass powder

Table – 5.2

Spot No.	Color of the spot	Rf value (calculated)	Rf value (Reported)	Component (Identified)
1.	Light green	0.07	---	Unknown
2.	Yellow	0.21	---	Unknown
3.	Grey	0.27	---	Unknown
4.	Yellow	0.35	0.32	Xanthophyll c
5.	Yellow	0.40	---	Unknown
6.	Light green	0.47	---	Unknown
7.	Yellow	0.54	0.53	Xanthophyll a
8.	Green	0.57	0.58	Chlorophyll b
9.	Blue green	0.61	0.62	Chlorophyll a
10.	Light grey	0.64	---	Unknown
11.	Light grey	0.69	0.69	Pheophytin b
12.	Grey	0.74	0.75	Pheophytin a
13.	Orange	0.92	0.91	β carotene

Chlorophyll a was found to be present in the maximum quantity in fresh Wheatgrass. The degraded product pheophytin was the least in fresh wheatgrass extract. The content of chlorophyll was least in spray-dried powder. The shade dried powder showed maximum quantity of pheophytin. Except chlorophyll a, 12 components were found present in shade-dried powder. Spot no. 4, 5, 6, 9 and 10 were absent in freeze-dried and spray-dried powders. β-Carotene was present in all formulations. Thus, shade drying, the natural method of drying, was found to preserve all components of wheatgrass and hence, the most suitable one.

5.2.3 HPTLC finger printing of wheatgrass formulations (qualitative) -

Based on the resolution, as observed in UV light at 254 nm and at 354 nm, the solvent system containing hexane: acetone (60: 40) was found to be the most suitable one. Chromatograms of methanolic extracts of standard β Carotene, fresh wheatgrass, shade-dried wheatgrass powder, freeze-dried wheatgrass powder, spray dried wheatgrass powder were subjected to HPTLC study. The chromatograms were scanned in combined as well as in individual modes at 254 nm and 354 nm wavelengths (Fig 5.14 to 5.25). Among individual chromatograms of wheatgrass formulations (Fig 5.16-5.19), the chromatogram of fresh wheatgrass extract (Fig 5.16) exhibited poor resolution in hexane: acetone solvent system. This may have happened due to the fact that there was presence of water content in the fresh grass, whereas the extracts of other formulations were prepared from dried powders. Spot number 1 and 2 showed maximum prominence in chromatogram of spray-dried powder. Low RF values of these two constituents in the non polar solvent system indicate their polar and therefore water soluble and thermostable character. It is possible that high temperature (55 ^{o}C) used in preparation of spray-dried powder resulted in degradation of non polar and thermolabile constituents of Wheatgrass, resulting into dominance of thermostable and watersoluble constituents. In consonance with TLC study, in qualitative HPTLC also, the chromatogram of shade dried powder (Fig 5.17) showed maximum number of peaks indicating that shade drying method, the natural method of drying, preserved all components of Wheatgrass.
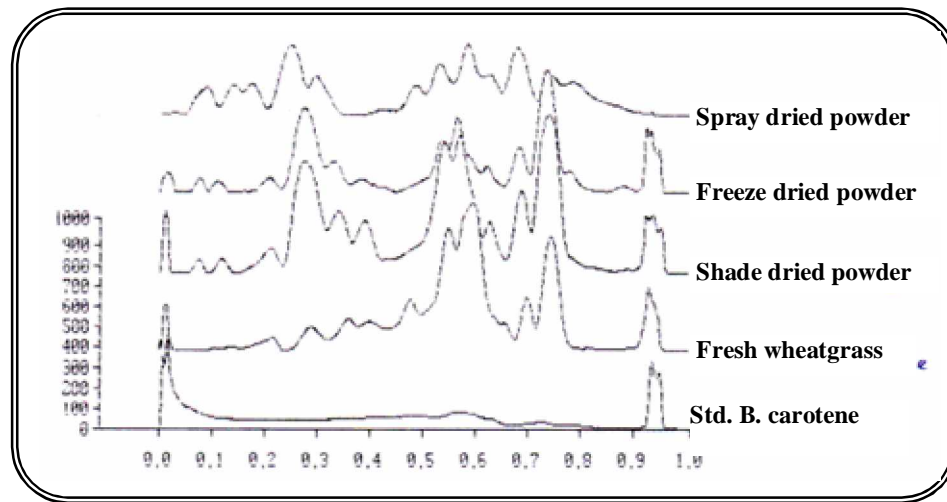
Figure 5.14 HPTLC (Qualitative) - Combined (overlapping) chromatograms of B, W, Sh, Fr and Sp at 354 nm wavelength.
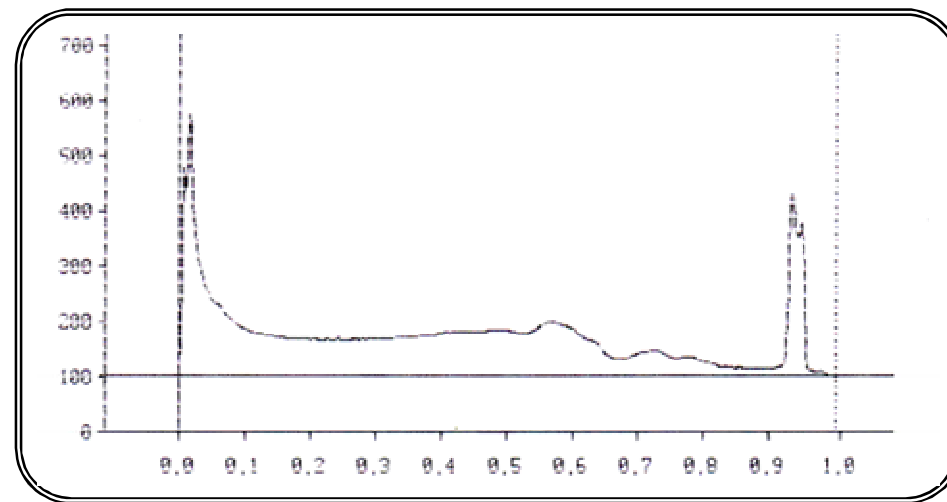

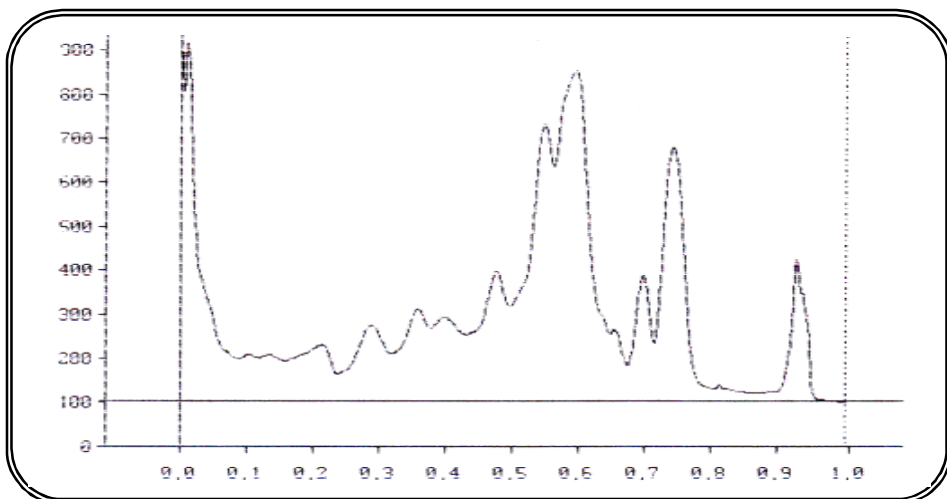**Figure 5.15 HPTLC (Qualitative) - Chromatogram of β carotene at 354 nm wavelength**

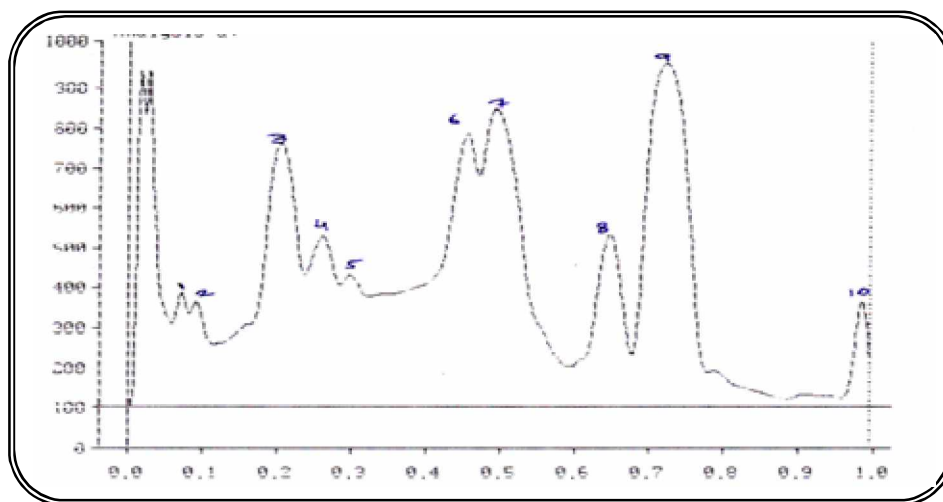**Figure 5.16 HPTLC (Qualitative) - Chromatogram of Fresh wheatgrass at 354 nm wavelength**

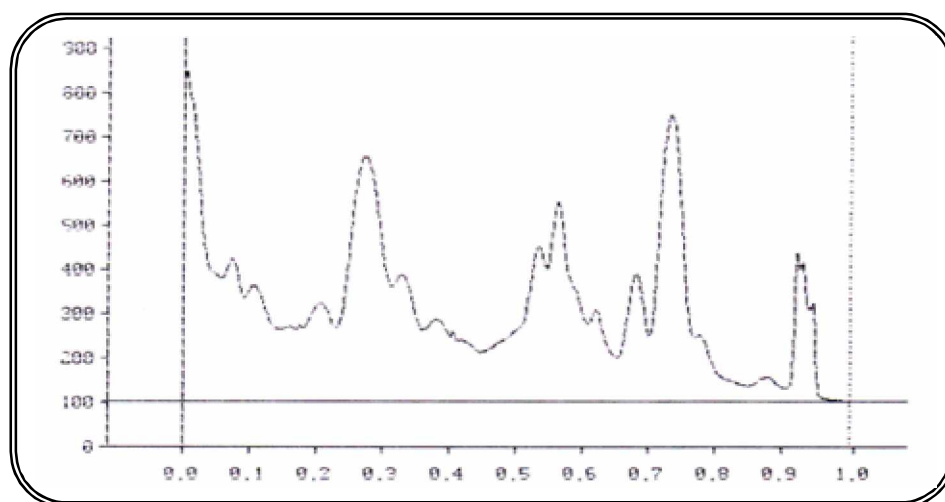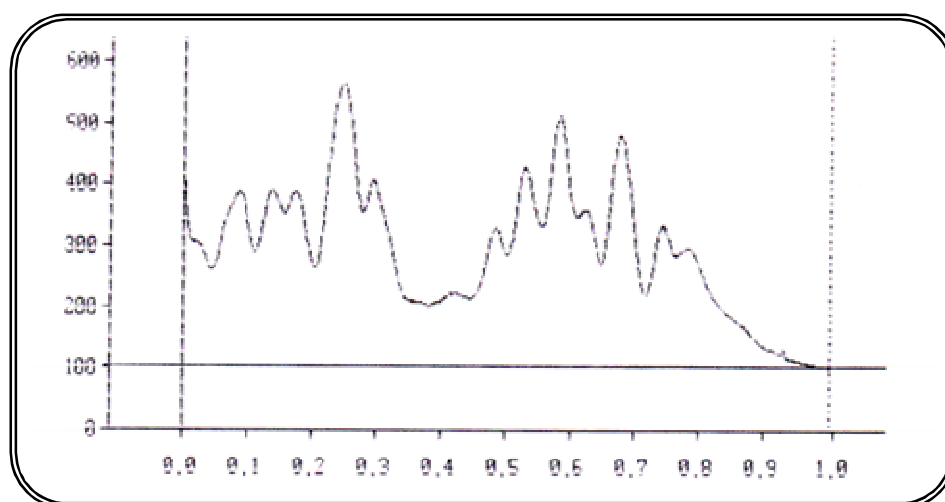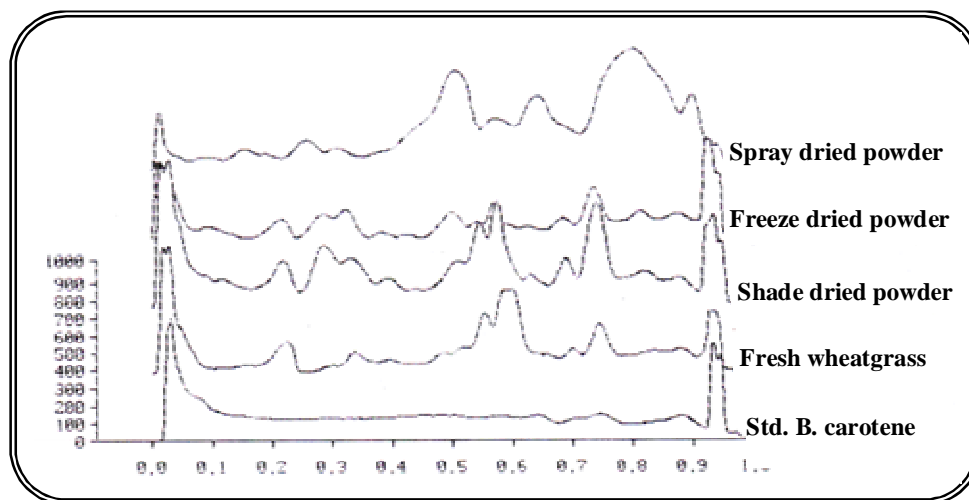**Figure 5.17 HPTLC (Qualitative) - Chromatogram of shade-dried powder at 354 nm wavelength**

Figure 5.18 HPTLC (Qualitative) - Chromatogram of freeze-dried powder at 354 nm wavelength

Figure 5.19 HPTLC (Qualitative) - Chromatogram of spray-dried powder at 354 nm wavelength

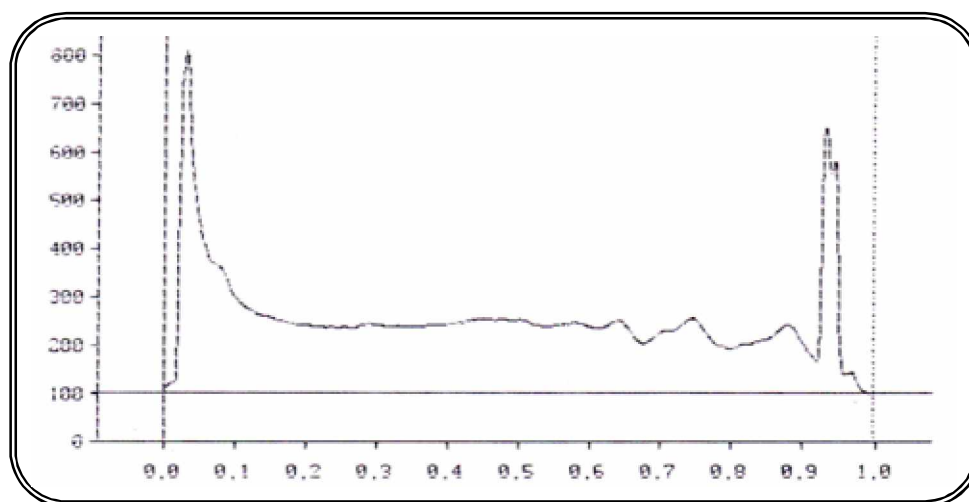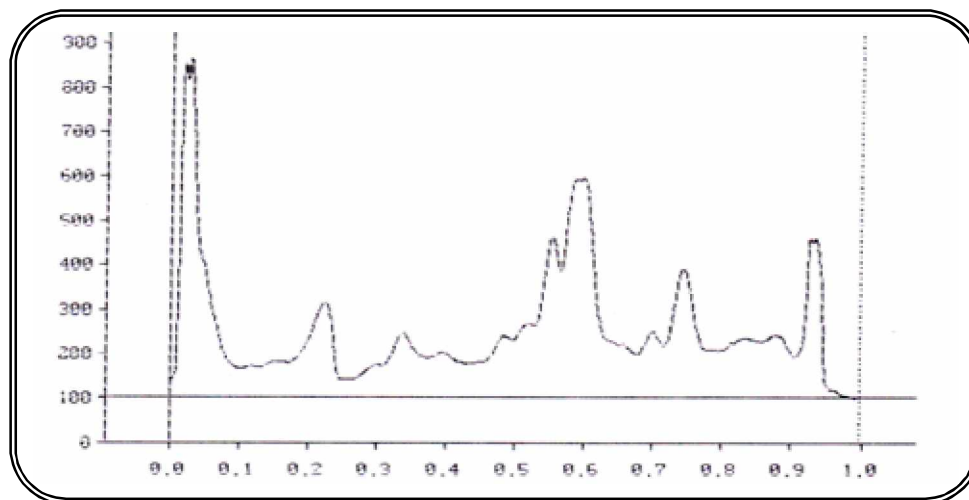**Figure 5.20 HPTLC (Qualitative) - Combined chromatograms of B, W, Sh, Fr and Sp at 254 nm wavelength**

Figure 5.21 HPTLC (Qualitative) - Chromatogram of β carotene at 254 nm wavelength

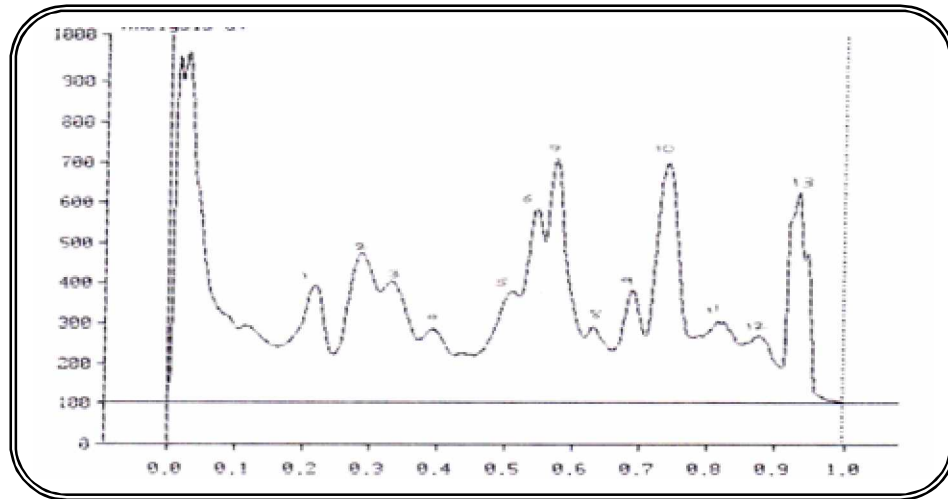**Figure 5.22 HPTLC (Qualitative) - Chromatogram of Fresh wheatgrass at 254 nm wavelength**

Figure 5.23 HPTLC (Qualitative) - Chromatogram of shade-dried powder at 254 nm wavelength
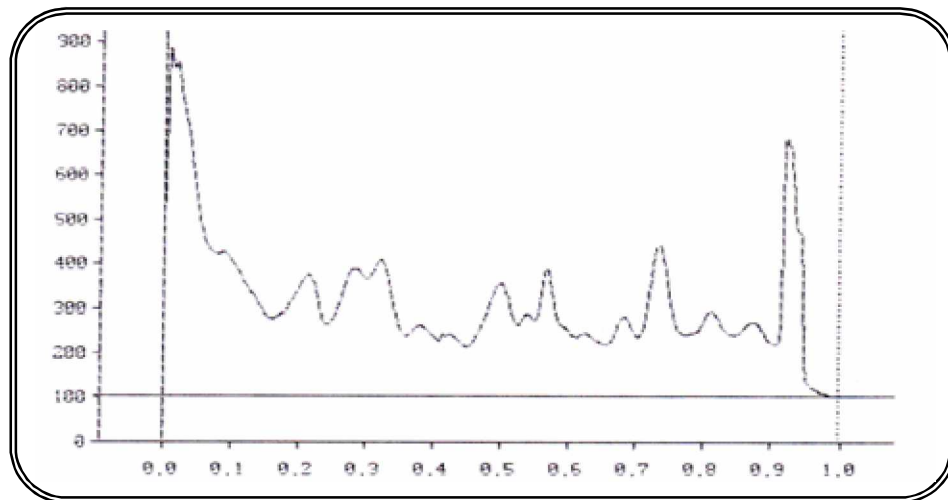

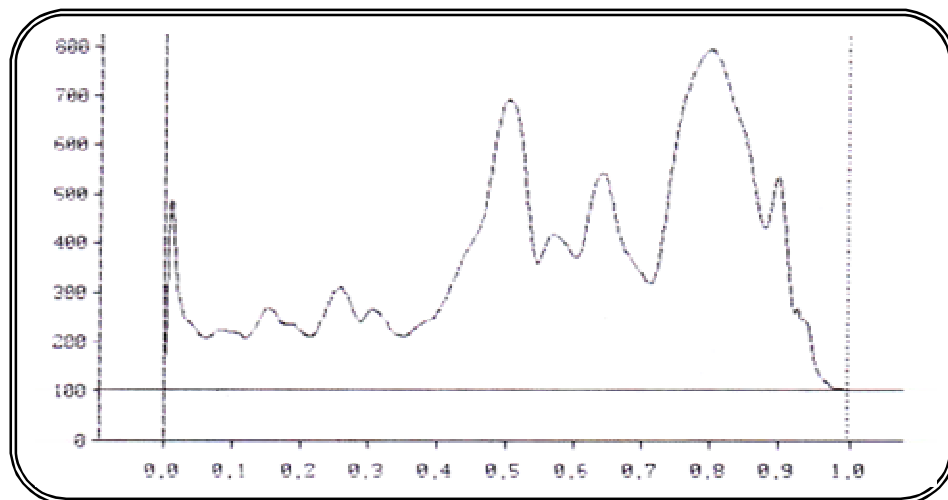**Figure 5.24 HPTLC (Qualitative) -Chromatogram of freeze-dried powder at 254 nm wavelength**

Figure 5.25 HPTLC (Qualitative) - Chromatogram of spray-dried powder at 254 nm wavelength

Since the chromatogram of shade dried powder exhibited best resolution and maximum number of prominent peaks, individual spots (components) were scanned at 254 nm wavelength to obtain their spectra. Some of these spectra were used for identification of the components of wheatgrass (Fig 5.26-5.38).
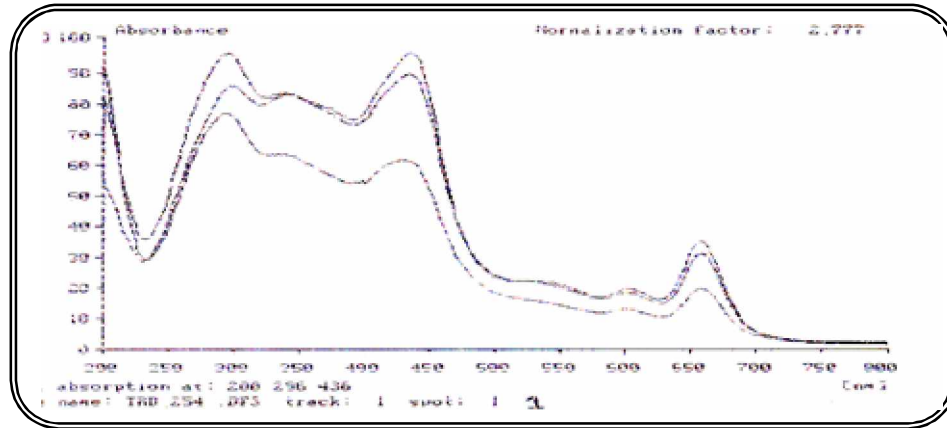
Individual spectra of components of Wheatgrass -

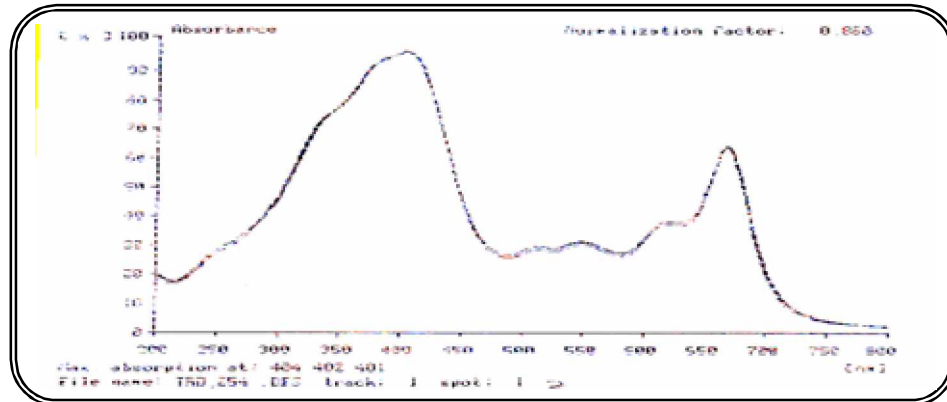**Figure 5.26 HPTLC (Qualitative) - UV spectra of spot 1 at 254 nm wavelength**

Figure 5.27 HPTLC (Qualitative) - UV spectra of spot 2 at 254 nm wavelength

Figure 5.28 HPTLC (Qualitative) - UV spectra of spot 3 at 254 nm wavelength

Figure 5.29 HPTLC (Qualitative) - UV spectra of spot 4 at 254 nm wavelength

Figure 5.30 HPTLC (Qualitative) - UV spectra of spot 5 at 254 nm wavelength
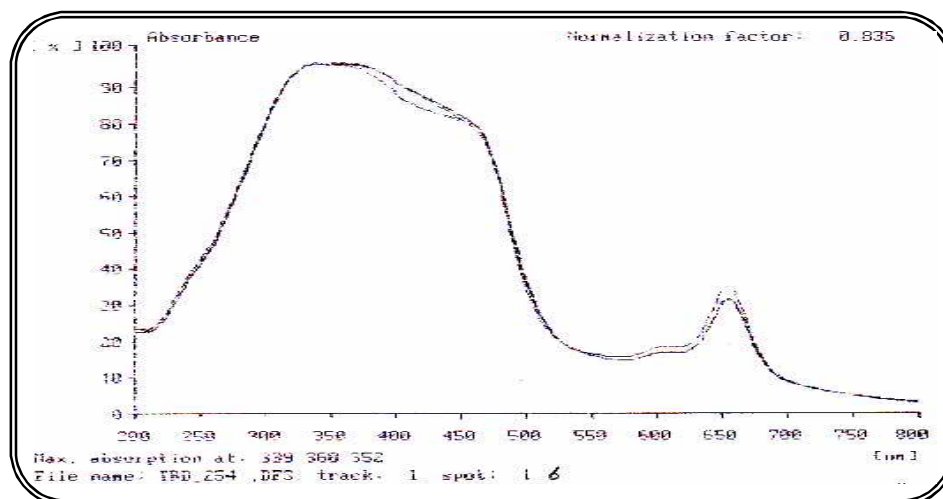
Figure 5.31 HPTLC (Qualitative) - UV spectra of spot 6 at 254 nm wavelength
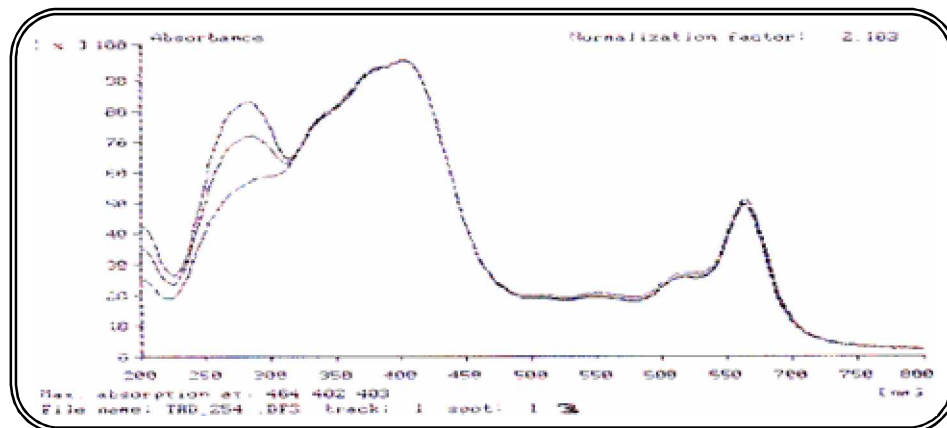
109

Figure 5.32 HPTLC (Qualitative) - UV spectra of spot 7 at 254 nm wavelength

Figure 5.33 HPTLC (Qualitative) - UV spectra of spot 8 at 254 nm wavelength

Figure 5.34 HPTLC (Qualitative) - UV spectra of spot 9 at 254 nm wavelength
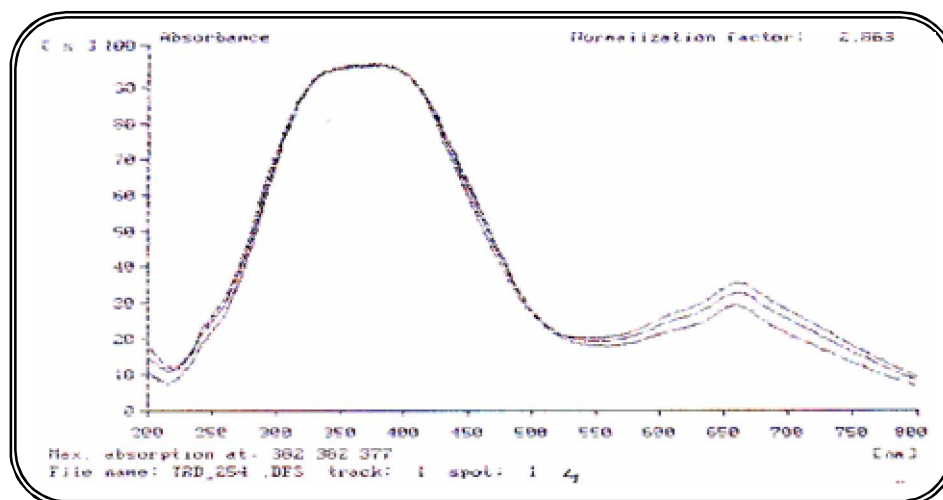
110
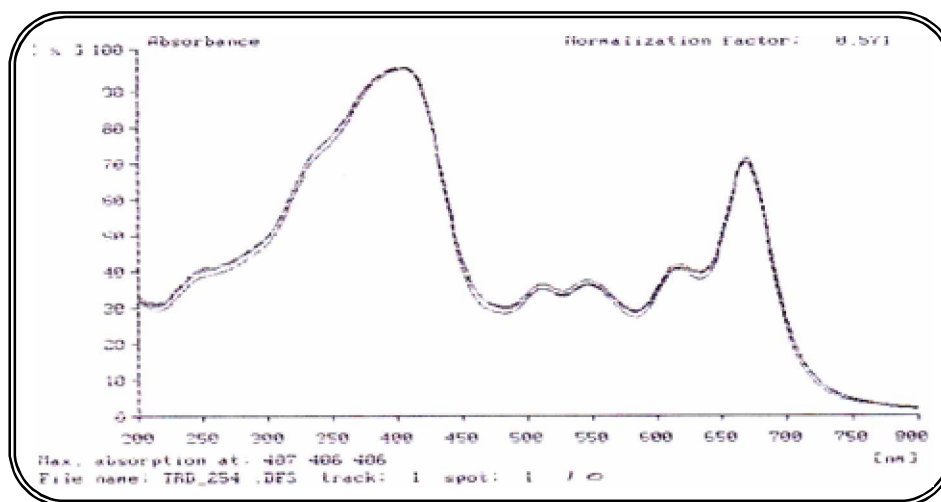
Figure 5.35 HPTLC (Qualitative) - UV spectra of spot 10 at 254 nm wavelength
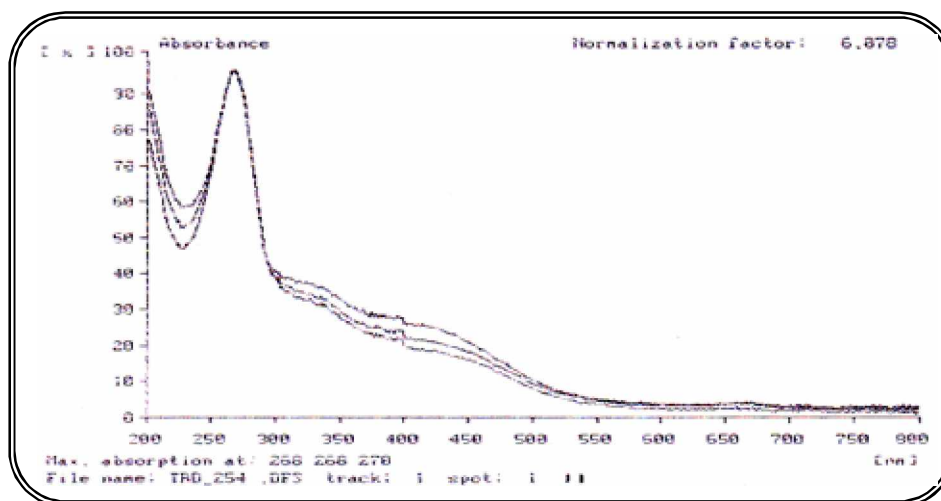
Figure 5.36 HPTLC (Qualitative) - UV spectra of spot 11 at 254 nm wavelength
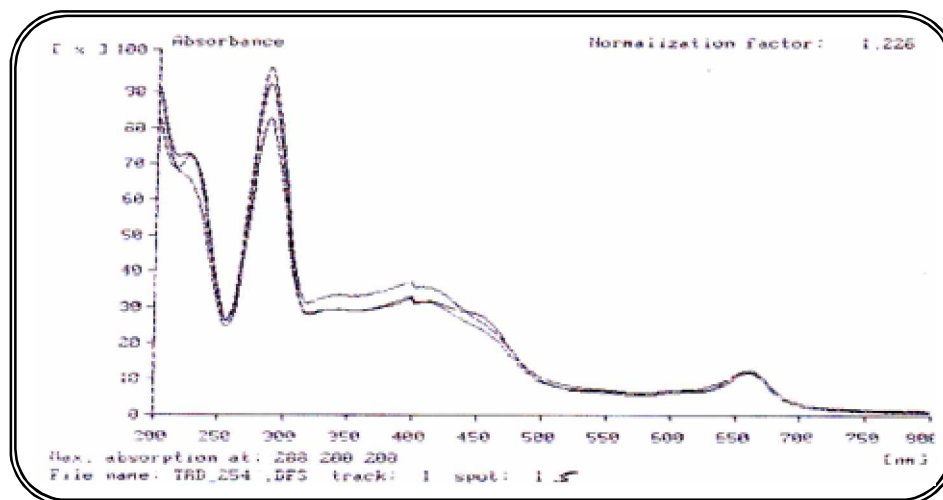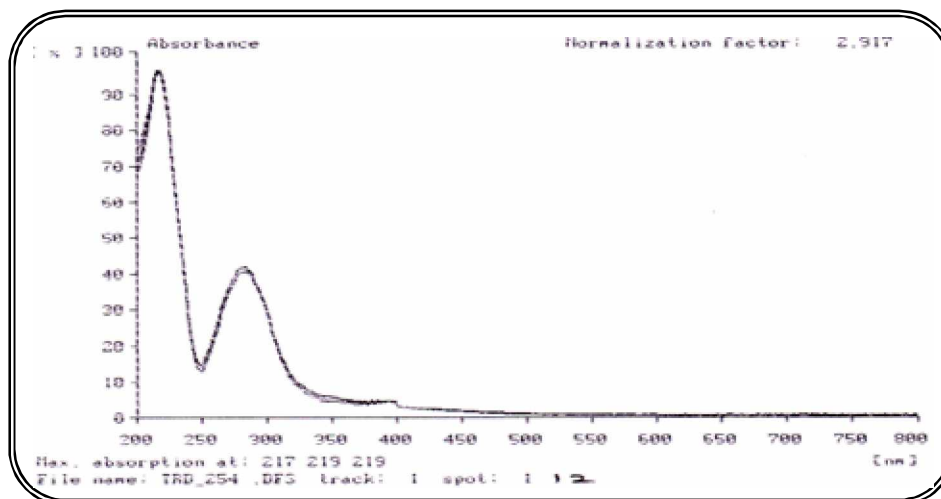

Figure 5.37 HPTLC (Qualitative) - UV spectra of spot 12 at 254 nm wavelength

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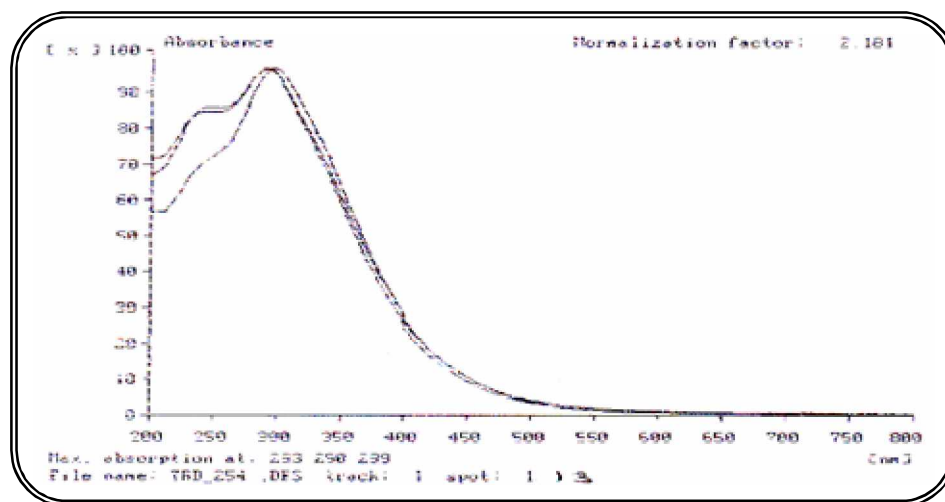
Figure 5.38 HPTLC (Qualitative) - UV spectra of spot 13 at 254 nm wavelength
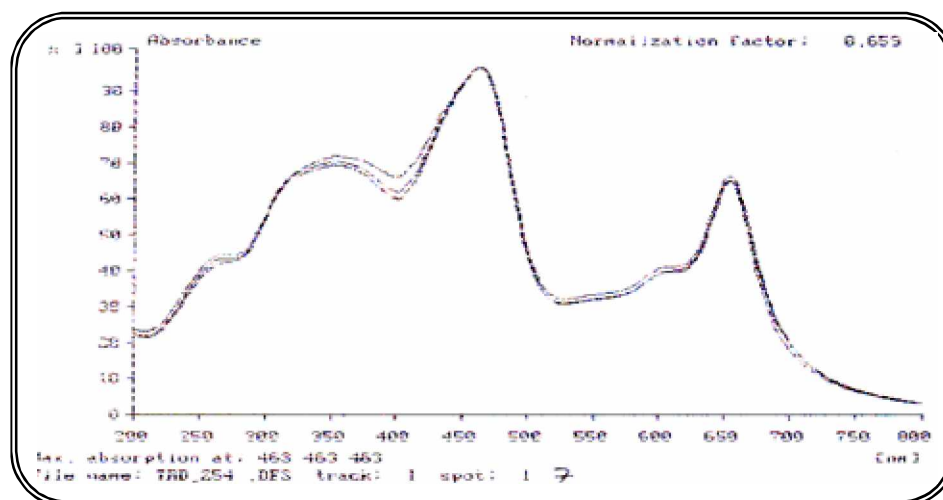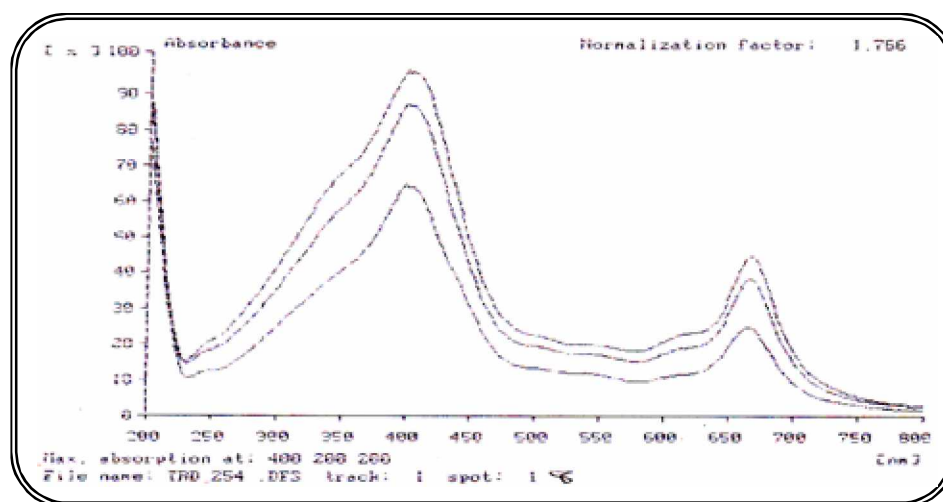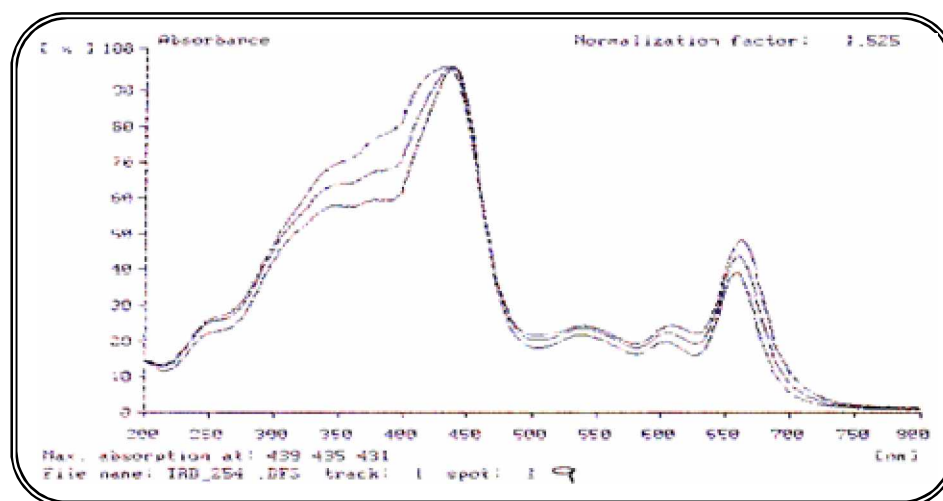
5.2.4 HPTLC fingerprinting of wheatgrass species and formulations (Quantitative) -

The quantitative HPTLC was carried out on wheatgrass formulations for presence of various constituents. For this purpose methanolic extracts were prepared at maximum concentration. Considering the weight of fresh wheatgrass as 100% the yields of shade dried, freeze dried and spray dried powders were found to be 10%, 6% and 10% respectively at the end of drying process. For quantitative estimation, methanolic extracts of equivalent amounts of these formulations were subjected to quantitative HPTLC along with those of different species of wheatgrass. Chromatograms (tracks) of the formulations or species were recorded (Table - 5.3).

Table – 5.3

Track no.	Extract
1	Standard β carotene
2	T. durum fresh grass (compost fertilizer)
3	T. durum traditional juice
4	T. durum shade-dried powder
5	T. durum freeze dried powder
6	T. durum spray dried powder
7	Gel
8	T. aestivum fresh grass (compost fertilizer)
9	T. dicoccum fresh grass (compost fertilizer)
10	T. durum (plain soil)
11	T. durum (Organic fertilizer)
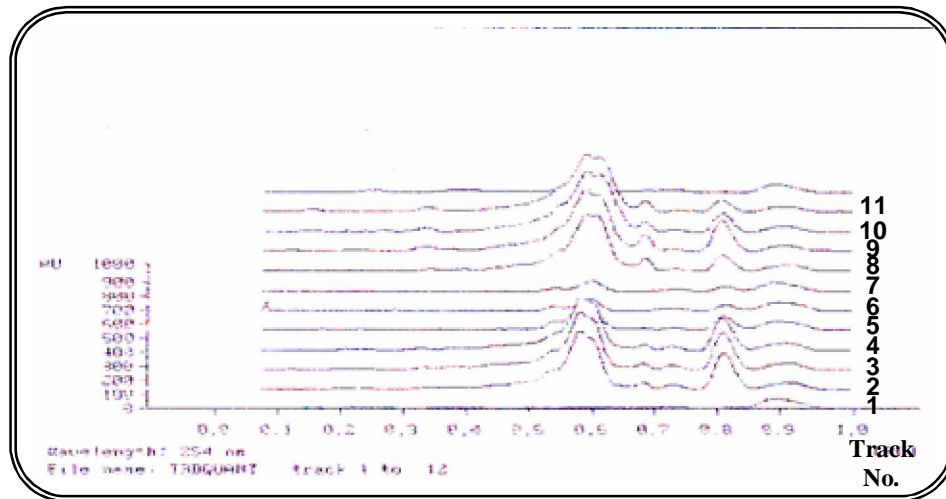
Figure 5.39 HPTLC (Quantitative) - Combined chromatograms of various extracts at 254 nm wavelength (Table 5.2)

Figure 5.40 HPTLC (Quantitative) - Chromatogram of track 1 at 254 nm wavelength
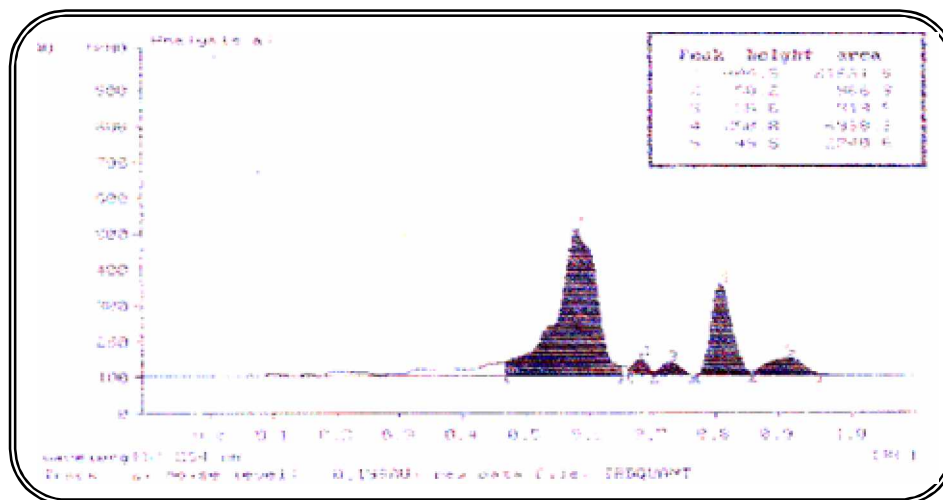
Figure 5.41 HPTLC (Quantitative) - Chromatogram of track 2 at 254 nm wavelength
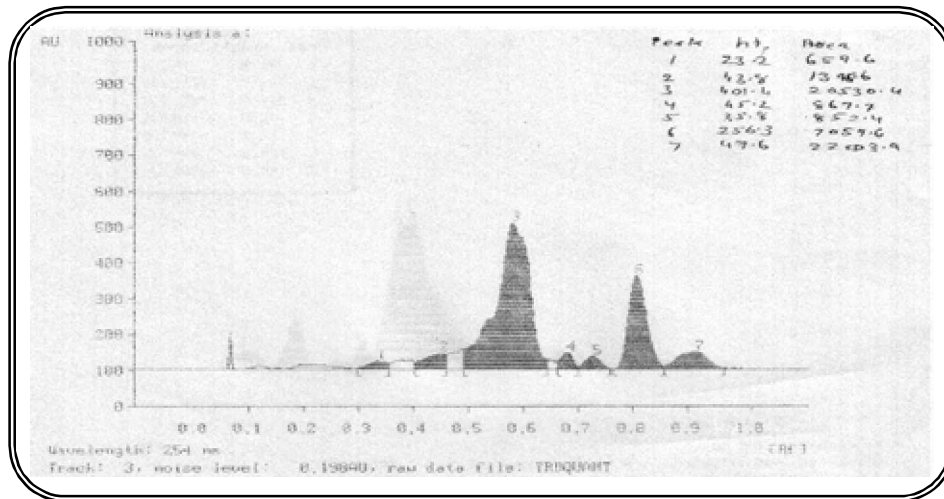
Figure 5.42 HPTLC (Quantitative) - Chromatogram of track 3 at 254 nm wavelength
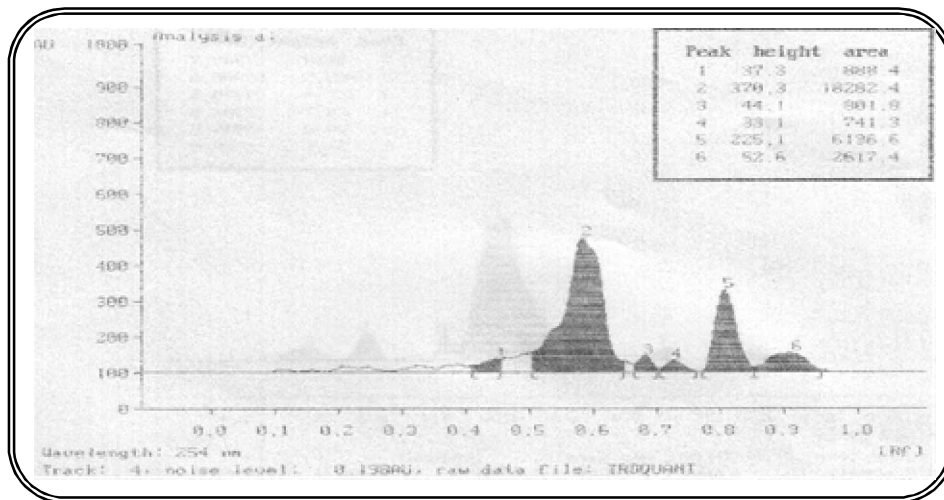
Figure 5.43 HPTLC (Quantitative) - Chromatogram (Quantitative) Of track 4 at 254 nm wavelength
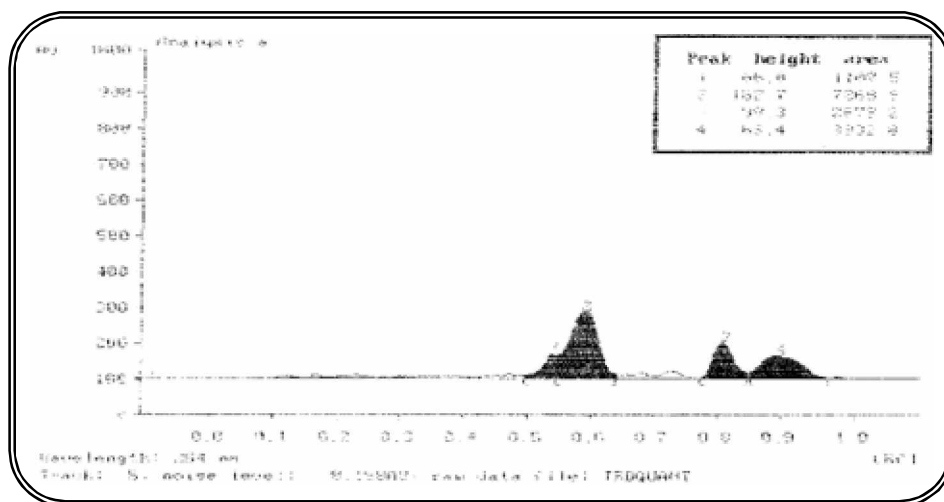
Figure 5.44 HPTLC (Quantitative) - Chromatogram of track 5 at 254 nm wavelength
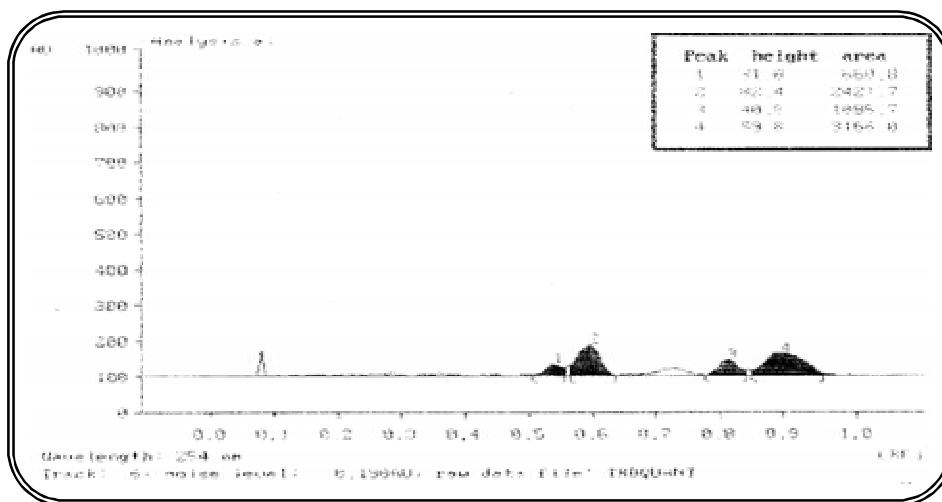
Figure 5.45 HPTLC (Quantitative) - Chromatogram of track 6 at 254 nm wavelength
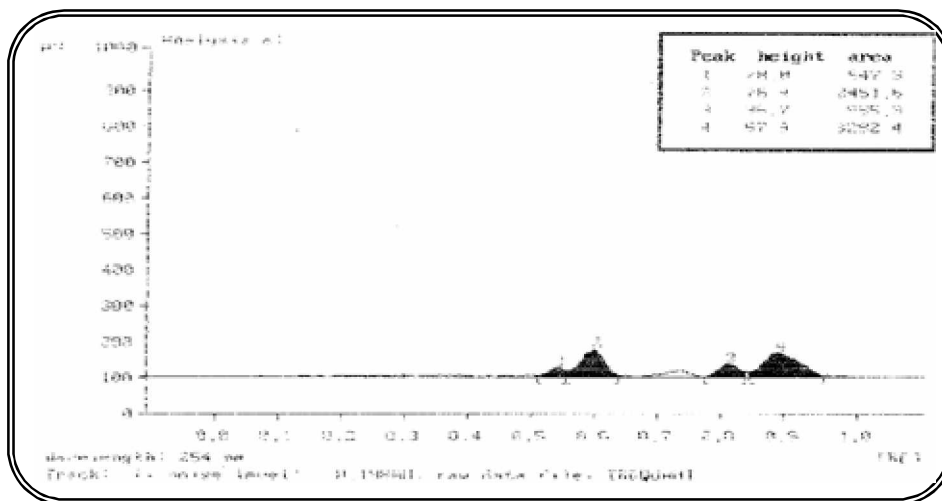
Figure 5.46 HPTLC (Quantitative) - Chromatogram of track 7 at 254 nm wavelength
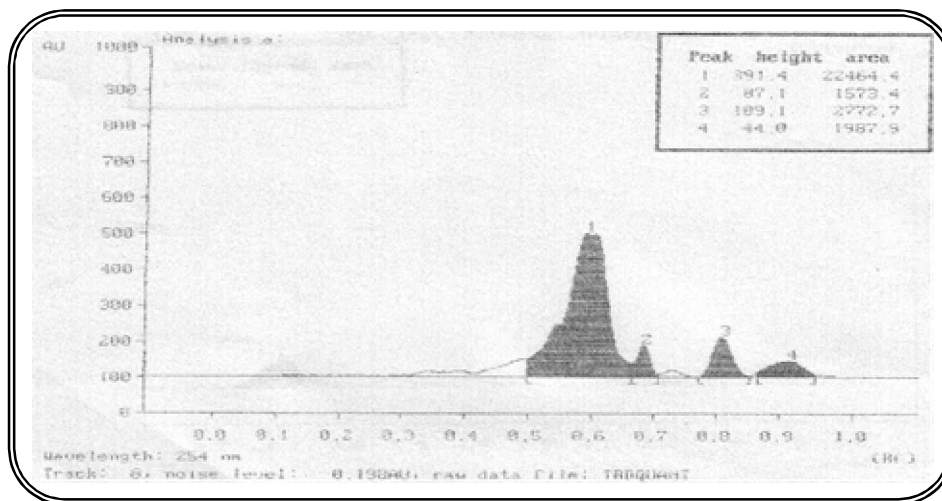
Figure 5.47 HPTLC (Quantitative) - Chromatogram of track 8 at 254 nm wavelength

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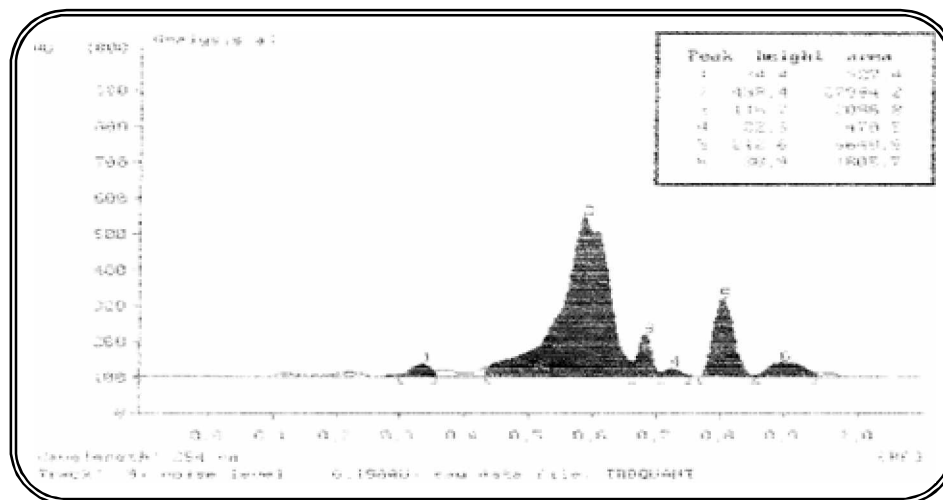
Figure 5.48 HPTLC (Quantitative) - Chromatogram of track 9 at 254 nm wavelength
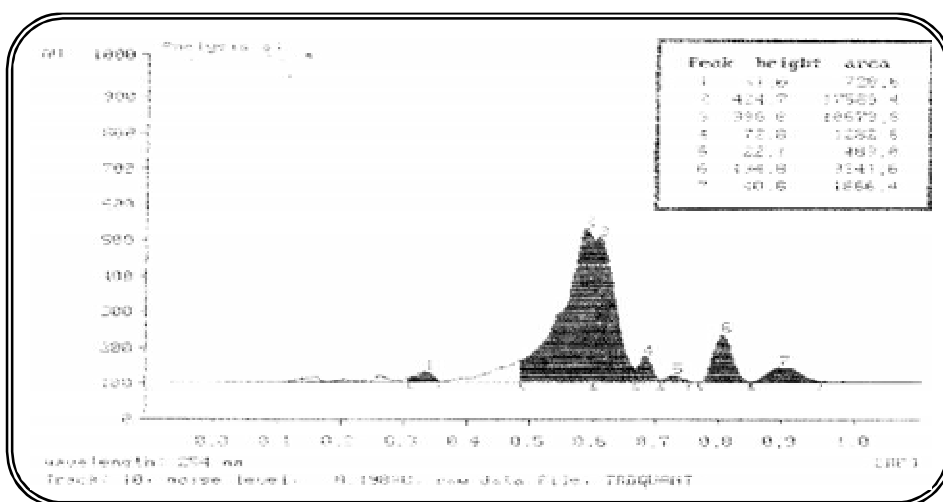
Figure 5.49 HPTLC (Quantitative) - Chromatogram of track 10 at 254 nm wavelength
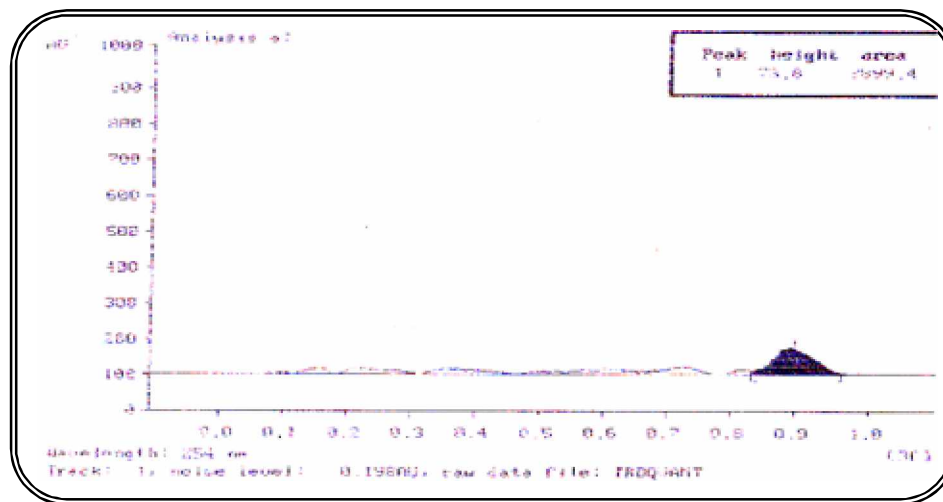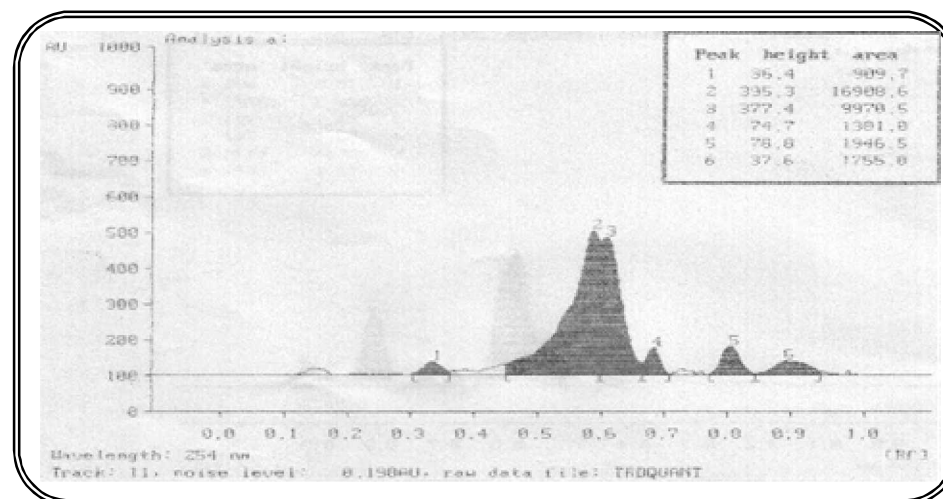

Figure 5.50 HPTLC (Quantitative) - Chromatogram of track 11 at 254 nm wavelength

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Area under curve of different components were measured in chromatogram (track) of each formulation or species (Fig. 5.39 – 5.50) and analyzed for comparison with that of fresh wheatgrass (Table – 5.4 - 5.7). Since all the constituents of wheatgrass could not be identified, the AUCs of only chlorophyll, pheophytin and beta-carotene were used for the purpose of comparison.

Wheatgrass grown from three major varieties of wheat, when subjected to quantitative HPTLC analysis revealed that *Triticum aestivum* had lowest contents (76.8%) as compared to the contents of *Triticum durum* (86.92%) and *Triticum dicoccum* (100 %) varieties (Table – 5.4) (Figure 5.51). Thus, the data indicate that wheatgrass grown from tetraploid species of wheat, especially *Triticum dicoccum* and *T. durum,* are more suitable for medicinal use as compared to the hexaploid species i.e. *T. aestivum* or common wheat.

Table – 5.4

Effect of wheat variety on contents of wheatgrass (HPTLC)					
Wheat variety	AUC (Chlorophyll)	AUC (Pheophytin)	AUC (B-carotene)	Total AUC	% AUC to T. dicoccum
T. dicoccum (fresh grass)	27994.2	5648.5	1805.7	35448.4	100.00
T. durum (fresh grass)	21531.8	6938.3	2340.6	30810.7	86.92
T. aestivum (fresh grass)	22464.4	2772.7	1987.9	27225	76.80

Fig. 5.51 Effect of Wheat Variety On Contents of Wheatgrass
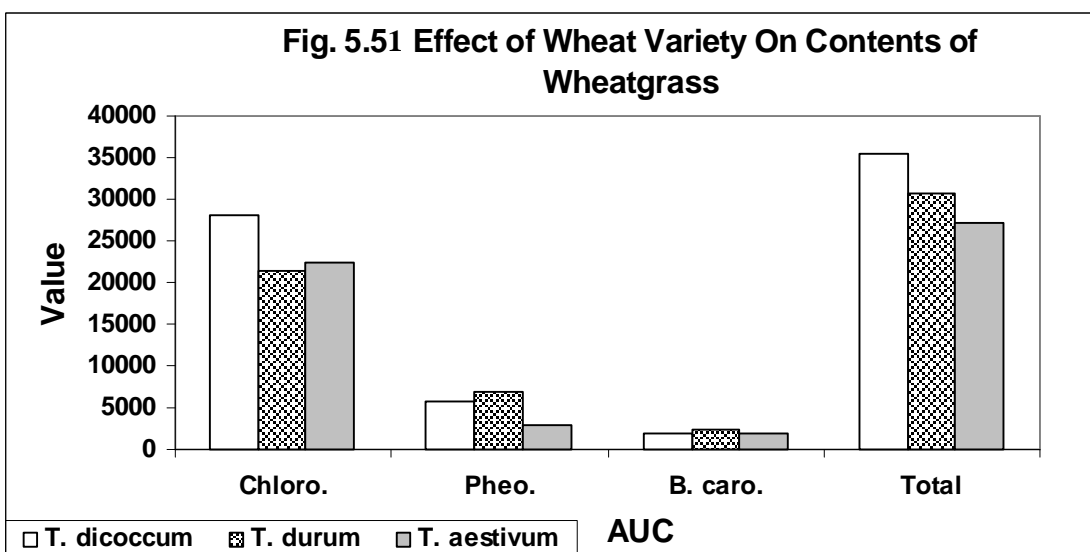
When subjected to quantitative HPTLC analysis, contents of wheatgrass (*Triticum durum)* grown in organic fertilizer (91.36%) and that of wheatgrass grown in compost fertilizer (92.05%) were nearly similar while the contents of wheatgrass grown in plain soil (100%) were highest among the three (Table – 5.5) (Figure 5.52). Thus, presence of a fertilizer seems to hamper or interfere with the sensitive phytochemical processes being carried out in the young growing grass in early stages of growth.

Table – 5.5

Effect of fertilizer on contents of wheatgrass (HPTLC)					
Fertilizer	AUC (Chlorophyll)	AUC (Pheophytin)	AUC (B.carotene)	Total AUC	% AUC to plain soil
Wheatgrass grown in plain soil	28263.2	3341.6	1866.4	33471.2	100.00
Wheatgrass grown with compost fertilizer	21531.8	6938.3	2340.6	30810.7	92.05
Wheatgrass grown with organic fertilizer	26879.1	1946.5	1755	30580.6	91.36

Fig. 5.52 Effect of Fertilizer On Contents Of Wheatgrass
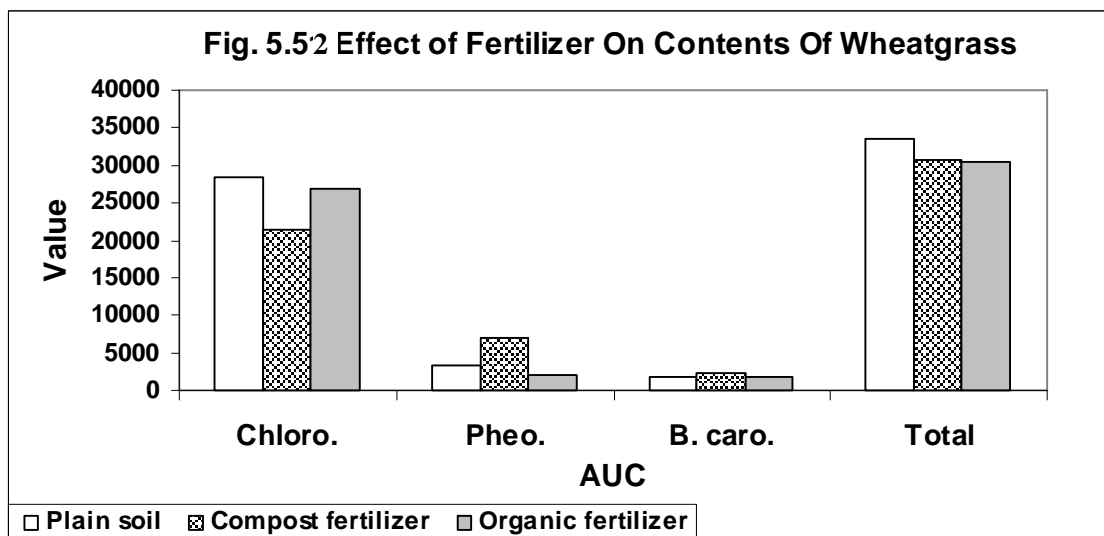
When wheatgrass juice prepared by traditional method was subjected to quantitative HPTLC analysis, it was found to have 96.7% contents considering the contents of methanolic extract of fresh wheatgrass as 100% (Table – 5.6) (Figure 5.53). Thus, the traditional method seems to be the best method for wheatgrass therapy compared to any formulations of wheatgrass (Table - 5.6) that were made in our study.

Table – 5.6

Evaluation of traditional method of wheatgrass therapy (HPTLC)					
Method	AUC (Chlorophyll)	AUC (Pheophytin)	AUC (B.carotene)	Total AUC	% AUC to meth. ext.
Meth. Extract of fresh wheatgrass	21531.8	6938.3	2340.6	30810.7	100.00
Fresh wheatgrass juice prepared by traditional method	20530.4	7059.6	2203.9	29793.9	96.70

Fig. 5.53 Evaluation of traditional method of wheatgrass therapy (HPTLC)
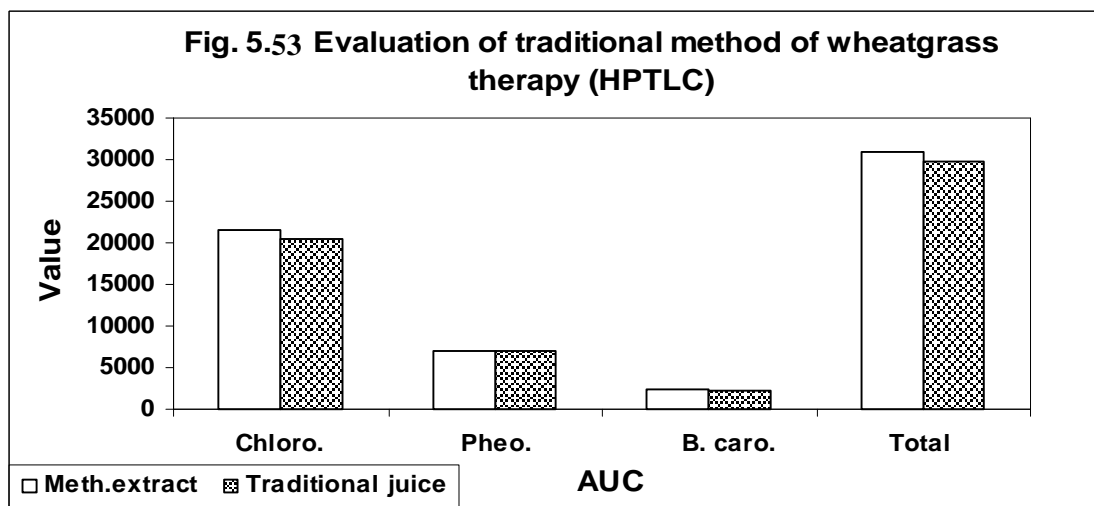
When subjected to quantitative HPTLC analysis spray-dried powder of wheatgrass was found to have lowest contents (21.66%) as compared to the contents of freeze-dried powder (46.68%) and shade-dried powder (87.75%) considering the contents of methanolic extract of fresh wheatgrass as 100% (Table – 5.7) (Figure 5.54). Thus, the potency of wheatgrass appears to be maintained at a substantially higher level in shade drying technique compared to other two techniques.

Table – 5.7

Evaluation of wheatgrass formulations (HPTLC)					
Formulation	AUC (Chlorophyll)	AUC (Pheophytin)	AUC (B.carotene)	Total AUC	% AUC to meth. ext.
Meth.extract of fresh wheatgrass	21531.8	6938.3	2340.6	30810.7	100.00
Shade-dried powder	18282.4	6136.6	2617.4	27036.4	87.75
Freeze-dried powder	8376.4	2673.2	3332.8	14382.4	46.68
Spray-dried powder	2421.7	1085.7	3166	6673.4	21.66

Fig. 5.54 Evaluation of Wheatgrass Formulations (HPTLC)
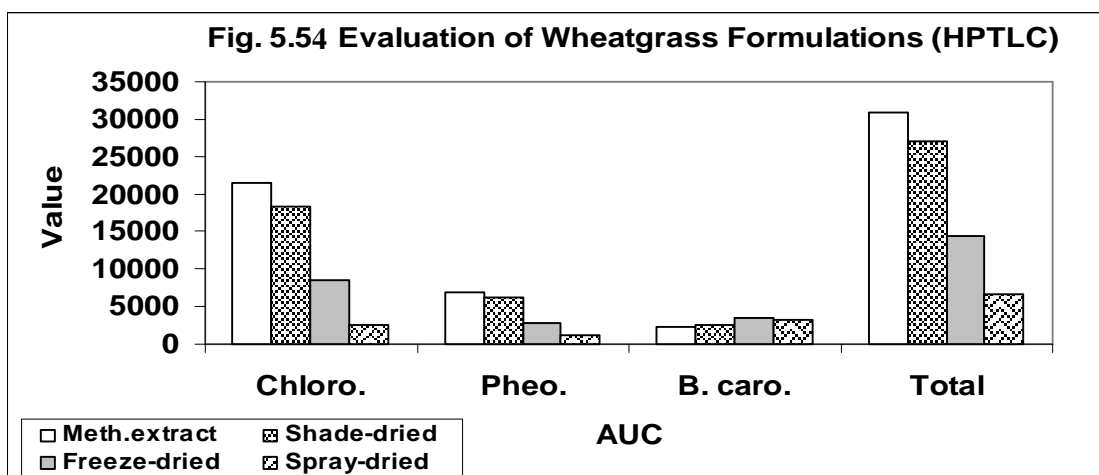
Meth. extract = Methanolic extract of fresh wheatgrass

Shade-dried = Shade-dried tablets

Freeze-dried = Freeze-dried tablets

Spray-dried = Spray-dried tablets

AUC = Area under curve

Chloro. = Chlorophyll

Pheo. = Pheophytin

B-caro. = Beta-carotene

Total = Total of AUC of Chlorophyll, Pheophytin, Beta-carotene

Traditional juice = Fresh Wheatgrass juice prepared by traditional method

Plain soil = Wheatgrass grown in plain soil

Compost fertilizer = Wheatgrass grown with compost fertilizer

Organic fertilizer = Wheatgrass grown with organic fertilizer

5.3 Wheatgrass formulations and tablets -

Various wheatgrass formulations (dried powders), tablets prepared wherefrom and market packs, prepared for the first time in our study, now commercially available in market (Figure 5.55 – 5.57) -

Figure 5.55 Display of freeze dried wheatgrass powder, tablets and bottle pack

Figure 5.56 Display of spray-dried wheatgrass powder, tablets and bottle pack

Figure 5.57 Display of shade-dried wheatgrass powder, tablets and bottle pack

Formulation data of wheatgrass tablets -

Wheatgrass powders obtained after shade drying, freeze drying and spray drying were used to make tablets using suitable excipients and binders. The preparations were done in pharmaceutical company and the method has been kept secret for the purpose of patent registration. Random samples of these tablets were subjected to analysis of pharmaceutical parameters (Table 5.8). There was consistency in weight of these tablets. Among the three types of tablets, spray dried tablets exhibited hygroscopic character and showed least hardness (3.2 kg/cm^2) compared to those of freeze dried tablets (4.5 kg/cm^2) and shade dried tablets (5.3 kg/cm^2). Spray dried tablets also showed least friability (1.11%) compared to those of freeze dried tablets (2.36%) and shade dried tablets (2.71%). Freeze dried tablets showed lowest dissolution rate (55.69%) compared to those of shade dried tablets (66.15%) and spray dried (73.96%). Shade dried tablets disintegrated at the fastest rate (12.25 min.) compared to spray dried tablets (15 min.) and freeze dried tablets (17min.) (Table 5.8).

Table – 5.8 Comparative characteristics of tablets prepared from different forms of wheatgrass formulations -

Sr. No.	Freeze-dried Tablet Wt. in mg	Shade-dried Tablet Wt. in mg	Spray-dried Tablet Wt. in mg
1	0.430	1.1100	0.5771
2	0.438	1.2258	0.5482
3	0.459	1.2109	0.5786
4	0.410	1.1354	0.5825
5	0.427	1.1621	0.5538
6	0.445	1.0088	0.5477
7	0.457	1.1858	0.5700
8	0.396	1.1777	0.5805
9	0.380	1.2116	0.5640
10	0.450	1.1999	0.5690
Average wt. in mg	0.4292	1.1549	0.5670
Disintegration time	17 min.	12.25 min.	15 min.
Dissolution rate	55.69 %	66.15 %	73.96 %
Friability	2.36 %	2.71 %	1.11 %
Hardness kg./cm^2	4.5 kg./cm2	5.3	3.2

5.4 Antibacterial activity -

The *in-vitro* antibacterial activity of undiluted juice as well as acetone and methanolic extracts of wheatgrass was studied by agar – well diffusion method. Acetone and methanolic extracts did not show any kind of antibacterial activity. Fresh and undiluted wheatgrass juice exhibited mild antibacterial activity against *Staphylococcus* aureus, *Bacillus cereus, Salmonella typhimurium* and *Kleibsella pneumoneae* at 2.7 gm/ml concentration. In terms of zone of inhibition, the diameter of the zone was found to be 12.1, 12.4, 13.2 and 12.2 % respectively (Table 5.9)*. Staphylococcus* aureas causes skin lesions such as boils, styes, and furuncles. Availability of excess iron in body e.g. in thalassemia, increases host susceptibility to *K pneumoniae* and *S. typhimurium* infections.

Table – 5.9

Sr. No.	Bacteria	Undiluted juice	Acetone Extract	Methanol Extract
1	*Bacillus subtilis*	- ve	- ve	- ve
2	*Staphylococcus aureus*	12.1	- ve	- ve
3	*Staphylococcus epidermidis*	- ve	- ve	- ve
4	*Escherichia coli*	- ve	- ve	- ve
5	*Pseudomonas aeruginosa*	- ve	- ve	- ve
6	*Bacillus cereus*	12.4	- ve	- ve
7	*Bacillus megaterium*	- ve	- ve	- ve
8	*Salmonella typhimurium*	13.2	- ve	- ve
9	*Kleibsella pneumoneae*	12.2	- ve	- ve

Agar-well diameter=8.5 mm

Results are expressed as inhibitory zone in mm.

Diameter of zone of inhibition (mm): - < 8.5; + = 12-16

5.5 Antiproliferative activity–

Comparison of seed germination rates of different seeds in presence of wheatgrass juice and distilled water revealed that the rates were considerably reduced in presence of wheatgrass juice. There was 50% reduction in the rate of wheat germination while those of green gram and groundnut were inhibited to the extent of 70% and 90% respectively. The antiproliferative activity exhibited by wheatgrass juice may be a clue to further investigation for possible anticancer activity of wheatgrass.

5.6 Clinical trial of wheatgrass on patients with anemic condition–

After 1-month treatment with wheatgrass, hemoglobin % (Hb gm%) was significantly increased (before treatment: 11.75 ± 0.245, after treatment: 12.63 ± 0.307), while RBC count was significantly decreased (before treatment: 4.72 ± 0.132, after treatment: 4.37 ± 0.087). The decrease in RBC count indicates

that wheatgrass may have suppressed haemopoietic process while rise in hemoglobin content indicates stimulation of hemoglobin synthesis in individual RBC. WBC count (before treatment: 8.615 ± 0.593, after treatment: 7.725 ± 0.44), TLC (before treatment: 2.6615 ± 0.083, after treatment: 2.483 ± 0.12), neutrophils count (before treatment: 6.22 ± 0.151, after treatment: 6.08 ± 0.17) and lymphocyte count (before treatment: 3.225 ± 0.162, after treatment: 3.37 ± 0.186) were not affected to any significant level (Table – 5.10) (Figure 5.58). This shows that either wheatgrass has no effect on body's defense mechanism or the duration of treatment i.e.1 month, was insufficient for such an effect.

Table – 5.10

Effect of one month treatment with wheatgrass on patients with anemia with respect to various hematological parameters -

Parameter	Before Treatment	After Treatment
Hb (gm%)	11.75 ± 0.245	12.63 ± 0.307*
RBC (/cmm)	4.72 ± 0.132	4.37 ± 0.087*
WBC (/cmm)	8615 ± 593	7725 ± 440
Total Lymphocyte Count (/cmm)	2661.5 ± 83	2483 ± 120
Neutrophil (%)	62.2 ± 1.51	60.8 ± 1.7
Lymphocyte (%)	32.25 ± 1.62	33.7 ± 1.86

* Significantly different from initial value (P<0.05)

Fig 5.58 Effect of Wheatgrass on patients with anemic condition
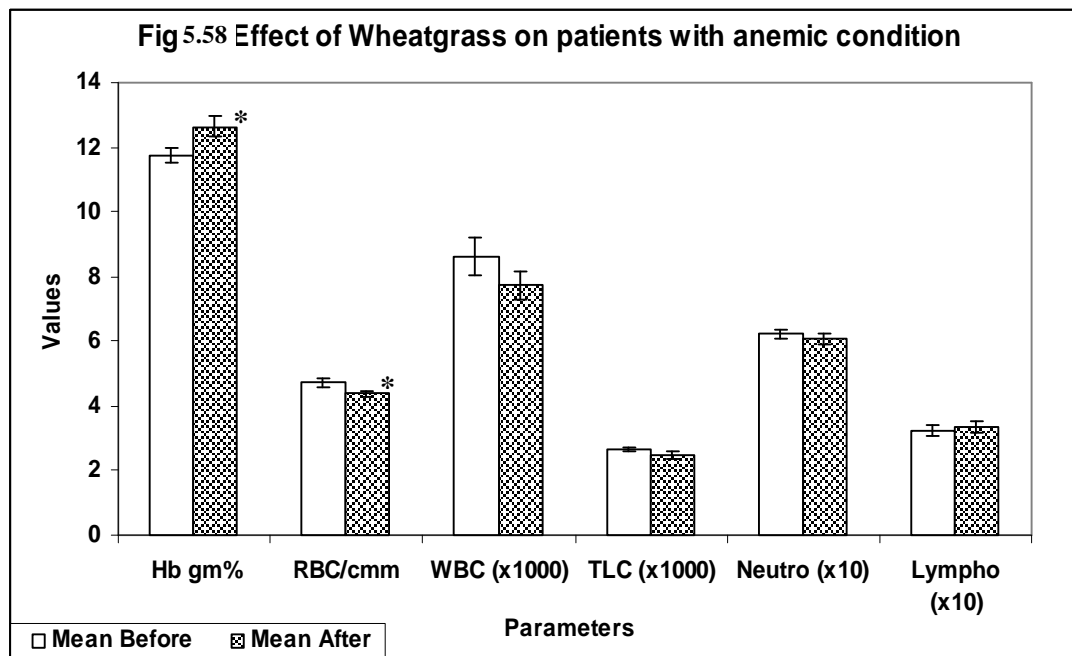
* Significantly different from initial value (P<0.05)

5.7 Clinical trial of wheatgrass on patients with β thalassemia (major)–

Table – 5.11

Profile of patients in the treatment group (ß-thalassemia (major) -

Total number of patients	20
Patients below 12 years age	8
Patients above 12 years age	12
Patients with S. Ferritin below 2500 (low iron load)	7
Patients with S. Ferritin above 2500 (high iron load)	13
Patients with spleen	13
Patients without spleen (spleenectomized)	7
Male/Female	12/8

After 9 months treatment with wheatgrass, Hemoglobin % (Hb gm%) in normal-range subgroup (i.e. Male-13.5-18 gm%, F–12-16 gm%) was significantly decreased (before treatment: 12.87 ± 0.10, after treatment: 10.77 ± 0.36). RBC count in normal-range subgroup (Male-4.5-6.5/cmm, Female-4.2-5.4/cmm) was also significantly decreased (before treatment: 4.55 ± 0.13, after treatment: 3.74 ± 0.20). Decrease in hemoglobin content and RBC count indicate possible haemopoietic suppression. The decrease in reticulocyte count was highly significant. Reticulocyte count in abnormal-range subgroup was significantly decreased (before treatment: 10.8 ± 2.55, after treatment: 2.8 ± 1.21). Decrease in reticulocyte count after wheatgrass treatment is an indication of increase in tissue oxygenation and improvement in erythropoiesis. Mean corpuscular volume (MCV) in normal-range subgroup was significantly increased (before treatment: 86.26 ± 1.06, after treatment: 77.02 ± 2.58) and that in abnormal-range subgroup was also significantly increased (before treatment: 77.63 ± 0.94, after treatment: 82.99 ± 1.74). Mean corpuscular hemoglobin concentration (MCHC) in abnormal-range subgroup was significantly increased (before treatment: 28.85 ± 1.06, after treatment: 32.63 ± 1.06) (Table 5.12) (Figure 5.59). Positive changes in MCV and MCHC reflect correction of both hypochromia and microcytosis, thus exhibiting reversal of abnormal condition in thalassemia.

Table – 5.12

Effect of wheatgrass on patients with β-thalassemia (major) with respect

to changes in parameters related to erythropoetic activity -

Parameter	Normal Value	Subgroup	Number of patients	Mean value before treatment with wheatgrass	Mean value after treatment with wheatgrass
Hb gm %	M - 13.5-18 F –12-16	Normal	3	12.87 ± 0.10	10.77 ± 0.36 *
		Abnormal	17	8.68 ± 0.54	8.83 ± 0.43
RBC m/cmm	M - 4.5-6.5 F - 4.2- 5.4	Normal	8	4.55 ± 0.13	3.74 ± 0.20 **
		Abnormal	12	3.08 ± 0.21	3.28 ± 0.19
Reticulocyte%	0.1-2	Normal	7	1.06 ± 0.06	1.00 ± 0.00
		Abnormal	4	10.80 ± 2.55	2.80 ± 1.21 *
PCV %	42-50	Normal	0	-	-
		Abnormal	20	29.49 ± 1.83	28.22 ± 1.39
MCV cu mic	82-92	Normal	6	86.26 ± 1.06	77.02 ± 2.58 *
		Abnormal	14	77.63 ± 0.94	82.99 ± 1.74 *
MCH pic gm	27-31	Normal	6	28.29 ± 0.16	26.73 ± 0.48 *
		Abnormal	17	24.96 ± 0.56	26.34 ± 0.49
MCHC %	32-36	Normal	12	33.83 ± 0.51	32.60 ± 0.42
		Abnormal	8	28.85 ± 1.06	32.63 ± 1.06 *

* Significantly different from initial value (P<0.05)

** Significantly different from initial value (P<0.01)

Fig. 5.59 Effect of Wheatgrass on patients with β-thalassemia (major) with respect to changes in parameters related to erythropoetic activity

* Significantly different from initial value (P<0.05)

** Significantly different from initial value (P<0.02)

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After 9 months treatment with wheatgrass, eosin count (Eosin %) in abnormal
-range subgroup was significantly decreased (before treatment: 3.22 ± 1.14,
0.33 ± 0.22) (Table – 5.13) (Figure 5.60). Reversal of eosinophilia may be an
indicator of detoxifying or antiallergic property of wheatgrass.

Table – 5.13

Effect of wheatgrass on patients with β-thalassemia (major) with respect

to changes in parameters related to natural defense mechanism of body-

Parameter	Normal Value	Subgroup	Number of patients	Mean value before treatment with wheatgrass	Mean value after treatment with wheatgrass
Platelet	1.5-4.5	Normal	11	273182 ± 12107.5	285091 ± 29245.2
		Abnormal	2	199000 ± 126572.1	258000 ± 154149.3
WBC/cmm	4000-10000	Normal	12	6008.3 ± 485.84	5769.2 ± 457.19
		Abnormal	8	13875.0 ± 2902.79	16057.1 ± 2033.11
Neutro %	60-70	Normal	7	62.14 ± 1.11	58.71 ± 2.63
		Abnormal	13	51.69 ± 2.04	54.23 ± 3.39
Lympho %	20-30	Normal	1	23.00 ± 0.00	43.00 ± 0.00
		Abnormal	19	40.00 ± 1.83	40.53 ± 2.00
Total lympho Count	800-3000	Normal	13	1963.85 ± 132.08	2293.92 ± 161.40
		Abnormal	7	5274.00 ± 916.94	6968.29 ± 1364.91
Eosin %	1.0-4.0	Normal	1	2.18 ± 0.36	0.64 ± 0.23
		Abnormal	9	3.22 ± 1.14	0.33 ± 0.22 *

* Significantly different from initial value (P<0.05)
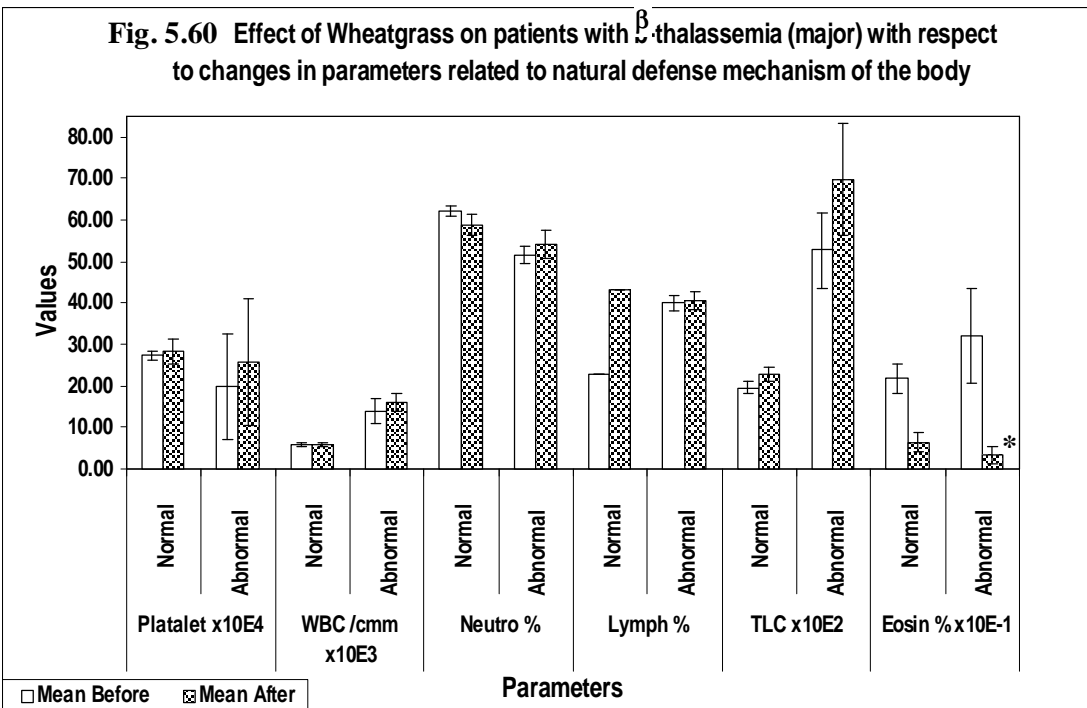
Fig. 5.60 Effect of Wheatgrass on patients with β-thalassemia (major) with respect to changes in parameters related to natural defense mechanism of the body

* Significantly different from initial value (P<0.05)

After 9 months treatment with wheatgrass, serum ferritin was significantly decreased (before treatment: 6846 $\pm$ 1222, after treatment: 3877 $\pm$ 676) indicating decrease in iron load. In absence of substantial rise in hemoglobin content, the only possible explanation for decreased iron load is increased excretion of iron. Treatment with wheatgrass had no influence on serum magnesium, serum iron and TBARS (Table 5.14) (Figure 5.61). These, data indicate that although wheatgrass treatment increases hemoglobin content of RBC in thalassemia, the mechanism of action is neither iron or magnesium supplementation nor replenishment of antioxidants.
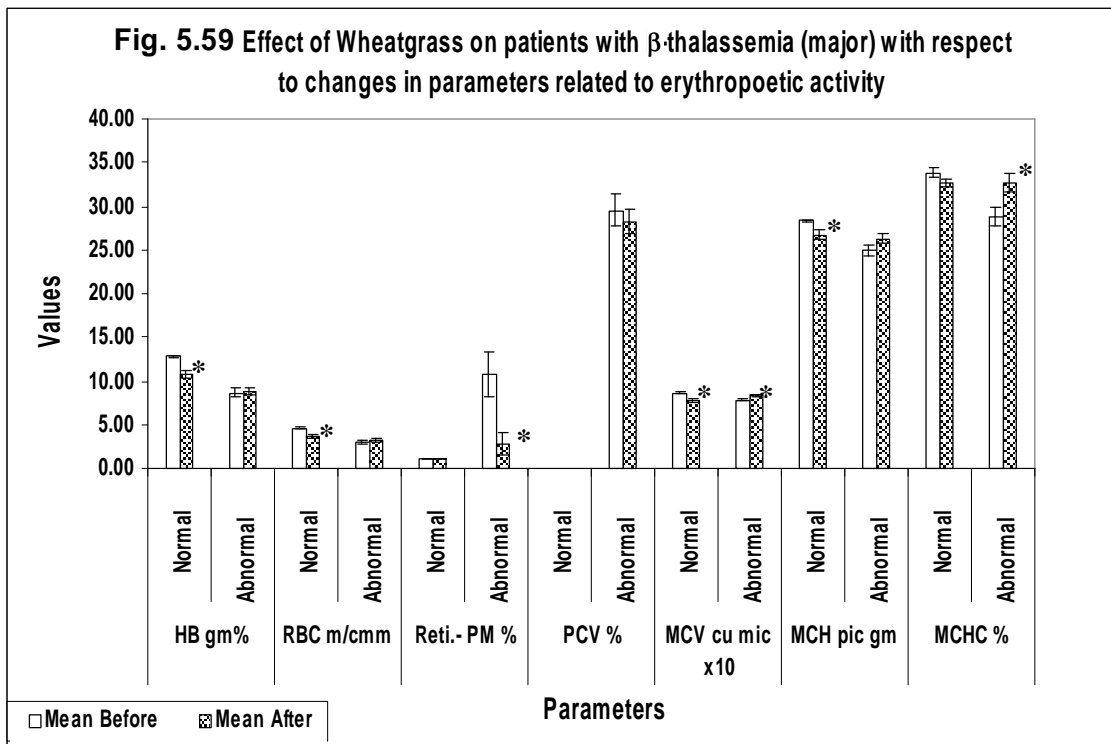
Table – 5.14

Effect of wheatgrass on patients with β-thalassemia (major) with respect to changes in parameters related to mechanism of action of wheatgrass-

Parameter	Normal Value	Subgroup	Number of patients	Mean value before treatment with wheatgrass	Mean value after treatment with wheatgrass
S. iron mic/dl	90-150	Normal	3	130.00 $\pm$ 12.25	274.00 $\pm$ 46.68
		Abnormal	14	225.51 $\pm$ 15.57	229.68 $\pm$ 22.34
S. ferritin ng/dl	12-140	Abnormal	16	6846 $\pm$ 1222	3877 $\pm$ 676*
S. mag meq/l	1.3-2.5	Normal	18	1.93 $\pm$ 0.10	1.92 $\pm$ 0.05
		Abnormal	1	3.54 $\pm$ 0.00	2.48 $\pm$ 0.00
TBARS x10^5 M/cmm	1.3-2.5	Normal	0		
		Abnormal	12	0.63 $\pm$ 0.05	0.60 $\pm$ 0.05

* Significantly different from initial value (P<0.05)
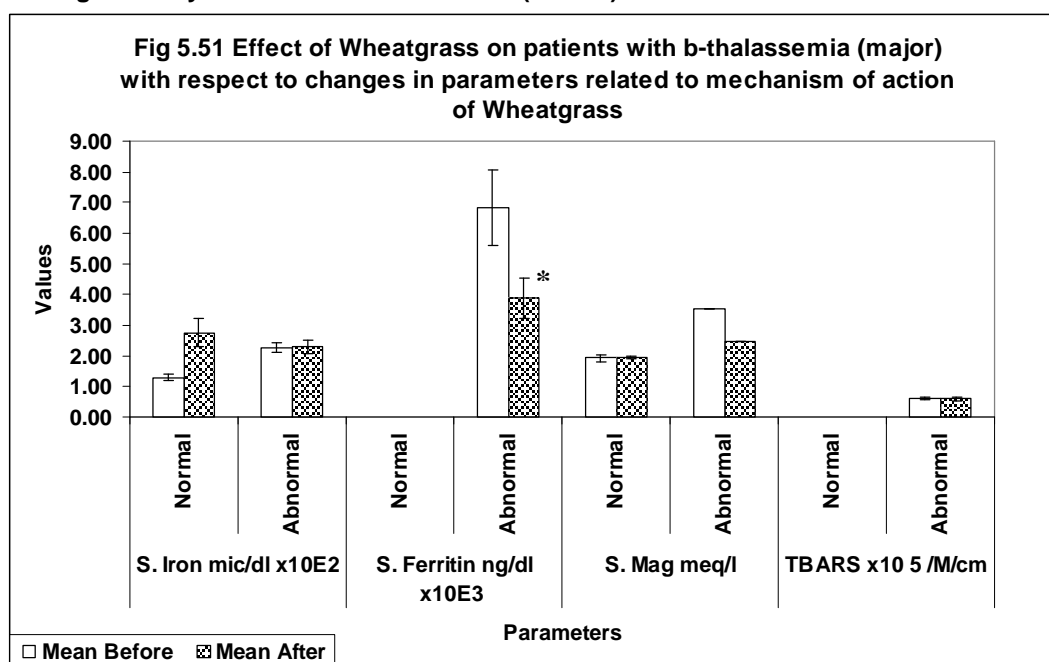
Fig 5.51 Effect of Wheatgrass on patients with b-thalassemia (major) with respect to changes in parameters related to mechanism of action of Wheatgrass

* Significantly different from initial value (P<0.05)

After 9 months treatment with Wheatgrass, different parameters in patients of above 12 years age group showed significant changes. In this group, the hemoglobin content in normal range subgroup was significantly decreased (before treatment: 12.86 ± 0.1, after treatment: 10.77 ± 0.36). RBC count in normal range subgroup was significantly decreased (before treatment: 4.54 ± 0.14, after treatment: 3.71 ± 0.22). Reticulocyte count in abnormal range subgroup was significantly decreased (before treatment: 10.8 ± 2.55, after treatment: 2.8 ± 1.21). MCV in normal range subgroup was significantly decreased (before treatment: 86.73 ± 1.37, after treatment: 76.2 ± 3.52). MCHC in normal range subgroup was significantly increased (before treatment: 28.43 ± 1.37, after treatment: 33.34 ± 1.28). Total lymphocyte count in normal range subgroup was significantly increased (before treatment: 1768.5 ± 151.04, after treatment: 2351.8 ± 157.97). In bellow 12 years age group, the serum iron content in normal range subgroup was significantly increased (before treatment: 149 ± 2.83, after treatment: 287 ± 7.78). Comparison of the effects of wheatgrass treatment in patients of both these age groups revealed that patients of above 12 years age group had experienced more beneficial effects of wheatgrass treatment compared to the patients of below 12 years age group. (Table 5.15- 5.17).

Table – 5.15

Comparison of effects of wheatgrass on patients of different age groups with β-thalassemia (major) with respect to changes in parameters related to erythropoetic activity -

Parameter	Subgroup	Below 12 years age group		Above 12 years age group	
		Before	After	Before	After
Hb gm %	Normal	-	-	12.86 ± 0.1	10.77 ± 0.36 **
	Abnormal	9.13 ± 0.18	8.63 ± 0.41	8.43 ± 0.7	8.94 ± 0.62
RBC m/cmm	Normal	4.57 ± 0.0	3.93 ± 0.0	4.54 ± 0.14	3.71 ± 0.22 **
	Abnormal	3.34 ± 0.23	3.28 ± 0.19	2.89 ± 0.3	3.28 ± 0.3
Reti.- PM %	Normal	1.0 ± 0.0	1.0 ± 0.0	1.08 ± 0.08	1 ± 0.0
	Abnormal	-	-	10.8 ± 2.55	2.8 ± 1.21 *
PCV %	Normal	-	-	-	-
	Abnormal	27.85 ± 2.7	26.92 ± 1.36	30.19 ± 2.33	28.77 ± 1.88
MCV cu mic	Normal	85.30 ± 1.42	78.65 ± 2.9	86.73 ± 1.37	76.2 ± 3.52 **
	Abnormal	74.53 ± 2.17	80.63 ± 4.2	78.87 ± 0.67	83.93 ± 1.66
MCH pic gm	Normal	-	-	28.29 ± 0.23	26.73 ± 0.63
	Abnormal	25.61 ± 0.56	25.67 ± 0.94	24.61 ± 0.79	26.7 ± 0.53
MCHC %	Normal	34.36 ± 0.88	32.97 ± 0.36	33.56 ± 0.6	32.42 ± 0.59
	Abnormal	30.09 ± 0.01	30.48 ± 0.31	28.43 ± 1.37	33.34 ± 1.28 *

Table – 5.16

Comparison of effects of wheatgrass on patients of different age groups with β-thalassemia (major) with respect to changes in parameters related to natural defense mechanism of the body –

Parameter	Subgroup	Below 12 years age group		Above 12 years age group	
		Before	After	Before	After
atelet	Normal	284250± 23495	221000 ± 9753	266857 ± 12884	321714 ± 39421
	Abnormal	-	-	258000 ± 154149	199000 ± 126572
WBC /cmm	Normal	6383 ± 536	6166 ± 740	5633 ± 781	5833 ± 343
	Abnormal	-	-	14025 ± 2639	14275 ± 2724
Neutro %	Normal	62.67 ± 2.18	62.33 ± 2.13	61.75 ± 1.02	56 ± 3.79
	Abnormal	50.33 ± 2.6	55.0 ± 0.82	52.1 ± 2.52	54 ± 4.4
Lymph. %	Normal	30 ± 0.0	40 ± 0.0	28.25 ± 1.52	34.5 ± 4.26
	Abnormal	42 ± 3.3	40 ± 2.15	44 ± 2.45	42.5 ± 2.5
Total lympho. Count	Normal	2276 ± 166	2345 ± 343	1768.5 ±151.04	2351.8 ± 157.97 *
	Abnormal	3744 ± 0.0	2970 ± 0.0	7505 ± 1483	6034 ± 960
Eosin %	Normal	1.75 ± 0.45	1 ± 0.35	0.8 ± 0.34	1 ± 0.3
	Abnormal	7 ± 2.12	1.5 ± 0.35	6.5 ± 1.06	0.5 ± 0.35

Table – 5.17

Comparison of effects of wheatgrass tablets on patients of different age groups with β-thalassemia (major) with respect to changes in parameters related to mechanism of action of wheatgrass -

Parameter	Subgroup	Below 12 years age group		Above 12 years age group	
		Before	After	Before	After
S. iron mic/dl	Normal	149 ± 2.83	287 ± 7.78 **	124.33 ± 10.71	231 ± 57.91
	Abnormal	281 ± 32.51	258 ± 56.79	225.8 ± 13.9	221.84 ± 27.48
S. mag meq/l	Normal	1.85 ± 0.07	1.87 ± 0.04	1.95 ± 0.11	1.95 ± 0.07
	Abnormal	1.96 ± 0.57	1.89 ± 0.03	3.54 ± 0.0	2.48 ± 0
TBARS	Normal	-	-	-	-
X10^5 M/cm	Abnormal	0.68 ± 0.09	0.45 ± 0.01	0.61 ± 0.08	0.66 ± 0.06

6. DISCUSSION

Wheatgrass has been traditionally used, since ancient times, to treat various diseases and disorders. Presently, there are a number of wheatgrass suppliers, in almost all cities of India, supplying fresh wheatgrass daily to their regular customers who use it as a traditional remedy against various ailments and also as a health tonic. There are several claims 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 (Wigmore 1985). Scientific reports on nutritional analysis of wheatgrass have been published (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, which have significant nutritious and medicinal value (Chan et al., 1999).

Thalassemia is one of the most common groups of genetic blood disorder. β-thalassemia is characterized by impaired production of β globin chain. The β-globin gene is present, but produces little β-globin protein (Weatherall 1997, Jandl 1982, Steinberg 1988). 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, precipitates and cause cell membrane damage. These deformed cells undergo premature destruction either in the bone marrow (extravascular hemolysis) or the peripheral circulation (intravascular hemolysis) (Festa 1985). Management of ß-thalassemia major (*Cooley's Anemia)* requires patients to have life-long regimen of regular blood transfusions coupled with iron chelation therapy. 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 desferrioxamine 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). Data indicate that dietary magnesium supplementation improves some of the characteristic cellular function abnormalities of β-thalassemia. The deficiency of magnesium in serum or erythrocytes has also been reported in human β-thalassemia. These deficiencies may play a significant role in various cellular abnormalities characteristic of this disorder (De Franceschi et al., 1998). Thus, in thalassemia, there is a vicious cycle of iron overload leading to oxidative stress with consequent increase in hemolysis and increase in blood transfusion requirement causing further iron overload and its toxicities on other organs.

In a chronic disease like thalassemia, the drug treatment is of long duration, may even be for years. In such a circumstances the factor of patient compliance becomes very important. Outcome of the therapy depends upon regular supply (round the year and in all seasons) and acceptability of the drug by patient. Recently, Marvaha et al (2004) reported an improvement in the patients using wheatgrass juice. About 100 ml of freshly extracted wheat grass juice was consumed daily. There was decrease in requirement of blood transfusion to atleast 25% in 19 patients. No specific variety of wheat grass was used. Poor compliance, indiscipline in intake and an insufficient duration of intake in 14 of 38 patients (37%) could be attributed to lack of positive effect observed in some cases. The process of growing, harvesting and extracting juice was too laborious, especially in summer months for most parents of defaulters . Thus, we could forsee non-compliance of the patients as a major hurdle in our tiral. As a pharmaceutical scientist, preparation of a suitable dosage form is prime area of research in the development of new drug formulations. Hence, in the present investigation we decided to prepare suitable formulation that is as effective as juice per se. While preparing dry wheatgrass powder for manufacture of tablets, it was noticed that the potency of the powders from different batches was affected by wheat species, fertilizer used to grow wheatgrass and the method adopted for drying or extraction.

Therefore, it was decided to carry out complete profiling of wheatgrass formulation (i.e. preformulation studies) by ascertaining most suitable species of wheat, fertilizer used to grow wheatgrass and also the method of extraction to prepare the powder.

Wheat species differ from one another both morphologically and genetically. Triticum species can be placed in three groups, according to whether their body cells contain 14, 28 or 42 chromosomes. The basic haploid number being 7, these groups are described as Diploid, Tetraploid and Hexaploid respectively (Percival 1974). Diploid species comprises of *Triticum boeoticum* or Wild Eincorn, which is the most ancient variety of wheat and *T. monococcum* Eincorn. *T. monococcum* is now grown to a limited extent in the mountainous region of Yugoslavia, Asia Minor and North Africa. Diploid species can be readily crossed to yield Tetraploid group. Tetraploid species comprises of *T. dicoccum* or emmer wheat and *T. durum* or macaroni wheat. *T. dicoccum* is one of the most ancient of cultivated cereals today. It was formerly grown in the United States for feed on a limited acreage but now has substantially disappeared from cultivation. *T. durum*, next important species to *T. aestivum*, is used mainly for the manufacture of semolina which is made into macaroni, spaghetti and related products (Percival 1974). It is grown to a considerable extent in parts of Gujarat and central peninsular India. When crossed with Diploid species, Tetraploid species yield Hexaploid group. Hexaploid species comprises of *T. aestivum* or common wheat is the most highly evolved and widely cultivated of all wheat species. In India, *T. aestivum* is the most widely grown wheat species.

In our investigation, certified samples of three major species of wheat viz. *Triticum aestivum*, *Triticum durum* and *Triticum dicoccum* were acquired form the Wheat Research Center, Gujarat Krushi University, Junagadh, Gujarat. These wheat varieties were grown in plastic trays as per the standard procedure. Microscopic studies of transverse sections, surface preparations and powder studies of the three species of wheatgrass were conducted using high-resolution microscope. In conformation with the description in literature (Percival 1974); 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). The leaf blade was linear and parallel –veined with mid rib projecting on the back continuing someway along the sheath. In *T. Dicoccum* the hairs on the swollen base of leaf were longer than those of other species. The longest leaves were possessed by *T. durum*.

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. the green assimilating parenchyma, 3. the 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 vary much in length and stoutness. On the leaves of *T. aestivum,* ample numbers of hair were present, while in *T. dicoccum* and *T. durum* they were sparsely distributed on the surface of the leaf. Stomata were observed at the base of the ridge arranged in single or double lines. Each stoma on the leaf consists of four cells, the two guard cells being narrow, with specially thickened walls round the stomatal pore and thin-walled widely dilated ends. Pores of the stomata are 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 is about 10:7, the number on the upper surface examined being 7000 per square centimeter. In the furrow between two ridges is a band of three to seven rows of motor cells. vascular bundles are 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 except in *T. durum*.

Phytochemical analyses of wheatgrass revealed that wheatgrass is a rich source of chlorophyll, various minerals like iron, magnesium, calcium, phosphorus, antioxidants like beta carotene and insoluble dietary fibers. Thus,

our analysis supports the scientific reports on nutritional analysis of wheatgrass (Kohler 1953, Hamilton et al., 1988, Laboratory Analyses 1989).

Three different techniques were adapted to prepare wheatgrass powder including spray drying, freeze drying and shade drying. In freeze drying technique, fresh wheatgrass was frozen to sub-zero temperature and subsequently subjected to low-temperature heating (10 ^{o}C -15 ^{o}C) in vacuum to evaporate crystallized water content. Dried wheatgrass was then milled to obtain powder. In spray-drying technique, fresh wheatgrass was pressed in a hydraulic press to obtain juice. The juice was then sprayed in aerosol form through a nebulizing nozzle in a conical vessel from top. A hot air counter current (55 ^{o}C) was passed from bottom of the vessel. The nebulized juice settled on the bottom of the vessel in the form of powder. In shade-drying technique, fresh wheatgrass was dried at room temperature in a well-ventilated dark room. The dried wheatgrass after 3-4 days of drying period was powdered in a mill.

TLC of the various extract of fresh wheatgrass and its formulations was carried out in hexane: acetone (60:40) solvent system. In all, 13 spots were observed at different R_f values viz. (1) 0.07(Light green), (2) 0.21(Yellow), (3) 0.27(Grey), (4) 0.35(Yellow), (5) 0.40(Yellow), (6) 0.47(Light green), (7) 0.54(Yellow), (8) 0.57(Green), (9) 0.61(Blue green), (10) 0.64(Light grey), (11) 0.69(Light grey), (12) 0.74(Grey), (13) 0.92(Orange). By comparing standard Rf values and colors reported in literature, out of 13 spots representing 13 different components of wheatgrass, seven components at spot number 4, 7, 8, 9, 11, 12 and 13 were identified as Xanthophyll c, Xanthophyll a, Chlorophyll b, Chlorophyll a, Pheophytin b, Pheophytin a and β carotene respectively. In comparative TLC study, chlorophyll a was found to be present in significant quantity in fresh wheatgrass and was absent in rest of the formulations. Intense grey-colored spots of pheophytin seem to have compensated the absence of chlorophyll in these formulations, as pheophytin is the degradation product of chlorophyll. Except chlorophyll a, 12 components were found present in shade dried powder. Spot no. 4, 5, 6, 9

and 10 were absent in freeze-dried and spray-dried powders. β-carotene was present in all formulations. Thus, shade drying, the natural method of drying, was found to preserve all components of wheatgrass and hence, appear to be the most suitable. The TLC study of wheatgrass extract has not been reported earlier to our knowledge.

In qualitative HPTLC chromatograms of methanolic extracts of Standard beta carotene, fresh wheatgrass, shade-dried wheatgrass powder, freeze-dried wheatgrass powder, spray dried wheatgrass powders were subjected to HPTLC study. The chromatograms were scanned in combined as well as individual modes at 254 nm and 354 nm wavelengths. Findings of qualitative HPTLC confirmed the patterns obtained in TLC study. Individual spots (components) in chromatogram were scanned at 254 nm wavelength to obtain their spectra for the purpose of identification of these components of wheatgrass in further investigations.

Qualitative HPTLC was carried out on extracts wheatgrass formulations at maximum concentration, for presence of various constituents. Methanolic extracts of equivalent amounts of these formulations were subjected to quantitative HPTLC along with those of different species of wheatgrass. Chromatograms of the formulations or species were recorded. Area under curve (AUC) of different components were measured in chromatogram of each formulation or species and analyzed for comparison with that of fresh wheatgrass. Since all the constituents of wheatgrass could not be identified, the AUCs of only chlorophyll, pheophytin and beta carotene were used for the purpose of comparison.

The HPTLC analysis of wheatgrass grown from three major wheat species revealed that *Triticum aestivum* had lowest contents (76.8%) as compared to the contents of *Triticum durum* (86.92%) and *Triticum dicoccum* (100 %). Thus, it seems that wheatgrass grown from tetraploid species of wheat, especially *Triticum dicoccum* (local variety called as DDK) and *T. durum,* is

more suitable for medicinal use as compared to the hexaploid species i.e. *T. aestivum* or the common wheat.

To determine the effect of fertilizer on quality of wheatgrass, same species of wheat i.e. *T. durum* was grown separately in plain soil, in compost fertilizer and in organic fertilizer. When subjected to HPTLC analysis, contents of wheatgrass grown in organic fertilizer (91.36%) and that of wheatgrass grown in compost fertilizer (92.05%) were nearly similar while the contents of wheatgrass grown in plain soil (100%) were highest among the three. Thus, presence of a fertilizer does not seem to hamper or interfere much with the sensitive phytochemical processes being carried out in the young growing grass in early stages of growth.

It is well known that the stability of components present in wheatgrass, like chlorophyll, beta-carotene, vitamin A, vitamin E, vitamin C etc, are likely to be adversely affected upon exposure to changes in air, light, humidity and temperature. This fact is confirmed by the results of our analysis. When subjected to HPTLC analysis, wheatgrass tablets of spray-dried powder was found to have lowest contents (21.66%) as compared to the contents of tablets of freeze-dried powder (46.68%) and shade-dried powder (87.75%) considering the contents of methanolic extract of fresh wheatgrass as 100%. In spray drying technique the use of 55^0 C temperature might have adversely affected the stability of thermolabile constituents like chlorophyll, beta-carotene, vitamin A, vitamin E. In freeze drying process although wheatgrass is subjected to low-temperature heating (10° C -15° C), the heating is carried out in high vacuum to evaporate crystallized water content. It is possible that low temperature (15° C), when applied at high vacuum, has a similar drastic effect on constituents of wheatgrass as to that of high temperature e.g. 60^0 C applied at room temperature. In shade drying technique, wheatgrass is neither exposed to high temperature nor to sunlight or moisture. Thus, the potency of wheatgrass is maintained at a substantially higher level. Hence, we infer that the most suitable method of preparing dry wheatgrass powder, among above three techniques is shade drying technique.

Traditionally, wheatgrass juice is prepared by grinding fresh wheatgrass in mixer/grinder adding little water that is subsequently filtered through a cloth. When subjected to HPTLC analysis the fresh wheatgrass juice, prepared by traditional method, was found to have 96.7% contents considering the contents of methanolic extract of fresh wheatgrass as 100%. Thus, in the face of it, traditional method seems to be the best method for wheatgrass therapy compared to any formulations of wheatgrass that were made in our study. But, when the traditionally prepared green colored juice was passed through a filter paper the filtrate obtained was only a light yellow colored liquid while the residue on the filter paper was full of green color. This indicates that the traditional juice does not extract chlorophyll and other ingredients from the intact cells in to water but in fact; the juice is a suspension of tissue debris in water. In our experiment the residue was treated with methanol for estimation of the contents of juice. Thus the traditional juice ingested by a patient delivers important constituents of wheatgrass packed inside intact cells made up of cellulose, which in turn may not be digested by human intestinal tract. Under these circumstances, how much of wheatgrass contents contained in the traditional juice, is a matter of speculation. The same logic of bioavailability also applies to freeze dried and shade dried tablets since both contain intact grass.

Determination of dose of wheatgrass tablets is an important issue to be addressed. According to Ann Wigmore, considered to be an authority in wheatgrass therapy and who is believed to have treated thousands of patients with wheatgrass juice, the daily dose of wheatgrass for a patient suffering from a chronic ailment e.g. thalassemia should be: traditional juice prepared from 100 gm. grass. In the light of foregoing discussion on partial bioavailability (probably 50%) afforded by the traditional juice, 50 gm. of methanolic extract of wheatgrass would be equivalent to traditional juice prepared from 100 gm. wheatgrass. In our investigation, the extractive value of wheatgrass was found to be 6%. Thus, 3 gm. of methanolic extract obtained from 50 gm. wheatgrass i.e. 2 tablets of 500 mg. methanolic extract, given three times a day might be the therapeutically useful dose.

Investigation in to antibacterial profile of wheatgrass revealed two important features. Aqueous extract of wheatgrass exibited mild to moderate antibacterial activity against four common opportuinistic pathogenic bacteria viz. *Staphylococcus aureus, Klebsiella pneumoneae, salmonella typhi* and *Bacillus cereus*. *Staphylococci* are Gram-positive spherical bacteria that occur in microscopic clusters resembling grapes. *Staphylococci* are found in cultures of the nose and skin of humans. *Staphylococcus aureas* causes skin lesions such as boils, styes and furuncles. Thus, our observation supports the claim made by Wigmore about wheatgrass juice being useful in treatment of skin infections (Wigmore 1985). Klebsiellae are nonmotile, rod-shaped, gram-negative bacteria with a prominent polysaccharide capsule. *Klebsiellae* may be regarded as normal flora in many parts of the colon and intestinal tract. *K pneumoniae* is the most medically important species of the group. Availability of iron increases host susceptibility to *K pneumoniae* infection. It would be worth recalling that thalassemic patients are chronic sufferers of iron overload. The presence of invasive devices, contamination of respiratory support equipment, use of urinary catheters, and use of antibiotics are factors that increase the likelihood of nosocomial infection with *Klebsiella* species. Hence, thalassemic patients undergoing regular blood transfusions are succeptible to nosocomial infections. Further, withholding iron from potential pathogens is one strategy used in host defense (Weinberg, 1978). Transferrin's extremely high affinity for iron, coupled with the fact that two-thirds of the iron-binding sites of the protein normally are unoccupied, essentially eliminates free iron from plasma and extracellular tissues. Both transferrin and the structurally related protein, lactoferrin, are bacteriostatic in vitro for many bacteria (Reiter et al., 1975). The very high transferrin saturations attained in patients with iron overload compromise the bacteriostatic properties of the protein. Iron sequestration is not a frontline defense against microbes. Therefore, iron overload does not produce the susceptibility to infection seen with defects in more central systems (e.g., chronic granulomatous disease.) Nonetheless, a number of infections, often with unusual organisms, including *Salmonella typhimurium* have been reported in patients with iron overload (Abbott et al., 1986) (Bullen et al., 1991). Thus, the antibacterial activity of wheatgrass could be beneficial for thalassemic patients, in addition to its clinical effects on

blood picture. The antibacterial activity was exibited only by undiluted extract of wheatgrass. Also, the antibacterial activity was lost within 2 hours of preparing the extract. This leads us to conclude that topical application of only fresh and undiluted wheatgrass extract could be beficial in treatment of skin infections.

The results of our investigation into effect of wheatgrass on seed germination reveal that wheatgrass exibits strong antiproliferative activity which could be a clue to its potential anticancer activity. It has been claimed that the wheatgrass contains abscisic acid and laetrile, both of which may have anti-cancer activity (Wigmore 1985). Chlorophyll-rich plant extracts, as well as water solutions of a chlorophyll derivative (chlorophyllin), dramatically inhibit the carcinogenic effects of common dietary and environmental chemicals (Kimm et al, 1982, Ong et al, 1986). However, some evidence suggests that the observed medical effects of chlorophyll in plants are actually the result of the total effect of the interaction between different components of the whole plant, in addition to the sole effects of chlorophyll (Lea 1999, Waladkhani 1998). Other phytochemicals that may work in therapeutic combination with chlorophylls include carotenoids, flavonoids, indole, isothiocyanate, poyphenolic compounds, protease inhibitors, sulfides, and terpens (Waladkhani 1998). Chlorophylls and chlorophyll-related products, both natural and man-made, have been shown to support normal cellular function, as demonstrated experimentally through in vitro, in vivo, and animal studies (Higashi 1998). Studies demonstrate that chlorophyll and chlorophyll-related products support normal cellular health through anti-mutagenic activity. Studies have found that chlorophyll binds carcinogens and reduces their uptake (Harttig 1998, Nakamura 1996). Thus, there is ample evidence to indicate that wheatgrass may useful in prevention and treatment of cancer. Results of our investigation support these evidences.

Iron deficiency anemia affects approximately one third of the world's population. On a worldwide basis, diet is the major cause of iron deficiency. In countries where little meat is in the diet, iron deficiency anemia is 6-8 times more prevalent than in America and Europe. This occurs despite consumption

of a diet that contains an equivalent amount of total dietary iron because heme iron is absorbed well from the diet than non-heme iron. Anemia due to deficiency of folic acid and vitamin B_{12} are not uncommon. Wheatgrass contains iron, folic acid and vitamin B_{12}. To assess effect of wheatgrass on anemia an open clinical trial of wheatgrass on anemia was carried out at Ayurved Hospital functioning under Civil Hospital, Rajkot. Necessary permission for conducting the clinical trial was obtained from the concerned ethical committee. 20 anemic patients, visiting the hospital were enrolled for the trial, after taking informed consent. 20 patients suffering from anemia whose age ranged from 15 to 50 years were included in the trial. The patients were given wheatgrass tablets with dosage regimen of 2 tablets (wheatgrass powder 250 mg.) 3 times in a day for 1 month. Blood samples were collected at the start and at the end of period of clinical trial (i.e. 1-month period). To assess effect of wheatgrass on haemopoietic activity, hemoglobin gm%, RBC count, WBC count were recorded while, to assess effect of wheatgrass on defense mechanism neutrophil count, lymphocyte count and total lymphocyte count were recorded. After 1-month treatment with wheatgrass, Hemoglobin (Hb gm%) was significantly increased. RBC count was significantly decreased. WBC count, neutrophil count, lymphocyte count and total lymphocyte count were not affected to any significant level.

An increase in hemoglobin content coupled with increase in RBC count indicates stimulation of erythropoiesis i.e. increase in overall hemoglobin content of blood due to increased production of RBC. In our investigation, we found a decrease in RBC count, which indicates that wheatgrass might have suppressed the haemopoietic process. The rise in hemoglobin content indicates stimulation of hemoglobin synthesis in individual RBC, probably by replenishment of deficient nutritional factors like iron as the major cause of anemia. WBC count, neutrophil count, lymphocyte count and total lymphocyte count were not affected significantly. This showed that wheatgrass had no effect on body's defense mechanism. The results of wheatgrass on anemic patients also suggest that it may also have beneficial effect in patients with thalassemia.

A randomized clinical trial of wheatgrass on β-thalassemia was carried out at K. T. Children Hospital working under Civil Hospital, Rajkot. Necessary permission for conducting the clinical trial was obtained from the concerned ethical committee. Twenty patients with thalassemia, visiting K. T. Children Hospital regularly for blood transfusion and registered at the hospital were enrolled for the trial, after taking informed consent. The patients above 12 years age were given wheatgrass tablets with dosage regimen of 2 tablets (wheatgrass powder 250 mg.) 3 times in a day for 9 months. Blood samples were collected at the start, after 6 months and at the end of period of clinical trial. The parameters recorded were Hemoglobin, Total RBC count, PCV, MCV, MCH, MCHC, Total WBC count, Neutrophils, Eosinophils, Basophils, Lymphocytes, Serum iron, Serum ferritin, Serum magnesium and Thiobarbituric acid reacting substances (TBARS).

After 9 months treatment with wheatgrass, hemoglobin in patients of normal-range subgroup, was found to be significantly decreased (before treatment: 12.87 ± 0.10, after treatment: 10.77 ± 0.36). RBC count in normal range subgroup was also significantly decreased (before treatment: 4.55 ± 0.13, after treatment: 3.74 ± 0.20). This may be an indication of haemopoietic suppression effect of wheatgrass.

After 9 months treatment with wheatgrass, hemoglobin content and RBC count in abnormal range were not affected significantly by wheatgrass treatment. Treatment with wheatgrass juice in thalassemia patients for a period of 6 months showed that the 'response' to wheatgrass juice was slow for couple of months (Marwaha et al., 2004). In our study also, the transfusion requirements remained unchanged even after a period of 9 months of ingesting wheatgrass tablets. However, there was a significant improvement in some hematological parameters related to thalassemia. After 9 months treatment with wheatgrass, reticulocyte count in abnormal-range subgroup was significantly decreased. The reticulocyte is an immature erythrocyte. Normally, the reticulocyte count ranges from 1 to 2% and reflects daily replacement of 1% of red cell population of red cells (Adomson and Longo

2001). The rate of reticulocyte release from the marrow into the peripheral circulation is governed primarily by the rate at which O_2 is being supplied to the tissues. A decrease in PO_2 (hypoxia) is recognized by the kidney, which is stimulated to release erythropoietin (Adomson and Longo 2001). The chronic hypoxic condition in thalassemia, due to persistent low hemoglobin content of blood stimulates secretion of erythropoietin, which in turn increases ineffective erythropoiesis and reticulocyte count. Decrease in reticulocyte count after wheatgrass treatment is an indication of increase in tissue oxygenation and decrease in ineffective erythropoiesis.

Thalassemia is characterized by hypochromic microcytic anemic condition. After wheatgrass treatment mean corpuscular volume (MCV) was significantly increased. Mean corpuscular volume (MCV) expresses the mean volume of each red cell. Similarly mean corpuscular hemoglobin concentration (MCHC) was also significantly increased. Positive changes in both these parameters reflect correction of both hypochromia and microcytosis, thus exhibiting reversal of abnormal condition in thalassemia. MCHC provides the mean concentration of hemoglobin in each red cell. It is most valuable in evaluating therapy for anemia because it takes in to consideration hemoglobin and hematocrit (PCV) and not the RBC count. The MCHC reflects defects in hemoglobin synthesis (Adomson and Longo 2001). Increase in MCHC indicates rise in hemoglobin concentration in individual RBC. In the face of increased MCV this rise in MCHC signifies a substantial increase of hemoglobin in RBC. In our investigation the increase in MCHC i.e. increase in hemoglobin content of individual RBC was offset by haemopoietic suppression effect of wheatgrass i.e. decrease in number of RBC. Thus, the reduction in overall transfusion requirement was not observed after 9 months treatment with wheatgrass.

Increase in MCHC could be an indication of increased synthesis of hemoglobin in RBC. Since, total adult hemoglobin comprises of hemoglobinA_1 - $\propto_2\beta_2$ (97%), hemoglobin A_2 - $\propto_2\delta_2$ (2.5%) and hemoglobin - $\propto_2\gamma_2$ (0.5%), it follows that rise in MCHC was contributed by any of these three type of

hemoglobin or in other words due to increased expression of β, γ or δ gene. It is known that RNA splicing mutations are fairly common and represent a large portion of all mutations resulting in beta thalassemia (Benz 2001). Probability of increasing expression of a mutated gene is very low. It is also known that a critical control region of the delta globin gene (promoter) is known to be defective. It inhibits messenger RNA (mRNA) processing, resulting in only a small amount of Hb A2 (alpha2/delta2) production, which accounts for less than 3% of total Hb in adult RBC. Further, it has been found that stimulation or induction of fetal hemoglobin in thalassemia can improve the patient's clinical condition (Jones and Taylor 1980). Thus, increase in MCHC after wheatgrass treatment may be an indication of induction of HbF in thalassemic patients.

Further, consequence of diminished beta-chain production is the formation of excess alpha chains that form tetramers and inclusion bodies because they are less soluble than normal Hgb. These inclusion bodies are lethal to developing erythroid precursors and are responsible for most of the severe clinical effects of thalassemia. The inclusion bodies cause the destruction of approximately 95% of red blood cell precursors prior to release into the circulation (i.e. intramedullary hemolysis), either as a consequence of arrest in the G1 phase of the cell cycle or by apoptosis during later phases of maturation (Weatherall 1997, Festa 1985). The only reasonable chance a red blood cell has for a somewhat normal morphology and lifespan occurs if significant amounts of non–beta-chain Hgb (e.g. gamma, delta) are available to bind the excess alpha chains. Thus, increased synthesis of gamma chain due to wheatgrass treatment can increase the life span of RBC, reduce severity of clinical picture and inhibit ineffective erythropoiesis in thalassemia. In our investigation, 90% patients taking wheatgrass treatment reported feeling of well-being increase in vitality and appetite, which is in concurrence with the forgoing discussion.

After 9 months treatment with wheatgrass, eosinophil count was significantly decreased. Eosinophilia is seen in allergic disorders. Thalassemic patients on regular blood transfusion therapy are subject to various allergens hence,

eosinophilia is commonly found in thalassemic patients. Thus, reversal of eosinophilia may be an indicator of much acclaimed detoxifying or antiallergic property of wheatgrass.

It is well known that management of ß-thalassemia major (*Cooley's Anemia)* requires patients to have life-long regimen of regular blood transfusions coupled with iron chelation therapy (Modell 1994, Cao et al., 1997). Blood transfusion produces on long term, serious and unavoidable side effects because 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. Iron overload may be treated or prevented with a chelating agent capable of complexing with iron and promoting its excretion. At present, only two iron-chelating agents are available for clinical use in thalassemia viz. desferrioxamine (Desferal - given subcutaneously) and deferiprone (Kelfer - given orally) (Olivieri et al., 1994, Zurlo et al., 1989). Painful administration, muscle aches, arthralgia and high cost are major disadvantages of desferrioxamine while neutropenia, agranulocytosis and arthropathy, are noted side effects of deferiprone (Al-Refaie et al., 1992, Al-Refaie et al., 1995, Cohen et al., 2000, Agarwal et al., 1992). Thus, the search continues for an oral, safe, effective, easily available and economical iron chelating agent.

As wheatgrass contains substantial quantity of iron, which is contraindicated for thalassemic patients, the major apprehension expressed by patients and doctors, at the outset of this study, was the possibility of increase in iron load with wheatgrass treatment. The measurement of plasma or serum ferritin is the most commonly used indirect estimate of body iron stores (Brittenham et al., 2001). In our investigation serum ferritin level, against all predictions, was significantly decreased at the end of 9 moths treatment with wheatgrass. In absence of substantial rise in hemoglobin content, the only possible explanation for decreased iron load is increased excretion of iron. Thus, the most notable outcome of our investigation, apart from ascertaining the ability of wheatgrass to increase hemoglobin content, is the detection of its probable ability to decrease iron load in thalassemia. Wheatgrass may well be the long

sought-after safe, oral and economical iron-chelating agent for thalassemic patients.

The deficiency of magnesium in serum or erythrocytes has also been reported in human β-thalassemia and that dietary magnesium supplementation improves some of the characteristic cellular function abnormalities of β-thalassemia (De Franceschi et al., 1998). Clinical data suggests that the iron-induced liver damage in thalassemia may play a major role in the depletion of lipid-soluble antioxidants (Livrea et al., 1996). 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). Hence, logical mechanism of action of wheatgrass in thalassemia, at the onset of the clinical trial, was either replenishment of magnesium and/or antioxidants that can break this vicious cycle. In our study, treatment with wheatgrass had no influence on serum magnesium and thiobarbituric acid reactive substances (TBARS). Both the parameters were included to investigate probable mechanism of action of wheatgrass. Thus, our data indicate that although wheatgrass treatment exhibits beneficial therapeutic effects in thalassemia, the mechanism of action is neither magnesium supplementation nor replenishment of antioxidants.

In the past few years, several new approaches to the treatment of thalassemia have included the use of drugs such as hydroxyurea, 5-AzaC and butyrate compounds to elevate fetal hemoglobin (HbF) (DeSimone et al., 1982). Mechanism of induction of HbF by butyrate is that it Inhibits histone deacetylases and thereby increasing histone acetylation, which opens up the chromatin structure and makes DNA more accessible to transcription factors. This results in increased transcription of the γ-globin gene by making its DNA more accessible to transcription factors. Early studies demonstrated that

treatment of tissue culture cells with the cytidine analog 5-azacytidine (5-azaC) led to cellular differentiation and DNA hypomethylation (Jones and Taylor 1980). Incorporation of 5-azaC into baboon DNA resulted in a 40- to 70-fold increase in γ-globin gene expression in the adult (DeSimone et al., 1982), and to hypomethylation of the γ-globin promoter (Lavelle et al., 1986). Thus, DNA hypomethylation and histone deacetylation seem to be the chief mechanism of action of the agents that induce HbF synthesis. This suggests that inhibitors of DNA methyl transferase and histone deacetylase may activate transcription of HbF (Bird 2001).

Is there any constituent in wheatgrass that has properties of gene regulation and ability to cause DNA hypomethylation and histone deacetylation? Wheat seeds contain substantial amount of abscisic acid (Banowetz, et al., 1994). That, wheatgrass contains abscisic acid has also been claimed by Ann Wigmore (Wigmore 1985). Abscisic acid is a known plant growth regulator (Banowetz, et al., 1994), which inhibits the process of seed priming in adverse climatic condition thus, allowing germination only under favorable circumstances. Gene regulating activity of abscisic acid has been further investigated by Schultz et al (1996) who report that nuclear extract of wheat enhances binding of the recombinant transcription factor EmBP-1 to Em1a by 80-fold. (Em1a is an ABA response element in the promoter of the Em gene from wheat.) ' Fractionation of nuclear extracts led us to identify histone H1 and HMGb as two factors that can enhance the ability of EmBP-1 to bind to Em1a. Furthermore, our study points to these chromosomal proteins (i.e. histones) as potential targets of an ABA- mediated modification (e.g. acetylation) that could affect the regulation of Em gene expression'. It is thus possible that wheatgrass contains abscisic acid, which has proven ability to regulate transcription of a gene through acetylation of chromosomal proteins or transcription factors. Ability of wheatgrass to Induce transcription of HbF in thalassemic patients may be through acetylation of chromosomal proteins (histones) by abscisic acid, present in wheatgrass. Abscisic acid may have the potential to emerge as the much sought-after HbF inducer for benefit of

thalassemic patients. However, further studies are required to substantiate the results.

In our investigation Hb% and RBC count within normal range were significantly decreased indicating possible haemopoietic suppression (i.e. suppression of proliferation of erythroid progenitors) which, when viewed with antiproliferative activity in the seed germination experiment, points once again to the involvement of abscisic acid in effects or actions of wheatgrass.

Comparison of effects of wheatgrass treatment in different groups of patients reveals that patients above 12 years age had more beneficial effects of wheatgrass treatment as compared to the patients below 12 years age. This was probably due to lower dose of wheatgrass (two tablets twice in a day) administered to patients' bellow 12 years age as compared to higher dose (two tablets three time in a day) to patients above 12 years age. Significant difference in the clinical effectiveness between these two dosage regimen indicate that the minimum dose of wheatgrass should be two tablets (250 mg tablets) three times in a day, for all thalassemic patients.

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. Hypersplenism results in to trapping of a variety of blood cell types causing cytopenia (Henry and longo 2001). Reversal of ineffective erythropoiesis and consequent reversal of spleenomegaly with subsequent release of trapped lymphocytes may be the cause of significantly increased total lymphocyte count in patients of above 12 years age grouping our investigation. Reversal of splenomegaly after wheatgrass treatment is further endorsed by the fact that lymphocyte% was significantly increased in patients with spleen, while it remained unaffected in spleenectomized group.

Results of clinical study indicate wheatgrass formulation to be one of the effective treatments for thalassemia. Although direct molecular studies are required to confirm, it is hypothesized that wheatgrass produces direct

beneficial effect in thalassemia patients by stimulating hemoglobin synthesis in RBC. Wheatgrass was found to decrease significantly the iron load and suppress the ineffective erythropoiesis.

7. SUMMARY AND CONCLUSIONS

1. The pharmacognostic characteristics of three varieties of wheat (*T. dicoccum, T. durum* and *T. aestivum)* used in the study were in confirmation with that reported in the literature.

2. The phytochemical analysis of wheatgrass by TLC revealed 13 spots at 254 and 354 nm. Out of these 13 some of the constituents identified were chlorophyll a and b, pheophytin a and b, xanthophyll a and c and beta-carotene.

3. For quantitative evaluation of different formulations, chlorophyll, pheophytin and beta-carotene contents were taken in to consideration.

4. Among three major varieties of wheat *T. dicoccum* variety may have highest concentration (100%) of constituents followed by *T. durum* (86.92%) and *T. aestivum* (76.8%).

5. Wheatgrass grown in plain soil may have highest concentration (100%) of constituents followed by wheatgrass grown in compost fertilizer (92.05%) and organic fertilizer (91.36%).

6. Wheatgrass powder obtained by shade drying technique seems to have highest concentration (87.75%) of constituents compared to that obtained by freeze-drying (46.68%) and spray drying (21.66%) techniques.

7. The fresh wheatgrass juice prepared traditionally seems to offer highest contents (96.7%) of wheatgrass, compared to its formulations i.e. shade drying (87.75%), freeze drying (46.68%) and spray drying (21.66%).

8. Aqueous extract of wheatgrass may have mild to moderate antibacterial activity against four common opportuinistic pathogenic bacteria viz. *Staphylococcus aureus*, *Bacillus cereus, Salmonella*

typhimurium and *Kleibsella pneumoneae*. The antibacterial activity may be lost on standing or on dilution of the fresh juice.

9. Wheatgrass juice exhibits antiproliferative activity. This may be a clue for further investigation into possible anticancer activity of wheatgrass.

10. Treatment with wheatgrass on patients with anemia may have beneficial effects in the form of increase in hemoglobin content and decrease in erythropoietic activity.

11. Treatment with wheatgrass on patients with ß-thalassemia (major) produced a significant decrease in reticulocyte count, especially in patients having higher reticulocyte count suggesting that treatment is effective in producing a decrease in ineffective erythropoiesis.

12. There was a significant decrease in MCHC in patients with abnormally low MCHC levels. This was also observed in MCH and MCV. All these results suggest a beneficial effect in patients with thalassemia possibly by stimulating hemoglobin synthesis in RBC.

13. The beneficial effect of wheatgrass was not associated with increase in iron overload. In fact the iron overload seems to have decreased by the treatment with wheatgrass as revealed by significant decrease in serum ferritin levels from 6846 ± 1222 to 3877 ± 676 ng/dl.

14. There was on change in the serum iron content, serum magnesium or TBARS. Thus, the mechanism of beneficial effect of wheatgrass appears to be independent of oxidative stress or nutritional supplementation. Some unidentifiable constituent appears to stimulate the hemoglobin synthesis and further studies are required to be carried out in this direction.

15. Treatment of thalassemic patients with wheatgrass did not produce any effect on platelet count or total lymphocyte count. However, a

significant decrease in eosinophil count was observed.

16. A significant increase in lymphocyte count was observed only in patients wherein splenectomy was not done. It is possible that wheatgrass produces a reversal of splenomegaly.

In nutshell, our studies support, scientifically, some of the therapeutic claims for wheatgrass. The potential benefit of wheatgrass in thalassemia appears to be convincing and opens new vista of investigation.

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