"PHYTOPHARMACOLOGICAL EVALUATION OF VARIOUS FORMULATIONS IN ANIMAL MODEL OF ANEMIA WITH AND WITHOUT INFLAMMATION"

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BY

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CERTIFICATE

This is to certify that the dissertation work entitled "Phytopharmacological evaluation of various formulations in animal model of anemia with and without inflammation" submitted by Mr. Rajeev Tiwari with Regn. No. (10MPH 205) in partial fulfillment for the award of Master of Pharmacy in "Pharmacology" is a bonafide research work carried out by the candidate at the Department of Pharmacology, Institute of Pharmacy, Nirma University under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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Dedicated to my beloved Mom, famlily, and my blu bans

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Date- *Rajeev Tiwari*

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C. ABBREVIATIONS

AI - Anemia of inflammation

WHO - World Health Organization

IL-6 - Interleukin-6

EPO – Erythropoietin

MCV- Mean corpuscular volume

MCH -Mean corpuscular haemoglobin

IFN – Interferon

TNF – Tumor necrosis factor

RBC – Red blood cell

Hb – Hemoglobin

AID- Anemia of inflammatory disease

IMHA- Immune-mediated hemolytic anemia

MDS - Myelodysplastic syndrome

TfR - Transferin

NO - Nitric oxide

AS - *Angelica sinensis*

HMF - Herbomineral formulation

MIP - Macrophage inflammatory protein

SF - Sodium ferulate

VEGF - Vascular endothelial growth factor

HUVEC - Human umbilical vein endothelial cell

MDA - Malondialdehyde

GSH – Glutathione

GSH-Px - Glutathione peroxidise

Abstract

1. ABSTRACT

Aim and Objective:

Anemia is the leading cause of morbidity and mortality in patients with chronic disease and iron deficiency. The present study was undertaken to evaluate anti-anemic potential of formulation of *Angelica sinensis* in ovalbumin induced anemia of inflammation in Balb/c mice and evaluation of herbomineral formulation in agar gel diet and phlebotomy induced iron deficiency anemia in Wistar rats.

Materials and Methods:

Formulation of *A. sinensis* and herbomineral formulation were subjected to pharmacognostic studies which involved organoleptic, physicochemical and preliminary phytochemical evaluations. Asthma was induced by intraperitonial injection of 50 mg ovalbumin in 4 mg aluminum hydroxide in 0.2 ml saline. On day 1^{st} and 14^{th} ovalbumin was given by intraperitonial injection. Control animals were induced with equal volume of vehicle. Treatment of *A. sinensis* 100 mg/kg, folic acid 2 mg /kg was given from day 1 to 35 days. Weakly food, water and body weight gain was observed. On day 28, 29 and 30 and 35 animals were exposured to 1% Ovalbumin in PBS for 20 minutes each day. 24 hours after last exposure blood was collected and haematological and biochemical parameter were determined like blood cell count, hematocrit, haemoglobin, reticulocyte, ferritin, total serum iron, iron binding capacity, transferin saturation.

Iron deficiency anemia was induced by agar gel diet for 30 days. On 10^{th},20^{th},28^{th}, 30 days phlebotomy was performed by snipping the tail vein of each rat. Treatment of herbomineral formulation (100 mg/kg) standard folic acid 2 mg /kg was given from day 1 to day 30. After 30 days blood was collected and haematological and biochemical parameter were determined like blood cell count, hematocrit, haemoglobin, reticulocyte, ferritin, total serum iron, iron binding capacity, transferin saturation.

Results:

Ovalbumin produced inflammation which cause reduction of hemoglobin, red blood cell count, hematocrit, reticulocyte and low serum iron, decreased total iron-binding capacity, decreased transferrin saturation, elevated serum ferritin. Treatment with formulation of *A. Sinensis* significantly ($P<0.05$) prevented ovalbumin induced reduction in hemoglobin, red blood cell count, hematocrit, reticulocyte. The formulation also significantly ($P<0.05$) reduced the elevated serum ferritin levels in inflammation induced anemic mice and elevated the serum iron, total iron-binding capacity, and transferrin saturation levels.

Agar gel diet and phlebotomy which cause reduction of hemoglobin, red blood cell count, hematocrit, reticulocyte and low serum iron, increased total iron-binding capacity, decreased transferrin saturation. Treatment with herbomineral formulation significantly increased ($P<0.05$) agar gel diet and phlebotomy induced reduction in hemoglobin, red blood cell count, hematocrit and reticulocyte levels. Herbomineral formulation also significantly ($P<0.05$) reduced the elevated total iron binding capacity in agar gel diet and phlebotomy induced anemic rats and elevated the serum iron and transferrin saturation levels.

Conclusion:

Formulation of *A. sinensis* showed beneficial effect on anemia associated with chronic inflammatory disease such as allergic asthma. The formulation showed modulation in haematological and biochemical parameters indicates that *A. sinensis* may produce its effect by acting on hepcidin pathway by increasing iron absorption from microphage and acting on inflammatory cytokines pathway. The herbomineral formulation also showed modulation in haematological and biochemical parameters in animal model of iron deficiency anemia, indicates that it may be beneficial in preventing iron deficient disease by releasing iron storage. From above experiments we can conclude that these formulations from herbal origin are efficient for the management of anemia with and without inflammation.

Introduction

Anemia is a reduction in the red blood cell mass with the hematocrit value less than 40% (37% in women), or hemoglobin below normal by more than two standard deviations. Anemia causes signs and symptoms of pallor, shortness of breath and fatigue since all result from reduced hemoglobin failing to provide sufficient oxygen (Page *et al.*, 2009).

Iron deficiency is the most prevalent nutritional deficiency and the most common cause of anemia. Iron deficiency anemia is characterized by a defect in hemoglobin synthesis, resulting in red blood cells that are abnormally small (microcytic) and contain a decreased amount of hemoglobin. The capacity of the blood to deliver oxygen to body cells and tissues is thus reduced (Provan, 1999).

Anemia of inflammation (AI), also known as anemia of chronic inflammation or anemia of chronic disease, was described over 50 years ago as a normo to microcytic anemia typically of mild severity, characterized biochemically by a low plasma iron, decreased total iron-binding capacity, decreased transferrin saturation, and on bone marrow examination, decreased sideroblasts and increased reticuloendothelial iron. Despite the heterogeneity of underlying infectious, malignant and inflammatory diseases described in conjunction with this type of anemia, there was a remarkable degree of similarity in terms of the severity of the anemia, correlating directly with the degree of inflammation present, and it, features which remain characteristic of AI today (Barry, 2010).

The World Health Organization (WHO) definition of anemia (hemoglobin concentration <12 g/dL in women and <13 g/dL in men) is most often used in epidemiologic studies. More than 10% of adults of 65 years age and older has WHO-defined anemia. After age 50 years, prevalence of anemia increases with advancing age and exceeds 20% in those 85 years and older. Among older adults with anemia, approximately one-third have evidence of iron, folate, and or vitamin B12 deficiency, another third have renal insufficiency and/or chronic inflammation, and the remaining third have anemia that is unexplained (Patel, 2008). Approximately 50% of the anemia is attributed to iron deficiency (Stoltzfus, 2003; Stoltzfus *et al*., 2007) while chronic inflammatory diseases

such as rheumatoid arthritis, inflammatory bowel disease, bronchial asthma and chronic kidney disease are the second leading cause of anemia worldwide.

Iron homeostasis is controlled by a regulatory network involving four main components: bone marrow erythropoiesis, tissue oxygen delivery, iron stores and inflammation (Bergamaschi and Vilani 2009). Iron overload (after a meal or an iron supplement) and an inflammation are the major cause of anemia leads to increased production of hepcidin, a liver-derived peptide regulator of iron homeostasis (Elizabeta and Ganz, 2006). Over the past two years, a variety of experiments have converged to establish a role for hepcidin, in iron homeostasis (Campbell *et al*., 2001; Hu Y., *et al* 2002). Hepcidin causes a decrease in serum iron (hypoferremia). It blocks iron flow from macrophages recycling iron, from iron stores in the liver and from enterocytes absorbing dietary iron. These different tasks are accomplished through a unique biochemical mechanism: the interaction of hepcidin with ferroportin. Hepcidin binds to ferroportin and causes its internalization and degradation in lysosomes, thus effectively blocking the export of iron from the cells. Due to this reason hepcidin represents a negative regulator of intestinal iron absorption and macrophage iron release (Bergamaschi and Vilani 2009). Expression of hepcidin increased by inflammation is mediated by inflammatory cytokines like interleukin-6 (IL-6) (Elizabeta and Ganz, 2006; Cartwright and Wintrobe, 1952). The clinical symptoms of anemia often do not stand in the foreground in patients with chronic inflammatory disease (Silverberg, 2001). Iron supplements and vitamins are generally not effective in anemia of chronic inflammation. For the unusual severe case of anemia of chronic inflammation, blood transfusions may be helpful. Additionally, drugs that help stimulate growth of new red blood cells, like erythropoietin, may be given temporarily but all these drugs do have risks that are important to consider before treatment (Kaltwasser, 2001). The treatment of chronic inflammatory diseases demands a long-term and systematic approach. Therefore, there is an urgent need to identify new types of pharmacological agents that can treat anemia of chronic inflammation with greater efficacy and fewer side effects. Management of expression of hepcidin is therefore can be investigated as a potential therapeutic procedure in patients with anemia of chronic inflammation.

WHO has approved the use of traditional medicines as a part of health programme. To pursue research in these systems of medicine, several USA agencies and institutions such as FDA and National Institute of Health have setup separate wings. According to the WHO survey 80% of the populations living in the developing countries rely almost exclusively on traditional medicine for the primary health care needs. In almost all the traditional form of medicine, the medicinal plants play a major role and constitute the backbone of the traditional medicine.

India has an ancient heritage of traditional medicine. Materia medica of India provides lots of information on the folklore practices and traditional aspects of therapeutically important natural products. Indian traditional medicine is based on various system including Ayurveda, Siddha and Unani. Also China and UK have got their own traditional system of medicine. These traditional systems have their individual uniqueness but there is a common thread running through these systems in their fundamental principles and practices. With the emerging interest in the world to adopt and study the traditional system and to exploit their potentials based on different healthcare systems, the evaluation of the rich heritage of the traditional medicine is essential. In the light of the above facts, present study was carried out for pharmacological evaluation of marketed formulation of *A. sinensis* and herbomineral formulation for the treatment of anemia.

Numerous scientific studies have reported several herbal drugs or plant extracts have their antianemic activity. University of Maryland medical center have reported use of siruline and stinging nettle in experimental animal models. They also reported effect of Alfalfa, raspberry leaf in animal models of anemia in rats.

A. sinensis, belongs to family Apiaceae is well known in the literature as dong quai, danggui, tang-kuei, Chinese angelica etc. Traditionally *A. sinensis* has been employed as a remedy for wide spectrum of diseases. It has been proved to have anti-inflammatory, anti-cancer, anti-cardiovascula, Immunomodulatory, Neuroprotective, anti-oxidant, anti-hepatoprotective effects etc. (Chao *et al*., 2011).

The chemical constituents present in Angelica roots include ferulic acid, Z-ligustilide, butylidenephthalide and various polysaccharides. Among these compounds, ferulic acid reported to exhibits many bioactivities especially anti-inflammatory and immunostimulatory effects (Chao *et al*.,2011). Z-ligustilide reported to have anti-inflammatory, anti-cancer, neuroprotective and hepatoprotective effects and n-butylidenephthalide exerts anti-inflammatory, anti-cancer and cadioprotective effects (Chao *et al*., 2011). Plant flavonoids like quarcetin, catechin epigenin have shown anti-inflammatory activity *in-vivo* and *in-vitro* (Kim, 2004). It has been well documented that inflammation plays a crucial part in pathogenesis of anemia of inflammation (Andrews, 2004). In the previously carried out research work, *A. sinensis* has been evaluated in iron deficiency anemia. It is reported that *A. sinensis* have anti-inflammatory activity by inhibition of and suppression of TNF-α, IL-6 and IL-8 (Andrews., 2004). Therefore, within the context of modern scientific framework, the present study was undertaken to substantiate claims made of this plant in traditional system of medicine especially for its antianemic role in experimental animal model of inflammation.

The science and practice of Ayurveda has been in India for many centuries now (Gogtay *et al*., 2002). More than 80% of the worlds' population is estimated to use herbal medicines out of which many of them are in the developing countries (Kamboj *et al*., 2000). There are various formulations available in Ayurveda, one of which is the Bhasma -meaning "ash". Bhasma, as described in the Ayurvedic literature, is a metallic preparation with organic macromolecules used in the treatment of various human disorders (Steiner *et al*., 1986).

The empirical use of different herbomineral preparations of iron in the treatment of anemia dates from ancient times. The calcined iron preparation of *Ayurveda*, the ancient Indian traditional medicine, is known as '*Louha Bhasma*'. The preparation of *Louha Bhasma* was followed according to a unique method described by the ancient Indian chemist Nagarjuna around 100 A.D. (Pandit *et al*., 1999). Herbomineral formulation contains Dhatri Loha, Kasis Bhasma, Mandur Bhasma, Yashad Bhasma, *Boerhaavia diffusa*, and *withania somnifera.* Bhasma, *Boerhaavia*, and *withania somnifera* present in herbomineral formulation also reported to possess anti anemic activity (Santosha *et al*., 2011; Lokhande *et al*.; 2010). Therefore, within the context of modern scientific

framework, the present study was undertaken to substantiate claims made of this formulation composition in traditional system of medicine especially for its anti anemic role in experimental iron deficiency anemia.

OBJECTIVES OF THE STUDY:

1. Pharmacognostic evaluation of formulation of root powder of *A. sinensis.*
2. Pharmacognostic evaluation of herbomineral formulation.
3. To study pharmacological effect of formulation of *A. sinensis* on animal *model* of anemia of chronic disease.
4. To study pharmacological effect of herbomineral formulation on animal model of iron deficiency anemia.

3. Review of Literature

3.1 Introduction to anemia

Anemia is the term given to the reduction in the number of erythrocytes, in the concentration of hemoglobin and/or in the hematocrit as long as the total blood volume is normal. Shortly after acute major blood loss, in dehydration, or in hyperhydration the blood volume must first be normalized before anemia can be diagnosed. Using the erythrocyte parameters mean *corpuscular* volume (MCV) and mean corpuscular hemoglobin (MCH) anemias can be classified according to cell volume (MCV: microcytic, normocytic, or macrocytic) and according to the ratio of Hb concentration/erythrocyte count (MCH: hypochromic, normochromic, or hyperchromic). Pathogenetic division of the anemias reflects the individual steps of erythropoiesis as well as the life span of the erythrocytes circulating in blood .Acute or chronic blood loss can also lead to anemia (Silbernagal *et al*., 2000).

3.2 Epidemiology

Anemia is a common, multifactorial condition among older adults. The World Health Organization (WHO) definition of anemia (hemoglobin concentration <12 g/dL in women and <13 g/dL in men) is most often used in epidemiologic studies of older adults. More than 10% of community dwelling adults age 65 years and older has WHO defined anemia. After age of 50 years, prevalence of anemia increases with advancing age and exceeds 20% in those 85 years and older. In nursing homes, anemia is present in 48–63% of residents. Incidence of anemia in older adults is not well characterized. Among older adults with anemia, approximately one third have evidence of iron, folate, and/or vitamin B12 deficiency, another third have renal insufficiency and/or chronic inflammation, and the remaining third have anemia that is unexplained. Several studies demonstrate that anemia is associated with poorer survival in older adults (Patel., 2008). Survey data covered 48.8 % of the global population, 76.1 % of preschool aged children, 69.0 % of pregnant women and 73.5 % of non pregnant women. The estimated global anemia prevalence is 24.8 % (95 % CI 22.9, 26.7 %), affecting 1.62 billion people (95 % CI 1.50, 1.74 billion). Estimated anemia prevalence is 47.4 % (95 % CI 45.7, 49.1 %) in preschool aged children, 41.8 % (95 % CI 39.9, 43.8 %) in pregnant women and 30.2 % (95 % CI 28.7, 31.6 %) in

non pregnant women. In numbers, 293 million (95 % CI 282, 303 million) preschool aged children, 56 million (95 % CI 54, 59 million) pregnant women and 468 million (95 % CI 446, 491 million) non pregnant women are affected (MacLean *et al*., 2009).

3.3 Risk Factors

Although nutritional iron deficiency anemia has declined in industrialized nations, it affects an estimated 2 billion people worldwide. In the U.S., iron deficiency is the most prevalent nutritional deficiency. It is highly associated with poverty. People in lower socioeconomic groups have double the risk of those who are middle or upper class. Among Americans with iron deficiency anemia, young children have the highest risk followed by premenopausal women. Adolescent and adult men and postmenopausal women have the lowest risk. Men, in fact, are at risk for iron overload, probably because of their higher meat intake and their reduced iron loss.

3.3.1 Infants and Children

General Risk Factors for Anemia in Infants and Children- Up to 20% of American children and 80% of children in developing countries become anemic at some point during their childhood and adolescence. Iron deficiency is the most common cause in children, but other forms of anemia, including hereditary blood disorders, can also cause anemia in this population. Hispanic American children have double the rates of iron deficiency as African-American and Caucasian children. Iron deficiency affects about 9% of children younger than 2 years. About 3% of children in this age group are anemic as a result. Children in lower-income homes are at higher risk than those in higher income homes. However, children in any income group can develop iron deficiency.Young children 9 - 18 months have the highest risk for iron deficiency anemia in the U.S. Such children also are at great risk for problems in mental development from anemia. Infant boys may have 10 times more risk than baby girls. In general, full-term, breast-fed infants have enough iron stores for their first 6 months of life (Gosz *et al*, 2009).

3.3.2 Premenopausal Women

Up to 10% or more of adolescent and adult women under 49 years are iron deficient. Hispanic American and African-American women have double the prevalence for anemia compared to Caucasian women. The risk for anemia in adolescent girls is about 3%. Anemia is generally mild in young women, however, and is more likely to occur with one or more of the following conditions: Heavy menstruation for longer than 5 days Abnormal uterine bleeding, such as from fibroids pregnancy. About 20% of women in industrialized countries have iron deficiency during pregnancy. Multiple pregnancies and births significantly increase the risk (Cuervo., 2007).

3.3.3 Older Adults

About 10% of adults age 65 years and older have anemia. For patients in nursing homes, about 50% are anemic. Causes of anemia in older adults include nutritional deficiencies, chronic inflammatory disease, and chronic renal disease.

3.3.4 Iron Poor Diet

Vegetarians who avoid all animal products may have a slightly higher risk for deficiencies in iron and vitamins B. Although dried beans and green vegetables often contain iron, it is less easily absorbed from plants than from meat. Fortunately, most commercial cereals are fortified with vitamin B12 and folic acid (the synthetic form of folate) (Horne *et al*, 2009).

3.3.5 Chronic or Critical Illnesses

A chronic disease that causes inflammation or bleeding is at risk for anemia. Critical illness in the intensive care unit is also highly associated with anemia (Doqai, 2007).

3.3.6 Excessive Exercise

Working out regularly may cause some iron loss, which is comparable to that from menstruation and rarely worrisome. Dietary choices may account for most cases of sports anemia. Sustained exercise, such as that performed by marathon runners, may cause a condition called sports anemia, which may be due to slight gastrointestinal bleeding, damaged red blood cells, low iron intake (Horne *et al*., 2009).

3.3.7 Pregnancy

Iron deficiency occurs in 20% of pregnant women in developed countries. Even worse, 50% or more of women in nonindustrialized nations become iron deficient, and 30 - 50% are deficient in folic acid. Severe anemia is associated with a higher mortality rate among pregnant women. Mild-to moderate anemia, however, does not pose any elevated risk. Pregnancy increases the risk for anemia in different ways: It increases the body's demand for folic acid and, therefore, poses a risk for deficiencies and an increased risk for megaloblastic anemia. Low levels of folate during pregnancy increase the risk of neural tube defects in newborns. It increases the body's demand for iron, thus posing a risk for iron deficiency anemia. Pregnant or nursing women need 30 mg of iron per day. Maternal iron deficiency anemia is associated with increased weight or size of the placenta, a condition that may later pose a risk for high blood pressure in the offspring. Pregnant women with low hemoglobin levels (the iron bearing component in the blood) have an elevated risk for pre term or low birth weight infants. Pregnancy is also associated with fluid retention, which in turn may produce high volumes of plasma (the fluid component of blood). This can dilute red blood cells, which may lead to anemia. During delivery, heavy bleeding or multiple births can cause postpartum anemia, which occurs in about 10% of women. Postpartum anemia can last 6-12 months after giving birth (Gynecol., 2008).

3.4 Etiologies

3.4.1 Acute Blood Loss

Anemia may be nonregenerative in the early phases of blood loss, before a peripheral reticulocyte response develops. Immediately following hemorrhage, the hematocrit may be normal, reflecting a loss of RBCs and plasma in equal proportions. However, a shift of water from the interstitial to the intravascular space ensues within 12 to 24 hours (more quickly with volume resuscitation). The result is a decrease in the hematocrit and the total protein level. At this point, the RBCs will likely appear normal in morphology, and anemia is normocytic and normochromic. As hypoxia stimulates the release of erythropoietin, reticulocytes are formed and released from the bone marrow, and the anemia becomes regenerative 4 to 5 days after acute blood loss.

3.4.2 Inflammatory Disease

Chronic inflammatory disease is the most common cause of anemia in veterinary patients, occurring with conditions such as infection, trauma (bony or soft tissue), immune mediated disease, and neoplasia (focal or disseminated). The resulting anemia, anemia of inflammatory disease (AID), is usually mild to moderate, normocytic, normochromic, and nonregenerative. The pathogenesis of AID is multifactorial, but a key mediator is hepcidin, a type 2 acutephase protein that is produced by the liver in response to inflammatory stimuli (Falzacappa *et al*., 2005).Specifically, interleukin-6, which is produced early during host defense, induces hepcidin synthesis (Ganz *et al*., 2006). Hepcidin inhibits iron export from duodenal enterocytes and macrophages, resulting in decreased iron absorption and the accumulation of iron in macrophages (Falzacappa *et al*., 2005). This reduces the serum iron level and results in decreased iron availability, which is thought to be a protective mechanism to deprive infectious agents of iron but can also decrease the iron available for erythropoiesis (Stone *et al* ., 1990). Hepcidin production is decreased in hypoxic or iron deficient states (Feldman *et al*., 2005).

3.4.3 Renal Disease

The association between progressive renal disease and anemia is well recognized in animals (Cowgill *et al*., 1992). There are four main components to the pathogenesis of anemia associated with renal disease. The most important is a deficiency in erythropoietin. Renal disease inhibits the ability of the kidneys to increase erythropoietin production in response to hypoxia. Thus, patients with renal disease have decreased erythropoietin levels relative to their degree of anemia. The second component involves the suppressive effects of uremic toxins on the bone marrow. These effects appear to be confined to the erythroid cell line, as leukocytes and platelets do not seem to be similarly suppressed gastrointestinal (Remuzzi *et al*., 1991). The third component, blood loss, is often overlooked as a cause of anemia in patients with renal disease. Bleeding can occur chronically from the GI tract, skin, and other sites due to qualitative platelet dysfunction induced by uremic toxins and to GI ulcers caused by the effects of uremia on the GI mucosa. The fourth potential component is shortened RBC survival due to mild hemolysis. Renal disease most often causes a normocytic, normochromic, nonregenerative anemia. Initially, the

anemia is often mild, but as renal function declines and the hematocrit drops, it can become severe enough to necessitate blood transfusion. Bone marrow cytology may reveal erythroid hypoplasia with an increased myeloid:erythroid ratio (Eschbach *et al*., 1989).

3.4.4 Feline Leukemia Virus(FeLV)

FeLV can induce anemia by causing bone marrow suppression, myelodysplastic syndromes, neoplasia (lymphoma or leukemia), or a secondary immune-mediated hemolytic anemia (IMHA). There are three FeLV subgroups (A, B, C); infection with subgroup C is most often associated with nonregenerative anemia secondary to bone marrow suppression (Abkowitz *et al* ., 1987). The virus is believed to affect RBC precursors near the stem cell level, as suppression of other cell lines can also be seen with this disease. Erythropoietin levels can be increased in anemic with FeLV, but ferrokinetic studies and bone marrow cultures have revealed decreased erythropoiesis and an impaired response of the bone marrow to anemia. All strains of FeLV undergo cell-associated viral replication and can stimulate IMHA. In a survey of 21 cases of feline IMHA, 52% were positive for FeLV. A condition known as *panleukopenia-like syndrome* can occur with FeLV infection and is characterized by leukopenia, anemia, thrombocytopenia, and enteritis with destruction of intestinal crypt epithelium. The anemia seen with FeLV infection is most often mild to moderate and normocytic−normochromic or macrocytic−normochromic, with reticulocytopenia. The macrocytosis is thought to result from skipped mitoses during erythropoiesis. 21 Megaloblastic anemia has been associated with FeLV infection and carries a poor prognosis (Hirsch *et al*., 1982).

3.4.5 Immune Mediated Hemolytic Anemia(IMHA)

IMHA occurs in cats less frequently than in dogs. It can be primary (idiopathic) or secondary to infectious diseases such as mycoplasmosis or FeLV, toxins, medications, neoplasia, and systemic lupus erythematosus (Werner *et al*., 1984). Hemolysis in cats with IMHA is complement mediated and extravascular; no cases of intravascular hemolysis have been reported in feline patients. Approximately 50% of feline IMHA patients have nonregenerative anemia (Kohn *et al*., 2006).

3.4.6 Pure Red Cell Aplasia

Pure red cell aplasia is a rare syndrome that is characterized by severe nonregenerative anemia with a lack of RBC precursors in the bone marrow, despite normal leukocyte and platelet counts. Pure red cell aplasia can be primary (idiopathic) or secondary to FeLV infection, most likely with subgroup C, which can impair maturation of erythroid progenitors. Primary pure red cell aplasia has been reported with severe normocytic, normochromic to hypochromic anemia and hematocrits ranging from 6% to 15%. The leukocyte and platelet counts of these patients were within the normal reference range. Bone marrow aspiration revealed normocellular to hypocellular marrow in most cases, and small, mature lymphocytes were found to account for 12% to 45% of the total marrow cells (Stokol *et al*., 1999).

3.4.7 Myeloproliferative Syndromes

Myeloproliferative disorders are interrelated dysplastic and neoplastic conditions that originate from clonal transformation of nonlymphoid stem cells and their progeny (Raskin *et al*., 1996). This group of disorders includes myelodysplastic syndrome (MDS), acute and chronic myeloid leukemias, and acute undifferentiated leukemia. A discussion of leukemia is beyond the scope of this article. *Dysmyelopoiesis* is a general term referring to bone marrow disorders that originate in the hematopoietic stem cells and result in a reduction of one or more types of circulating blood cells. The anemia is most often nonregenerative and can be macrocytic and normochromic as well. MDS is considered preleukemic because it can progress to acute myelogenous leukemia. However, due to severe cytopenia,MDS is often lethal without progression to leukemia (Weiss *et al*., 2005).

3.4.8 Mycoplasma Infection

Hemobartonella spp have been reclassified as hemotrophic mycoplasmas (hemoplasmas) based on phylogenetic evidence and 16S ribosomal RNA gene sequences (Messick *et al*., 2004). Theseorganisms do not infect other species. *M. haemofelis* and "*Candidatus* Mycoplasma haemominutum" are gram-negative,

epicellular RBC parasites. They may be rod shaped, ringshaped, or spherical and may exist individually or in chains . The anemia associated with *Mycoplasma* infection occurs via direct RBC damage and (more importantly) immunemediated RBC injury, as suggested by positive Coombs test results. Macrophages in the spleen, liver, lungs, and bone marrow cause extravascular hemolysis. Intravascular hemolysis has been infrequently reported (Harvey *et al*., 1998). The anemia varies from mild to severe, and icterus usually is not observed. If the hematocrit drops acutely, the anemia may appear nonregenerative. In most cases, the anemia is regenerative.

3.4.9 Iron Deficiency

Iron exists in the body as hemoglobin (the most common form), myoglobin, labile iron, tissue iron, and transport iron. Each molecule of hemoglobin has four atoms of iron, representing 0.34% of its total weight. Each milliliter of RBCs contains 1.1 mg of iron (Andrews *et al*., 2000). Fleabite anemia tends to be most severe that develop iron deficiency secondary to blood loss combined with low iron stores at birth (Yaphe *et al*., 1993). Iron deficiency is uncommon in adult cats. However, conditions resulting in chronic gastrointestinal (GI) blood loss (e.g., ulceration, neoplasia, endoparasitism) can cause iron deficiency. Other possible etiologies, although rare include genitourinary disease (e.g., transitional cell carcinoma) and diseases resulting in thrombocytopenia (Smith *et al*., 1992). The anemia associated with iron deficiency can range from mild to life threatening. Early on, the anemia is often regenerative and is characterized by anisocytosis, polychromasia, and reticulocytosis. Subsequently, it can progress to become nonregenerative, characterized by a lack of reticulocytes and by microcytosis. Other laboratory findings in feline patients with iron deficiency may include poikilocytosis, leptocytosis, RBC fragmentation, and thrombocytosis (Mahaffey *et al*., 1986).

3.4.10 Nutritional Deficiency

Nutritional deficiency anemias are rarely seen in today because of the improved quality of foods and increased awareness of nutritional requirements. Such anemias are most likely to occur as a result of error (such as feeding an improperly balanced homemade diet) or a GI problem affecting nutritional absorption. A normocytic,

normochromic, nonregenerative anemia can be seen in severely malnourished due to deficiencies in protein, calories, vitamins, or minerals (Watson *et al*., 2000). Although iron deficiency is a well documented cause of nonregenerative anemia, most cases of iron deficiency anemia are not nutritional in origin (Fyfe *et al*., 1991).

3.5 Classification of anemia

Anemia is defined as a reduced concentration of haemoglobin in the blood. It may give rise to fatigue but, especially if it is chronic, is often surprisingly asymptomatic. The commonest cause is blood loss related to menstruation and child bearing, but there are several different types of anaemia and several different diagnostic levels. Determining indices of red cell size and haemoglobin content and microscopical examination of a stained blood smear of blood allow classified in to following classes (Rang *et al*., 2006)

3.5.1.Microcytic anemias (MCV < 80 fL

• Iron deficiency anemia
• Thalassemia
• Sideroblastic anemia (hereditary, lead poisoning)
• Anemia of chronic disease

3.5.2.Normocytic anemias (MCV 80 100 fL)

Increased RBC loss/detruction/sequestration (RPI > 2)

• Acute hemorrhage
• Hemolytic anemia
• Hypersplenism

3.5.3.Decreased RBC production (RPI < 2)

• Nutritional deficiencies (iron, vitamin B12, folate)
• Renal insufficiency
• Anemia of chronic disease
• Endocrine dysfunction
• Bone marrow disorders (*e.g.*, drugs, infections, aplastic anemia, myelodysplastic syndrome, multiple myeloma, and other infiltrative diseases)

3.5.4.Macrocytic anemias (MCV > 100 fL)

• Drugs (methotrexate, zidovudine, hydroxyurea)

• Megaloblastic (vitamin B12 or folate deficiency)

• Liver disease

• Alcohol excess

• Reticulocytosis

• Bone marrow disorders

MCV: Mean corpuscular volume

RPI: Reticulocyte production index

RBC: Red blood cell (Benjamin *et al*., 2004)

3.6 Introduction to anemia of inflammation

Anemia of chronic disease (ACD) occurs in patients with acute or chronic immune activation such as infection, cancer, autoimmune disease, or chronic kidney disease. ACD is characterized by normocytic or microcytic iron deficiency anemia, low serum iron, and preserved marrow iron. Although the underlying mechanisms of ACD are not completely understood, there is broad consensus that pro inflammatory cytokines have an important role in this syndrome. Accordingly, ACD is also known as “anemia of inflammation”. In patients with ACD, pro inflammatory cytokines and cells of the reticuloendothelial system (RES), including monocytes and macrophages, induce characteristic alterations in iron metabolism and erythropoiesis. The pathologic changes leading to ACD are mediated by several interrelated factors, including interleukin-6 (IL- 6), hepcidin and hypoferremia (Dominic., 2009).

3.7 Pathophysiology of anemia of inflammation

From our current view anemia of inflammation is primarily an immunity driven disease. Various pathways involved in erythropoiesis are affected to a certain extend by immune effector molecules and thus contribute to the pathogenesis of anemia of inflammation.

3.7.1 Disturbances of iron homeostasis

The cause–effect relationship between immunity and iron homeostasis has been nicely demonstrated by experiments showing that application of TNFα and IL-1 to mice resulted in both, hypoferraemia in serum and induction of ferritin synthesis with the reticuloendothelial system, a condition which is also seen in ACD (Hernandez *et al*., 1989). Thus, TNFα and IL-1

induce ferritin synthesis, iron acquisition and iron storage by the reticuloendothelial system which limits the availability of the metal to the erythron and results in development of ACD (Konjin *et al*., 1981). The biological effect of TNFα and IL-1 has been referred to stimulation of ferritin synthesis in macrophages and the liver by the cytokines via transcriptional primarily of H-ferritin gene expression (Torti *et al*.,1988). Moreover, IL-1 and IL-6 also modulate ferritin translation which has been referred to the presence of a target region within the 50 untranslated region of ferritin mRNA, termed as acute phase box (Rogers *et al*., 1990). At the same time these cytokines down-regulate TfR mRNA expression by as yet uncharacterized mechanisms. This leads to the question of how cells stimulated with these pro inflammatory cytokines then acquire iron for incorporation into ferritin. One very likely explanation is that these cytokines may stimulate the expression and/or transport capacity of the divalent metal transported-1 (DMT-1) which has been first characterized in duodenum as the transmembrane protein being able to take up ferrous iron from the luminal side of the duodenum by a proton coupled process (Gunshin *et al*., 1997). DMT- 1 has been detected in almost every tissue and recent data also provided evidence that its expression is up regulated by cytokines (Wardrop *et al*.,2000). It stimulates ferritin transcription but at the same time it inhibits its translation. The latter can be referred to the induction of NO and presumably also of radical synthesis by the cytokine, which then activates IRP-1 binding affinity thus inhibiting IRE-dependent ferritin translation. Moreover, like pro-inflammatory cytokines IFNγ inhibit TfR mRNA expression by an IRE/IRP-independent process (Byrd *et al*., 1993). Although, IFNγ blocks TfR-mediated iron uptake it now becomes evident that this cytokine may increase uptake and retention of molecular/ferrous iron in cells. This is achieved by up regulation of DMT-1 expression with consecutive stimulation of ferrous iron influx into human monocytes. Moreover, IFNγ down regulates the mRNA and protein expression of another transmembrane protein known as IREG-1 or ferroportin (Aigner *et al*., 2001). This transporter is responsible for the transfer of iron from the enterocyte to the basolateral site of the duodenum where iron is then transferred to transferrin after being oxidized by a membrane bound ferroxidase, called hephastein (Donovan *et al*., 2000). Ferroportin may also be responsible for the export of iron from cells. The reduction in ferroportin expression by may result in retaining of iron within monocytes which will then be stored within ferritin. Moreover, anti inflammatory cytokines are also able to modulate iron homeostasis in activated macrophages. This is primarily achieved by counteracting the effects of IFNγ towards ferritin and TfR expression. In activated murine macrophages, IL-4, IL-10 and IL-13 inhibit NO formation and thus increase ferritin translation

by opposing IFNγ /NO-mediated activation of IRP-1. Moreover, TfR mRNA levels are increased in the presence of these anti-inflammatory cytokines which is most likely due to reversing the inhibitory effect of IFNγ on TfR mRNA expression (Weiss *et al*., 1997). Thus, Th-2-derived cytokines are able to increase TfR-mediated iron uptake and storage in activated macrophages. The in vivo relevance of these effects has recently been shown by a study in Mb. Crohn patients received therapy with human recombinant IL-10 as part of a placebo controlled study. Patients receiving the highest dose levels of IL-10 developed a normocytic anaemia which was preceded by a significant increase in serum ferritin levels while reticulocyte counts were not affected as compared to placebo-treated controls (Tilg *et al*.,). Thus both Th-1- and Th-2-derived cytokines, participate in the induction of hypoferraemia/hyperferritinaemia during chronic inflammatory processes(Feelders *et al*., 2002). This is achieved by divergent and subtle effects on uptake of transferrin-bound iron via modulation of TfR expression and on acquisition of ferrous iron via regulation of DMT-1 formation, on iron retention via reduction of ferroportin expression and iron export and towards iron storage via induction of ferritin synthesis. Moreover, acute phase proteins such as 1-antitrypsin and 2 macroglobin contribute to the diversion of iron traffic in inhibiting TfR-mediated iron uptake into erythroid progenitor cells (Graziadei *et al*., 1994).

3.7.2 Inhibition of erythroid progenitor proliferation and differentiation

Cytokines directly affect erythropoiesis by inhibiting the growth of erythroid progenitor cells. Mainly proinflammatory immune modulators, such as TNFα, IFNγ and Type I Interferons, block BFU-e and CFU-e colony formation. However, IFNγ appears to be the most potent inhibitor of erythropoiesis in directly blocking CFU-e proliferation and this notion is also reflected by an inverse correlation between IFNγ levels with haemoglobin concentrations and reticulocyte counts (Fuchs *et al*., 9191). It was suggested that cytokines may interact with the formation and/or function of central growth factors for erythropoiesis, such as stem cell factor or erythropoietin, or that they may induce apoptosis/growth arrest in stem cells. It will thus also be interesting to see if part of the inhibitory effects of TNFα or IFNγ towards erythropoiesis can be referred to their ability to induce the formation of NO or toxic oxygen radicals. NO can directly block erythropoiesis by inhibiting the proliferation of erythroid progenitor cells in the bone marrow (Maciejewski *et al*., 1995). This is due to an inhibitory effect of NO on haem biosynthesis. Thereby, NO blocks erythroid aminolevulinate synthase mRNA translation by a

mechanism involving activation of IRPs but also inhibits the final step of the haem biosynthetic pathway in reducing ferrochelatase activity by targeting its central iron–sulphur cluster (Furukawa *et al*., 1995). Moreover, the diversion of iron traffic which subsequently limited availability of iron to erythroid progenitor cells is also an important factor contributing to the impaired proliferation of erythroid cells at least by impairing haem biosynthesis. This can be monitored by increased Zn-protoporphyrin IX levels in serum of ACD patients indicating limited iron availability to erythroid progenitor cells. Thus an insufficient amount of circulating levels of erythropoeitin may also play an important role.

3.7.3 Bone marrow infiltration

Suppression of bone marrow infiltration can be exerted also directly by invading tumour cells or microorganisms and also by toxic products derived from them as shown for HIV or malaria plasmodia (Yap *et al*., 1994). Finally, antiproliferative effects towards erythropoiesis have also been described for ferritin. This may be due to limitation of iron availability to erythroid progenitor cells by the protein.

3.7.4 Blunted erythropoietin response

Although erythropoietin (Epo) levels in patients with ACD are mostly increased as compared to healthy subjects, however, it is assumed that Epo levels are too low for the degree of anaemia. This notion refers to comparison of erythropoietin levels between subjects with iron deficient anaemia and ACD, showing that erythropoietin levels are significantly higher in iron deficient anaemic patients(Miller *et al*., 1990). However, at least in subjects with autoimmune disorders such as juvenile chronic arthritis but also in children with cancer, this is not the case (Corazza *et al*., 1998).This implies that Epo levels may vary with the disease underlying ACD and may further depend on other factors, such as bone marrow infiltration, iron availability or proliferative capacity of erythroid cells. Moreover, Epo responsiveness may also be related to the severity of the disease and the amount of circulating cytokines. This is also indicative from in-vitro data demonstrating that in the presence of high concentrations of IFNγ or TNFα much higher amounts of erythropoietin are needed to restore CFU-E colony formation (Means *et al*., 1991). Finally, the response to Epo is largely dependent on the presence of a sufficient amount of iron for cell proliferation and haemoglobin synthesis, a condition which is violated in ACD. (Rodriguez *et al*., 2001).

3.7.5 Erythrocyte life cycle

There is indication but insufficient evidence in humans as to whether cytokines may impair erythrocyte survival and whether or not this may stimulate erythrophagocytosis by macrophages (Karle *et al*.,1974). One single report showed that administration of sublethal doses of TNFα or endotoxin to mice reduced red blood cell (RBC) half life, decreased the incorporation of iron into RBC, induced hypoferraemia and thus caused anaemia (Moldawer *et al*., 1989). The amount of circulating erythrocytes may also be affected by erythrophagocytosis, a physiological pathway designed to re. move senescent or damaged RBC, the latter possibly being due to effects exerted by cytokines, endotoxin and subsequent radical formation. Erythrocytes are taken up by phagocytosis and are then destroyed within monocytes/macrophages. Within the macrophage iron is then released from haemoglobin and rapidly shifted to re utilization via incorporation into iron proteins. Moreover, a high proportion of erythrocyte iron is released by macrophages in different forms, namely as haemoglobin, ferritin or low molecular weight iron which then binds to plasma transferring (Custer *et al*.,1982). However, since many of such patients develop splenomegaly an increase in erythrophagocytosis and shortening of erythrocyte half life would not be unlikely. This is compatible with the observation of increased amounts of erythrocyte derived iron in splenic macrophages andKupffer cells under inflammatory conditions. Haemolysis is quite an unusual event in the course of ACD but may contribute to shortening of erythrocyte survival in certain chronic infections such as subacute endocarditis or tuberculosis (Fillet *et al*., 1989).

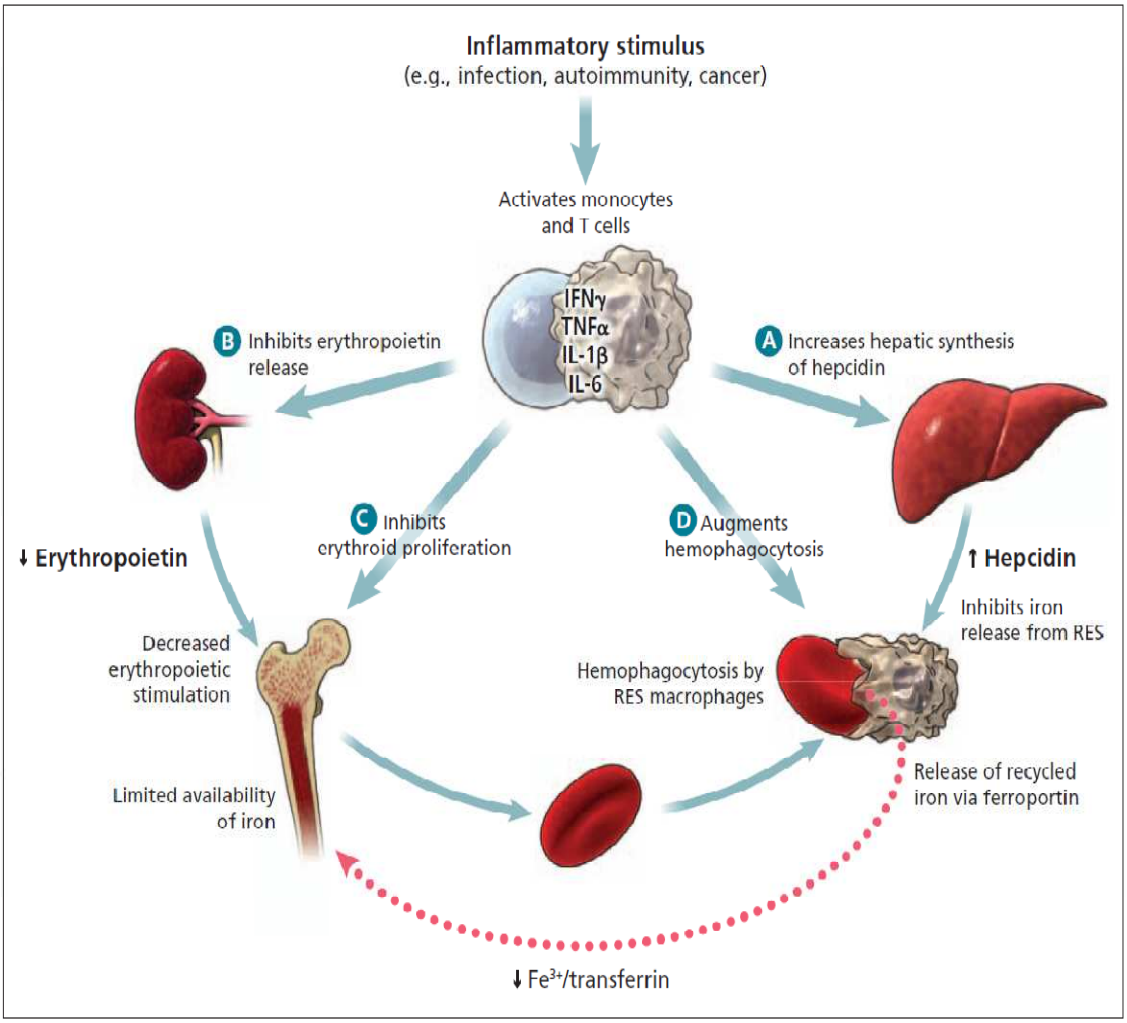

Fig 3.1 Pathophysiology of anemia of inflammation (Ryan *et al*., 2008)

3.8 Iron Deficiency Anemia

Iron (Fe) content in the body (2 g in females, 5 g in males) ca. 2⁄3 is bound to *hemoglobin* (Hb), 1⁄4 is *stored iron* (ferritin, hemosiderin), the rest is *iron with diverse functions* (myoglobin, Fe-containing enzymes). Loss of iron is 1mg/d in males and up to 2 mg/d in females (menstruation, pregnancy, birth). Iron taken up in food, 3–15% is absorbed in the duodenum in cases of Fe deficiency it can be up to 25% . Iron intake with food should therefore be at least 10–20mg/d (women > children > men). Fe can be absorbed relatively efficiently as heme ferrous (found in meat and fish). The Fe (split off from heme) gets into the blood or remains in the mucosa as ferritin-F and returns to the lumen on mucosal cell disintegration. Non heme Fe can be absorbed only in the form of Fe2+, which is absorbed by a Fe2+-H+-symport carrier (DCT1) (in competition with Mn, Co, Cd, etc.). A *low pH of the chyme* is essential for absorption, because it will increase the H+ gradient that drives Fe2+ into the cell via DCT1

release Fe from compounds in food. Non-heme Fe3+ in food must be reduced by *ferrireductase* (+ ascorbate) to Fe2+ on the surface of the luminal mucosa FR). Fe uptake by blood is *regulated by the intestinal mucosa*: in Fe deficiency mucosal ferritin translation is inhibited by binding the Fe-regulating protein IRP1 to ferritin-mRNA, so that more of the absorbed Fe2+ can reach the blood. There it is oxidized by ceruloplasmin (+copper) to Fe3+ and bound to *apotransferrin*, which transports Fe in plasma. Transferrin (= apotransferrin with 2 Fe3+) is taken up, via *transferrin receptors*, endocytotically in erythroblasts and in hepatic, placental, and other cells. After Fe has transferred to the target cells, apotransferrin again becomes available for Fe absorption from the intestine and macrophages Iron storage . *Ferritin* (in the intestinal mucosa, liver, bone marrow, erythrocytes, and plasma), which has a "pocket" for 4500 Fe3+ ions, is a rapidly available iron reserve (600mg), while Fe from *hemosiderin* is more difficult to mobilize (250 mg Fe in macrophages from liver and bone marrow). Hb-Fe and heme-Fe, released from malformed erythroblasts (so-called *inefficient erythropoiesis*) and hemolyzed erythroblasts, is bound to *haptoglobin* and *hemopexin* respectively, and taken up by the macrophages in bone marrow or by liver and spleen by endocytosis, 97% being reused. Iron deficiency (serum Fe < 0.4 mg/L; serum ferritin ") inhibits Hb synthesis so that hypochromic microcytic anemia develops: MCH < 26 pg, MCV < 70 fL, Hb < 110 g/L. *Blood loss* (gastrointestinal tract, increased menstrual bleeding) in adults is the *most common cause* of iron deficiency (0.5 mg Fe lost with each mL of blood). *Fe recycling is decreased*; this form of anemia (the second most common worldwide) occurs with *chronic infections*. In this situation the Fe regained by the macrophages is no longer adequately released and thus cannot be reused. *Fe uptake is too low* in malnutrition, especially in the developing countries. Achlorhydria atrophic gastritis, after gastrectomy; *malabsorption* in diseases of the upper small intestine or in the presence of Fe-binding food components (phytate in cereals and vegetables; tannic acid in tea, oxalates, etc.) *There is increased Fe requirement* during growth, pregnancy, breast-feeding and in apotransferrin defect. If Fe overloading occurs in the body, damage is caused mainly to the liver, pancreas and myocardium (hemochromatosis) (Silbernagal *et al*., 2000).

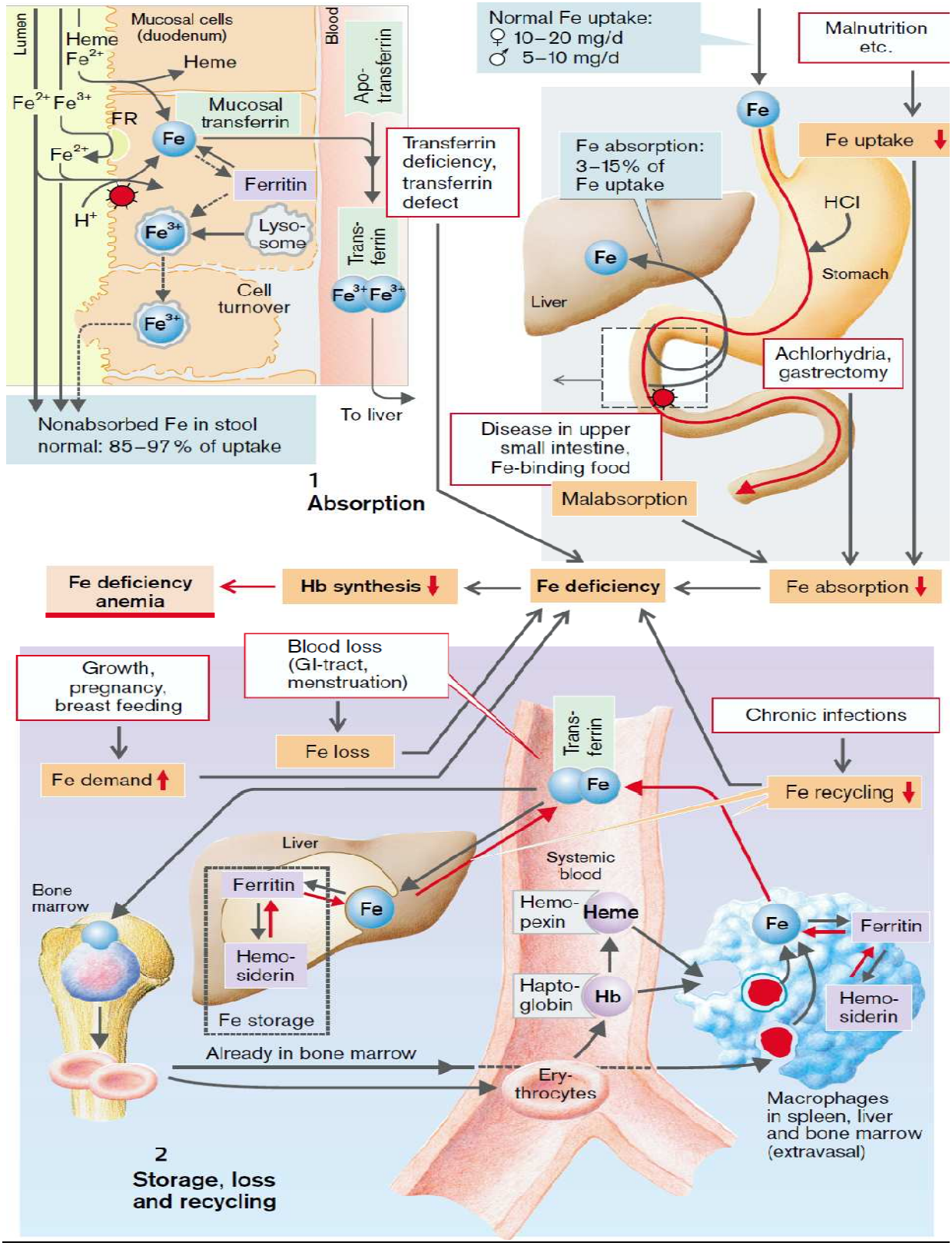

Fig. 3.2 iron deficiency anemia (Silbernagal *et al*., 2000)

3.9 Formulation profile

A. sinensis (dong quai , danggui , tang-kuei , Chinese angelica)

Fig. 3.3 *Radix A. sinensis* (Sun, 2005)

3.9.1 Introduction

In ayurvedic system of medicine, the root of *A. sinensis* (Fam: Apiaceae) are commonly known as dong quai,danggui,tank-kuei,Chinese angelica. The tree is widely distributed throughout China. Its use has been widely uses in Ayurvedic, Unani systems of medicine.

3.9.2 Composition

It contains dried root powder of *A. sinensis*.

2.9.3 Botanical Description

It is fragrant, perennial herb, 0.5–1.0 m high. Stem is glabrous and purplish, with light, linear striations. Inferior leaves tripinnate; superior leaves are often pinnate; segments oval, dentate-incised, teeth obtuse. Petiole 3–11cm long, sheathed; bracts rudimentary, not prominent. Umbels 10–14, radiate on top of the plant, rays irregular, interior margin uneven; bracteoles, narrow-linear 2–4; pedicels slender; carpophore bipartite; each umbel multiflorous (12–36 flowers); umbel stem 0.3–1.5 cm long. Flowers white, 5 petals, glabrous, incurvate at the tips. Carpels dorsally compressed, square-elliptical, the base cordiform, the tip rounded or lightly notched; dorsal veins 5, closely placed, projecting; central vein barely winged, marginal veins with very large wings; ducts oleaginous, 1 in each sinus, 2 in the commissure.(Farnsworth *et al*., 1958)

3.9.4 Chemical constituents

3.9.4.1 Phthalides

Phthalides consist of monomeric phthalides such as Z-ligustilide and phthalide dimers. In 1990 Danggui was reported in the literature when the Z-ligustilide dimer E-232 was isolated (Lin *et al* ., 1998). The majority of the phthalides identified is relatively non-polar, the fraction of which can be extracted with solvents such as hexanes, pentane, petroleum ether, methanol, 70% ethanol and dichloromethane. The amount of Z-ligustilide in Danggui varies between 1.26 and 37.7 mg/g dry weight (Yi *et al*., 2001) . Z-ligustilide facilitates blood circulation, penetrates the blood brain barrier to limit ischemic brain damage in rats and attenuates pain behaviour in mice (Du *et al*., 2007). Preclinical studies have indicated that AS and Z-ligustilide may also relax smooth muscle in the circulatory, respiratory and gastrointestinal systems (Wedge *et al*., 2009).

3.9.4.2 Organic acids

Danggui contains many organic acids. For example, ferulic acid isolated from Danggui is widely used as the marker compound for assessing the quality of Danggui and its products. Methanol, methanol-formic acid (95:5), 70% methanol, 70% ethanol, 50% ethanol or diethyl ether-methanol (20:1) is used as the initial extraction solvent. The amount of ferulic acid in Danggui varies between 0.21 and 1.75 mg/g dry weight (Yi *et al*., 2001) Abundant in rice bran, wheat, barley, tomato, sweet corn and toasted coffee, ferulic acid is an antioxidant, anti inflammatory and anti cancer agent and apart from its effects against Alzheimer's disease, it possesses antihyperlipidemic, antimicrobial and anti carcinogenic properties (Yan *et al*., 2001).

3.9.4.3 Polysaccharides

Biochemical and medical researchers have recently been interested in the anti tumor and immunomodulatory effects of polysaccharides (Ooi VE *et al*., 2000). The efficacy of AS is associated with its various polysaccharides which are extracted with water as the initial extraction solvent. Polysaccharides from AS consist of fucose, galactose, glucose, arabinose, rhamnose and xylose (Wang *et al*., 2003). Danggui contains a neutral polysaccharide and two kinds of acidic polysaccharides (Sun *et al*., 2005).

3.9.5 Pharmacological activities

3.9.5.1 Anti-inflammatory effects

Ferulic acid and isoferulic acid inhibit macrophage inflammatory protein-2 (MIP-2) production by murine macropharge RAW 264.7 cells, suggesting that these compounds contribute to the anti-inflammatory activity of AS(Skai *et al*., 1997) . Z-ligustilide also shows anti-inflammatory effects, probably related to inhibition of the TNF-α activities(Liu *et al*., 2005) . AS suppresses NF-β luciferase activity and decreases NO and PGE2 production in lipopolysaccharide (LPS)/IFN-g-stimulated murine primary peritoneal macrophages. Ferulic acid and Z-ligustilide, two major compounds in AS, decrease NF-β luciferase activity, which may contribute to the anti-inflammatory activity of AS . Using a murine air pouch model, Jung *et al*. reported that the leukocyte count in the pouch exudate decreases in the BALB/c mice fed with 100 mg/kg body weight of a root extract accompanied by a decrease in the neutrophil count, IL-6 mRNA level and TNF-α mRNA level in the pouch membrane and by decreased IL-6 and PGE2 concentrations in the pouch fluid and that the concentration of anti-inflammatory PGD2 in the pouch fluid increases as well(Jung *et al*., 2007). (Fu *et al*., 2006) reported that n-butylidenephthalide decreases the secretion of IL-6 and TNF-α during LPS stimulated activation of murine dendritic cells 2.4 via the suppression of the NF-β dependent pathways.

3.9.5.2 Antiasthmatic activity Ligustilide had significant antiasthmatic and spasmolytic activities. In guinea pigs at dose of 0.14 mg/kg (ip), the constituent inhibited the asthmatic reaction induced by acetycholine and histamine, at intravenous dose of 0.08 mg/kg, it inhibited histamine induced asthmatic reaction. In vitro test, ligustilide exhibited spamolytic action on isolated guinea pig trachea constricted by acetycholine, barium chloride of histamine and produced relaxation of the uncontracted trachea. Ligustilide exhibited antisoasmedic effect on isolated trachea strip of the guinea pig contracted by acetycholine, histamine and barium chloride, and a relaxation effect on trachea strip under normal tension. (Sun, 2005)

3.9.5.3 Antithrombotic effect

Using extracoporeal shunt method the aqueous extract of *A. sinensis* (AS) 20 g/kg wit intravenous injection was found to have inhibitory effect on rat arterial thrombus formation. The rate of inhibition was 30%. Sodium ferulate (SF) was shown to be one of the active antithrombotic constituents present in AS (Sun, 2005)

3.9.5.4 Immune Support and Hematopoiesis

Lymphocyte proliferation assays indicate dong quai consistently exerts an immunostimulatory effect (Wilasrusmee *et al*., 2002). A high molecular weight polysaccharide found in dong quai has demonstrated immunostimulating activity and a blood tonifying effect by inducing hematopoiesis in the bone marrow. This is accomplished, in part, by either direct or indirect stimulation of macrophages, fibroblasts, erythrocytes, granulocytes, and lymphocytes, and can induce an increased secretion of human growth factors from muscle tissue. Hematopoiesis is further supported by the presence of significant amounts of vitamin B12, folic acid, and biotin in dong quai (Huang *et al*., 1999).

3.9.5.5 Antispasmodic Activity

Ligustilide, butylidenephthalide, and butylphthalide were found to have antispasmodic activity against rat uterine contractions and in other smooth muscle systems. The components were characterized as non specific antispasmodics with a mechanism different from papaverine(Marderosian *et al*., 2004).

3.9.5.6 Anti cancer effects

AS extract induces apoptosis and causes cell cycle arrest at G0/G1 in brain tumor cell lines(Tsai *et al*., 2005). AS extract also decreases the expression of the angiogenic factor vascular endothelial growth factor (VEGF) in brain astrocytoma (Lee *et al*., 2006) . Moreover, n-butylidenephthalide and Z-ligustilide are cytotoxic against brain tumor cell lines and leukemia cells (Tsai *et al*., 2006).The three main AS phthalides, namely n-butylidenephthalide, senkyunolide A and Z-ligustilide, decrease cell viability of colon cancer HT-29 cells dose dependently (Kan *et al*., 2008) It is reported that pretreatment of the PC12 cells with Z-ligustilide attenuates H2O2- induced cell death, attenuates an increase in intracellular reactive oxygen species (ROS) level, decreases Bax expression and cleaves caspase-3 and novel polysaccharide (50 mg/kg, 100 mg/kg) isolated from AS inhibits the growth of HeLa cells in nude mice via an increased activity in the caspase-9, caspase-3 and poly (ADP-ribose) polymerase (PARP) (Cao *et al*., 2010).

3.9.5.7 Nephrotic Syndrome

An herbal preparation of Astragalus and AS has long been used in China to treat nephrotic syndrome, as it was thought to elicit antifibrotic effects. In a recent animal study the AS

mixture was found to retard the progression of renal fibrosis and deterioration of renal function with an effect similar to the drug enalapril.(Wang *et al*., 2004).

3.9.5.8 Cardiovascular effects

Pre treatment with AS (15 g/kg daily for 4 weeks) decreases doxorubicin-induced (15 mg/kg) myocardial damage and serum aspartate aminotransferase levels in male ICR mice (Xuan *et al*., 2007) . Human umbilical vein endothelial cells (HUVECs) treated with AS water extract activate VEGF gene expression and the p38 pathway, thereby increasing angiogenic effects of HUVECs both in vitro and in vivo (Lam *et al*., 2008) . Excess adipose tissue can lead to insulin resistance and increases the risk of type 2 diabetes and cardiovascular diseases. Water and 95% ethanol extracts of AS effectively decrease fat accumulation in 3T3-L1 adipocytes and reduce triglyceride content (Guo *et al*., 2009). Demonstrated that n-butylidenephthalide is anti-angiogenic and is associated with the activation of the p38 and ERK1/2 signaling pathways (Yeh *et al*., 2000)

3.9.5.9 Neuroprotective effects

Z-ligustilide treatment decreases the level of malondialdehyde (MDA) and increases the activities of the antioxidant enzymes glutathione peroxidise (GSH-Px) and superoxide dismutase (SOD) in the ischemic brain tissues in ICR mice; meanwhile there is a decrease in Bax and caspase-3 protein expression (Kuang *et al*., 2006). Z-ligustilide increases the choline acetyltransferase activity and inhibits the acetylcholine esterase activity in ischemic brain tissue from Wistar rats (Kuang *et al*., 2008). Extract protects Neuro 2A cell viability against b-amyloid (Ab) peptide induced oxidative damage by ROS, MDA and glutathione (GSH) and rescues mitochondrial transmembrane potential levels (Huang *et al*., 2008). Z-ligustilide inhibits the TNF-α-activated NFβ signaling pathway, which may contribute to Z-ligustilide's protective effect against Ab peptide-induced neurotoxicity in rats (Kuang *et al*., 2009).

3.9.5.10 Anti oxidative activities

BALB/c murine peritoneal macrophages pretreated with various AS polysaccharide fractions alleviate the decrease in cell survival caused by tert-butylhydroperoxide, with an increased intracellular GSH content(Yang *et al*., 2007). Furthermore, acidic polysaccharide fraction is also reported the most active fraction in terms of inhibiting the decrease in cell viability caused by H2O2. Acidic polysaccharide fraction also decreases the MDA formation, reduces the

decline in SOD activity and inhibits the depletion of GSH in murine peritoneal macrophages caused by H_2O_2 (Yang *et al*., 2007).

3.9.6 Contraindications

Radix Angelicae Sinensis should not be administered to children or patients with diarrhoea, haemorrhagic diseases or hypermenorrhoea, and should not be used during pregnancy or lactation (Zhu, 1987).

3.9.7 Drug interactions

Decreased prothrombin times were reported in rabbits that received both a single subcutaneous dose of warfarin (2 mg/kg body weight) and a repeated oral dose of Radix Angelicae Sinensis (2 g/kg body weight twice daily for 3 days) . Therefore, patients receiving anticoagulant therapy should be advised against taking Radix Angelicae Sinensis without medical supervision.(Lo A *et al*., 1995).

3.9.8 Adverse reactions

Oral administration of Radix Angelicae Sinensis is generally regarded as having few side effects; however, headaches may occur in sensitive individuals(Hirata *et al*.,1997). No adverse reactions were reported in 40 people who received an aqueous root extract by intravenous administration (240 ml/person) for 30 days .(Chang *et al*., 1997)

3.10 Herbomineral formulation Profile

3.10.1 Name Bloom Up

3.10.2 Composition

It contains Dhatri Loha,Kasis Bhasma,Mandur Bhasma,Yashad Bhasma, Punarnava, Ashwagandha.

3.10.3 Biological source of plants

3.10.3.1 Ashwagandha

It obtained from dried root of *Withania somnifera* belonging to family *Solanaceae*.

3.10.3.2 Punarnava

It obtained from wlole plant of *Boerhaavia diffusa* belonging to family *Nyctaginaceae.*

3.10.4 Chemical constitutes

3.10.4.1 Ashwagandha

The main constituents of Ashwagandha are Alkaloids, Steroidal lactones, Within alkaloids. Withanine is the main alkaloid. Other constituents are amino acids, choline, beta-sitosterol, chlorogenic acid, scopoletin, withaferin etc (Lokhande *et al*., 2010).

3.10.4.2 Punarnava

Generally whole plant consists the following phytochemical constitiutents, those are punarnavine (*Alkaloids)*, B-sitosterol (Phytosterols), Liriodendrin (lignans), Punarnavoside (Rotenoids), Boerhavine (Xanthones) and Potassium nitrate (Salts). The roots contain the rotenoids boeravinones AI, BI, C2, D, E and F besides the new dihydroisofurenoxanthin, Alanine, Arachidic Acid, Aspartic Acid, Behenic Acid, Beta-Sitosterol, Boeravinone A - F, Boerhaavic Acid, Borhavine, Borhavone, Campesterol, Daucosterol, Beta-Ecdysone, Flavone,5-7-dihydroxy-3'-4'-dimetho, Xy-6-8-dimethyl, Galactose, Glutamic Acid, Glutamine, Glycerol, Glycine, Hentriacontane N, Heptadecyclic Acid, Histidine, Hypoxanthine-9-l-arabinofuranoside, Leucine, Liriodendrin, Methionine, Oleaic Acid, Oxalic Acid, Palmitic Acid, Proline, Proline,hydroxy, Serine, Sitosterol Oleate, Sitosterol Palmitate, Stearic Acid, Stigmasterol, Syringaresinol-mono-beta-d-glucoside, Threonine, Triacontan-1-OL, Tyrosine, Ursolic Acid, Valine, Xylose, triacontanol hentriacontane, ß-sitosterol, ursolic acid, 5,7-dihydroxy-3,4-dimethyoxy-6,8-dimethyl flavone, and an unidentified ketone (mp 86°). The roots contain the rotenoid boeravinones AI, BI, C2 , D, E and F besides the new dihydroisofurenoxanthin and an antifibrinolytic agent, Two lignans, liriodendrin and syringaresinol mono-ß-D-glucoside, have also been reported in the roots (Santhosha *et al*., 20011).

3.10.5 Uses

3.10.5.1 Ashwagandha

It has been used in diseases such as rheumatism, leprosy, arthritis and intestinal infections. Used to treat general debility, arthritis, depression, chronic fatigue, insomnia, anxiety, depressed immunity, infertility and memory loss. It is used as a general tonic, Blood purifier, increases the iron content in the blood. It is useful in sexual & general weakness. It gives vitality and vigour and helps in building greater endurance. It is diuretic, i.e. it promotes urination, and removes functional obstruction of the body (Lokhande *et al*., 2010).

3.10.5.2 Punarnava

This plant rejuvenates liver, male reproductive system and other organ system; detoxifies liver and skin; aphrodisiac; increases libido, erection and quality and quantity of semen; reduces cough, asthma etc. It is used in Vajikarana preparations. This plant cleanses the kidneys and helps to get rid of renal calculi (kidney stones). Mainly, the roots and the whole plant is used for the medicinal purpose, externally punarnava is used for alleviate the pain and swelling. The fresh juice of its roots instilled into eyes, mitigates the ailments of the eyes like night blindness and conjunctivitis. The paste applied on the wounds, dries up the oozing. Internally, punarnava is beneficial to treat a wide range of diseases. Punarnava is the most commonly used and the best herb to alleviate swelling, due to its potent diuretic property. It boosts up the filteration, rejuvenates the renal functions and takes out the excessive fluids and kleda by augmenting the urinary output. The sesame oil, medicated with punarnava is very useful as an adjunct to oleating enemas in the treatment of ascites of vata type and flatulence. In large doses, punarnava acts as a purgative. Punarnava effectively reduces fever, especially in malaria.The decoction of rasna, sunthi and punarnava is the best panacea for rheumatic swollen joints, as rasna alleviates the pain and vata, sunthi destroys ama and punarnava reduces the swelling. Punarnava enhances the quality of 6 of the 7 categories of bodily tissues, including nutrient plasma (Rasa Dhatu), blood (Rakta Dhatu), muscle (Mamsa Dhatu), fat (Meda Dhatu), bone marrow and nerves (Majja Dhatu), and reproductive fluids (Shukra Dhatu). For liver disorders (jaundice, hepatitis, cirrhosis, anemia, flukes, detoxification, chemical injury, etc), for

gallbladder disorders (stones, sluggish function, low bile production, emptying, and detoxification), for kidney and urinary tract disorders (stones, nephritis, urethritis, infections, renal insufficiency/injury, etc), for menstrual disorders (pain, cramps, excessive bleeding, uterine spasms, water retention), to tone, balance, and strengthen the adrenals (and for adrenal exhaustion and excess cortisol production and in the treatment of following disorders (Santhosha *et al*., 2011).

3.10.6 Bhasma

Bhasma (calcified powder or ash) are herbo mineral or herbo metallic formulation. The section of ayurveda which deals with Bhasma is referred as 'Rasa Sastra'. Buddhist Philosopher Nagarjuna who was considered as father of metallic medicine in India used metals and minerals in the form of Bhasma as therapeutic agent in ayurvedic system of medicine from prior 8thcentury A.D. Metals in Bhasma are believed to provide an enhanced bioavailability to the herb, also act as carrier and catalyst for many process (Kumar *et al*., 2011).

3.10.6.1 Pharmacological actions:

Lauha Bhasma (incinerated iron) possesses *Vrishya* (aphrodisiac), *Vayasthapana* (antiageing), *Lekhana* (emaciating) and *Rasayana*(immunomodulator) properties. It increases potentiality *(Valya),* complexion *(Kantijanana)* and appetite *(Agni Vardhana).* It eradicates diseases caused by vitiated *Kapha andPitta* (Sarkar *et al*., 2010).

3.10.6.2 Therapeutic uses:

Lauha Bhasma is used in *Pandu* (anaemia), *Prameha* (diabetes), *Yakshma* (tuberculosis), *Arsha* (piles), *Kustha* (skin disorders), *Krimi Roga* (worm infestation), *Kshinatwa* (cachexia), *Sthaulya* (obesity), *Grahani* (bowel syndrome), *Pliha Roga* (spleenic disorders),*Medoroga* (hyperlipidemia), *Agnimandya* (dyspepsia), *Shula* (spasmodic pain), and in *Visha* (poisoning) (Sarkar *et al*., 2010).

3.10.6.3 Adjuvant:

Lauha Bhasma is given with honey, ghee, powder of *Terminalia chebula, Terminalia belerica* and *Emblica officinalis* fruits *(Triphala),* etc. It is also prescribed with other plant drugs acting on particular disease (Sarkar *et al*., 2010).

3.10.6.4 Dose:

1/4th to 2 *Ratti* (30 to 250mg)/day (Sarkar *et al*., 2010).

3.10.6.5 Unwholesome Diet:

Some food stuffs like *Kushmanda (Benincasa cerifera* fruit), sesame oil, *Masha (Phaseolus mungo* seeds), *Rajika {Brassica juncea*seeds and oil), wine, sour foods, fish, brinjal, *Karbellaka (Momordica charantia* fruit), etc. are forbidden during iron therapy (Sarkar *et al*., 2010).

3.10.6.6 Adverse reactions:

The raw iron or improperly prepared *Lauha Bhasma* may cause various ill effects like *Hritpida* (angina), *Kustha* (skin disorders), *Ashman*(urolethiasis), *Shula* (spasmodic pain), *Daha* (burning sensation), weakness *(Vala Vinasha),* etc. even death *{Ayu Nasha)* (Sarkar *et al*., 2010).

3.10.6.7 Management of adverse effect:

Powder of *Embelia ribes* fruit *(Vidanga)* should be given repeatedly with the Juice of *Sesbania grandiflora* leaves *(Munirasa)* and patients should be exposed to sunlight. If worm infestation occurs, then patient should be purgated by giving the fruit pulp of *Cassia fistula (Aragvadha Phala Mafia).* If patients complain of spasmodic pain *{Shula),* then *Abhraka Bhasma* (incinerated biotite mica) and Powder of*Embelia ribes* fruit *{Vidanga)* with juice of *Embelia ribes* fruit *{Vidanga)* or Powder of *Elletoria cardamomum* seeds *{Ela Churna)* should be given repeatedly (Sarkar *et al*., 2010).

4. MATERIALS AND METHODS

4.1 Pharmacognostical Evaluation:

4.1.1 Collection of Formulations

The capsule of *A. sinensis* root powder (Family-Apiaceae) was obtained from a vendor Vitamin Shoppe Industries Inc. New Jersey U.S. and herbomineral formulation "Bloom up" from UAP Pharma Pvt. Lit. Ahmedabad Gujarat India. The capsule was stored in cool and dry place.

4.1.2 Organoleptic Properties

The organoleptic characters of the samples were evaluated based on the method described by Siddiqui *et al*. Organoleptic evaluation refers to evaluation of the formulation by color, odor, taste and texture etc.

4.1.3 Physiochemical Evaluation

Physicochemical parameters such as moisture content, total ash, acid insoluble ash, water-soluble ash, were determined for formulation of *A. sinensis* and herbomineral formulation according to the methods described in guidelines of WHO.

4.1.3.1 Determination of moisture content

The powdered material (1gm) was placed in a moisture dish and dried to a constant weight in an oven at 100 °C-105 °C. The loss of weight in mg/g of air-dried material was calculated (Ali., 2009).

4.1.3.2 Determination of total ash content

The powdered material (2gm) was accurately weighed and placed in a crucible. The material was ignited to a constant weight by gradually increasing the heat to 500 °C-600 °C until it was white. The residual ash was allowed to cool in a desicator. The content of total ash in mg/g of air dried material was calculated (Ali, 2009).

4.1.3.3 Determination of acid insoluble ash content

Hydrochloride acid (2 N; 25 mL) was added to the crucible containing the total ash, covered with a watch glass and boiled gently for 5 min. The watch glass was rinsed with 5 mL of hot water and the rinsed contents were added to the crucible. The acid insoluble matter was collected on an ashless filter paper and washed with hot water until the filtrate was neutral. The filter paper containing acid insoluble matter was

transferred to the original crucible, dried and ignited to a constant weight. The residue was allowed to cool in a desicator and weighed. The content of the acid insoluble ash in mg/g of air dried material was calculated (Ali, 2009).

4.1.3.4 Determination of water soluble ash content

Water (25mL) was added to the crucible containing the total ash, covered with a watch glass and boiled gently for 5 min. The watch glass was rinsed with 5 ml of hot water and added to the crucible. The water insoluble matter was collected on an ashless filter paper and washed with hot water. The filter paper containing the water insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to a constant weight. The water soluble ash content was calculated (Ali, 2009).

4.1.4 Phytochemical Analysis

Both the formulation extracted with water or alcohol and extracts was screened qualitatively for the major groups of chemical constitutes using standard reagents **(**Cocate *et al*., 2009).

4.1.4.1 Alkaloids

A few ml of (2-3 ml) of solution was evaporated in a test tube. One ml of dilute hydrochloric acid and a few drops of Mayer's reagent were added.

A few ml of (2-3 ml) of solution was evaporated in a test tube and a few drops of Dragendorff's reagent were added.

4.1.4.2 Amino acids

A few ml of (2-3 ml) of solution was evaporated in a test tube and about 2 ml of millons reagent were added to the solution.

A few ml of (2-3 ml) of solution was evaporated in a test tube and Ninhydrine solution about 2 ml of was added to the solution.

4.1.4.3 Carbohydrates

A few ml of (2-3 ml) of solution was evaporated in a test tube and added few drops of alcoholic ∞-nephthol, few drops of sulphuric acid through side of the test tube were added.

A few ml of (2-3 ml) of solution was evaporated in a test tube. To the solution crystals of resorcinol was added and equal volume of HCL and heated on water bath.

A few ml of (2-3 ml) of solution was evaporated in a test tube. To the solution the equal volume of HCL containing a small amount of phloroglucinol was added and heated on water bath.

4.1.4.4 Proteins

A few ml of (2-3 ml) of solution was evaporated in a test tube added and heated the test solution in a boiling water bath.

A few ml of (2-3 ml) of solution was evaporated in a test tube. To the solution biuret reagent was added.

4.2 Pharmacological Evaluations

4.2.1 Study of effect of formulation of *A. sinensis* on animal model of anemia of chronic inflammation

4.2.1.1 Experimental Animals

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of Institute Of Pharmacy, Nirma University, Ahmedabad as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Protocol number is IPS/PCOL/MPH11-12/1008. Healthy Balb/c mice of either sex (20-30gm) were selected for the study. Animals were housed in a pathogen-free environment at the animal house of Institute of pharmacy, Nirma University. Animals were maintained under well controlled temperature 22±5 ^{o}C & humidity 55±5% and 12h/12h light/dark cycle. They kept in well ventilated animal house under natural photoperiodic condition in polypropylene

cages with free access to food and water *ad libitum.* Animals were acclimatized for one week before starting the experiment.

4.2.1.2 Experimental Protocol

Allergic asthma was induced by intraperitonial injection of 50 mg ovalbumin + 4 mg $Al(OH)_2$ in 0.2 ml saline. On day 1^{st} ,14^{th} i.p. injection was given. Control animals were induced with equal volume of vehicle. Treatment of *A. Sinensis* 100 mg/kg, folic acid 2 mg /kg was given from day 1 to 35 days. Weakly food, water and body weight gain was observed. On day 28, 29 and 30 and 35 animals were exposured to 1% Ovalbumin in PBS for 20 minutes each day. 24 hours after last exposure blood was collected and haematological and biochemical parameter were determined like blood cell count (R.B.C.), hematocrit (ht), haemoglobin(hb), reticulocyte , ferritin, total serum iron, iron binding capacity, transfeerin saturation (Chen *et al*., 2010).

4.2.2 Study of effect of herbomineral formulation on animal model of iron deficiency anemia.

4.2.2.1 Experimental Animals

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of Institute Of Pharmacy, Nirma University, Ahmedabad as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Protocol number is and IPS/PCOL/CONS11-12/2006. Healthy Wistar rat (250-300 gm) were selected for the study. Animals were housed in a pathogen-free environment at the animal house of Institute of pharmacy, Nirma University. Animals were maintained under well controlled temperature 22±5 ^{o}C & humidity 55±5% and 12h/12h light/dark cycle. They kept in well ventilated animal house under natural photoperiodic condition in polypropylene cages with free access to food and water *ad libitum.* Animals were acclimatized for one week before starting the experiment.

4.2.2.2 Experimental Protocol

Iron deficiency anemia was induced by agar gel diet for 30 days. On 10^{th} ,20^{th} ,28^{th}, 30 days phlebotomy was performed by snipping the tail vein of each rat. Treatment of herbomineral formulation (100 mg/kg, p.o.), standard folic acid (2mg /kg p.o.)was given from day one to day 30. After 30 days blood was collected from retroorbital sinus and haematological parameters like blood cell count (R.B.C.), hematocrit (ht), haemoglobin(hb), reticulocyte and biochemical ferritin, total serum iron, iron binding capacity, transferin saturation determined (Pandit *et al*., 2009).

4.2.3 Estimation of hematological parameters

4.2.3.1 Red blood cell count

Principle

The number of red cells in bloods are too many and the size of cells is too small. It is therefore not possible to count the cells even under high power. This difficulty is partially overcome by diluting the blood with a suitable dilution fluid to know as degree. The dilute blood is placed in a capillary space of known capacity in between counting chamber and coverslip. The number of the cells in the small capillary space of known volume is then counted under the high power of the microscope. The count can be calculated by multiplying the number with the dilution factor (Goyal *et al*., 2008)

Procedure

The Thomas coverslip, counting chamber and the lenses of the microscope are cleaned first with the help of the xylol and then absorbent cotton. The Thomas counting chamber is adjusted and observed for RBC squares under low power of the microscope ,keeping the Thomas coverslip resting on the plate form of the slide . The objective of microscope is raised and then it is adjusted for high power and the chamber of the RBC square under high power. The RBC pipette is cleaned and dried. The blood is sucked in the RBC pipette up to the mark 0.5. Immediately the RBC dilution fluid is sucked up to the mark 101. The pipette is brought to a horizontal position and the finger placed over the pipette. A simple knot is given to the rubber

tube. The pipette is rolled between the palm to mix the blood with dilution fluid for one minute. Few drops are discarded and then pipette is held at at the angle of 45^0 to the surface of the counting chamber and tip is applied to the narrow slit between the counting chamber and coverslip. A drop is allow to come out from the pipette . The fluid was run in to the capillary space because of capillary action and it is filled. The drop shouled not flow in to the moat and cover glass. The fluid is allowed to settle for 3 minutes on the stage of microscope. The RBC chamber is located and RBC are counted in one smallest square. This is repeated in another four such chamber.

4.2.3.2 Haemoglobin Estimation

Principle

When blood is mixed with N/10 HCL RBC'^{S} are haemolysed and Hb is liberated. This Hb is converted in to acid haematin which is radish brown color.The solution is diluted with distilled water till it matches with the standard glass(comparison) tubes. The Hb % can directly be read from the graduated tube (Goyal *et al*., 2008).

Procedure

The graduated diluting tube and the micropipette are cleaned thoroughly and dried. The graduated diluting tube is filled with N/10 HCL up to the mark 2 gm or till the micropipette touches the level of the acid in to the tube. Blood is sucked in the micropipette upto the mark 20 cmm. The blood is immediately deposited at the bottom of the graduated tube .The pipette is rinsed two to three times in HCL. The blood is mixed with the help of the stirrer and then solution is allowed to stand for 10-15 minutes so that all acid is converted in to acid haematin. The mixer is then dituted with distilled water . Distilled water is added drop by drop and every time it is stirred till the exact match with standard glass tubes is obtained. When the matching is complete, stirrer is taken out from the diluting tube and the scale is read on the side of tube.

4.2.3.3 Haematocrit (Packed Cell Volume PCV) determination

Principle

A volume of anticoagulated blood is placed in a glass tube which is centrifuged so the blood will be separated into three layers: Red cells, Buffy coat (WBC and platelets) and plasma. Ideally there should be complete separation of the three layers.

Haematocrit is the ratio of the height of red cells column to that of the whole blood in the tube.

Procedure

The blood sample should be used as fresh as possible, and well mixed. Using the capillary action, allow blood to enter the tube stopping at 10-15 mm from one end. Wipe the outside of the tube. Seal the dry end by pushing into the plasticine two or three times. If heat sealing is used rotate the dry end of the tube in a fine Bunsen Burner flame. Placed the tube into one of the centrifuge plate slots, with the sealed end against the rubber gasket of the centrifuge plate. Centrifuged for five minutes. Read the PCV in the micro haematocrit reader.

4.2.3.4 Reticulocyte count

Principle

Reticulocytes are immature RBCs that contain remnant cytoplasmic ribonucleic acid (RNA) and organelles such as mitochondria and ribosomes. Reticulocytes are visualized by staining with supra-vital dyes (stain living cells) that precipitate the RBC and organelles. This stain causes the ribosomal and residual RNA to coprecipitate with the few remaining mitochondria and ferritin masses in living young erythrocytes to form microscopically visible dark-blue clusters and filaments (reticulum). An erythrocyte still possessing RNA is referred to as a reticulocyte. The reticulocyte count is a means of assessing the erythropoietic activity of the bone marrow (Harmening *et al.*,2004)

Procedure

Preparation of smears

Added 5 drops of new methylene blue solution to 5 drops of thoroughly mixed EDTA anticoagulated blood to a glass 10 x 75 mm test tube.

Mixed the contents by gently shaking and allowed to incubate at room temperature for a minimum of 10 minutes. At the end of 10 minutes, gently mixed the blood/stain solution. Using a capillary tube, placed a drop of the mixture on each of three slides near the frosted edge as you would when making a peripheral smear. Using the wedge smear technique, made acceptable smears not too thick or thin. Labeled the slides.

Allow to air dry. (Do not blow to hasten to drying.)

Method using the Miller disc

Use a 100x objective and a 10x ocular secured with a Miller disc. The Miller disc imposes two squares (one 9 times the area of the other) onto the field of view. Find a suitable area of the smear. A good area was showed 3-10 RBCs in the smaller square of the Miller disc. Counted the reticulocytes within the entire large square including those that are touching the lines on the left and bottom of the ruled area. Counted RBCs in the smaller square whether they contain stained RNA or not. A retic in the smaller square should be counted as an RBC and a retic. Record RBC # counted and retic # counted separately. Continue counting until a minimum of 111 RBCs have been observed (usually 15-20 fields). This would correspond to 999 RBCs counted with the standard procedure.

Reagents and equipment

Commercially prepared liquid new methylene blue solution. It should be stored in a brown bottle. If precipitate is a problem on the smear, the stain should be filtered prior to use.

Microscope slides

Microscope

10 x 75 mm test tubes

Pasteur pipets (with bulb if pipets are glass)

Capillary tubes

Miller ocular (if available)

4.2.4 Estimation of biochemical parameters

4.2.4.1 Ferritin determination

Principle

The FERRITIN CLIA test utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the intact FERRITIN molecule. Mouse monoclonal anti- FERRITIN antibody is used for solid phase (microtiter wells) immobilization and a goat anti-FERRITIN antibody is in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the two antibodies, resulting in the FERRITIN molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 60 minutes incubation

at room temperature, the wells are washed 5 times by wash solution to remove unbound anti-FERRITIN conjugate. A solution of chemiluminescent substrate is then added and read relative light units (RLU) in a Luminometers. The intensity of the emitting light is proportional to the amount of enzyme present and is directly related to the amount of FERRITIN in the sample (White *et al* .,1986).

Procedure

Secure the desired number of coated wells in the holder. Dispensed 50µl of standards, specimens, and controls into appropriate wells. Dispensed 100µl of Enzyme Conjugate Reagent into each well. Thoroughly mixed for 30 seconds. It is very important to have complete mixing in this step. Incubated at room temperature (18-22°C) for about 60 minutes. Rinsed and flicked the microtiter wells 4 times with washing solution and final 1 time with distilled water. Striked the wells sharply onto absorbent paper to remove residual water droplets. Dispensed 100 µl Chemiluminescence substrate solution into each well. Gently mix for 5 seconds. Read wells with a chemiluminescence microwell reader 15 minuters later. (between 10 and 20 min. after dispenced the substrates.

Reagent Preparation

1. All reagents should be brought to room temperature (18- 22°C) before use.
2. To prepared Chemiluminescence Substrate solution, make a 1:1 mixing of Reagent A with Reagent B right before use. Discard the excess after use.
3. Prepared the washing solution by diluting 1 part of the 20X PBS concentrate to 19 parts of distilled water.

4.2.4.2 Total serum iron

Principle

The iron in serum is dissociated from its Fe (III) - transferrin complex by the addition of an acidic buffer containing hydroxylamine. This addition reduces the Fe (III) to Fe (II). The chromogenic agent, Ferrozine, forms a highly colored Fe (II) -complex that is measured photometrically at 560 nm(Tietz, 1976)

Procedure

Labeled test tubes/cuvettes: “Blank”, “Standard”, “Control”, “Sample”, etc. Added 2.5 ml Iron Buffer reagent to all tubes. Added 0.5 ml (500µl) sample to respective tubes and mix. *NOTE: Added 500 µl iron-free water to blank.* Zero spectrophotometer at 560 nm with the reagent blank. Read and record the absorbencies of all tubes (A1 reading). Added 0.05 ml (50 µl) Iron color reagent to all the tubes. Mixed Placed all

the tubes in the heating bath at 37°C for 10 minutes. Zero the instrument at 560 nm with the reagent blank. (Wavelength range: 520-560 nm). Read and recorded absorbencies of all the tubes (A2 reading).

Reagents

1. Iron buffer reagent: Acetate buffer containing 220 mM Hydroxylamine hydrochloride, pH 4.5 with surfactant.
2. Iron color reagent: Ferrozine (16.6 mM) in Hydroxylamine hydrochloride.
3. Iron standard (500 µg/dl): 500 µg Ferrous chloride in Hydroxylamine hydrochloride.

4.2.4.3 Total iron binding capacity

Principle

The unsaturated iron binding capacity (UIBC) is determined by adding Fe (II) ion to serum so that they bind to the unsaturated iron binding sites on transferrin. The excess Fe (II) ions are reacted with Ferrozine to form the color complex, which measured photometrically. The difference between the amount of Fe (II) added and the amount of Fe (II) measured represents the unsaturated iron binding. The total iron binding capacity (TIBC) is determined by adding the serum iron value to the UIBC(Tietz, 1976).

Procedure

UIBC (Unsaturated Iron-Binding Capacity)

Labeled test tubes/cuvettes, "Blank", "Standard", "Control", "Test", etc. Added 2.0 ml UIBC buffer reagent to all tubes. To "'Blank" add 1.0 ml iron-free water. Mixed To "Standard' add 0.5 ml (500 µl) iron-free water plus 0.5 ml (500 µl) Iron standard. Mixed To "Test" add 0.5 ml (500 µl) respective sample plus 0.5 ml (500 µl) Iron Standard. Mixed Zero spectrophotometer at 560 nm with reagent blank. Read and recorded the absorbance of all tubes (A_1 reading). Added 0.05 ml (50 µl) of Iron Color Reagent to all tubes. Mixed Placeed all tubes in a heating bath at 37°C for ten (10) minutes. Zero spectrophotometer at 560 nm with reagent blank. Read and recorded the absorbance of all tubes. (A_2 reading).

TIBC (Total Iron-Binding Capacity):

Iron Level + UIBC = TIBC (µg/dl)

Reagents

1. UIBC buffer reagent: Tris buffer 0.5 M, pH 8.0, with surfactant, and sodium azide as a preservative.
2. Iron color reagent: Ferrozine (16.6 mM) in Hydroxylamine hydrochloride.
3. Iron standard (500 µg/dl): 500 µg Ferrous chloride in Hydroxylamine hydrochloride.

4.2.4.4 Transfferin saturation

Principle

Transferrin is protein which carry iron molecule for erythropoiesis. Transferrin saturation which denoted the number of iron binding sites on transferrin. Which are determine by ratio of total serum iron and total iron binding capacity multiply by 100.

Calculation

Percent transferrin saturation (serum iron × 100 divided by TIBC) [TSAT]

4.3 Stastical Analysis

Results are presented as mean ± SEM. Statistical differences between the means of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test. Data were considered statistically significant at P value ≤ 0.05 and highly significant at $P<0.001$. Statistical analysis was performed using Graph pad Prism 5 statistical software.

6. DISCUSSION

Anemia is most prevalent disease. Present world wise classified majorly in to iron deficiency anemia and anemia of chronic disease. Anemia of chronic disease defined as the anemia occurring in chronic infectious or inflammatory disorders characterized by hypoferremia in the presence of adequate iron stores (Means *et al*., 1992). While iron deficiency anemia is characterized by a defect in hemoglobin synthesis, resulting in red blood cells that are abnormally small (microcytic) and contain a decreased amount of hemoglobin (Provan., 1999). Anemia is common in older adults, and its prevalence rises with age. The National Health and Nutrition Examination Survey (NHANES III) reports that just over one of every ten adults >65 years of age is anemic. The prevalence rate is 20% in women and 25% in men.

The world health organization has recognized herbal medicine as an essential building block for primary health care for country like India and China. It has recognized the potential of traditional and folk medicine in the management self reliance of health care system and currently it is encouraging and promoting the traditional system in national health care program of various countries (Shiv prakash *et al*., 2005). Today, there is a novel interest in traditional medicine in all over the world as an alternative to pure synthetic medicine because of various problem associated with them (Manimaram *et al*., 2002). These problem include high cost (almost 200 million dolor) long time 15-20 years taken in development of new drug. The several side effects and adverse reaction and poor success are also associated with them. Their fore it is necessary to develop a safe, cost effective treatment strategies for the management of certain chronic disease like anemia.

In the present study, pharmacognostic evaluation of formulation of *A. sinensis* and herbomineral formulation were carried out. The phytochemical analysis showed presence of carbohydrates, organic acids, phalides in formulation of *A. sinensis* which correlates with previous reports by (Wan *et al.,* 2011). Herbomineral formulation also show presence of alkaloid, glycoside. A comparatively low ash value indicates presence of less inorganic matter for *A. sinensis* powder formulation and high ash value indicates presence of high inorganic matter for herbomineral formulation. Both the formulation contains low amount of moisture content and high amount of solid content. The physicochemical and

phytochemical data of both the formulations correlates well with different standard texts and floras. These data supports of traditional and folkloric use of both the formulations.

Various complementary and alternative medicines including herbal drugs have been reported to possess antianemic activity. *A. sinensis* play potential and beneficial role in the prevention of various diseases including inflammation, cancer, ulcers, and cardiovascular disease. The main constituents of the root of *A. sinensis* reported are polysaccharide, organic acids, phalides (Wan *et al*., 2011).

This study aimed to evaluate the anti anemic effect of *Angelica sinenis* root powder on ovalbumin induced anemia of inflammation and herbomineral formulation on agar gel diet and phlebotomy induced iron deficiency anemia.

Anemia of chronic disease (ACD) is very frequently associated with chronic disease. Anemia of chronic disease (ACD), sometimes called anemia of inflammation, is due to the effects of chronic inflammation, which result in release of mediators like interleukins and tumor necrosis factor. Ovalbumin which cause inflammation and release of inflammatory cytokines which activate T cells and microphage. Activated cells as leads to increase hepatic hepcidin synthesis, inhibits erythropoietin release, erythroid proliferation and hemophagocytosis which finally cause anemia (Rayan *et al*., 2008). In present investigation decrease in hemoglobin level was observed in ovalbumin induced asthmatic animals. Which may be due to inflammation and release of inflammatory cytokines (Weiss *et al*., 2005). In the present study treatment with formulation of *A. sinensis* significantly ($P<0.05$) prevented ovalbumin induced reduction in hemoglobin. Rayan *et al*. (2008) reported that IL-6 and hepcidin play role in negative iron homeostasis thus reduced hemoglobin synthesis. Thus treatment with formulation of *A. sinensis* produced improvement in hemoglobin may be due to decrease in negative regulators of iron homeostasis by inhibition of TNFα, IFNγ, IL-6 and hepcidin.

The mature red blood cell (also known as an erythrocyte) carries oxygen attached to the iron in hemoglobin. The clinical importance of the test is that it is a measure of the oxygen carrying capacity of the blood (Zargchanski *et al*., 2008). Mainly proinflammatory immune modulators, such as TNFα, IFNγ and Type I Interferons, block

BFU-e and CFU-e colony formation. However, IFNγ appears to be the most potent inhibitor of erythropoiesis in directly blocking CFU-e proliferation and this notion is also reflected by an inverse correlation between IFNγ levels with R.B.C. counts (Fuchs *et al*., 9191). In the present study treatment with formulation of *A. sinensis* produced significant ($P<0.05$) elevation in red blood cell count in ovalbumin induced animals. NO can directly block erythropoiesis by inhibiting the proliferation of erythroid progenitor cells in the bone marrow (Maciejewski *et al*., 1995). The improvement in R.B.C. level observed with treatment with formulation of *A. sinensis* may be due to stimulation of proliferation of erythroid progenitor cells in bone marrow by *A. sinensis* because polysaccharide of *A. sinensis* have inhibitory action on NO level (Cai., 2009).

The hematocrit is one of the most precise methods of determining the degree of anemia. A low hematocrit and hemoglobin usually indicates decreased production, or destruction of red blood cells. In the present study treatment with formulation of *A. sinensis* significantly ($P<0.05$) prevented ovalbumin induced reduction in hematocrit. Inflammatory immune modulators, which, appears to be the most potent inhibitor of hematocrit (Fuchs *et al*., 9191). Inflammatory mediator can directly block erythropoiesis by inhibiting hematocrit (Chao *et al*., 2011). The improvement in hematocrit levels was observed in our study. These improvement may be due to inhibition of inflammatory mediators (Cai., 2009).

Reticulocytes are juvenile red cells. They contain remnants of ribosomes and ribonucleic acids which were present in larger amounts in their nucleated precursors. Because the number of reticulocyte in the peripheral blood is afairly accurate reflection of erythropoietic activity (Blinder *et al*., 2002). In the present study treatment with formulation of *A. sinensis* significantly ($P<0.05$) prevented ovalbumin induced reduction in reticulocytes. Cai., 2009 has been reported that in inflammatiory conditions reticulocytes level wre found to be decreased. The decrease in reticulocyte is may be due to inflammation which leads to release IFNγ which is reported to be have inverse correlation with reticulocyte count. There fore improvement may be attributed to inhibition of IFNγ.

Iron is the vital component of hemoglobin which are responsible for carrying the oxygen to the tissues. Ganz (2003) reported that IL-6 and hepcidin play role in negative iron homeostasis and thereby produces reduction in total serum iron. In the present study treatment with formulation of *A. sinensis* significantly ($P<0.05$) prevented ovalbumin induced reduction in serum iron. It is reported that *A. sinensis* polysaccharide have inhibitory action on hepcidin and IL-6 (Wang *et al*., 2011). Thus improvement in serum iron levels may be due to inhibition of IL-6, hepcidin and TNF-α.

Allergic asthma leads to release of serum ferritin. results in both, hypoferraemia in serum and induction of ferritin synthesis with the reticuloendothelial system, a condition which is also seen in ACD (Hernandez *et al*., 1989). Therul *et al*. (2012) reported that IL-6 mediates release of hepcidin which leads to release of serum ferritin. In the present study treatment with formulation of *A. sinensis* significantly ($P<0.05$) prevented ovalbumin induced increase in serum ferritin. The improvement may be due to suppression of ferritin level from reticuloendothelial system which is reprted to be stimulated in various inflammatory conditions (Bergamaschi and Vilani 2009).

Total iron binding capacity which indirectly indicated transferrin. Transferrin is protein which carry the iron to erythrocyte precursors for erythropoiesis. (Gao *et al*., 2012) In the present study treatment with formulation of *A. sinensis* significantly ($P<0.05$) prevented ovalbumin induced reduction in total iron binding capacity. Gao *et al*. (2012) reported that inflammation which leads to release of IL-6 mediated hepcidin production leads to reduction in the total iron binding capacity. Similar improvement in total iron binding capacity has been previously reported by due to inhibition of IL-6 and hepcidin. Thus *A. sinensis* may be producing this effect by improvement in IL-6, and hepcidin levels.

Transferrin saturation is the ratio of total serum iron over total iron binding capacity. It represented the number of iron binding site on the transferrin. (Gao *et al*., 2012) In the present study treatment with *A. sinensis* root powder formulation significantly ($P<0.05$) prevented ovalbumin induced reduction in transferrin saturation. It has been reported that increased *Hfe* and hepcidin mRNA and lowered hepatic iron and transferrin saturation (Gao *et al*., 2012). Thus elevation in transferrin saturation may be due to inhibitory activities of *A. sinensis* on hepcidin pathway.

Iron deficiency is the commonest cause of hypochromic microcytic anaemia. In iron deficiency, the amount of iron lost from the body exceeds the amount absorbed. The physiological demand for iron exceeds iron uptake. First there is depletion of the iron store of the body followed by reduction in the plasma level of iron and development of hypochromic microcytic anaemia. Hypochromic microcytic anaemia can be interpreted based on reduction of haemoglobin content, total RBC count, reticulocyte count, haemaocrit, total serum iron and increase iron binding capacity and decrease in transferrin saturation and that is why we precise the estimation of these haematological and biochemical parameters (Godkar & Godkar., 2004).

Bhasma used to use since ancient time for treatment of iron deficiency anemia. Thus the herbomineral formulation containing Dhatri loha, Kasis bhasma, Mandur bhasma, Yashad bhasma, Punarnava, Ashwagandha was evaluated on animal model of iron deficiency anemia. The pharmacological evaluation of herbomineral formulation was carried out because in market herbomineral formulations are commonly used rather than individual herbal formulation.

It has been reported that agar gel diet cause iron deficiency anemia due to hemolysis of red blood cells (Shiv kumar *et al.*, 1985). It is also reported that phlebotomy is the most common cause of iron deficiency which leads to loss of hemoglobin in blood (Roy., 2000; Pandit et al., 1998). In the present study we observed decrease in R.B.C., hemoglobin, hematocrit, reticulocyte level in animal model of iron deficiency anemia which correlates with previous studies. In the present study treatment with herbomineral formulation significantly ($P<0.05$) prevented agar gel diet and phlebotomy induced reduction in hemoglobin, red blood cell count, hematocrit and reticulocyte. It has been reported that lauha bhasma, mandura bhasm, increased blood iron due to depletion of iron storage, may be due to stimulation of progenitor cells, release of erythropoietin from kidney (Sarkar *et al*., 2007; Santosha *et al*., 2011; Lokhande *et al*., 2010). Herbomineral formulation containing bhasma may be responsible for improvement in R.B.C., hemoglobin levels due to stimulation of progenitor cells (Mohapatara *et al*., 2010). Previous reports suggests that punarnava and ashwagandha plays important role in treatment of anemia. (Lokhande *et al*., 2010; Rajputa *et al*., 2011). Therefore we can

conclude that all constitute present in herbomineral formulation participates in improvement of hematological parameters.

In the present investigation agar gel diet and phlebotomy induced high total iron binding capacity reduced transferrin saturation and total serum iron. It is in correlation with previous studies which reported high total iron binding capacity, reduced transferrin saturation, serum iron level (Pandit *et al*., 1999). It is reported that bhasma who contributed role in prevention of iron deficiency anemia improves total iron binding capacity and transferrin saturation (Santosha *et al*. 2011). Lokhande *et al*. (2010) reported that punarnava is excellent in improvement of blood count and quality of blood by removal of toxins. It has been also reported that aswagandha is high in iron and has been shown to help increase hemoglobin levels and red blood cell counts (kulkarni *et al*., 2008). Thus, the improvement in anemia may be due to presence of aswagandha in herbomineral formulation.Thus we can conclude that herbomineral formulation plays important role in maintenance of iron homeostasis in iron deficiency anemia.

7. CONCLUSION

Formulation of root powder of *A. sinensis* showed beneficial effect on anemia associated with chronic inflammatory disease such as asthma induced by ovalbumin which accomplished through improvement of haematological and biochemical parameter like blood cell count, hematocrit, haemoglobin, reticulocyte , ferritin, total serum iron, iron binding capacity, transferin saturation. The formulation showed modulation of haematological and biochemical parameters indicates that *A. sinensis* may produce its effect by interaction with hepcidin which is a negative regulator of intestinal iron absorption acting on inflammatory pathway.

The herbomineral formulation also showed modulation in haematological and biochemical parameters in animal model of iron deficiency anemia. Modulations on all these parameters indicate that it may be beneficial in preventing iron deficient anemia by releasing iron storage. These beneficial effects may be due to presence of constitute present in formulation. Research on herbal medicines is necessary to improved utilization by the public would benefit from strong scientific evaluation. Very limited scientific data are available on herbomineral and herbal medicines with respect to efficacy in animal models. From above experiments we can conclude that these formulations from herbal origin are efficient for the management of anemia with and without inflammation.

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