EVALUATION OF *AYABRINGARAJA PAANIDHAM* IN THE MANAGEMENT OF *VELUPPU NOI* (ANAEMIA)

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DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that the thesis entitled "EVALUATION OF *AYA BRINGARAJA PAANIDHAM* IN THE MANAGEMENT OF *VELUPPU NOI* (ANAEMIA)" submitted for the degree of Doctor of Philosophy in Siddha by Dr.V.VIJAYAKUMAR is the record of research work carried out by him during the period of 2011-2015 at National Institute of Siddha, Chennai, Siddha Central Research Institute, Chennai and Siddha Regional Research Institute, Puducherry, under my guidance and supervision and this work has not formed the basis for the award to the candidate of any degree, diploma, associateship, fellowship or other similar titles. This thesis represents the independent work done by him under my supervision and guidance.

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DECLARATION BY THE CANDIDATE

This is to certify and declare that the thesis entitled **"EVALUATION OF *AYA BRINGARAJA PAANIDHAM* IN THE MANAGEMENT OF *VELUPPU NOI* (ANAEMIA)"** submitted for the degree of Doctor of Philosophy by me is the record of research work done by me during the period of 2011-2015 at National Institute of Siddha, Chennai, Siddha Central Research Institute, Chennai and Siddha Regional Research Institute, Puducherry, under the guidance of **Prof. Dr. M. LOGAMANIAN** and has not formed the basis for the award to the candidate of any degree, diploma, associateship, fellowship or other similar titles.

Date:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABP	Aya Bringaraja Paanidham
ALP	Alkaline Phosphatase
ALT	Alanine transaminase
ANOVA	Analysis Of Variance
AOAC	Association Of Analytical Communities
AST	Aspartate Aminotransferase
AYUSH	Ayurveda, Yoga, Unani, Siddha, Homeopathy
CCl_4	Carbon tetra chloride
g	Gram
Hb	Haemoglobin
IDA	Iron Deficiency Anaemia
L	Litre
LFT	Liver Function Test
MAO	Monoamine Oxidase
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
ml	millilitre
µl	microlitre
µg	microgram
µm	micrometre
ppm	parts per million
RDA	Recommended Daily Allowance
RDW	Red cell Distribution Width
RFT	Renal Function Test
SSM	Siddha System of Medicine
sTfR	Soluble Transferrin Receptor
TIBC	Total Iron Binding Capacity
UIBC	Unsaturated or Latent Iron Binding Capacity
CFU	Colony Forming Unit
cm	centimeter
mm	millimeter

W	Watts
meV	millielectron volt
eV	electron volt
ms	millisecond
s	second
kg	kilogram
hr	hour
dl	decilitre
IU	International Units
WHO	World Health Organization
nm	nanometer
ng	nanogram
SGPT	Serum Glutamate-Pyruvate Transaminase
SGOT	Serum Glutamic Oxaloacetic Transaminase
OR	Odds Ratio
CI	Confidence Interval
SD	Standard Deviation
SEM	Standard Error of Mean
ISO	International Organization for Standardization
UV	Ultra-Violet
USFDA	United States-Food and Drug Administration
BAM	Bacteriological Analytical Manual
GMP	Good Manufacturing Practice
v/v	volume by volume
m/v	mass by volume
r.d	relative density
ESR	Erythrocyte Sedimentation Rate
cu. mm	cubic millimeter

1. INTRODUCTION

Nutritional deficiency diseases affect the humankind with adverse impacts on their socio-economic status. Even with greater advancements in biomedical research, certain diseases are riddled with inconsistent treatment modalities and difference of opinion among practitioners or researchers. Anaemia pertains to be one such disease condition with multi-faceted treatment modalities. Globally, anaemia remains as a persistent health problem due to various known and unknown factors. It is one of the common public health problems that indirectly affects the socio-economic status of populations in both developed and developing countries. Although the primary cause is iron deficiency, more frequently it coexists with a number of other causes, such as malaria, parasitic infections, malnutrition and haemoglobinopathies. Iron deficiency affects at least one third of the world's population or two billion persons and is therefore, a critical nutritional problem worldwide.

Iron deficiency anaemia is considerably more prevalent in the developing countries than in the industrialized world (36%-or about 1400 million persons-out of an estimated population of 3800 million in developing countries, versus 8%-or just under 100 million persons out of an estimated population of 1200 million in developed countries). Africa and South Asia have the highest overall regional prevalence rates.(1)

In general, if iron deficiency is the main etiological factor, additional iron intake is usually provided through iron supplements to vulnerable groups, in particular pregnant women and young children. Weinberg (1984) in his review has proposed that iron administration itself might predispose a person to infection.(2) There are some experimental evidences to suggest that iron-binding proteins protect animals from infection by withholding iron from the invading organisms that require it for growth. This phenomenon would explain why the administration of large doses of iron by injection could be harmful. In routine clinical practice, any patient suspected of being anaemic is tested for and, if the presence of anaemia is confirmed, they are treated with medicinal

iron supplements. Treatment generally depends upon medicinal iron supplements, since dietary changes alone cannot significantly correct iron deficiency anaemia. Even today, general treatment for anaemia is the oral administration of ferrous fumarate, gluconate or sulfate and parenteral administration being reserved for patients who are completely intolerant of oral iron. Blood transfusion is sought only in most severe cases (i.e. haemoglobin concentration less than 3 g/dl). There are many iron-containing syrups and liquid preparations commercially available particularly in paediatric care. Usually they are expensive, deteriorate in storage, and contain minerals and vitamins that are unnecessary for most patients. But liquid formulations are of much use when the patients are averse to solid drugs or unable to swallow. Elemental iron thus used in many forms is poorly absorbed in most cases and also cause certain side effects like constipation, gastric irritation, nausea etc.

In Indian traditional systems of medicine several combinations of herbal drugs with iron supplements are in practical use which are proven to be safe and comparatively effective. Based upon these basic concepts, modern phytochemistry incorporated active principles in such formulations. For example, since ascorbic acid is a known enhancer of iron absorption, it has been incorporated into many iron preparations. When enough is added (at least 200 mg) ascorbic acid increases medicinal iron absorption by about 30%.(3) But later it was however proved by Hallberg et al (1966) and Mathan et al (1979) that not only is ascorbic acid relatively expensive, but also it increases the frequency of side-effects and thus higher the risk of poor compliance.(4,5)

In current status, the Biomedicine finds it very difficult to treat anaemia not only because of unpleasant taste and side effects of elemental iron based formulations but also due to poor understanding of their mode of action. Thus the contemporary treatment modules mostly fail in the management of anaemia, which can be holistically treated by traditional medical systems. Rational thinking over the basic principles of Siddha System of Medicine (SSM) and its application on pathogenesis of the disease and pharmacological aspects of any drug will definitely solve this problem.

SSM is important among ancient Indian traditional systems of medicine and dates back to a period of minimum five thousand years developed by Siddhars. Siddha system is known and has also gained popularity in South India and among Tamil speaking regions of the World. Siddha Medicine classifies disease and disorders into 4448 types. Its *Materia Medica* mentions about 64 commonly prescribed types of dosage forms (32 types of internal and 32 external medicines/ therapies). *Siddhars* also developed the knowledge of converting inorganic substances into organic or inorganic complexes, in atomic and ionic form. For this they have used certain medicinal plants and alchemical techniques like *putam* (a traditional method of calcination) that may be easily absorbed by the body at molecular levels. As mentioned earlier, the application of Siddha basic concepts of *trithodam* in contemporary medicine may pave way for effective management of anaemia.

Siddha scriptures elaborate different signs and symptoms of *veluppu noi* which shall be correlated with that of anaemia. Many studies in the recent past have validated herbal remedies in the management of various types of anaemia. In particular, iron deficiency anaemia (IDA) is effectively treated with herbal supplements or herbo mineral iron complexes, broadly dealt in SSM, in current practice. Though the efficacy of SSM is obvious from the routine clinical practice, there are notable drugs which are to be explored scientifically paving a way for the optimal use of these therapeutic sources, meanwhile to serve for the betterment of mankind.

The *Materia Medica* of SSM deals with drugs of plant, mineral, metal or animal origin. Speciality of Siddha lies in conglomeration of herbs, herbo-mineral or herbo-metallic formulations. *Aya Bringaraja Paaanidham* (ABP) is an iron based formulation mentioned in the text, that belongs to the *manapagu* group among 32 types of internal medicines. Since the ingredients in this formulation are proven for their hematinic activity and also possess activities like laxative, carminative, digestive, anthelmintic, etc., ABP was selected and the study was planned to scientifically evaluate the herbo-metallic siddha formulation, ABP.

2. AIM AND OBJECTIVES

AIM

The aim of this study is to evaluate the safety and efficacy of a Siddha herbo-mineral drug – *Aya Bringaraja Paanidham* (ABP) in the management of *Veluppu noi* (Anaemia).

OBJECTIVES

- To review the Siddha and Modern Literatures with respect to *Veluppu Noi* (Anaemia).
- To prepare the classical formulation, ABP as per the scriptures of Siddha system and establish the physico-chemical characterization of ABP.
- To evaluate the safety and efficacy of the traditionally prepared ABP in experimental animal models.
- To evaluate the clinical efficacy and safety of ABP in human through a open label prospective clinical study.

3. REVIEW OF LITERATURE

SIDDHA ASPECTS OF ANAEMIA

In Siddha system of medicine, all the systemic diseases are classified under three major categories :

I. Based on the vitiation of three humors (Vali, Azhal, Iyam)
II. Based on the predominant symptoms
III. Based on the line of treatment

Siddha System of Medicine (SSM), besides the above classification of various diseases, also describes their total numbers as 4448.(6) "*Veluppu Noi*" is one among the diseases classified based on the symptoms which literally means the pallor that can be exactly correlated with modern classification of anaemia. A detailed description of signs and the symptoms, etiological factors and their management are found to be described in detail in literature. As per siddha text, this clinical entity is classified into six types: four, based on vitiation of humours, one under toxic anaemia and one due to consumption of ash and soil (Pica).

Etiology

According to the ancient Siddha scripture, "Yugi Vaithiya Chinthamani," the word *Veluppu* or *Paandu* literally means 'pallor'. (7) In this clinical condition the conjunctiva, tongue, nail bud turn into pallor and thus termed as *Paandu Noi* or *Veluppu Noi* or *Venmai Noi* in the scriptures. The etiological clusters, like nutritional deficiency, haemorrhages, worm infestation and other secondary causes like tuberculosis, chronic sprue and the diseases like piles, menorrhagia are also described in the scriptures. Some of the major etiological factors of the disease include:

- Excessive intake of salt, sour foods, mud, ashes, toxic drugs
- Haemorrhagic conditions like Mennorrhagia (*Perumpaadu*), Hypertension (*Pithathikkam*), Haemorroids (*Moolam*), Haematemesis (*Kuruthivaandhi*)
- Worm Infestations
- Hepatic disorders

The disease commonly affects young children, adolescent girls, women, pregnant and lactating women. The prevalence of anaemia is reported to be higher in *Kurinchi thinai* (Hilly tract) compared to other types of land as mentioned in siddha scriptures. Among the six main classification of seasons, *Elavenil* (mid-April to mid-June) and *Muthuvenil* (mid-June to mid-August) aggravates the disease. (6)

Signs and Symptoms

The premonitory symptoms of *Veluppu noi* are due to dietary changes leading to vitiated *Azhal* that affects the color and consistency of the blood, which will prevent the proper supply of nutrients to the body, thus leading to change of body colour to pale. Dyspnoea while walking even small distance and weakness of the lower limbs, anorexia, nausea, giddiness, blackouts, frequent fainting, palpitation and emaciation are major symptoms.The general symptoms of this disease as per Siddha scriptures include: weakness of the body, headache, palpitation, blackouts, giddiness, fainting, dyspnea, anorexia, vomiting, pallor, shrinkage of the skin, emaciation and shining of the body, clubbing, fissures, redness and softening of the tongue etc. (8)

Siddha Classification of Anaemia

As stated earlier, *Veluppu noi* is classified into six types. Based on humoral pathology, there are four types of anaemia, described as follows:

- *Vali Paandu*
- *Azhal Paandu*

- *Iyya Paandu*
- *Mukkutra Paandu*.

Based on Toxaemia, only one type termed *Nanju Paandu* is mentioned to describe anaemia due to consumption of toxic materials. The symptoms described in *Nanju Paandu* as per Siddha text, may be easily correlated with toxic anaemia which is described in modern sciences. Haemolytic anaemia, thalassemia can be grouped under this classification. In addition, *Mannun Paandu* is another type caused by consumption of ash, mud, etc., which induces worm infestations leading to anaemia and dropsy is also mentioned. (7)

The main symptoms related to each types of *Paandu* and the text describing the specific types are summarized in the Table 3.1.

Table 3.1: Classification of types & symptoms of *Paandu noi*

Classification based on	Type of *Paandu*	Associated Symptoms
Humoral pathology	*Vali*	Anorexia, stomach ache, thirst, blackish discoloration of blood vessels, redness of eye, constipation, pallor and anasarca
	Azhal	yellowish discoloration of body, pallor of tongue, hand and foot, eye vision diminished, thirst, dyspnea and giddiness
	Iya	Whitish coloration of body, pylo erection, coughs with expectoration, syncope and low backache
	Mukkutra	Dyspnea, bronchial asthma, frequent micturition, sneezing and anasarca
Toxemia	*Nanju*	Excessive thirst, vomiting, hiccough, cough and anasarca
Others	*Mannun*	Flatulence, indigestion, vomiting and diarrhea

The following quotes from Yugi Vaithiya Chinthamani (7) describes the types of *Paandu:*

வளி வெளுப்பு நோய்

“கொள்ளவே வாதபாண்டு ரோகம் கேளாய்
குடல்புரட்டி யடிவயிறு தான்வ லிக்குந்
தள்ளவே தாகமொடு பசியு மில்லை
தழலான சரசாப் பாகத் தானும்
நள்ளவே நரம்பெல்லாங் கறுப்பு மாகும்
நடுக்கலொடு கண்சிவப்பு மலபந் தந்தான்
விள்ளவே தலைவலிக்கு மேனி வீங்கும்
வெளுப்பாகும் வாதத்தின் பாண்டு வாமே. - 522

அழல் வெளுப்பு நோய்

வாமென்ற மேனியெலா மஞ்ச ளித்து
மகாவெளுப்பு உண்டாகி மந்தக் கண்ணாந்
தாமென்ற தாகமொடு மூர்ச்சை யாகுந்
தனிவாயில் மிளகுபோற் றானு றைக்கும்
நேமென்ற நெஞ்சுமுள் தானு முண்டாய்
நெருக்கியே மூச்சுமுட் டதுவே யாகுங்
கோமென்ற கிறுகிறுத்து வாய்கைப் பாகுங்
கிளர்பித்த பாண்டுவெனக் கூற லாமே. - 523

ஐய வெளுப்பு நோய்

கூறியதோர் நரம்புதோல் மிகவெ ளுப்பு
கிளர்நாவு உப்புறைக்கு மயிர்க்கூச் சாகும்
வாறியதோர் வாந்தியாங் குரலுங் கம்மும்
மகத்தான தும்மலுடன் கோழை யாகும்
ஈறியதோ ரிருமலொடு மயக்க முண்டா
மிடுப்பசதி யிந்திரிய நட்ட மாகுஞ்
சீறியதோர் சோபமொடு தாப மாகுஞ்
சிலேட்டுமத்தின் பாண்டென்னச் செப்பலாமே. - 524

முக்குற்ற வெளுப்பு நோய்

செப்பவே அருசியோடு சோப தாபம்
செயலான சுவாசமொடு இளைப்பு மாகும்
வெப்பவே மோகனத்தில் நீர்தான் வீழும்
மிடுக்கான பெலவீன மார்பி டித்தல்
துப்பவே சூட்டோடு தியக்க மாகுந்
தும்மலா யுடம்பெங்கு மூதிக் காணுந்
திப்பவே தேகமெங்கு மசதி யாகுந்
திரிதோடப் பாண்டெனச் செப்பு நூலே. - 525

நஞ்சு வெளுப்பு நோய்

நூலாக நலத்தோடே யுடல் வெளுக்கும்

நோய்நரம்பு சூடாகுந் தாக மாகும்

ஆலாக அருசியொடு சர்த்தி விக்கல்

அதட்டியே இருமலுட னதிசு வாசம்

வாலாக மேனியெங்கும் மிகவே ஊதல்

விடப்பாண்டு அசாத்யமென்றே விளம்ப லாமே. - 526

Pathogenesis of *Veluppu noi*

In *Veluppu noi*, the changes in *Mukkutram* are due to alteration in digestion which leads to derangement of *Ranjaga pitham,* followed by derangement of *Vyaanan* and *Kapham*. Generally, the *Naadi nadai* observed in this disease are *Kapham, Kapha vatham, Pitha vatham* and *Kapha pitham*.

Line of treatment

First priority in the line of treatment is to alleviate the deranged *Trithodam*. Second is to stimulate appetite and later improve blood.(8) Treatment in the SSM by administering iron based formulations orally is in common practice. Herbomineral formulations like Kadukkai mathirai, Ayabringarajakarpam, Ayachenduram etc., commonly used in Siddha system of medicine contain traditionally processed iron. Kantha chenduram (calcined oxide of magnetite) is a popular siddha preparation indicated for microcytic anaemia, anaemia, chlorosis, obesity, edema, scrotal swellings and rheumatic diseases, enlargement of liver and spleen and even abdominal tumors.

From a recent unpublished data, Annabedhi chenduram and Vediannabedhi chenduram, used in Siddha system of medicine, are validated for efficacy with multi-centric open clinical trials conducted by Department of AYUSH, Government of India.

Pararasasekaram, a classical siddha text, describes the symptoms related to anaemia under the classification *Vellai pitham / Sokai pitham.* (9) It has mentioned about the main clinical symptoms like dropsy, pallor, headache, vomiting, increase body temperature, pain in all bone joints, anorexia, loss of appetite, etc.

வெள்ளைப் பித்தம் அல்லது சோகைப் பித்தம்

மறஞ்செயும் வெள்ளைப் பித்த மயக்கிடுஞ் சோப மாகும்
நிறங்குன்றித் தலைவ லித்து நின்றுநின் றுவாந்தி செய்யும்
உறுங்கைகால் கனன்று வெப்பாம் பொருத்தெலா மூதைவிம்மும்
புறந்தரு சோகைப் பித்தம் பொருதிடுங் குணங்க டாமே. - 19

காலொடு வயிற்றி லுற்றாற் காமொடு கண்கள் தாழும்
சாலவே சிரத்தில் நொந்து தன்னுடல் கொள்ளாதன்னம்
மேலது வருந்தி நொந்து மின்னென மயங்குங் கண்ணும்
ஆலவேல் விழியி னாளே யறிந்துகொள் பித்தமாமே. - 20

The famous Tamil-English Dictionary of Medicine compiled by T.V. Sambasivam Pillai and published by Govt. of Tamil Nadu in 1994 defines *Pandu* as follows (10): "பாண்டு, dropsy which is generally defined as the abnormal accumulation of the serous fluid in the tissues or a body cavity. It consists of swelling arising, from the escape of watery fluid of the blood through the coats of the vessels to the surrounding parts due to the impediment to the circulation of the blood, causing stagnation of that fluid. The usual position of the dropsy is the leg and the lower extremities. It may even extend to the abdomen, in some cases (heart, lungs) chest and the face or the entire body.

The malady is recognized by pressing the affected part with finger when depression is left which only gradually fillsup. Various are the causes ascribed to it viz. exposure to cold or sitting or lying in a drought of cold air, from diseases of kidneys, heart on lungs, liver or spleen. It may also be due to other diseases as congenital or acquired diseases, syphilis, anaemia, chronic drunkeness, etc. in females from menstrual disorders, the ovarian tumor giving rise to gradual distension of the parts affecting the womb;

The author of this book described பாண்டு (pandu) as dropsy. In my opinion Pandu (பாண்டு) which means only வெண்மை (white or paleness) refer merely to anaemia where the patient turns pale or white."

Selection of Drug for the present study

Aya Bringaraja Paanidham (ABP)

A preliminary survey was done based on selected siddha literatures, interview with traditional siddha practitioners and experts. It was observed that much number of formulations of plant origin and metallic origin (especially iron based) are in common use currently.

The test drug *Aya Bringaraja Paanidham* (ABP) is a classical siddha formulation not of enough common use, till now scientifically unexplored for its efficacy and safety. It is a unique combination of herbs and iron prepared by traditional method. Since the formulation belongs to the *agamarunthu* category (internal medicine) and contains a balanced combination of herbs, processed iron, palm jaggery, honey, etc., it should be palatable to all age groups. The common dosage forms mentioned in the siddha scriptures deals with "paanidham" as "manapagu" or syrup. The word "Paani" is supposed to be of Sanskrit origin, but it is also mentioned in Tamil-English Dictionary (T.V. Sambasivam Pillai, 1994) as follows (11):

பாணி = இலைச்சாறு, சர்க்கரைப்பாகு (any syrup, leaf juice)

பாகுவண்டல் (sediment of sweet syrup)

பாணிதம் = கருப்பஞ்சாறு (sugarcane juice); குழம்புப்பாகு (a treacle boiled down to a sticky consistent).

Review of ingredients in the formulation

Iron in medicine

According to modern system of medicine the current usage of iron is first reported in 1681 by Sydenham. But only in third and fourth decades of nineteenth century iron was started to be used commonly.

P C Ray in his book, History of Hindu chemistry, states that *Siddhars* flourished even before 7^{th} or the 6^{th} century B.C. and the use of iron and other metals in SSM is grossly traced back to that period. (12) Iron is commonly known as *Ayam* in Siddha literatures. It is considered to be one of the *Pancalogam* (group of five basic metals). The native metal is very rare. But most of the iron is found in the form of oxide, in ochres, bog-ores & friable earthy substances. It is generally obtained by melting black sand and pebbles containing iron.

It is in grey colour with a strong metallic lustre, susceptible of being heightened by polishing and exceeds all metals in tenacity. Iron obtained by smelting is not pure, except in the 3 following states - white crude iron, grey crude iron and black cast iron. It is generally obtained from the mines and then smelted.

The purified or bar iron is soft, ductile, flexible and malleable and the same is converted into steel by being exposed to the action of heat and by being tampered when red hot by immersion in water. It exists in several forms and the descriptions from Indian Pharmacopoeia articles are numerous such as Iron dross or Oxidised iron, iron rust, Iron sulphate or green vitriol, Iron filings, Iron pyrites with a golden lustre, Iron pyrites with

silvery radiated crystals, Haematite, magnetic iron ore, protocarbonate of iron etc. (14) The same dictionary also describes that iron possesses medicinal use.

இரும்புப்பொடி, அரப்பொடி – iron filings: It is medicinally used as a tonic, emmanagogue and anthelmintic.(14) A compilation of drugs used in siddha, the Siddha Materia Medica – Mineral and animal kingdom by R.Thiyagarajan (1992) describes the details of iron including its properties, identification by traditional methods, formulations, etc. (15,16,17)

The following details are reproduced from the compilation.

Synonym – Tamil :

அகி, அயசு, அயில், இடி, இரும்பு,ஈசசெயம், கருங்கொல், கருப்பி, கரும்பி, கரும்பு, கருப்பு, கருமணல், கரும்பொன், கயசு, இருணவையம், காலில் நெகிளம், ஆதி, சத்து, சிநோசரம், சிட்டம், திரும்பி, துண்டம், பிண்டம், பொன்மணல்,லோகம், வாழ்பூமிநாதம், கருந்தாது.

Agi, Ayasu, Ayil, Idi, Irumpu, Issacheyam, Karunkol, Karuppi, Kariumpi, Karumphu, Karuppu, Karumanal, Karumpon, Kayasu, Krishnavayam, Kalilnegilam, Adhi, Chathu, Cinosaram, Chittam, Thirumphi, Thundam, Pindum, Ponmanal, Logam, Vazhbhoominatham, Karunthathu.

Taste : Predominantly Astringent

Potency : Hot

Action : பசியுண்டாக்கி - Digestive stimulant , உடல் உரமாக்கி - Tonic, குருதிபெருக்கி - Hematinic , உடல் தேற்றி- Alterative.

பொதுக்குணம் (Properties):

“பாண்டுவெண் குட்டம் பருந்தூல நோய்சோபை

மாண்டிடச் செய் மந்தங்கா மாலைகுன்மம் பூண்ட

பெருந்தாது நட்டமும்போம் பேதிபசி யுண்டாங்

கருந்தாது நட்டமிடுங்காண்”

Deficiency of iron results in diseases like *Pandu* (Anaemia), *Venkuttam*(Lecuoderma), *Barunthoolanoi* (Ascitis), *Cobi* (Dropsy), *Kamalai* (Jaundice), *Kunmam* (Ulcer), *Kalichal* (Dysentery), *Pasi* (Appetite), *Thoonduthal* (Stimulating the organs for effective functioning).

The therapeutic use of iron in SSM through many centuries is evident from the ancient Siddha literatures. Iron based formulations are commonly used for disease conditions like anaemia, jaundice, ascitis, etc. Some of the siddha formulations prepared from iron are Aya parpam, Aya chenthuram, Aya veera chenthuram, Aya kantha chenduram, Aya melugu, Aya bringaraja panitham, Aya jambira karpam, Aya bringarajakarpam, etc.

Physical properties of mineral specimens

Generally, *ayam* used in SSM is a mineral complex or natural oxide ore of iron. Iron is the second most abundant metallic element in the Earth’s crust and accounts for 5.6% of the lithosphere. The principal minerals of iron are the oxides (haematite and magnetite), hydroxide (limonite and goethite), carbonate (siderite) and sulphide (pyrite). Iron, like most other metals, is found in the Earth's crust only in the form of an ore, i.e., combined with other elements such as oxygen or sulphur. Haematite and magnetite are the two important iron ores from which iron is extracted.

Geological Setting

India has large reserves of good quality iron ore. These iron ores occur in different geological rock groups/ formations in different time domains but the largest concentration of economic deposits are found associated with volcano-sedimentary Banded Iron Formation (BIF) of Precambrian age. The BIF, mainly comprising of banded haematite quartzite / banded haematite jasper (BHQ/ BHJ) contains iron in the range of 25 – 40%. Magnetite dominant deposits are generally associated with banded magnetite quartzite (BMQ) and contain about 25 – 40% iron. These magnetite ores are often utilized by appropriate beneficiation, making the ores suitable for the consumer industries.**(**18-22)

Genetic Type

On the basis of mode of occurrence and origin, the iron ore deposits of India are divided into five groups; viz. (i) Banded Iron Ore Formation, (ii) Sedimentary iron ore deposits of sideritic and limonitic composition, (iii) Lateritic ores derived from sub-aerial alteration of gneiss, schists etc., (iv) Titaniferous and Vanadiferous magnetite deposits and (v) Fault and fissures filling deposits. Amongst these, the larger deposits are from the Banded iron ore formation of Precambrian age followed by Titaniferous and Vanadiferous magnetite deposits.

Geological distribution and brief description of deposit

Haematite and magnetite are the most prominent of the iron ores found in India. The iron ores occurs in different geological rock groups/ formations in India but the largest concentration of economic deposits are found associated with volcano-sedimentary Banded Iron Formation (BIF) of Precambrian age. About 60% of haematite ore deposits are found in the Eastern sector and about 80% magnetite ore deposits occur in the Southern sector, especially in Karnataka. Large resources of low grade magnetite ores occur in Karnataka, Goa, Tamil Nadu, Rajasthan and Andhra Pradesh. Karnataka has the highest resources of magnetite ore.

Each mineral is different in its composition and varies by chemical formula. The following enlists the important iron ores available in India:

1. Magnetite - $Fe_3 O_4$
2. Ilmentie - $FeOTiO_2$
3. Hematite - Fe_2O_3
4. Siderite - $FeCO_3$
5. Pyrite - FeS_2
6. Wad Marcasite - $(Mn, Fe) O_2$
7. Columbite/ Tantalite - FeS_2
8. Pyrrhotite - $(Fe, Mn) (Nb, Ta)_2O_6$
9. Pyrrhotite - Fe_5S_6

Magnetite

Magnetite is one of the most ubiquitous minerals, occurring in a great variety of igneous, metamorphic and sedimentary rocks, typically as disseminated crystals or grains comprising less than one percent of their host rock. Most plutonic igneous rocks commonly contain magnetite as disseminated grains. In some igneous melts, magnetite grains may concentrate by magmatic segregation thereby becoming the main or only constituent, thus forming ore bodies of economic importance.

Magnetite often occurs in metamorphic rocks which are formed from ferruginous sediments in both regional and contact metamorphic settings, by the reduction of hematite and ferric hydroxide minerals. Banded Precambrian iron formations commonly contain magnetite. Magnetite also can form from volcanic gases, and from high temperature hydrothermal sulfide solutions, and has also been found in some meteorites. Magnetite detrital grains are commonly the main constituent of black sands. Magnetite is characteristically recognized by its strong magnetism, color and streak.

Hematite

Hematite occurs in many types of igneous, metamorphic and sedimentary rocks. The largest and most economically important hematite deposits are mainly of sedimentary origin, forming from the weathering of iron bearing minerals. In these sedimentary deposits, hematite is thought to have precipitated from lakes or seas by organic and/or chemical processes. The hematite often occurs with intermixed layers of quartz or chert (sometimes as the chert variety jasper).

Hematite often occurs in metamorphic rocks which are formed from ferruginous sediments, and in contact metamorphic deposits. In igneous rocks hematite occurs as accessory grains in granite. It also occurs as euhedral crystals associated with quartz deposited by volcanic gases and by high temperature hydrothermal solutions. Hematite also occurs as inclusions in a variety of other minerals, sometimes forming highly valued gem and lapidary materials such as sunstone, which is a transparent gem variety of plagioclase feldspar which can contain minute hematite or goethite inclusions which reflect light creating a sparkling sheen. Hematite is characteristically recognized by its red streak and habit.

DRUGS OF PLANT ORIGIN USED IN *ABP*

i) Kadukkai - *Terminalia chebula* Retz.

Katukkai consists of the pericarp of mature fruits of *Terminalia chebula* Retz. (Fig. 3.1)

(Fam. Combretaceae), a moderate sized or large tree found throughout India, chiefly in deciduous forests and areas of light rainfall, but occasionally also in slightly moist forests, upto about 1500 m altitude, throughout India; flowers appear from April-August and fruits ripen from October-January.

Part used **:** Fruit (Pericarp)

Synonyms

Tamil : Ammai, Amutam, Aritaki, Pattiyam

Sanskrit : Abhaya, Haritaki

English : Myrobalan

Hindi : Harre, Harad, Harar

Kannada : Alalekai

Malayalam : Katukka

Telugu : Karaka, Karakkaya

Constituents- Tannins, anthraquinones and polyphenolic compounds.

Properties and Action

Cuvai : Mainly *Thuvarppu* (Astringent), slightly *Inippu* (Sweet), *Kaarppu* (Pungent), *Kaippu* (Bitter) and *Pulippu* (Sour)

Gunam : Ilaku, Varatchi

Virium : Veppam

Pirivu : Inippu

Ceykai : Digestive, Expectorant, Laxative, Appetiser, Nutrient

Therapeutic uses

Kamalai, Kan noykal, Kuruti alal, Malamilakki, Peruvayiru, Vitam.

ii) Thandrikkai - *Terminalia bellerica* Roxb.

Thandrikkai consists of pericarp of dried ripe fruits of *Terminalia bellerica* Roxb.(Fam. Combretaceae), a large deciduous tree, 10-12 m or more high, commonly found in plain and forests upto 900 m altitude, fruits ripen towards November. It grows in *kurinchi* and *marutham thinai.* (Fig. 3.2)

Part used **:** Fruit (Pericarp)

Synonyms

Tamil : Thanrikkai

Sanskrit : Bibhitaka, Vibhita

English : Beleric Myrobalan

Hindi : Bahera

Kannada : Tare kai, Shanti Kayi

Malayalam : Tannikka

Telugu : Thanikkaya

Constituents- Gallic acid, tannic acid and glycosides.

Properties and Action

Cuvai : Tuvarppu (Astringent)

Gunam : Ilaku, Varatchi

Virium : Veppam

Pirivu : Inippu

Ceykai : Expectorant, Laxative, Astringent, Tonic

Therapeutic uses

Aankuri pun, Cilanti nancu, Kuruti alal

iii) Nellikkai - *Emblica officinalis* Gaertn.

Nellikkai consists of fresh fruit pulp of ***Emblica officinalis*** Gaertn. (Fam.Euphorbiaceae); a small or medium sized tree, found in mixed deciduous forests, ascending to 1300 m on hills and cultivated in gardens and homeyards. (Fig. 3.3)

Part used **:** Fruit

Synonyms

Tamil : Nellikkai, Nelli

Sanskrit : Amalaki, Amrtaphala, Dhatriphala

English : Emblic Myrobalan

Hindi : Amla, Aonla

Kannada : Nellikayi

Malayalam : Nellikka

Telugu : Usirika

Constituents- Ascorbic acid and tannins.

Properties and Action

Cuvai : Inippu (Sweet), Pulippu (Sour), Tuvarppu (Astringent)

Gunam : Ilaku, Varatchi

Virium : Tatpam

Pirivu : Inippu

Ceykai : Diuretic, Rejuvenator, Coolant, Laxative

Therapeutic uses

Iya noi, Mayakkam, Peenisam, Piramekam, Vanti, Verinoi.

Fig.3.1 *Terminalia chebula*

Fig.3.2 *Terminalia bellerica*

Fig.3.3 *Emblica officinalis*

iv) Chukku - *Zingiber officinale* Rosc.

Chukku consists of dried rhizome of ***Zingiber officinale*** Rosc. (Fam.Zingiberaceae), widely cultivated in India, rhizomes dug in January-February, buds and roots removed, soaked overnight in water, decorticated, and sometimes treated with lime and dried. (Fig. 3.4)

Part used **:** Dried rhizome

Synonyms

Tamil : Sukku, Chukku

Sanskrit : Ardraka, Ausadha, Visva

English : Ginger root, Ginger

Hindi : Sonth

Kannada : Shunthi

Malayalam : Chukku

Telugu : Sonthi, Sunti

Constituents–Gingerols, shogaols, dihydrogingerol, gingerdione, lipids, proteins, fats, waxes and starch.

Properties and Action

Cuvai : Karppu (Pungent)

Gunam : Ilaku, Noimai

Virium : Veppam

Pirivu : Karppu

Ceykai : Carminative, Appetiser, Heat enhancer.

Therapeutic uses

Ceriyamai, Irumal, Gunmam, Nencerippu, Paciinamai, Talai vali, Vata kunmam, Yeppam.

v) Thippili - *Piper longum* Linn.

Thippili consists of the dried, immature, catkin-like fruits with bracts of ***Piper longum*** Linn. (Fam. Piperaceae), a slender, aromatic climber with perennial woody roots, occurring in hotter parts of India from central Himalayas to Assam upto lower hills of West Bengal and ever green forests of Western Ghats as wild, and also cultivated in Northeast and many parts of the South. (Fig. 3.5)

Part used **:** Fruit, Stem

Synonyms

Tamil : Arisi Tippali, Thippili

Sanskrit : Pippali, Kana, Magadhi, Krsna

English : Long Pepper

Hindi : Pipar

Kannada : Hippali

Malayalam : Pippali

Telugu : Pippalu

Constituents- Essential Oil and Alkaloids

Properties and Action

Cuvai : Kaippu (Bitter), Tuvarppu (Astringent)

Gunam : Ilaku, Noimai

Virium : Veppam

Pirivu : Inippu

Ceykai : Carminative, Heat enhancer.

Therapeutic uses

Cuvaiinamai, Iraippu, Irumal, Iyappini, Kan, Kathu, Mooku noigal, Gunmam.

vi) Milaku - *Piper nigrum* Linn.

Milaku consists of fully mature dried fruit of ***Piper nigrum*** Linn. (Fam. Piperaceae); a climber, cultivated from Konkan Southwards, especially in North Konkan Kerala, and also in Assam; fruits ripen from December to March, depending upon climatic conditions; fruits harvested from December to April. (Fig. 3.6)

Part used **:** Fruit

Synonyms

Tamil : Milagu, Kari, Maricam

Sanskrit : Vellaja, Maricha, Usana

English : Black Pepper

Hindi : Kalimirch

Kannnada : Karimonaru, Menaru

Malayalam : Karumulaku

Telugu : Miriyalu, Marichamu

Constituents- Piperine, Chavicine, Piperidine, Piperetine, pipercide and essential oils.

Properties and Action -

Cuvai : Kaippu (Bitter)

Gunam : Ilaku, Koormai, Varatchi

Virium : Veppam

Pirivu : Karppu

Ceykai : Carminative, Acrid, Periodic febrifuge, Antidote, Anti Vatha, Heat enhancer, Deobstruent

Therapeutic uses

Alal noi, Ceriyamai, Suram, Suvaiinmai, Kalalai, Timirvatham, Vali noi.

vii) Karisalangani - *Wedelia calendulacea* LESS. (Syn. *W.chinensis*)

This herb is found abundantly throughout India in wet places. It has yellow flowers. (Fig. 3.7)

Part used **:** Herb-roots and leaves.

Synonyms

Tamil : Pottralai kaiantagarai

Sanskrit : Bhringaraj, Kesharaj

Hindi : Bhungra, Phila bhangra

English : Chinese wedelia

Kannnada : Gargari

Malayalam : Kadal-kayyonni,Mannakkannunni

Telugu : Guntagalagara

Constituents **:** Wedelactone

Action **:** Cholagogue, emetic, tonic.

Therapeutic uses

Jaundice, liver disorders, premature graying of hair.

Fig. 3.4: *Zingiber officinale Rosc.*

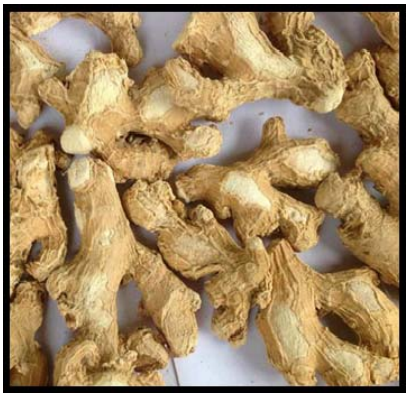

Fig. 3.5: *Piper longum Linn.*

Fig. 3.6: *Piper nigrum Linn.*

Fig. 3.7: *Wedelia calendulacea LESS.*

REPORTED BIOLOGICAL ACTIVITIES FOR THE INGREDIENTS IN *ABP*

- Dwivedi et al (2008) have reported that the alcoholic extract of *Terminalia chebula* Retz. at higher concentration of 100mg/ml was found to be exhibiting more potent anthelmintic activity in comparison with standard drug albendazole and aqueous extract of *Terminalia chebula* Retz. (23)

- Shyamkumar et al (2003) have reported that hepatoprotective compound chebulic acid and its isomer neochebulicacid has been isolated from the ethanolic extract of the fruits of *Terminalia chebula* Retz.(24)

- Tasduq et al (2006) have found that the extract of *T.chebula* prevents the hepatotoxicity caused by the administration of rifampicin, isoniazid and pyrazinamide (in combination) in a sub-chronic mode.(25)

- Sarkar et al (2012) investigated both reducing power and iron chelating activity of 70% methanolic extract of *Terminalia chebula* and showed that it can reduce the toxic level of iron in iron overloaded mice and hence protect liver from oxidative stress and fibrosis.(26)

- Saleem et al (2002) have reported that *T.Chebula* contains more phenolics than any other plant.(27)

- Pinmai et al (2010) have reported that the water extract of *T.chebula* showed antiplasmodial activity *in vitro* and *in vivo.*(28)

- Bagavan et al (2011) have reported the antiplasmodial activity of acetone seed extract of *T.Chebula.*(29)

- Chen et al (2011) have isolated an aglycone from *T.chebula,* triethylchebulate which significantly inhibited $FeSO_4$ / Cys- induced microsome lipid peroxidation and

protected both H_2O_2 induced RBCs haemolysis and RBCs auto-haemolysis in a dose dependent manner.(30)

- In a recent study, Padma V et al (2014) have reported that the *Phyllanthus emblica* fruit juice increased the dialysable iron three times more than the $FeSO_4$ alone control in the cell-free digestion model.(31)

- Shoba Benjwal (2013) has reported that if Amalaki (*Emblica officinalis)* churna is added with iron tablets, it increases its absorption and reduces the side effects in pregnant women with iron deficiency anaemia.(32)

MODERN ASPECTS OF ANAEMIA

Definition

Anaemia is defined as reduction in Haemoglobin concentration or other red cell indices in the blood. (33) Anaemia refers to a decrease in the total number of circulating red cells with decrease in haemoglobin when compared with normal for the same age group and sex. WHO criteria for anaemia are as follows:

Adults:

Adult males	-	Hb < 13g/dl
Adult females	-	Hb < 12g/dl

Infants and children:

Upto 12 years	-	Hb < 11g/dl
Pregnant women	-	Hb < 11g/dl

Severity of anaemia

Based on haemoglobin levels, the severity of anaemia is classified as follows:

- Mild anaemia : Hb 9.1-10.5g/dl
- Moderate anaemia : Hb 6-9g/dl
- Severe anaemia : Hb < 6g/dl

In severe anaemia hyper dynamic cardiac circulatory system may fail and as a result there is fluid retention leading to edema.

Manifestations of anaemia

Symptomatology of anaemia depends upon: haemoglobin, change in blood volume, severity of the disease contributing to anaemia and the rate at which the above changes occur.

- **Dyspnea:** Dyspnea and palpitation on exertion are common complaints of patients with moderate to severe anaemia.
- **Pallor:** Pallor is the most evident feature of anaemia. It is best detected in conjunctiva, mucous membrane of tongue/lips and nailbeds.
- **CNS signs:** Patients of severe anaemia may complain of headache, vertigo, tinnitus, lack of concentration and muscle weakness.
- **Glossitis:** In severe anaemia, there is atrophy of the tongue papillae, glossitis, stomatitis and fissures at the angle of mouth.
- **Cardiac features:** Tachycardia on exertion. In severe anaemia, pulse is bounding type and systolic bruits are heard over the carotid arteries.

General examination

- Pallor – conjunctival, tongue, skin
- Nails – platynchia/ koilonychias

- Jaundice
- Signs of infection/ bleeding due to neutropenia/ thrombocytopenia
- Lymphadenopathy
- Spleenomegaly
- Hepatomegaly
- Cardiac assessment for murmur
- Chest examination for tuberculosis, infection, bronchiectasis, etc.
- Assessment of kidneys for chronic renal failure

Investigations

a. **Hematologic**
   - Haemoglobin
   - Hematocrit
   - Red cell count
   - Red cell indices – MCV, MCH, MCHC
   - Reticulocyte count
   - Red cell morphology
   - Differential leucocyte count
   - Platelet number
   - Leucocyte count
   - ESR
   - Serum iron, ferritin and transferring saturation

b. **Urine analysis**
   - Color, pH, specific gravity
   - Protein, sugar, ketones
   - Bilirubin, urobilinogen
   - Haemoglobin in urine
   - Microscopic examination for red cells - hematuria

c. Stool examination

- Occult blood
- Ova and cysts

d. Other investigations

- Renal function tests: Blood urea nitrogen, Serum creatinine
- Liver function tests: Serum Bilirubin, SGOT, SGPT, Serum Alkaline phosphatase
- X-Ray chest
- Ultrasound abdomen for any lump, kidneys, spleen and liver

Classification of anaemia:

i. Morphological classification

- Microcytic anaemia – MCV < 80 fl
- Normocytic anaemia – MCV 81-99 fl
- Macrocytic anaemia – MCV > 100 fl

ii. Etiological classification

a. Anaemia due to impaired red cell production – Nutrients deficiency anaemia, Aplastic anaemia, Anaemia due to chronic disorders, etc.

b. Haemolytic anaemia due to increased red cell destruction – Hereditary, Haemoglobinopathies like Thalassemia, Sickle syndromes, Immune haemolytic anaemia, Drugs and chemicals (34-38)

IRON DEFICIENCY ANAEMIA (IDA)

Definition and history of IDA

Iron deficiency is the state in which the content of iron in the body is less than normal. Iron depletion is the earliest stage of iron deficiency, in which storage iron is decreased or absent but serum iron concentration and blood haemoglobin levels are normal. Iron deficiency without anaemia is a somewhat more advanced stage of iron deficiency, characterized by decreased or absent storage iron, usually low serum iron concentration and transferrin saturation, without anaemia. Iron deficiency anaemia is the most advanced stage of iron deficiency. It is characterized by decreased or absent iron stores, low serum iron concentration, low transferrin saturation, and low haemoglobin concentration or hematocrit value.

The clinical manifestations of iron deficiency anaemia appear to have been recognized in earliest times. A disease characterized by pallor, dyspnea, and edema was described in about 1500 BC in the Papyrus Ebers, a manual of therapeutics believed to be the oldest complete manuscript extent. This ancient disease may have been due to chronic blood loss from hookworm infestation. Chlorosis, or “green sickness,” was well known to European physicians after the middle of the sixteenth century. In France, by the middle of the seventeenth century, iron salts and other remedies like phlebotomy were used in the treatment of this disease. Later iron was recommended by Sydenham as a specific remedy for chlorosis. For about 100 years preceding 1930, iron was used in the treatment of chlorosis, often in ineffective doses, although the mechanism of action of iron and the appropriateness of its use were highly controversial. Only by the beginning of the twentieth century, it had been established that chlorosis was characterized by a decrease in the iron content of the blood and by the presence of hypochromic erythrocytes. Most of the fundamental research work on iron metabolism and iron deficiency has been carried out only during this century.

Prevalence

On a worldwide basis, caloric insufficiency, manifested as hunger, famine, starvation, appears to be the dominant nutritional problem. Iron deficiency affects at least a third of the world's population, or 2 billion persons, and is therefore, second only to hunger as a major, worldwide nutritional problem. In tropical areas, where hookworm infestation is common, iron deficiency anaemia has notable high prevalence. In India, where hookworm disease is prevalent and vegetarianism is common by religion, iron deficiency is especially common. The prevalence of iron deficiency in children varies from 35-45% in various Indian studies. Its frequency is higher in females and in both urban and rural population.

Etiology and Pathogenesis

Iron deficiency may occur as a result of chronic blood loss, inadequate dietary iron intake, malabsorption of iron, diversion of iron to fetal and infant erythropoiesis during pregnancy and lactation, intravascular haemolysis with haemoglobinuria, or a combination of these factors.Blood loss may be due to various conditions like Diaphragmatic (hiatal) hernia, Gastritis due to drug ingestion, Colon cancer, colonic diverticula, periampullary tumors, leiomyomas, adenomas, and other malignant or benign neoplasms of the intestine, rheumatoid arthritis, ulcerative colitis, haemorrhoidal bleeding, intestinal parasitism, haemoptysis, menstrual bleeding etc.

The pathogenesis involved in IDA occurs due to various etiological factors. Early iron deficiency (iron depletion) is usually not accompanied by any abnormalities in blood; at this stage, serum iron concentration is occasionally below normal values and storage iron is markedly depleted. As iron deficiency progresses, development of anaemia precedes appearance of morphologic changes in blood, although some cells may be smaller and paler than normal; serum iron concentration is usually low at this time, but it may be normal. With advanced iron depletion, classic changes of hypochromic, microcytic, hypoferremic anaemia become manifest.

Iron-containing proteins

Though the mortality rate due to IDA is very low, its health impacts especially among underprivileged women and children of under developed and developing countries are highly significant. IDA is caused due to insufficient balance between iron intake, its store and loss, eventually affecting the erythrocyte production. In human, 4 main proteins are conjugates with iron such as:

(1) Mononuclear iron proteins (e.g., superoxide dismutase)

(2) di, iron-carboxylate proteins (e.g., ribonucleotide reductase, ferritin),

(3) iron-sulfur proteins (e.g., aconitase)

(4) Heme proteins (e.g., haemoglobin)

Among these, the most abundant is the haemoglobin, containing more than one-half of total-body iron. The facts behind IDA can be unrevealed considering the concept of iron requirement for the production of erythrocytes. Erythropoiesis related demands for iron are naturally formed by three variables: tissue oxygenation, erythrocyte turnover, and erythrocyte loss from haemorrhage. Of these variables, the first two remains stable in normal adults and maintain the iron homeostasis. In a day, approximately 20 mL of senescent erythrocytes are cleared and 20 mg of iron in those cells is recycled for the production of new erythrocytes.

As the body becomes depleted of iron, changes occur in many tissues. Haemosiderin and ferritin virtually disappear from bone marrow and other storage sites. There is a decreased activity of many other important iron proteins such as: cytochrome c and cytochrome oxidase, succinic dehydrogenase, aconitase, xanthine oxidase, myoglobin. Reduced activity has also been reported for some enzymes which do not contain or require iron. Phosphocreatine content is decreased and inorganic phosphorus is increased in skeletal muscle of iron-deficient rats.Many of the affected enzymes are in the

oxidative glycolysis (Krebs) cycle of mitochondria. On the other hand, the activities of several mitochondrial matrix enzymes are increased in skeletal muscle of iron-deficient animals. (39)

Impact of anaemia

Anaemia being a global public health issue has its major consequences on socio-economic development in both developing and developed countries. Occurrence at all stages of human life primarily affecting reproductive stage women and young children forms the major characteristics of the disease. As per the WHO Global Database on Anaemia (1993–2005), in the past decade, its prevalence in Asian countries was found to be alarmingly increasing; thereby future research in all disciplines concerned to anaemia became mandatory. The report estimated the prevalence rate of anaemia in India at a severity level of > 40%. A wide range of causes for onset of anaemia coexists and the most significant factor among them is iron deficiency thus, the terms IDA and anaemia are often used synonymous, with a general assumption that 50% of anaemia cases are due to iron deficiency, but the proportion may vary among population groups. Main risk factors for IDA include low intake of iron, poor absorption of ironfrom diets high in phytate or phenolic compounds and period of life when iron requirements are especially high (i.e.growth and pregnancy). Among the other causes are, heavy blood loss as a result of menstruation, parasitic infections (hookworms, ascaris and schistosomiasis), acute and chronic infections (malaria, cancer, tuberculosis, and HIV) which can lower blood haemoglobin (Hb) concentrations. Moreover, presence of other micronutrient deficiencies, such as vitamins A and B12, folates, riboflavin and copper deficiencies can also increase its risk. Furthermore, the impact of haemoglobinopathies on anaemia and its prevalence is under main consideration in some global populations. (40)

IDA is a leading cause of morbidity and mortality worldwide. Recent advancements in the field of Bio-medicine have provided many elemental iron based formulations to treat anaemia. There is also some concern that these oral iron supplementation may lead to oxidative stress and exacerbation of inflammation. It is a

well-known fact that all iron preparations like exsiccated ferrous sulfate, ferrous gluconate, ferrousfumarate and ferrous succinate are probably equally toxic per unit mass of soluble iron. (35)

Anaemia affects 1.62 billion worldwide with its burden predominantly on Asia and Africa due to complex interplay of dietary factors, infectious disease, genetics, etc., that determines anaemia status. As per WHO reports, IDA contributes to approx. 1,20,000 maternal deaths globally; in low and middle income countries, 18% of maternal mortality is attributed to iron deficiency. (41) Women of reproductive age are physiologically more vulnerable to anaemia because of recurrent menstrual loss and due to the nutritional demands of pregnancy and repeated childbearing; global estimates suggest that the prevalence of anaemia is 41.8% among pregnant women and 30.2% among non-pregnant women.(42)

The significant increase in anaemia among Indian women during this recent period is a matter of concern, and in contrast to secular improvements in other markers of women's health and nutritional status. While socioeconomic inequalities in anaemia persist, the relative and absolute inequalities in anaemia have decreased over time. India's prevalence of anaemia among women of reproductive age has increased significantly over a recent 7-year period, even after adjustment for age, parity, wealth, education, caste and residence. Socioeconomic inequalities in anaemia (by wealth, education and caste), using both absolute and relative metrics, have decreased over time. Over the 7-year period, anaemia prevalence increased significantly from 51.3% to 56.1% among Indian women. This corresponds to a 1.11-fold increase in anaemia prevalence after adjustment for age and parity, and 1.08-fold increase after further adjustment for wealth, education and caste.(42)

Hematinic substances are essential for the proper formation of blood components. Iron being a hematinic substance is a very essential component of red blood cells and muscles that assist in the transportation of oxygen throughout the body. When the blood

is deficient in red blood cells (RBCs), in haemoglobin, or in total blood volume, it can cause a variety of complications, including fatigue and stress on bodily organs. (43)

There is no sole, reliable biochemical indicator that is consistently diagnostic of iron deficiency except the 'goldstandard', bone marrow iron aspirates. As iron status changes are sequential, we need to move beyond the current philosophy using a single assay to diagnose IDA but rather take an intentional approach that involves the systematic assessment of the underlying cause and use of multiple parameters. Current recommendations for adults with unexplained IDA include endoscopy procedures such as colonoscopy or esophagogastroduodenoscopy (EGD) and FOBT (Fecal occult blood test). The treatment modalities for managing IDA will dependon the underlying cause. Once the cause of IDA has been ascertained, either oral or parenteral iron therapy is commonly prescribed to correct the deficiency.

Hepcidin is currently highlighted biomarker in iron metabolism and considered as its key regulator; it regulates iron concentrations and tissue iron distribution via inhibition of intestinal iron absorption, iron reclamation by macrophages and iron mobilization from hepatic stores. Its production is decreased in IDA and increased during inflammation and iron overloading. The over production of hepcidin during an acute phase response results in reduced iron absorption, mobilization or both, contributing to the disease of anaemia. Kemna et al developed an algorithm to predict hepcidin levels. Results showed that soluble transferring receptor (sTfR) highly associates with erythropoietic activity that strongly interfered with the iron store regulation of hepcidin. A strong correlation between the predicted hepcidin values and the actual measured hepcidin levels was found. Despite the selected parameters used in this algorithm, each has shortcomings; the lab indices are readily available and less expensive than serum hepcidin. (44)

Iron deficiency anaemia is characterized by microcytic hypochromic red cells with MCV < 80 fl and MCH <25 pg. Morphologic changes of red cells appear as the iron stores get depleted and iron is not available in adequate amounts for Heme synthesis.

Iron absorption

Iron in the food in the presence of pepsin and low pH in stomach is broken in to Fe^{++} ions. At the mucosal cell surface Fe^{+++} is converted to Fe^{++} by duodenal cytochrome B and is transported across the cell membrane by divalent metal transporter 1 (DMT 1). Ferroportin 1 helps in transfer of Fe from mucosal cell into circulation. Hepcidin acts by inhibiting the expression or activity of one or more genes involved in intestinal absorption. Iron is absorbed as Fe^{++}. (Fig. 3.8)

Factors promoting iron absorption

- HCl in the stomach
- Ascorbic acid
- Haem iron is better absorbed

Factors inhibiting iron absorption

- Phytates of cereals
- Tannates of tea
- Phosphates of diet and drugs
- Milk

Transport of Iron

The transport of iron from diet in to our body involves a unique biochemistry dependent on body requirement. (Fig. 3.9) Iron in the blood is carried all over the body by a β-globulin-transferrin. Iron is released from transferring in the marrow for erythropoiesis and transferring is reutilized to carry iron.

Iron excretion

A small amount of iron is excreted through sweat and urine. Daily loss in males is about 1 mg and in females about 2 mg.

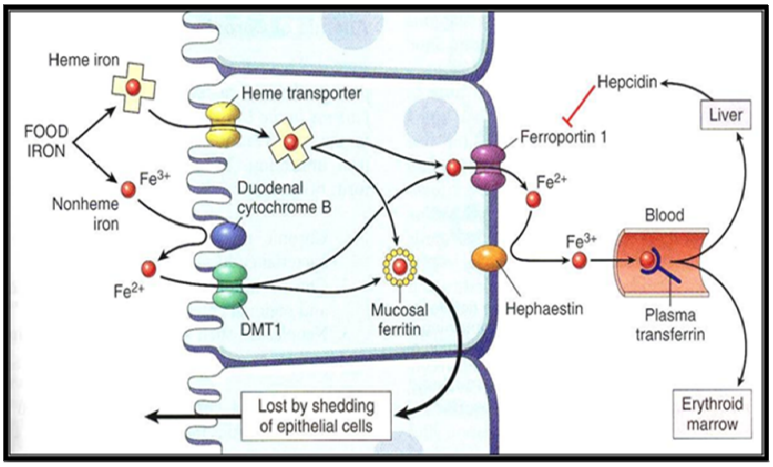

Fig. 3.8: Iron absorption and storage

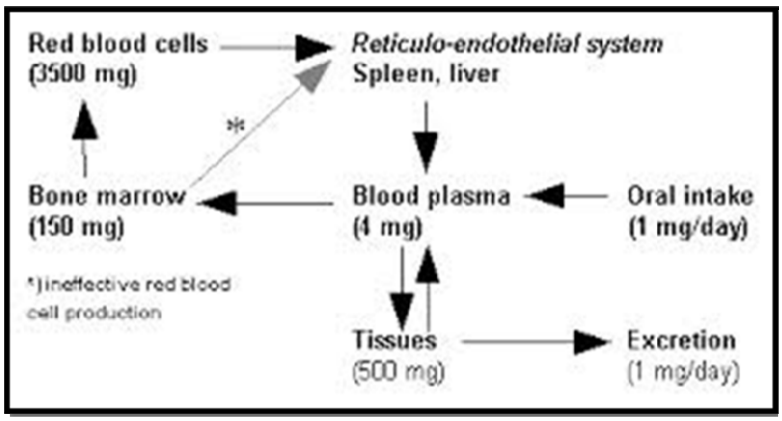

Fig. 3.9: Iron metabolism in human body

Storage of Iron

Iron is stored in the body in two forms – Haemosiderin and Ferritin.

Clinical features

1. Onset is insidious and symptoms appear with progressive anaemia
2. Fatigue: most of the patients complain of fatigue, palpitations, breathlessness and irritability.

3. Growth and development: Growth in infancy is impaired. The children become irritable and show lack of concentration.
4. Pica: Eating of items like ash, mud, etc. which may be the cause rather.
5. Koilonychia / platynychia: the finger nails become thin, flattened, brittle and finally spoon shaped. This is a rare feature.
6. Angular stomatitis: There are fissures and ulcerations at the angles of the mouth.
7. Intellect and IDA: Iron is essential for synthesis of myelin. IDA in childhood causes delayed mental development and changes in cognition appear. These changes are reversible if IDA is corrected early.

Common features of IDA are fatigue, weakness or palpitation. There is a poor correlation between severity of symptoms and blood haemoglobin concentration. Fatigue, irritability and headaches are common complaints of patients with iron deficiency. Depletion of storage iron and, to some extent, of tissue iron precedes the appearance of anaemia. These observations suggest that some of the symptoms may be caused by impaired function of iron enzymes or iron proteins other than haemoglobin. Headache, paresthesias, and a burning sensation of the tongue are symptoms of iron deficiency that are not due to anaemia but seem likely to be caused by deficiency of iron within tissue cells. Pica, the craving to eat unusual substances such as dirt, clay, ice, laundry starch, salt, cardboard, or hair, is a classic manifestation of iron deficiency and is usually cured promptly by iron therapy.

The physical findings in iron deficiency anaemia include, in an approximate order of frequency: pallor, glossitis (smooth, red tongue), stomatitis, and angular cheilitis. Koilonychia, once was a common finding, is now encountered rarely. The spleen is palpable in a small proportion of patients with iron deficiency anaemia.

In severe uncomplicated iron deficiency anaemia, the erythrocytes are hypochromic and microcytic, the plasma iron concentration is diminished, the iron-binding capacity increased, the serum ferritin concentration is low, the serum transferrin receptor and erythrocyte zinc protoporphyrin concentrations are increased, and the

marrow is depleted of stainable iron. Anisocytosis is the earliest recognizable morphologic change of erythrocytes in iron deficiency anaemia. As the iron deficiency worsens, there is often mild normochromic, normocytic anaemia (blood haemoglobin concentration greater than 11 g/dl, mean corpuscular volume, MCV, less than 80 fl). With further progression, haemoglobin concentration, erythrocyte count, MCV, and mean erythrocyte haemoglobin content (MCH) all decline together.

The red cell indices are consistently abnormal in adults only when iron deficiency anaemia is moderate or severe (e.g., in males with haemoglobin concentrations less than 12 g/dl or in women with haemoglobin concentrations less than 10 g/dl). Measurement of the distribution of erythrocyte volume (e.g., red cell distribution width, or RDW) is made easy by modern cell counters. It has been asserted that such measurements permit discrimination between iron deficiency anaemia and other microcytic anaemias.However, haemoglobinopathies and thalassemias commonly exhibit increased RDW, as do some anaemias that are due to chronic disease. Highest RDWs are observed in haemolytic disorders, in which the RDW appears to reflect reticulocytosis. Thus, the early expectation that RDW would permit diagnosis of iron deficiency anaemia has been disappointed.

The serum iron concentration is usually low in untreated iron deficiency anaemia; the normal range for males is between 13 and 31 μmol/liter (75 and 175 μg/dl); for women it is about 2 μmol/liter (10 μg/dl) lower.

The iron-binding capacity is a measure of the amount of transferrin in circulating blood. Normally, there is enough transferrin present in 100 ml serum to bind 4.4 to 8.0 μmol (250 to 450 μg) of iron; since the normal serum iron concentration is about 1.8 μmol/dl (100 μg/dl), transferrin may be found to be about one-third saturated with iron; i.e., one-third of the binding sites are occupied. TIBC may also be measured directly. Transferrin is normally 20 to 50 percent saturated with iron. In iron deficiency anaemia, UIBC and TIBC are often increased; transferrin saturation of 15 percent or less is often

found. A normal value for transferrin saturation often accompanies a low serum iron concentration in the anaemia of chronic disease.

Serum ferritin concentration correlates with total-body iron stores. Serum ferritin concentrations of 10 µg/l or less are characteristic of iron deficiency anaemia. For iron deficiency without anaemia, serum ferritin concentration is typically in the range 10 to 20 µg/l. Radiography and Ultra Sonogram may be helpful in finding out pathology involving internal vital organs like liver, spleen, kidneys, uterus etc.

Differential diagnosis

Other causes of microcytic hypochromic anaemia are:

- Thalassemia major: Iron stores are increased with hepatosplenomegaly and other parameters of haemolytic anaemia.
- Anaemia due to chronic diseases like chronic renal failure, jaundice, etc.
- Sideroblastic anaemia.
- Lead poisoning.
- Thalassemia minor

Iron deficiency and iron deficiency anaemia are common nutritional and hematologic disorders worldwide, affecting an estimated 2 billion people. In infants and young children iron deficiency is most commonly due to insufficient dietary iron. In young women it is most often the result of blood loss in menstruation or as a result of pregnancy. In older adults bleeding may be from the gastrointestinal tract, as from haemorrhoids, bleeding peptic ulcer, hiatus hernia, colon cancer, or angiodysplasia. Iron deficiency has adverse effects on activity of numerous enzymes and in infants can result in impairment of growth and intellectual development. A low serum ferritin concentration is an excellent indicator of iron deficiency. Other laboratory tests that may prove useful include assays for serum transferrin receptor, erythrocyte ferritin concentration, or serum ferritin iron saturation. Treatment of iron deficiency with ferrous salts, in doses of 100 to

200 mg of elemental iron daily, is superior to, much safer, and far less costly than parenteral therapy. Enteric-coated and prolonged-release preparations should be avoided. Complete correction of anaemia is expected in 8 to 12 weeks, depending on patient's age. If this response is not achieved, the patient and the diagnosis require reevaluation. Administration of iron should be continued for 8 months after correction of anaemia or as long as bleeding continues. (39)

The medical importance of *Emblica officinalis* is always being more emphasized in traditional system of medicine. Amla, a well-known source of Vitamin C or ascorbic acid, is also an essential ingredient that helps in the absorption of Iron. In addition to Vitamin C, it also contains calcium, iron, protein, tannic acids, sugar, phosphorus, carbohydrates etc. Its dried fruit with iron supplement for anaemia conditions is usually prescribed in clinical practice. Thus, supplements of Amla are very beneficial to IDA patients. (11)

Patrick-Iwuanyanwu et al (2007) reported that ginger might possess constituents that would trigger the erythropoietic system to produce red cells. (45) A study has shown that ethanol and aqueous extracts of *Eclipta prostrata* have significantly determined anthelmintic activity in which the ethanol extract has shown the significant activity as compared to Albendazole. (46) Since, ginger possesses ascorbic acid, reducingsugars, amino acids it might aid in absorption of iron.

Arollado and Osi in their work suggested and proved the presence of hematinic activity of *Alternantherasessilis* (L.) R. Br.,by monitoring the change in serum ferritin and haemoglobin levels of mice and rats, after treatment with the plant extract at different doses for a period of 14 days. Results of the study showed a significant increase in serum ferritin and haemoglobin level which was dose and time dependent but there was no significant difference on these parameters between mice and rats. (47)

Friel et al conducted a small (n=77) double blind randomized clinical trial (DBRCT) of a group of breastfed children supplemented with iron or placebo from ages 1

to 6 months. At 12–18 months of age, higher visual acuity scores and improved performanceon the Bayley Psychomotor Development Index (but not on the Mental Development Index) were observed (48), which led investigators to conclude that supplementation might have beneficial developmental effects. As noted by others (49), the study requires replication with larger group sizes before any definitive conclusions can be made.

A statistical analysis of data from studies examining this issue concluded that both daily and weekly iron supplementation reduced the prevalence of iron deficiency and anaemia (50). Daily supplementation was found to be more effective than weekly for increasing haemoglobin and ferritin. Although daily supplementation produced only a 2 g/L greater increase in haemoglobin across studies on average, it caused a 34% greater reduction in the risk of anaemia.

Global guidelines for iron supplementation have been published by the International Nutritional Anaemia Consultative Group/ World Health Organization/ UNICEF. The recommendationsare as follows: for age 6 to 24 months, 12.5 mg/d plus 50 µg/d folic acid until 12 months of age, with supplementation from 2 to 12 month for low birth weight cases; for age 2 to 5 y, 20 to 30 mg/d (2 mg iron/kg body); for age 6 to 11 y, 30 to 60 mg/d; for adolescents and adults, 60 mg/d (plus 400 µg folicacid for women of reproductive age); and for pregnant women, 60 mg/d plus 400 µg folic acid for 6 mo, continuing for 3 month of lactation where the prevalence of anaemia is high (<40%). These doses are doubled for the first 3 months in cases of severe anaemia (Hb<70 g/L).(51)

A recent clinical study showed that the trial drug Trikatrayadi Lauha suspension is effective to improve clinical features and hematological parameters significantly. The medicine is effective to increase the haemoglobin level 1.94 g/dL (8.52 -10.46 g/dL, $P < 0.001$) in 5 weeks and 3.33g/dL (8.52 -11.85g/dL, $P < 0.001$) in 10 weeks. No adverse effect of the trial drug was observed during the study. (52)

Currently, there is a substantial increase in the number of epidemiological studies focusing on women health, considering their work load, diet intake, nutrition deficiency etc., with respect to their economic status and life style. Increasing incidence of Chronic Energy Deficiency (CED) among Indian women is reported under health surveys. A recent cross-sectional study on rural south Indians of either sex (n=178; Age: ≥18 yrs.) concluded that low dietary iron intake may not be the only main cause of anaemia among them. Moreover, the authors also found a significant association between farming and CED (Women-OR: 2.20, 95%CI: 1.39-3.49; Men-OR: 1.71, 95%CI: 1.06-2.74), thus stating farming as a risk factor for CED. (53)

In a prospective, randomized, comparative, open-label, non-inferiority trial conducted in Europe and India, the non-inferiority of intravenous iron isomaltoside (n=225) to oral iron sulfate (n=113) was assessed. The primary endpoint of this trial was change in Hb levels from baseline (0 week) to 8th week. The change in serum ferritin and TSAT levels, patient intolerance or lack of response and total quality of life (QoL) were assessed as secondary endpoints. The trial drugs were found to be equally effective, significantly (P=0.04). (54)

In a cross-sectional survey among school girls (n=400; Age: 13-17 years) in Tamilnadu significant association ($p<0.05$) between prevalence of IDA and its contributing factors such as family type, socio-economic status and diet was reported. In this study, a gradual fall in Hb levels with increase in age was found and the authors predicts that its increasing effect on infant and maternal mortality rate. (55)

Even among North Indian adults, the prevalence of IDA among women is reported to be higher than that of in men. For an example, an epidemiological survey on North Indian adults (Age: 20-50 years) reported the prevalence rate as 70.1% among women compared to 53.2% in men. The study stated the wider problem of IDA in both gender, indicating that the problem is not only predominant among vulnerable population (children, pregnant and lactating women, etc.) but also the same in general. (56)

4. SCOPE AND PLAN OF WORK

Since it is evident from the review of literatures that there is a huge demand for traditional medicines in the management of diseases which are considered to be major public health issues, scientific evaluation of unique siddha formulations like the selected drug, ABP, may be helpful in exploring new areas of research on Siddha Medicines. Since diseases like anaemia are of Global importance, there are tremendous scopes for future research on Siddha formulations, for which the present study may form the basic platform.

PLAN OF WORK

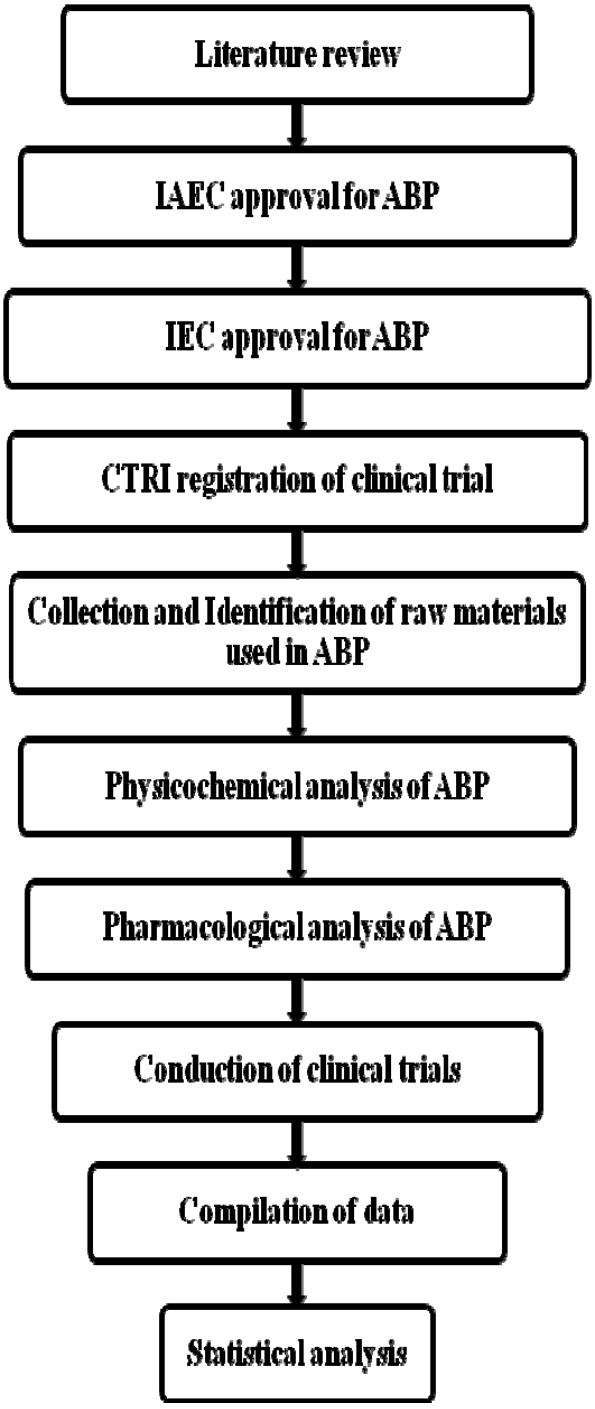

Fig. 4.1. Plan of work

5. MATERIALS AND METHODS

The study was done in three parts:

- Identification of raw materials and preparation of *ABP*
- Preclinical studies including Physicochemical analysis, Toxicity and Acticity studies
- Clinical trial to evaluate the efficacy and safety of *ABP*

The raw materials used for the preparation of *ABP* were procured from available market sources in Erode and Madurai districts. Fresh herbs were procured from Erode district. Both fresh and dry plant drugs were identified and authenticated by the Quality Control department of the GMP certified industry and Siddha Central Research Institute, Chennai and a voucher specimen of each drug were deposited at their herbarium. The Botanical identification of the plant drug used in ABP was done as per pharmacognostical standards. The iron ore used in the formulation was identified and authenticated by the Department of Geology, Government Arts College, Salem. The formulation was prepared as per Siddha scriptures, in strict adherence to GMP guidelines, under direct supervision using the infrastructure of a private Siddha Medicine manufacturing company (GMP certified).

The physicochemical analysis including organoleptic characters, ash values, extractive values, Microbial content, High performance thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC), Gas chromatography (GC-MS) and Atomic absorption spectrometry (AAS) were done at the Central Research Facility, Sri Ramachandra University, Chennai.

In addition, to know the status of iron in *ABP*, Electron spectroscopic chemical analysis (ESCA) was done at Indian Institute of Chemical Technology (CSIR), Hyderabad.

The Pharmacological studies were approved by the Institutional Animal Ethics Committee (IAEC), S.C.R.I, Chennai during 2011. The pharmacological studies, including toxicity (acute and sub-acute) and biological activity (Hepatoprotective and Erythropoietic activities) of the test drug *ABP* were done at Department of Pharmacology, Siddha Central Research Institute, Chennai and C.L.Baid Metha College of Pharmacy, Chennai.

The Clinical trial on the trial drug *ABP* was duely approved by the Institutional Ethics Committee (IEC), National Institute of Siddha, Chennai, during the year 2011. The Clinical trial to evaluate the efficacy and safety of *ABP* was done at two centres: National Institute of Siddha, Chennai and Siddha Regional Research Institute (under Central Council for Research in Siddha, Chennai), Puducherry. Patients visiting the out-patient departments of the selected trial centres were screened for anaemia using clinical examination and related diagnostic parameters as per Screening Proforma. The selected patients were issued an Information sheet explaining the purpose and procedure of the clinical trial. Informed Consent forms in English or Tamil duly signed by the patient was obtained before starting the Trial. The patients were selected as per the Inclusion, Exclusion criteria and the baseline diagnostic parameters were recorded before administering the trial drug ABP as per the protocol.The detailed history with respect to the patient was recorded in Case History Proforma. The Periodical Clinical assessment and assessment of diagnostic parameters was done in regular intervals as per protocol. A total no. of 192 patients were screened out for the trial including pilot study, out of which 92 were recruited with their consent. There were 10 drop-outs due to missed out treatment regimen or withdrawl and a total of 82 patients completed the trial successfully.

5.1. IDENTIFICATION OF RAW DRUGS

The raw drugs, both fresh and dry crude drugs were identified and authenticated by the Quality Control department of the GMP certified industry and Siddha Central Research Institute, Chennai and a voucher specimen of each drug were deposited at their

herbarium. The iron ore used in the formulation was identified and authenticated by the Department of Geology, Government Arts College, Salem.

5.2. PREPARATION OF TRIAL DRUG

The trial drug *Aya Bringaraja Paanidham* (ABP) was prepared with strict adherence to the traditional methods mentioned in the Siddha text, Gunapadam – Thathu, Jeeva vaguppu (Thiagarajan, 1992) under direct supervision by following Good Manufacturing Practice (GMP) guidelines (13). The intermediate samples were collected and stored for physio-chemical analysis. Two batches of preparation of the trial drug were carried out based on which a Standard Operating Procedure (SOP) was evolved. The ingredients of *ABP* is shown in Table 5.1.

Table 5.1: Ingredients of *Aya Bringaraja Paanidham*

S.No	Name of the Ingredients	English / Botanical name	Quantity
1	Bhavana Ayam	Iron powder processed withthe juices of *Wedelia chinensis*(Wh.Pl.) and *Emblica officinalis* (Fr.Frt.)	350 g
2	Chukku	*Zingiber officinale* (Drd.Rz.)	17.5 g
3	Kadukkai	*Terminalia chebula* (Frt.)	35 g
4	Nellikai	*Emblica officinalis* (Drd.Frt.)	35 g
5	Thandrikai	*Terminalia bellerica* (Frt.)	35 g
6	Milagu	*Piper nigrum* (Frt.)	52.5 g
7	Thippili	*Piper longum* (Frt.)	52.5 g
8	Thippilimoolam	*Piper longum* (St.)	52.5 g
9	Thaen	Honey	700 g
10	Panaivellam	Palm jaggery	700 g
11	Karisalaisaru	Juice of *Wedelia chinensis* (Wh.Pl.)	1.3 L

Standard Operating Procedure (SOP):

The following steps of SOP were followed for the drug preparation. The process and the final product provided in dispensing packs of 100g. (Fig. 5.1 & 5.2)

Step-1:

Bhavana Ayam – Soak purified* Iron powder in *Karisalai saru* (Juice of *Wedelia chinensis* – whole plant) for three months in a porcelain vessel. Stir the contents regularly once in a fortnight. After three months soak the processed iron in *Nellikai saru* (Juice of *Emblica officinalis*- Fruit) again for three months. Repeat stirring as earlier. After the said period, take out the iron powder, dry it, grind and sieve it through a 40 No. Mesh. Then heat it in a pan till red hot and allow it to cool gradually.

Step-2:

Check the raw materials S.No. 1-11 as per formula.

Step-3:

Pulverize the drugs in S.No.2 to 8 and sieve them in 80 No. mesh to obtain *chooranam* (fine powder)

Step-4:

Take *Manjal karisalai* (*Wedelia chinensis*) juice in a vessel and add *Panaivellam* (Palm jaggery) to it.

Step-5:

Heat the vessel and mix them well till *Panaivellam* dissolves. Filter this liquid through a muslin cloth in to another vessel.

Step-6:

Pour the filtered liquid from Step-4 in to a stainless steel (SS) pan.

Step-7:

Heat the pan and add all the *chooranam* (S.No. 2 to 8) in to it. Stir them well.

Step-8:

Add Bhavana Ayam in to it and stir well till the mixture reaches *Kambipatham* (Single string like texture).

Step-9:

Add honey finally to the vessel and mix well.

Step-10:

Store in amber coloured glass bottles / High Density Poly-Ethylene (HDPE) containers.

(*__Purified Iron powder –__ *Suthi seitha ayam* –Traditional method of purifying iron powder. Raw iron powder should be soaked in a mixture of *Thiriphala chooranam* and *Naaval pazha charu* (Fruit juice of *Syzygium cumini*) in a ceramic jar for six months. Once in a month open the jar and stir the contents. This process should be repeated for a period of six months followed by frying the iron powder thus obtained to red hot in a pan, ground, sieved (40 No. mesh) and stored for further preparation.)

Fig.5.1: The process of preparation of Aya Bhringaraja Paanidhaan

Fig.5.2: Dispensing packs of Aya Bhringaraja Paanidhaan

5.3. PHYSICOCHEMICAL ANALYSIS

ORGANOLEPTIC STUDIES OF THE TRIAL DRUG – *Aya Bringaraja Paanidham*

The organoleptic characters such as colour, odour, taste and texture for the trial drug ABP were studied using traditional and standard techniques.

5.3.1. DETERMINATION OF FOREIGN MATTER IN HERBAL FORMULATIONS

Definition of foreign matter

Medicinal plant materials should be free from moulds, insects, animal faecal matter and other contminations such as soil, stones and extraneous material. Any foreign matter in the drugs must be removed before they are ground for testing and preparation of formulation.

Foreign matter is material consisting of any or all of the following:

1. In particular, parts of the organ or organs from which the drug is derived other than the parts named in the definition or for which a limit is prescribed in the individual monographs.

2. Any organ or part of organ, other than those named in the definition and description.

The amount of foreign matter shall not be more than the percentage prescribed in the monograph.

Experimental

100g of each herbal ingredients of trial drug ABP were weighed accurately as specified in the guidelines. It was spread in a thin layer and the foreign matter were sorted out by visual inspection and by using a magnifying lens (6X).

The trial drug *ABP* was subjected to physico chemical evaluation as per standards under the following headings:

i) Determination of moisture content

ii) Determination of Ash values

iii) Determination of pH

iv) Total sugar content

v) Identification using chromatographic techniques – HPTLC, HPLC and GC

vi) Estimation of iron concentration

vii) Test for heavy metals – Lead, Cadmium, Mercury, Arsenic

viii) Determination of microbial content

ix) Electron spectroscopic chemical analysis (ESCA)

For all the above physico chemical evaluations of the test drug, the standard protocols as per the guidelines described for testing of Indian medicines published by the department of AYUSH, India were followed. (57)

5.3.2. DETERMINATION OF MOISTURE CONTENT (Loss on drying)

About 10 g of ABP was accurately weighed in a tared evaporating dish. Then it was dried at 105^{o} C for 5 hours and weighed. The procedure was repeated at one hour interval until the difference between two consecutive weighings was 0.25 percent.

The formula used for calculating Loss on drying is W1/W2 x 100,

where W1 – weight of ABP after heating

W2 – weight of ABP before heating, i.e original weight of the sample

5.3.3. DETERMINATION OF ASH VALUES

TOTAL ASH

Apparatus

- Dish – Flat bottomed, having a surface areas of at least 15 cm made of silica unaffected by the conditions of the test.
- Muffle Furnace – regulated at 550 ± 25˚C
- Filter Paper – ashless, medium fine.

Procedure

Preparation of Sample – Weighed, to the nearest 0.001g, about 2g of ABP into the tared flat-bottomed dish.

Determination – Poured about 2ml of ethanol on the material in the tared dish and ignited it. When the ethanol is burnt off, the dish was heated carefully over a small flame to char the material. Then it was ignited in the muffle furnace at 550˚C ± 25˚C for 2 hours. After cooling down and several drops of water was added to the ash, evaporated carefully to dryness and heated in the muffle furnace for further 1 hour at 550˚C ± 25˚C. This was done until it was white, indicating the absence of carbon. Then the dish was removed and allowed to cool to room temperature in a dessicator and weighed without delay. Repeated these procedure until the difference in mass between two successive weighings was less than 0.001g. Recorded the lowest mass and reserved the toal ash for determining the water soluble ash and the acid insoluble ash.

Calculation

$$\text{Total ash (on dry basis), percent by mass} = (M_2 - M_0) \times \frac{100}{M_1 - M_0} \times \frac{100}{100 - H}$$

Where,

M_2 = mass in g of the dish and total ash;

M_0 = mass in g of the empty dish;

M_1 = mass in g of the dish and test portion; and

H = moisture content of the sample as received in percent

WATER SOLUBLE ASH

To the reserved ash in the dish, added 25 ml of distilled water, heated nearly to boiling, and filtered through an ashless filer paper. Washed the filter paper with hot water until the combined filtrate and washings measured about 60ml. Returned the filter paper and the contents to the dish, evaporated the water carefully on a water bath and ignited at 500 ± 25˚C for 1 hour. Cooled in the desiccators and weighed. Repeated the process of igniting, cooling and weighing till the difference in mass between two successive weighings was less than 0.001g.Noted the lowest mass.

Calculation

Water soluble ash was calculated by subtracting the weight of the residue remaining from the weight of the total ash.

ACID INSOLUBLE ASH

Reagents

1. Hydrochloric Acid Solution – Concentrated hydrochloric acid (r.d. 1.19 at 20˚C) diluted in water 2:5 (v/v)
2. Silver Nitrate Solution – 10 percent (m/v)

Procedure

Test Portion – Use the total ash obtained in the above procedure.

Determination – Added to the test portion 15 to 25 ml of the hydrochloric acid and boiled for 10 minutes, covering the dish with a watch glass to prevent sputtering. Allowed to cool and filtered the contents of the dish through the ashless filter paper. Washed the filter paper with hot water until the washings are free from hydrochloric acid, as tested by silver nitrate solution and returned it to the dish. Evaporated carefully on the water bath and ignited in the muffle furnace at 550 ±25˚C for 1 hour. The dish was allowed to cool in the desiccator and weighed. Repeated the operations of igniting for 1 hour, cooling and weighing till the difference in mass between two successive weighings was less than 0.001 g. Noted the lowest mass.

Calculation

$$\text{Acid insoluble ash (on dry basis), percent by mass} = (M_4 - M_0) \times \frac{100}{M_1 - M_0} \times \frac{100}{100 - H}$$

Where

M_4 = mass in g of the dish and acid insoluble ash;

M_0 = mass in g of the empty dish;

M_1 = mass in g of the dish and test portion; and

H = moisture content of the sample as received in percent.

ALCOHOL SOLUBLE EXTRACT

Test Portion – Weighed, to the nearest 0.001g, about 2 g of the prepared sample.

Determination – Transferred the test portion quantitatively with ethanol [95 percent (v/v) solution] to the 100 ml volumetric flask and filled to the mark with ethanol. The flask was stoppered and shaken at approximately 30 minutes intervals for about 4 hours and allowed to stand 16 hours longer without shaking. Filtered the extract through a dry filter paper, evaporated a 50 ml aliquot portion to dryness on the water bath and heated in the oven at 103 ± 2˚C to constant mass, that is, until two consecutive weighing separated by a period of 1 hour in the oven do not differ by more than 0.001g. Recorded the final mass.

Calculation

$$\text{Alcohol-soluble extract (on dry basis), percent by mass} = M_1 \times \frac{100}{50} \times \frac{100}{M_0} \times \frac{100}{100 - H}$$

Where

M_1 = mass in g of the dish reside obtained,

M_0 = mass in g of the test portion, and;

H = moisture content of the sample as received in percent

COLD WATER SOLUBLE EXTRACT

Test Portion – Weigh, to the nearest 0.001 g, about 2g of the prepared sample.

Determination –Transfered the test portion quantitatively with distilled water to the volumetric flask (100 ml) and filled to the mark with cold water. The flask was stoppered and shaken at approximately 30 minute intervals for 8 hours and allowed to stand for 16 hours longer without shaking. Filtered the extract through a dry filter paper, evaporated a 50 ml aliquot portion to dryness in the dish on the water bath and heated in the oven at 103 ± 2˚C to constant mass, that is, until two consecutive weighings separated by a period of 1 hour in the oven do not differ by more than 0.001 g. Recorded the final mass.

Calculation

$$\text{Cold water soluble extract (on dry basis), percent by mass} = M_1 \times \frac{100}{50} \times \frac{100}{M_0} \times \frac{100}{100 - H}$$

Where

M_1 = mass in g of the residue obtained,

M_0 = mass in g of the material taken for the test; and

H = moisture content of the sample as received in percent (see 9)

TOTAL SUGAR CONTENT [Ref.Annex C of IS 4941 : 1994]

1 g of prepared sample was accurately weighed and taken in a 250 ml volumetric flask and diluted with 150 ml of water. The contents were mixed thoroughly and the volume was made up to 250 ml with water. 5 ml each of standard copper sulphate solution and potassium sodium tartrate solution was taken in porcelain dish. To this solution 12 ml of the sample solution was added from a burette and heated to boiling on a asbestos gauze. To this 1 ml of methylene blue indicator was added and keeping the solution boiling the titration was completed within 3 minutes, the end point being change of colour from blue to red. Noted the volume of sample solution required for titration.

The overall above procedure was followed based on methods given in Annex C of IS 4941 : 1994 (58)

Calculation

$$\text{Total reducing sugars, percent by mass} = \frac{250 \times 100 \times S}{H \times M}$$

Where

S = strength of copper sulphate solution;

H = volume, in ml, of sample solution required for titration; and

M = mass, in g., of ABP

5.3.4. EVALUATION OF MICROBIAL CONTENT

The microbial content in the test drug *ABP* was performed as per standards as mentioned below:

i) Total Plate Count:

To enumerate viable aerobic bacteria in 1g or 1ml of the given sample by USFDA (BAM) 8th Edition Chapter 3. (59)

ii) Yeast and Mould:

To enumerate the number of Yeast and Mould in the given sample by USFDA (BAM) method. (60)

iii) Salmonella spp:

To isolate the Salmonella spp in the given sample by USFDA (BAM) method. (61)

iv) S.aureus:

To enumerate *Staphylococcus aureus* in the given sample by USFDA (BAM) method. (62)

v) *E.coli*

To determine the presence or absence of Escherichia coli by ISO: 7251-2005. (63)

vi) *Pseudomonas aeruginosa*

To detect the presence of *Pseudomonas aeruginosa* in the given sample by IS 13428-1998. (64)

5.3.5. ESTIMATION OF IRON CONCENTRATION [AOAC OFFICIAL METHOD 999.10]

The procedure for Atomic Absorption Spectrophotometry after Microwave Digestion as given by the AOAC OFFICIAL METHOD 999.10 was followed. (65)

Principle

Products are digested with HNO_3 and H_2O_2 under pressure closed vessel heated by microwaves. Solution is diluted with H_2O. Iron in the sample is determined by Flame Atomic Absorption Spectrophotometry (FAAS).

Procedures

Digestion

Weighed 2g of *ABP* accurately in to a digestion vessel. Added 5ml $HNO_3$and 2ml 30% H_2O_2.Closed the vessel, placed in microwave oven. Set oven program according to the parameters prescribed. Removed digestion vessels from microwave oven and let cool thoroughly before opening them. Opened the vessel and rinse down lid and walls into container. Transferred the solution to 25ml volumetric flask and diluted to mark with deionized water. Then, transfered solution to plastic container. Treat a blank in the same way as test.

Atomic absorption spectrophotometry

Use of flame or graphite furnace technique is determined by the concentration of the metal to be determined. Flame technique should be used as far as possible, since this technique is less sensitive to interference than the GFAAS. The most appropriate

wavelength, gas mixture/temperature program, and other instrumental parameters for each metal are found in the manual provided with the instrument.

(1) Flame technique – The concentration of Fe is usually at levels suitable for determination by FASS.

(2) Graphite furnace technique – This technique is generally required for determination of Pb and Cd.

Calculation and Evaluation of Results

Calculate the concentration (C) of metal in the test sample according to the formula:

$$C = \frac{(a - b)\ df \times 25}{m}$$

where C= concentration in the test sample (mg/kg); a = concentration in the test solution (mg/l); df=dilution factor; b= concentration in the blank solution (mg/l); m=weight of the test portion (g).

5.3.6. TEST FOR HEAVY METALS – Lead, Cadmium, Mercury, Arsenic

- Estimation of Lead & Cadmium – Same as above **[AOAC Official Method 999.10]**
- Estimation of Mercury [**AOAC Official Method 971.21** Flameless Atomic Absorption Spectrophotometric Method]
- Estimation of Arsenic [**AOAC Official Method 986.15]**

5.3.7. EXTRACTION PROCEDURE FOR THE TRIAL DRUG ABP

The final product of the trial drug prepared was selected as such for the extraction procedure using various solvents viz., ethanol, methanol, ethyl acetate and hexane with the help of Soxhlet apparatus. The conventional soxhlet extraction procedure was

followed as described for testing of Indian medicines published by the department of AYUSH, India were followed. (57)

The filtered and dried extract of ethyl acetate (EA) of all the above was stored at room temperature and used for further chromatographic analyses. The extracts obtained from other solvents were found to be inadequate in reproducibility.

5.3.8. HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) FINGER PRINTING OF *ABP*

HPTLC procedure for ABP was carried out to obtain a standard for the formulation. The following working conditions were observed and maintained throughout the procedure. The resulting chromatogram was analysed qualitatively to check the presence of peaks corresponding to that of its ingredients. This analysis was done by comparing the Rf values between the test sample and known standards of its ingredients obtained from other published data.

Chromatographic condition for HPTLC

Test item name	:	V Cbio-dec-2014-APB-II
Analysis type	:	HPTLC Profile
Instrument Name	:	HPTLC-CAMAG
		- Injector – Automatic TLC sampler 4
		- Scanner - Scanner -3
		- Documentation unit –reprostor-3
Stationary phase	:	TLC Silica gel $60F_{254}$
Mobile phase	:	n-Hexane: Ethyl acetate: Formic

		Acid: Aceticacid (60:40:2.0:2.0)
Scanning wave length	:	UV-254 nm
TLC-Development	:	Ascending
Injection volume	:	2.5, 5.0, 7.5, 10, 12.5,15,20 µl

5.3.9. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) FINGER PRINTING OF *ABP*

The ethyl acetate extract of ABP obtained above was utilized for HPLC analysis. HPLC procedure for ABP was carried out to obtain a standard for the formulation. The following working conditions were observed and maintained throughout the procedure. The resulting chromatogram was analysed qualitatively to check the presence of peaks corresponding to that of its ingredients. This analysis was done by comparing the Rf values between the test sample and known standards of its ingredients obtained from other published data.

Chromatographic condition for HPLC

Project code	:	255 a
Test item name	:	*ABP*
Analysis type	:	HPLC profile
Solubility	:	Ethyl acetate extraction
Instrument Name	:	HPLC-LC-2010 SHIMADZU
Stationary phase	:	C18-250×4.6 -5µ 100 A^0
Mobil phase	:	Buffer solution: ACN [82:18 (V/V)]
		Buffer (KH_2PO_4-0.04 (PH-7.0)
		Mobile phase
		Buffer solution (7.0 pH): ACN) -

		80:20 (V/V)]
Flow rate	:	1.0 ml /minutes
Column oven temperature	:	Ambient
Detector	:	UV-254 nm

5.3.10. ELECTRON SPECTROSCOPY FOR CHEMICAL ANALYSIS (ESCA)

ESCA analysis, also known as Electron Spectroscopy for Chemical Analysis or X-Ray Photoelectron Spectroscopy (XPS), is a surface analysis technique that provides elemental and binding energy information about a material's surfaces and interfaces.This technique is helpful in determining the elemental composition of the surface (top 0–10 nm usually) and chemical or electronic state of each element in the surface.

Analytical condition of ESCA:

Spectrum Lens Mode:Electrostatic Resolution:Pass energy 80

Acqn. Time(s): 376 Sweeps: 2 Anode:Al(75 W) Step(meV): 1000.0

Dwell Time(ms): 170 Charge Neutraliser :On

Three samples of iron obtained from different traditional processing methods were subjected to ESCA study. Mainly the raw iron and processed iron (Bhavana Ayam) were compared for the oxidation state of iron in the samples.

5.3.11. Gas Chromatography (GC)

GC procedure for ABP was carried out to obtain a standard for the formulation. The following working conditions were observed and maintained throughout the procedure. The resulting chromatogram was analysed qualitatively to check the presence of peaks corresponding to that of its ingredients. This analysis was done by matching the Retention time (RT) between the test sample and known standards available in the database of National Institute of Standard and Technology (NIST).

Chromatographic condition for GC

Acquisition parameters: Oven: Initial temp 50°C for 5 min, ramp 10°C/min to 280°C, hold 15 min, Inj=200°C, Volume= 1.0 µL, Split=25:1, Carrier Gas=He, Solvent Delay=0.00 min, Transfer Temp=220°C, Source Temp=220°C, Scan: 50 to 600 Da, Column 30.0m x 250µm

Test item name	:	APB-II
Analysis type	:	GC Profile
Instrument Name	:	Clarus 500 Perkin Elmer with autosampler
Running time	:	60 min

5.4. PHARMACOLOGICAL TOXICITY STUDIES

IAEC Approval

The project was accepted during the Institutional animal Ethics Committee meeting held on 08-07-2011 at Siddha Central Research Institute, Arumbakkam, Chennai, Tamil Nadu.

Proposal No. 117/PHARMA/SCRI, 2011 dated 08-07-2011.

5.4.1. ACUTE ORAL TOXICITY STUDIES

Objective

The objective of this study is to determine:

Maximum Tolerated Dose (MTD) for toxicity (mortality) in Albino mice after single oral dose administration.

Animal welfare

This study is performed as per the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for Laboratory Animal Facility after approved Institutional Animal Ethics Committee (IAEC).

Materials and Methods

All the procedures were followed as mentioned in Acute Oral Toxicity – Acute Toxic Class Method of OECD GUIDELINE FOR TESTING OF CHEMICALS (66)

Test item

Identity	: Semi-solid
Physical Appearance	: Blackish brown in colour
Storage Conditions	: Room temperature
Safety of Handling	: Appropriate safety precautions viz; gloves, masks and dangree/apron will be worn while handling.
Report of Standardization	: Received from Sponsorer

Vehicle

Distilled water was used as a vehicle for the formulation preparation. Details of vehicle used in the study are mentioned below.

Justification for Selection of Vehicle

Distilled water was used as the vehicle in the current study as it is the most commonly used Vehicle for oral toxicity studies.

Test system

Albino mice of 8 to 9 weeks old, males weighing 30-40 g and females weighing 25-35 g was received with health certificate. Only nulliparous and non-pregnant females were used in the experiment. The actual range of the body weight at receipt of animals was recorded in the raw data and reported in the final report. The body weight variation of the animals selected for the study on the day of randomization did not exceed ± 20% of the mean body weight of each group and sex.

Justification for Selection of Test System

- Mouse is one of the recommended species by regulatory agencies for conducting pre-clinical toxicity evaluation among the rodents.
- Availability of vast background data.

Feed

Autoclaved standard pelleted laboratory animal diet was provided ad libitum during study period except during scheduled both blood collection and terminal sacrifice.

Water

The animals were provided free access to autoclaved water purified with reverse osmosis during the study period.

Acclimatization

Mice were acclimatized for 7 days prior to the treatment and observed for clinical signs daily. Veterinary examination of all the animals was performed on day of receipt and body weights recorded on day of receipt and randomization.

Animal Identification

Each animal was identified uniquely by marking using within the cage during acclimatization period. Post randomization, animals will be identified uniquely using marking throughout the study. Each cage card was provided with Study No., Animals No., Cage No., Sex, Dose group, Dose level, Dosing start date and date of Necropsy. No other study related information was recorded on this cage cards hence they were discarded post terminal sacrifice of main study animals.

Grouping and Randomisation

A total of 10 mice (5 males + 5 females) were selected based on the body weight and randomly distributed into 2 dose groups (Group I and II). Each group will contain 5 animals/sex.

Experimental procedure

Study Design

An outline of the study design is presented in the following Table 5.2:

Table 5.2: Study design for grouping of animals acute toxicity study

Group No.	Treatment	Dose (mg/kg body wt)	Animals / sex (M/F)
I	Vehicle	0	5
II	HD	2600 mg/kg	5
HD: 10 time's higher therapeutic dose (single dose or dose administered within 24 hour)			

As females are more sensitive, 5 females/group will be used 1st, based on its results incorporation of males will be decided by the investigator. It is recommended to verify results in both the sexes.

Justification for Dose selection:

Doses selected for this study is, 2600 mg/kg, have been selected as it is the maximum feasible dose based on available human therapeutic dose. The dose is adequately derived from human therapeutic dose and converted in to dose for test species.

Dose Volume

The total volume of dose administration was calculated based on the body weight taken on the Day 1 of treatment.

Route of Administration

The test drug and vehicle was administered orally to the animals.

Justification for Selection of Route

Oral route has been selected, as it is the clinical route of administration.

Histopathological Analysis Not performed

Details of dose calculation

Human dose of the test drug = 2000 mg once a day

Conversion factor for mice of average body weight of 20 g = 0.0026

Therapeutic dose for a mice of 20 gram body weight = 2000 x 0.0026 = 5.2

Therapeutic dose in a mice per kg body weight = 1000 x 5.2/20 = 260 mg.

Average dose in a mice per kg body weight = 260 x 5 = 1300 mg.

High dose in a rat per kg body weight = 260 x 10 = 2600 mg / kg.

5.4.2. SUB-ACUTE TOXICITY STUDY (REPEATED DOSE 28-DAY)

All the procedures were followed as mentioned in Repeated Dose 28-Day Oral Toxicity Study in Rodents in OECD GUIDELINES FOR THE TESTING OF CHEMICALS (67)

Objective

The objective of this study was to evaluate the toxicological profile, the target organs of toxicity, reversibility of toxicological findings and No Observed Effect Level (NOEL) or No Observed Adverse Effect Level (NOAEL) in rats after oral (gavage) administration of *ABP* Formulation for 28 consecutive days.

Animal Welfare

This study was performed as per the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for Laboratory Animal Facility after approval of Institutional Animal Ethics Committee (IAEC).

Materials and Methods

Test item

Identity : Siddha drug

Physical Appearance : Semi-liquid

Storage Conditions : Room temperature

Vehicle

Distilled water was used as a vehicle for the formulation preparation.

Justification for Selection of Vehicle

Distilled water was used as the vehicle in the current study as it is the most commonly used vehicle for oral toxicity studies.

Test system

A total of 48 Wistar rats (24 male and 24 female) of 6 to 7 weeks age, was received from Animal Breeding station, TANUWAS, Madhavaram, Chennai, Tamil Nadu. The body weight variation of the animals selected for the study on the day of randomization did not exceed ± 20 % of the mean body weight of each sex. Only nulliparous and non-pregnant females were be used in the experiment.

Environment

Temperature and relative humidity were maintained at 18 to 25 ^{0}C and 30 to 65 % respectively and illumination was controlled to give approximately a sequence of 12 hours light and 12 hours dark.

Housing

Animal were housed 1animal /cage in each polycarbonate cage with rice husk bedding and metal tops. Each cage was identified with cage card, which displayed study number, cage number, sex and animal identification numbers. Cages were rotated once on Day 1, 6, 11, 16, 21 and 26 so that the variation in the exposure to light effects due to cage placement on the rack was minimized during the study period. The cage rotation schedule has been maintained with raw data. Results of microbial load analysis performed after fumigation of the Experimental Animal Room and periodic contaminant analysis of bedding material was done.

Feed

Autoclaved standard pelleted laboratory animal diet was provided *ad libitum*during study period except during scheduled both blood collection and terminalsacrifice.

Water

The animals were provided free access to autoclaved water purified with reverse osmosis during the study period.

Acclimatization

Male and female animals were acclimatized for 7 days, respectively before initiation of treatment. Veterinary examination and detailed clinical signs of all the animals were performed on the day of receipt followed by cage side clinical signs observations once daily during the acclimatization period. Body weights of all the animals were recorded on the day of receipt and randomization.

Animal Identification

Each cage was identified properly during the acclimatization period and throughout the study. Each cage card was provided with Study No., Animals No., Cage No., Sex, Dose group, Dose level, Dosing start date and date of Necropsy. No other study related information was recorded on this cage cards hence they were discarded post terminal sacrifice of main study animals.

Route, Frequency and Method of Administration

The test drug Siddha drug and vehicle (Distilled water) was administered to the animals once daily up to 28 days by oral (gavage) route using appropriate graduated disposable syringe and mouse gavaging cannula. The animals from Group II, III and IV received test item whereas the animals from Group I were dosed with vehicle control.

Mortality

Mortality and morbidity were recorded twice daily.

Body Weight

Weekly body weight of the animals was recorded on the day of receipt, randomization study animals.

Feed Consumption

Feed consumption was recorded weekly throughout the study for main study animals. The food intake was quantified once daily by weighing the left over feed on standard electronic balance. The feed was withdrawn overnight before scheduled blood collection (clinical pathology investigation) and terminal sacrifice respectively. Only feed left was recorded on these days before keeping the animals on fasting collection and terminal sacrifice.

Laboratory Investigations

General

The blood samples were analyzed for clinical chemistry and hematology on Day 28 for main study animals. The blood sampling procedure was randomized so that approximately equal numbers of males and females representing each dose group will be bled at similar times of the day throughout the sampling period. Blood was withdrawn from retro-orbital plexus (left eye) with light ether anesthesia from overnight fasted animals (approximately 16 hr). Approximately 1 ml of blood was collected in each tubes containing EDTA-K_2 (1mg/ml or 0.5 ml of blood) for analysis of hematological parameters. Approximately 3 ml of blood was collected for analysis of clinical chemistry parameters.

Hematology

The hematology parameters viz., total white blood cell count (WBC), total red blood cell count (RBC), haemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), Platelet count (PLT) and differential leucocytes count (DLC) were analyzed.

Clinical Chemistry

Following serum biochemical parameters were estimated using RA-50 auto analyzer (Bayer).

Parameter	**Unit**	**Method**
Creatinine (CREA)	mg/dl	Jaffee's method
Glucose (GLU)	mg/dl	Glucose oxidase/ peroxidase (GOD/POD) method
Total Protein (TP)	g/dl	Biuret end point reaction
Alanine aminotransferase	I.U./L	UV Kinetic (International Fedaration of Clinical Chemistry)
Aspartate aminotransferase	I.U./L	UV Kinetic (International fedaration of Clinical Chemistry)
Alkaline phosphatase	I.U./L	Paranitro Phenyl Phosphate (p-NPP)

Pathology

Necropsy

All surviving animals were euthanized on Day 29 (main study animals) using ether anesthesia followed by cervical dislocation.

Histopathology

The histopathology examination was carried out on all organs and tissues collected for vehicle control (Group I) and high dose (Group IV) of main study animals of both the sexes and gross lesions. Histopathology evaluations was not performed for Group II and III of main study, since no treatment related histopathology changes were noticed in high dose group animals.

Statistical Analysis

The data were analyzed statistically at each interval for each sex. Group II, III and IV were statistically compared with Group I in order to find the treatment related effects.

5.5. PHARMACOLOGICAL ACTIVITY STUDIES

The following procedure as adopted from previous studies by Mohan et al (2007) was followed in test animals. (68)

5.5.1. Hepatoprotective effect against CCl4-induced hepatotoxicity in rats

Animals were divided into five groups of six rats each. Group I served as normal control and Group II CCL4 (1:1 of CCl4 in olive oil) orally , GroupIII and IV animals were treated with low dose and high dose of extracts. GroupV, standard silymarin at an oral dose of 100 mg/kg. The treatment was continued for 7days, once daily. On the day of 7 for groups II-V,30 min post-dose of extract administration animals received CCl4 at the dose of 1.5 ml/kg(1:1 of CCl4 in olive oil) orally. The animals were sacrificed after 36 h after administration of acute dose of CCl4.

The blood was collected by retero orbital route and the serum was separated out and used for estimation of aspartate aminotransferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and total serum bilirubin using Span diagnostic kits.

5.5.2. Erythropoietic effect

The following procedure as adopted from previous studies by Oluyemi et al (2007) was followed in test animals. (69)

Basal RBC count and haemoglobin concentration of blood are determined in Sprague-Dawley rats. Animals were divided into three groups with 6 in each. Anaemia is induced using phenylhydrazine (PHZ) (60 mg/kg, i.p., in divided doses daily, for three consecutive days). Anaemia was considered induced when RBC level as well as haemoglobin concentration of the blood reduced by about ~30%. Anemic rats were put into groups and treated as follows: Group I-Normal control, group II and III receive ABP at low and high dose, respectively daily. The RBC number and haemoglobin concentration were determined using PE 6000 auto analyzer weekly for 28 days.

5.6. CLINICAL EVALUATION OF TRIAL DRUG *ABP*

Objective:

To scientifically and systematically evaluate the therapeutic efficacy and safety the selected traditional Siddha formulation *ABP* used in the treatment of anaemia.

Patients and Methods

The study protocol procedures needed to be followed were designed for proper conduction of the trial. The approval of Institutional Ethics Committee (IEC) of the concerned institutes were sought before the provisional registration of the study. After receiving the IEC's approval, the clinical trial was registered in the Clinical Trial Registry of India (CTRI). Following the approval of the IEC and CTRI registration, patients with anaemia were recruited as per inclusion and exclusion criterias. Ambulatory

patients of either sex, in the age group of 18-60, both vegetarians, all economic classes, with blood parameters Hb: 6 -10 g/dl, MCV < 80 fl, MCHC < 34 g/dl, Serum iron concentration < 50 µg/dl, Serum Ferritin < 30 ng/ml and patients willing to sign the informed consent were recruited.

As per the guidelines of the Tamil Nadu Dr.M.G.R Medical University, before starting the actual trial, a Pilot study on the trial drug ABP with a sample size of 10 was conducted. The patients were recruited as per Inclusion and Exclusion criteria. After assessing the feasibility of clinical trial on ABP through the above Pilot study, a Open labeled Clinical Trial was carried out as per protocol.

The trial centres National Institute of Siddha, Chennai and Siddha Regional Research Institute, Puducherry were chosen to conduct the clinical trial since they had a good patient census. Patients visiting the out-patient departments of the selected trial centres were screened for IDA using clinical examination and related diagnostic parameters as per Screening Proforma.

A total no. of 192 patients were screened out of which 92 were recruited with their consent. There were 10 drop-outs due to irregular treatment schedule and a total of 82 patients completed the trial successfully. The selected patients were issued an Information sheet explaining the purpose and procedure of the clinical trial. Informed Consent forms in English or Tamil duly signed by the patient was obtained before starting the Trial. The patients were selected as per the Inclusion, Exclusion criteria and the baseline diagnostic parameters were recorded before administering the trial drug ABP as per the protocol.The detailed history with respect to the patient was recorded in Case sheet History Proforma. The Periodical Clinical assessment and assessment of Diagnostic parameters was done in regularintervals as per protocol.

The diagnostic parameters – Physical examination and Laboratory investigations were done on 0 day (before treatment with ABP) and on 45th day (before treatment with ABP). The prevalence of *Veluppu Noi* / IDA was observed among all completed cases

with respect to their age, sex, height, weight, food habits, *Kaalam*(Seasonal variations), *Thinai* (Land tracts), *Udaliyal* (Body constitution), occupation, etiological factors etc. Among the trial participants the incidence of prominent clinical feature was observed. The eight fold diagnostic tools – *Naadi, Sparisam, Naa, Niram, Mozhi, Vizhi, Malam and Moothiram*, unique to Siddha system were recorded for all the patients at 0 day, 15th day, 30th day and 45th day of treatment with ABP.The prevalence of iron deficiency anaemia among both gender and different age groups were observed. The distribution of IDA in different *Udaliyal* (Body constitution), *Kaalam* (Seasons) and *Thinai* (Habitat) was also recorded.

i. IEC approval

The trial was approved by the Institutional Ethics Committee of National Institute of Siddha vide. **IEC approval No.NIS/IEC/2011/1/18,** before the initiation of trial.

ii. Registration of clinical trial in Clinical Trial Registry of India (CTRI)

Trial Registration No.: **CTRI/2014/07/004802**

Study design	**:**	Open – Labelled Clinical Trial.
Sample size	**:**	82
Study centres	**:**	i) National Institute of Siddha, Chennai.
		ii) Siddha Regional Research Institute, Puducherry.
Treatment period	**:**	45 days
Dosage	**:**	2g Twice a day (after food)

Observation period (Without medication):60 days; during this period physical examination including Siddha and Modern parameters was examined once in a month.

Inclusion criteria:

- Age group: 18 to 60 years of both sexes
- Classical symptoms of *Veluppunoi*– Pallor, Breathlessness, Weakness, Breathlessness on exertion, Fatigue, Swollen feet, Palpitation.
- Blood parameters – Hb: 6 -10 g/dl

  MCV < 80 fl

  MCHC < 34 g/dl

Serum iron concentration < 50 µg/dl

Serum Ferritin < 30 ng/ml

Exclusion criteria:

- Age < 18 and > 60 years
- Pregnant and lactating woman
- Any abnormality in the Blood Platelet count
- Bleeding disorders, Sickle cell Anaemia and structural anomalies of RBC
- Cardiac disorders, cancer, renal failure and other major ailments

Withdrawal criteria:

- Miss out the specified drug regimen.
- Development of any complications or Adverse Events during the trial period.

Clinical assessment parameters:

The clinical assessment of the signs and symptoms is done for all patients at 0^{th} day, 15^{th} day, 30^{th} day and 45^{th} day.

- Physical examination – Pallor, Tachycardia, Oedema, Fatigue, Breathlessness on exertion, Palpitation.
- Siddha parameters – *Naadi, Sparisam,Naa, Niram, Mozhi, Vizhi, Malam, Moothiram.*
- The laboratory investigations –Hb, Serum Ferritin, Serum Iron concentration, TRBC, TC, DC, ESR, MCV and MCHC were carried out before (O day) and after (45^{th} day) the treatment period with trial drug. To evaluate the safety of trial drug Liver Function Tests (LFT) and Renal Function Tests (RFT) were performed before (O day) and after (45^{th} day) the treatment period.

Routine diagnostic parameters:

For all recruited patients the following diagnostic parameters were done as per protocol:

Blood - TRBC, TC, DC, E.S.R, Hb, M.C.H.C, M.C.V, Serum Iron, Serum ferritin, PCV

Liver function tests - Serum Bilirubin, SGOT, SGPT, S. Alkaline phosphatase

Renal function tests - Blood Urea, S.Creatinine

Urine examination – Albumin, Sugar, Bile salts, Bile pigments, RBC, Pus cells, Epithelial cells

Stool examination - Ova cyst, Occult blood

5.7. STATISTICAL ANALYSIS:

- Sample size was determined prior to the start of the study through consultation with bio statistical expert and by using online sample size calculator, available on the website: www.kevinotto.com.
    - As per the protocol, the study has a prospective design with single group to be analyzed at two different time periods (paired group analysis).
    - A minimum difference of 2.0 was assumed to be significant between the average of pre and post treatment endpoints (Hb levels).
    - The standard deviation of the above expected difference was taken as 4 arbitrary.
    - The alpha error was set at 0.01 with 2-sided significance.
    - With 90% power expected, the sample size calculated to detect the required difference was 63.
    - Considering a dropout rate of 25%, the sample size was determined as, n=80.
- All statistical analyses were carried out using SPSS software (version20.0) and the graphical representations were plotted using GraphPad Prism software (Demo version 6.0).
- All data are expressed in mean with standard deviation or as median whichever seemed to be appropriate in use.
- The observed data showed normal distribution and parametric tests were employed to test the level of significant difference.
- One way ANOVA was used to compare and analyze safety and efficacy measures between control and test groups in laboratory animal experimentations.
- For clinical data, paired t test was used to analyze difference in pre and post treatment parameters.

6. RESULTS AND ANALYSIS

6.1. IDENTIFICATION OF RAW MATERIALS – BOTANICAL & GEOLOGICAL

The raw materials used for the preparation of *ABP* were procured from available market sources in Erode and Madurai districts. Fresh herbs were procured from Erode district. Both fresh and dry plant drugs were identified and authenticated by the Quality Control department of the GMP certified industry and Siddha Central Research Institute, Chennai and a voucher specimen of each drug were deposited at their herbarium. The Botanical identification of the plant drug used in ABP was done as per pharmacognostical standards. The iron ore used in the formulation was identified and authenticated by the Department of Geology, Government Arts College, Salem. The formulation was prepared as per Siddha scriptures, in strict adherence to GMP guidelines, under direct supervision using the infrastructure of a private Siddha Medicine manufacturing company (GMP certified).

The macroscopic and microscopic confirmatory paramenters observed in the identification of the herbs used in ABP are described below:

i) Kadukkai – *Terminalia chebula* Retz

Macroscopic:

Intact fruit yellowish-brown, ovoid, 20-25 mm long, 13-20 mm wide, wrinkled and ribbed longitudinally, pericarp fibrous, 3-4 mm thick, non-adherent to the seed; taste - astringent.

Microscopic:

Powder- Brownish in colour, under microscope shows a few fibres, vessels with simple pits and groups of sclereids. Transverse section of pericarp shows epicarp consisting of

one layer of epidermal cells inner tangential and upper portions of radial wall thick, mesocarp, 2-3 layers of collenchyma, followed by a broad zone of parenchyma in which fibres and sc1ereids in group and vascular bundles scattered, fibres with peg like out growth and simple pitted walls, sclereids of various shapes and sizes but mostly elongated, tannins and raphides in parenchyma, endocarp consists of thick-walled sclereids of various shapes and sizes, mostly elongated, epidermal surface view reveal polygonal cells, uniformly thickwalled, several of them divided into two by a thin septa, starch grains simple rounded oroval in shape, measuring 2-7 μ in diameter, found in plenty in almost all cells of mesocarp.

ii) Thandrikkai – *Terminalia bellerica* Roxb.

Macroscopic:

Fruit nearly spherical, 25-30 mm in diameter, mature fruits grey or grayish brown with slightly wrinkled appearance, rind of fruit shows variation in thickness from 3-5 mm; taste - astringent.

Microscopic:

Transverse section of fruit shows an outer epicarp consisting of a layer of epidermis, most of epidermal cells elongate to form hair like protuberance with swollen base, composed of a zone of parenchymatous cells, slightly tangentially elongated and irregularly arranged, intermingled with stone cells of varying shape and size, elongated stone cells found towards periphery and spherical in the inner zone of mesocarp ingroups of 3-10, mesocarp traversed in various directions by numerous vascular strands, bundles collateral, endarch, simple starch grains and some stone cells found in most of mesocarp cells, few peripheral layers devoid of starch grains, rosettes of calcium oxalate and stone cells present in parenchymatous cells, endosperm composed of stone cells running longitudinally as well as transversely.

iii) Nellikkai – *Emblica officinalis* Gaertn.

Macroscopic:

Fruit, globose, 2.5-3.0 cm in diameter, fleshy, smooth with six prominent lines; light yellowish or pinkish color; dry fruit pulp dark brown in color, wrinkled: taste, sour and astringent followed by delicately sweet taste.

Microscopic:

Transverse section of mature fruit shows an epicarp consisting of single layer of epidermis and 2-4 layers of hypodermis; epidermal cell, tabular in shape, covered externally with a thick cuticle and appear in surface view as polygonal; hypodermal cells tangentially elongated, thick-walled, smaller in dimension than epidermal cells; mesocarp forms bulk of fruit, consisting of thin-walled parenchymatous cells with intercellular spaces, peripheral 6-9 layers smaller, ovoid or tangentially elongated while rest of cells larger in size, isodiametric and radially elongated; several collateral fibrovascular bundles scattered throughout mesocarp consisting of xylem and phloem; xylem composed of tracheal elements, fibre tracheids and xylem fibres; tracheal elements show reticulate scalariform and spiral thickenings; xylem fibres elongated with narrow lumen and pointed end; mesocarp contains large aggregates of numerous irregular silica crystals.

iv) Chukku – *Zingiber officinale* Rosc.

Macroscopic:

Rhizome, laterally compressed bearing short, flat, oblique, branches on upper side each having at its apex a depressed scar, pieces about 5-13 cm long, 1.5-6.5 cm wide (usually 3-4 cm) and 1-1.5 cm thick, externally buff coloured showing longitudinal striations and occasional loose fibres, fracture short, smooth, transverse surface exhibiting narrow cortex (about one-third of radius), a well-marked endodermis and a wide stele showing

numerous scattered fibro-vascular bundles and yellow secreting cells, odour agreeable and aromatic, taste, agreeable and pungent.

Microscopic:

Transverse section of rhizome shows cortex. of isodiametric thin-walled parenchyma with scattered vascular strands and numerous isodiametric idioblasts, about 40-80 μ In diameter containing a yellowish to reddish-brown oleo-resin, endodermis slightly thick walled, free from starch immediately inside endodermis a row of nearly continuous collateral bundles usually without fibres stele of thin-walled, parenchymacells, arranged radially around numerous scattered, collateral vascular bundles, each consisting of a few unlignified, reticulate or spiral vessels upto about 70 μ in diameter,a group of phloem cells, unlignified, thin-walled, septate fibres upto about 30 μ wide and 600 μ long with small oblique slit, like pits, present, numerous scattered idioblasts, similar those of cortex, and associated with vascular bundles, also present, idioblasts about 8-20 μ wide and up to 130 μ long with dark reddish-brown contents: in single or in axial rows, adjacent to vessels, present, parenchyma of cortex and stele packed with flattened, rectangular, ovate, starch grains, mostly 5-15 μ - 30-60 μ long about 25 μwide and 7 μ thick, marked by five transverse striations.

v) Thipili – *Piper longum* Linn.

Macroscopic:

Fruit black in color, cylindrical, 2.5 to 3 cm long and 0.4 to 1 cm thick, consisting of minute sessile fruits, arranged around an axis; surface rough and composite; broken surface shows a central axis and 6 to 12 fruitlets arranged around an axis; taste, pungent producing numbness on the tongue; odour, aromatic.

Microscopic:

Catkin shows 6 to 12 fruits, arranged in circle on a central axis, each having an outer epidermal layer of irregular cells filled with deep brown content and coveredexternally with a thick cuticle; mesocarp consists of larger cells, usually collapsed, irregular in shape and thin-walled; a number of stone cells in singles or in groups present; endocarp and seed coat fused to form a deep zone, outer layer of this zone composed of thin-walled cells and colorless, inner layer composed of tangentially elongated cells, having reddish-brown content; most of endocarp filled with starchgrains, round to oval measuring 3 to 8 μ in dia.Powder - Deep moss green, shows fragments of parenchyma, oval to elongated stonecells, oil globules and round to oval, starch grains, measuring 3 to 8 μ in dia.

vi) Milaku – *Piper nigrum* Linn.

Macroscopic:

Fruits greyish-black to black, hard, wrinkled, 0.4-0.5 cm in dia.; odour, aromatic; taste, pungent.

Microscopic:

Fruit consists of a thick pericarp for about one third of fruit and an inner mass ofperisperm, enclosing a small embryo; pericarp consists of epicarp, mesocarp and endocarp; epicarp composed of single layered, slightly sinuous, tabular cells forming epidermis, below which, are present 1 or 2 layers of radially elongated, lignified stone cells adjacent to group of cells of parenchyma; mesocarp wide, composed of band of tangentially elongated parenchymatous cells having a few isolated, tangentially elongated oil cells present in outer region and a few fibro-vascular bundles, a single row of oil cells in the inner region of mesocarp; endocarp composed of a row of beaker shaped stone cells; testa single layered, yellow coloured, thick-walled sclerenchymatous cells; perisperm contains parenchymatous cells having a few oil globules and packedwith

abundant, oval to round, simple and compound starch grains measuring 5.5-11.0 µ in dia.; having 2-3 components and a few minute aleurone grains. Powder - Blackish-grey; shows debris with a characteristic, in groups, more or less isodiametric or slightly elongated stone cells, interspersed with thin-walled, polygonal hypodermal cells; beaker-shaped stone cells from endocarp and abundant polyhedral, elongated cells from peri sperm, packed tightly with masses of minute compound and single, oval to round, starch grains measuring 5.5-11.0 µ in dia.; having 2-3 component and a few aleurone grains and oil globules.

Iron (Ferrum)

The iron ore was procured from local raw drug market in Erode district. The sample was studied megascopically, microscopically and qualitatively and was authenticated as Magnetite (Fe_3O_4). Magnetite is characteristically recognized by its strong magnetism, color and streak.

6.2. PREPARATION OF *AYA BHRINGARAJA PAANIDHAM* (ABP)

The trial drug, *ABP* was prepared with strict adherence to traditional methods as per Siddha scriptures. Two different batches were prepared based on which a Standard Operating Procedure (SOP) was developed.

6.3. PHYSICOCHEMICAL ANALYSIS

6.3.1. Organoleptic studies of *Aya Bringaraja Paanidham* (ABP)

The organoleptic characters such as colour, odour, taste and texture for the trial drug *ABP* were studied using traditional and standard techniques.

Colour	:	Blackish brown
Odour	:	Agreeable pleasant odour

Taste : Sweet, Pungent, Astringent

Texture : Semi-liquid, thick syrupy consistency

6.3.2. Determination of Foreign matter

Presence of foreign matter was tested as per standard procedure and was found to be 0.01 g/ 100 g. The results were found to be within normal limits.

The presence of mould and insects were studied and it does not show presence of any mould or insects.

6.3.3. Determination of Ash values

Total ash

The total Ash value of the trial formulation, *ABP* = 7.62%

Acid insoluble ash

The acid insoluble ash value of the formulation, *ABP*= 1.56%

6.3.4. Determination of Extractive values

Alcoholsoluble extractive

The alcohol soluble extractive value for *ABP* = 71.24%

Water soluble extractive

The water soluble extractive value for *ABP* = 61.01%

6.3.5. Determination of Moisture Content/ Loss on drying (LOD) and pH

An excess of moisture/ water in medicinal plant materials will lead to unwanted microbial growth, increase the presence of fungi or insects and detioration of the quality of plant materials.

In this study, LOD of the trial formulation = 29.62%

The pH of the formulation was estimated using standard pH meter.

pH of *ABP* = 4.36

6.3.6. Determination of Total Sugar content

The total sugar content in the formulation, *ABP* = 18.42%

6.3.7. Determination of Microbial contamination

Medicinal plant drugs usually contain plenty of bacteria and moulds, often originating from soil and handling. While a large range of bacteria and fungi form the naturally occurring microflora of herbs, aerobic spore forming bacteria generally contaminate the drugs. The determination of *E.coli* and moulds may indicate the quality of production, since these are spread easily while handling and manufacturing.

Determination of Enterobacteriaceae and presence of *E.coli*

The formulation, *ABP* did not show any significant presence of micro organisms or *E.coli*.

Determination of total viable aerobic count

The total viable count of the material was determined as specified in the test procedure. Those batch of formulation having a count more than 10^4 are considered to be failed and unfit for human consumption. (Table 6.1)

Table 6.1: Results of Microbiological analysis of *ABP*

Microbiological analysis	Results	Unit	Procedure
Total Bacterial Count	1000	CFU/g	USFDA (BAM) Chapter-3
Total Fungal Count	< 10	CFU/g	USFDA (BAM) Chapter-18
E.coli	Absent/25g	-	ISO 7251 : 2005
Salmonella	Absent/25g	-	USFDA (BAM) Chapter-5
S.aureus	< 10	CFU/g	USFDA (BAM) Chapter-12
Pseudomonas aeruginosa	Absent/g	-	IS 13428 Annex D : 2005 (Reaff.2009)

6.3.8. Estimation of Iron Concentration

The concentration of iron present in the test drug, *ABP* was determined as a part of its standardization. The sample was digested as per standard procedures and the amount of iron in the formulation was estimated using Atomic absorption spectroscopy.

The concentration of iron in *ABP* was found to be 2.79 mg/kg (Ref. AOAC 19th Edition.2012 999.10).

6.3.9. Test for Heavy Metals – Lead, Cadmium, Mercury, Arsenic

Since the presence of heavy metals in a formulation is highly concerning parameter, regarding safety in human use, it is mandatory to test the heavy metal content in the trial drug. The concentration of heavy metals like Lead, Cadmium, Mercury and Arsenic were determined in the formulation using standard procedures in Atomic absorption spectroscopy. In the trial drug, *ABP* the heavy metals were found to be below detection limit. (Table 6.2).

Table 6.2. Analysis of Heavy Metal Content in ABP

Parameters	**Results**	**Permissible Limits (in mg/kg)**
Lead	BDL (DL:0.1)	10 ppm
Cadmium	BDL (DL:0.05)	0-3 ppm
Mercury	BDL (DL:0.05)	1 ppm
Arsenic	BDL (DL:0.05)	3 ppm

6.3.10. High Performance Thin Layer Chromatography (HPTLC) fingerprinting

Standardisation of the siddha formulation by HPTLC fingerprinting

Ethyl acetate extract (EA) of the sample ABP, was subjected to HPTLC analysis. Chromatographic information on the fingerprinting of the drug components can be observed in the Fig. 6.1. The Fig. 6.2 (a) shows a typical 2-D chromatogram of 12-17 peaks representing unknown substances of the test sample. The EA extract of *ABP* sample was analysed in seven different tracks of injection volumes: 2.5, 5.0, 7.5, 10, 12.5,15,20 µl. Among these, Track 5 exhibits maximum of 17 peaks of which 5 main peaks with R_f values 0.57, 0.61, 0.71, 0.78 and 0.97 were observed. Almost similar peaks were observed when the sample was subjected to densitometric scanning at 254 and 366 nm. (Fig. 6.2 b & c)

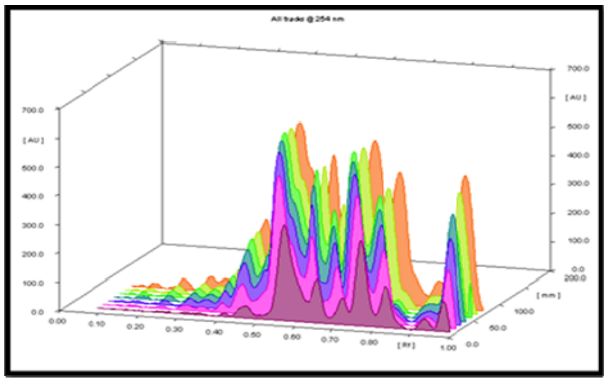

Fig. 6.1: 3-D chromatogram of HPTLC fingerprinting of *ABP*

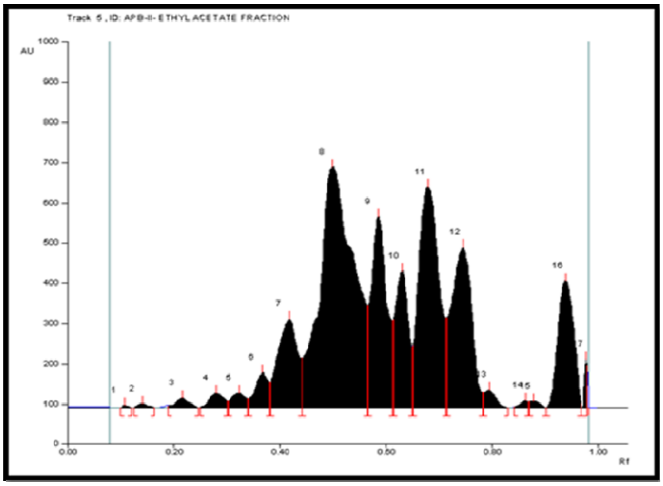

Fig. 6.2(a): HPTLC fingerprinting of *ABP*

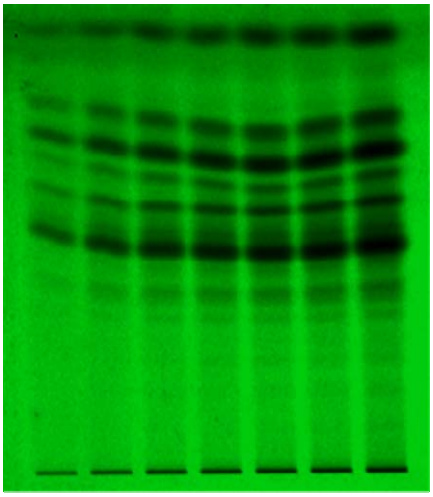

Fig.6.2(b) Scan at 254 nm

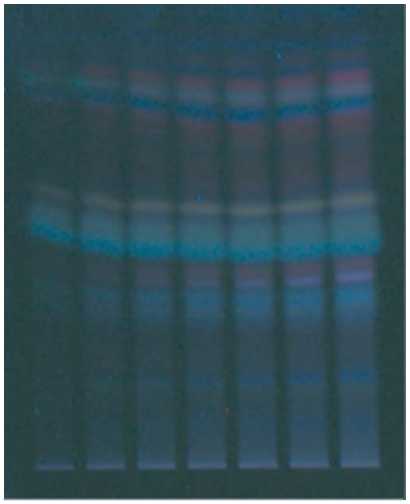

Fig.6.2(c) Scan at 366 nm

6.3.11. High Performance Liquid Chromatography(HPLC) fingerprinting of *ABP*

The same ethyl acetate extract of *ABP* was subjected to HPLC analysis under specified conditions. Three different sample injection volumes, 20, 40 and 50 µL were used and chromatographic profile was documented at 254 and 298 nm. In 40 µL sample injection, a single major peak at retention time 2.79 and 2.791 is observed for 254 and 298 nm detection, respectively. The Fig. 6.3 depicts the chromatogram exhibiting the above fingerprint of the sample.

6.3.12. GC Analysis

GC-MS chromatogram of EA extract of the trial drug *ABP* showed ten major peaks indicating the presence of ten unknown phytocomponents. These peaks were relatively compared with NIST database entities based on its structural similarities by the inbuilt software of the instrument. (Fig. 6.4) Medicinal properties between the ten unknown phytocomponents identified and the database entities were not significantly comparable.

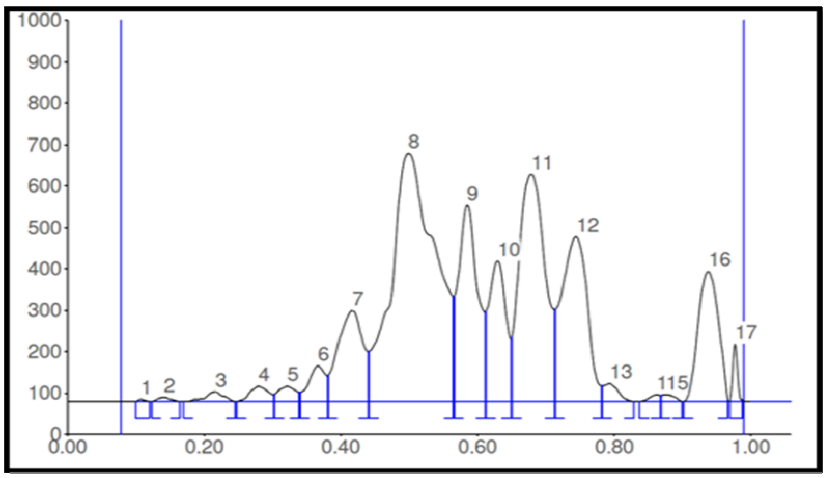

Fig 6.3: HPLC Fingerprinting of ABP

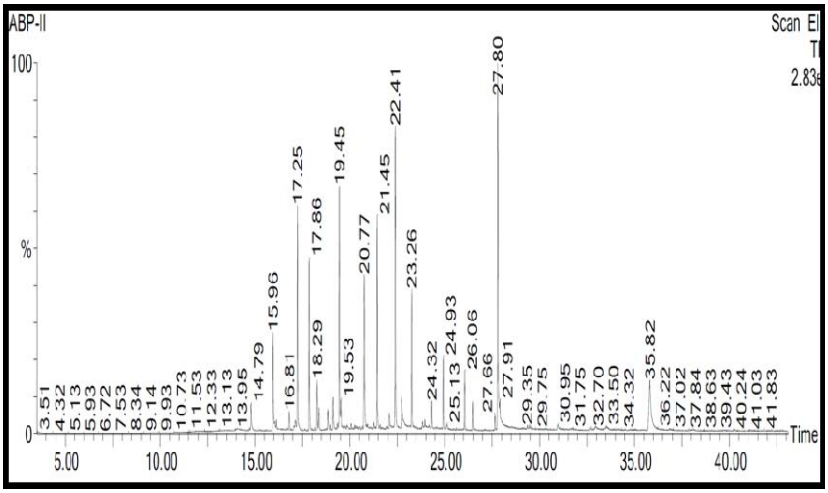

Fig 6.4: GC Profile of ABP

6.3.13. Electron Spectroscopy for Chemical Analysis (ESCA)

ESCA analysis, also known as Electron Spectroscopy for Chemical Analysis or X-Ray Photoelectron Spectroscopy (XPS), is a surface analysis technique that provides elemental and binding energy information about a material's surfaces and interfaces.This technique is helpful in determining the elemental composition of the surface (top 0–10 nm usually) and chemical or electronic state of each element in the surface.

Three samples of iron obtained from different traditional processing methods were subjected to ESCA study. Mainly the raw iron and processed iron (Bhavana Ayam) were compared for the oxidation state of iron in the samples. The scan of iron samples shows that elements such as Carbon , Oxygen and Fe $(CO)_5$ are found in the processed samples. Fe $(CO)_5$ was not found in raw iron sample. The Binding energies of Fe observed was as follows:

Fe^{0+} - 707 eV

Fe^{2+} - 709eV

Fe^{3+} - 712eV

The ESCA survey scan of iron samples showed presence of peaks in the range between 707 and 724 eV. The peaks obtained for the presence of iron before and after preparation process are given in the Fig. 6.5a & b.

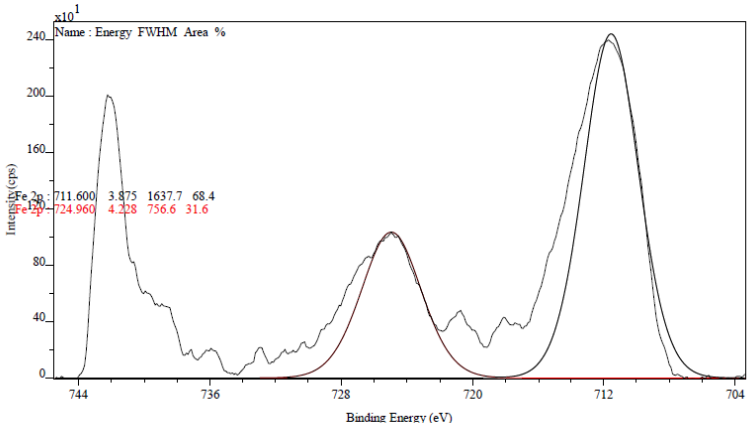

Fig. 6.5(a): ESCA survey scan of raw iron – Before processing

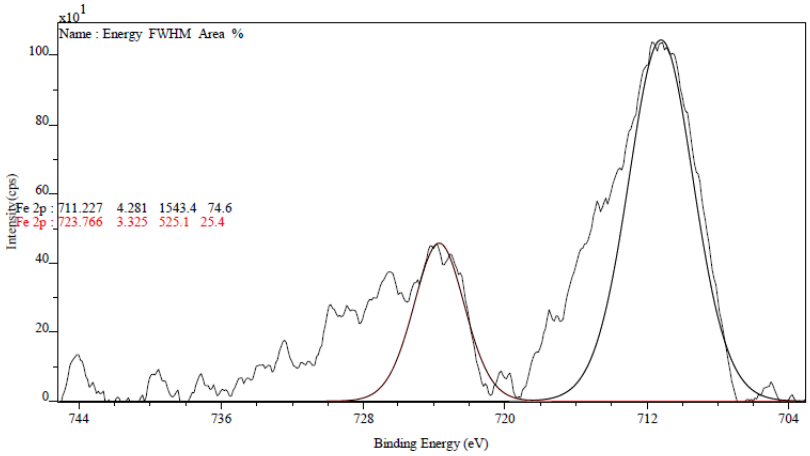

Fig. 6.5(b): ESCA survey scan of processed iron

6.4. PHARMACOLOGICAL TOXICITY STUDIES

6.4.1. Acute toxicity

To evaluate the toxicity of the trial drug, *ABP* the acute and sub-acute toxicity studies were carried out as per standard procedures. No mortality was observed upto 2600 mg/kg body weight of *ABP* in acute toxicity studies. Even the administration of high dose did not result in mortality or changes in behavior, body weight or blood parameters. All the

animals were found to be normal during and at the end of the observation period when studied in albino mice.

6.4.2. Sub-acute toxicity

In Sub-acute toxicity study, there was no death in the treatment period either in the control or in the treated groups. Food and water consumption also did not differ significantly. There was no change in the general behavior or other physiological activities of the animals. None of the organs in the treated rats showed any significant change in weight. Haematological analysis showed no significant increase in serum alkaline phosphatase, acid phosphatase, ALT, AST, Urea or Creatinine levels as compared to control. Gross histological examination of the organs did not reveal any pathological changes. These observations were similar in both male and female rats. The results show that a very high oral dose was tolerated by the animals without producing any toxicity symptoms.

The results of Sub-acute toxicity studies are tabulated in the following tables:
(Table. 6.3, 6.4, 6.5a & b, 6.6, 6.7)

Table 6.3: SUB ACUTE TOXICITY STUDIES: Biochemical Parameters - 15th day of treatment*

MALE Wistar albino rats:									
S. No.	**Group#**	**Sugar**	**Cholesterol**	**Total Protein**	**Serum Creatinine**	**TGL**	**SGOT**	**SGPT**	**SAP**
1	Control	88.84±12.92	49.28±12.97	6.41±0.89	0.68±0.1	118.82±23.79	129.68±13.79	47.85±6.99	214.84±33.93
2	I dose	86.38±3.71	44.10±4.73	7.25±0.61	0.66±0.12	176.06±46.42	136.75±24.27	57.49±8.47	278.58±51.12
3	II dose	70.53±11.75	54.06±6.47	6.58±0.81	0.49±0.08	153.06±39.42	125.27±17.55	56.57±6.04	273.66±32.68
4	III dose	90.12±10.89	54.14±7.98	6.45±0.45	0.45±0.06	123.13±3.9	116.68±7.8	53.62±4.38	225.92±45.48
FEMALE Wistar albino rats:									
1	Control	85.33±16.33	49.62±7.37	6.91±0.51	0.71±0.08	212.77±85.42	127.1±11.72	55.56±5.98	201.47±34.34
2	I dose	91.89±7.25	46.37±14.6	6.76±0.72	0.64±0.11	186.44±41.24	114.48±20.4	58.35±11.5	172.57±16.87
3	II dose	81.05±12.36	59.75±10.28	6.51±0.32	0.57±0.05	164.75±82.26	130.72±13.04	58.88±10.19	260.61±46.30
4	III dose	85.73±3.12	59.17±12.19	6.22±0.45	0.55±0.07	124.07±4.50	114.62±8.4	61.71±12.99	237.65±38.61

***Values are expressed as mean ± SD**
 # Data analyzed using One Way ANOVA; $p<0.01$ considered as significant.

Table 6.4: SUB ACUTE TOXICITY STUDIES: Biochemical Parameters - 30th day of treatment*

MALE Wistar albino rats:									
S.No	**Group#**	**Sugar**	**Cholesterol**	**Total Protein**	**Serum Creatinine**	**TGL**	**SGOT**	**SGPT**	**SAP**
1	Control	111.2±10.59	70±20.07	117.8±27.68	0.59±0.09	9.17±0.55	120.6±11.82	67±12.31	257.4±99.49
2	I dose	109.5±14.37	88.5±13.02	131.4±37.84	0.58±0.04	8.28±0.50	127.1±13.89	83.2±4.77	323.7±50.11
3	II dose	91.8±11.26	72.2±12.90	124.3±46.11	0.58±0.06	8.41±0.53	127.5±11.94	90.9±7.03	299.7±53.89
4	III dose	95.6±8.35	52.2±9.23	115.5±18.6	0.56±0.05	8.13±0.44	121.2±23.34	82±16.77	261.6±57.78
FEMALE Wistar albino rats:									
1	Control	114.47±8.21	68.86±13.52	154.13±34.47	0.45±0.08	7.92±0.70	113.14±23.60	70.63±23.96	175.13±15.50
2	I dose	110.39±10.39	81.41±9.61	159.93±26.25	0.55±0.04	8.38±0.76	155.91±25.05	77.55±13.15	198.89±17.79
3	II dose	104.3±16.49	76.8±9.10	157.6±58.64	0.51±0.06	9.17±0.59	156.5±36.21	74.4±7.98	185.6±14.54
4	III dose	99.89±6.86	72.26±10.90	145.96±27.65	0.48±0.06	9.33±0.59	148.98±44.82	71.15±21.69	180.73±17.10

***Values are expressed as mean ± SD**
 # Data analyzed using One Way ANOVA; $p<0.01$ considered as significant.

Table 6.5a: SUB ACUTE TOXICITY STUDIES: Organ Weights[*]

MALE Wistar albino rats:							
No.	**Group[#]**	**Heart**	**Liver**	**Lungs**	**Pancreas**	**Spleen**	**Kidney**
1	Control	1.37±1.42	8.03±1.23	1.79±0.55	0.64±0.16	0.54±0.14	1.83±0.30
2	I dose	0.63±0.13	6.85±1.18	1.23±0.37	0.62±0.26	0.41±0.14	1.49±0.29
3	II dose	0.68±0.17	5.87±1.41	1.30±0.32	0.26±0.03	0.40±0.12	1.53±0.21
4	III dose	0.60±0.09	5.85±1.14	1.12±0.20	0.20±0.05	0.41±0.11	1.43±0.29
FEMALE Wistar albino rats:							
1	Control	0.64±0.16	5.32±1.29	1.16±0.47	0.79±0.49	0.39±0.19	1.49±0.32
2	I dose	0.51±0.08	4.28±0.61	1.11±0.43	0.29±0.06	0.33±0.09	1.09±0.11
3	II dose	0.57±0.13	4.16±0.85	1.11±0.17	0.39±0.08	0.39±0.10	1.08±0.19
4	III dose	0.49±0.10	4.47±0.82	0.95±0.08	0.25±0.02	0.34±0.06	1.17±0.12

Table 6.5b: SUB ACUTE TOXICITY STUDIES: Organ Weights*

MALE Wistar albino rats:						
No.	**Group#**	**Stomach**	**Intestine**	**Eyes**	**Brain**	**Testes**
1	Control	1.97±0.46	14.05±2.81	0.26±0.06	1.46±0.26	2.98±0.89
2	I dose	1.60±0.40	11.42±1.30	0.28±0.10	1.23±0.23	2.31±0.42
3	II dose	1.60±0.32	11.08±2.04	0.28±0.02	1.23±0.25	2.59±0.79
4	III dose	1.58±0.34	10.38±1.96	0.24±0.02	1.13±0.17	2.66±0.85
FEMALE Wistar albino rats:						
1	Control	1.60±0.24	9.15±3.49	0.28±0.08	1.22±0.21	1.80±0.55
2	I dose	1.63±0.32	8.64±1.74	0.27±0.08	1.03±0.28	1.77±0.46
3	II dose	1.24±0.36	8.15±2.05	0.27±0.00	1.24±0.19	1.66±0.64
4	III dose	1.21±0.15	8.12±1.82	0.25±0.02	1.23±0.23	1.69±0.51

***Values are expressed as mean ± SD**
 # Data analyzed using One Way ANOVA; $p<0.01$ considered as significant.

Table 6.6: SUB ACUTE TOXICITY STUDIES: Body weight (weekly) [*]

MALE Wistar albino rats:						
S. No	**Group[#]**	**Initial**	**I[st] week**	**2[nd] week**	**3[rd] week**	**4[th] week**
1	Control	193.33 ± 28.75	192.5 ± 26.78	200.83 ± 26.53	207.5 ± 24.23	214.16 ± 22.01
2	I dose	206.66 ± 22.50	206.66 ± 23.59	211.66 ± 23.16	218.33 ± 22.28	220.83 ± 20.83
3	II dose	184.16 ± 15.62	187.5 ± 15.73	195 ± 13.78	203.33 ± 14.02	208.33 ± 16.32
4	III dose	174.16 ± 13.57	177.5 ± 12.54	182.5 ± 12.54	190.83 ± 12.01	203.33 ± 10.32
FEMALE Wistar albino rats:						
1	Control	133.33 ± 20.65	140.83 ± 18.81	146.66 ± 17.22	154.16 ± 16.85	163.33 ± 16.32
2	I dose	130 ± 7.07	133.33 ± 8.16	140.83 ± 9.17	148.33 ± 10.32	144.16 ± 9.17
3	II dose	126.66 ± 13.66	130.83 ± 13.58	135.83 ± 13.57	143.33 ± 13.29	153.33 ± 13.66
4	III dose	128.33 ± 14.71	132.5 ± 14.05	138.33 ± 14.71	145 ± 17.32	153.33 ± 20.89

***Values are expressed as mean ± SD**
 # Data analyzed using One Way ANOVA; p<0.01 considered as significant.

Table 6.7: SUB ACUTE TOXICITY STUDIES: Food Consumption (weekly) *

MALE Wistar albino rats:					
S. No	**Group#**	**I st week**	**2nd week**	**3rd week**	**4th week**
1	Control	13.06 ± 2.04	12.21 ± 1.85	15.5 ± 1.71	13.16 ± 2.22
2	I dose	14.12 ± 1.62	15.4 ± 1.12	13 ± 2.11	15.5 ± 2.07
3	II dose	13 ± 2.01	14.35 ± 1.44	14 ± 2.74	12.5 ± 2.16
4	III dose	13.4 ± 1.72	12.52 ± 1.94	16.33 ± 2.26	17.66 ± 1.50
FEMALE Wistar albino rats:					
1	Control	11.66 ± 1.50	11.66 ± 2.94	16.83 ± 2.85	14.33 ± 4.27
2	I dose	11 ± 1.09	17.33 ± 2.80	10.66 ± 1.63	14 ± 2.60
3	II dose	10.66 ± 1.03	14.5 ± 1.51	17.16 ± 1.83	13.33 ± 1.63
4	III dose	12.16 ± 2.40	11.33 ± 2.06	16.83 ± 2.71	15.5 ± 3.78

***Values are expressed as mean ± SD**
 # Data analyzed using One Way ANOVA; p<0.01 considered as significant.

6.5. PHARMACOLOGICAL ACTIVITY STUDIES

The Pharmacological studies involving experimental animal models revealed that no mortality was observed in animals receiving test compound ABP in therapeutic high dose by oral route. No significant treatment related effect on clinical signs or behavioral activity etc was observed in all the groups of animals that survived during the experimental period.

The below mentioned liver function marker were measured in the experimental animal models at the end of the treatment period. Plasma – SGOT, SGPT, Alkaline phosphatase, Total bilirubin, γ-GT, Total proteins, Lactate dehydrogenase; Hepatic tissue - Reduced glutathione, Super oxide dismutase, Catalase, Glutathione peroxidase, Lipid peroxidation, Nitrite/nitrate.

6.5.1. Hepatoprotective activity against CCl4-induced hepatotoxicity in rats

Hepato-protective activity was analyzed pre-clinically in 5 groups of Wistar rats (n=6 in each group) of either gender, with an initial normal body weight. No statistically significant difference ($F=0.95$, $p=0.41$) was observed between the 5 groups, among all the parameters assessed such as, ALT (IU/L), AST (IU/L), ALP (IU/L) and Total Bilirubin (mg/dl).

The mean findings with standard deviations are summarized in the Table 6.8. and depicted graphically in the Fig 6.6. These results confirmed the presence of hepato-protective activity in the test groups (Group III & IV) given with the test drug formulations.

Table 6.8: Hepato protective activity studies (n=6 in each group)*

Parameters# (units)	I (Control)	II (CCl_4+ olive oil)	III (Low dose)	IV (High dose)	V (Std-Silymarin)
ALT(IU/L)	20 ± 2.8	35.3 ± 1.9	24.2 ± 2.4	22.5 ± 2.3	21.6 ± 1.8
AST(IU/L)	132 ± 4.0	130 ± 4.2	122.4 ± 5.0	117.6 ± 5.2	118.4 ± 2.3
ALP(IU/L)	114 ± 7.8	189.4 ± 6.4	172.4 ± 4.6	186.7 ± 5.4	188.2 ± 4.5
TOTAL BILIRUBIN (mg/dl)	0. 39 ± 0.03	0.85 ± 0.06	0.52 ± 0.2	0.53 ± 0.2	0.58 ± 0.1

***Values are expressed as mean ± SD**
 # Data analyzed using One way ANOVA; p<0.01 considered as significant.

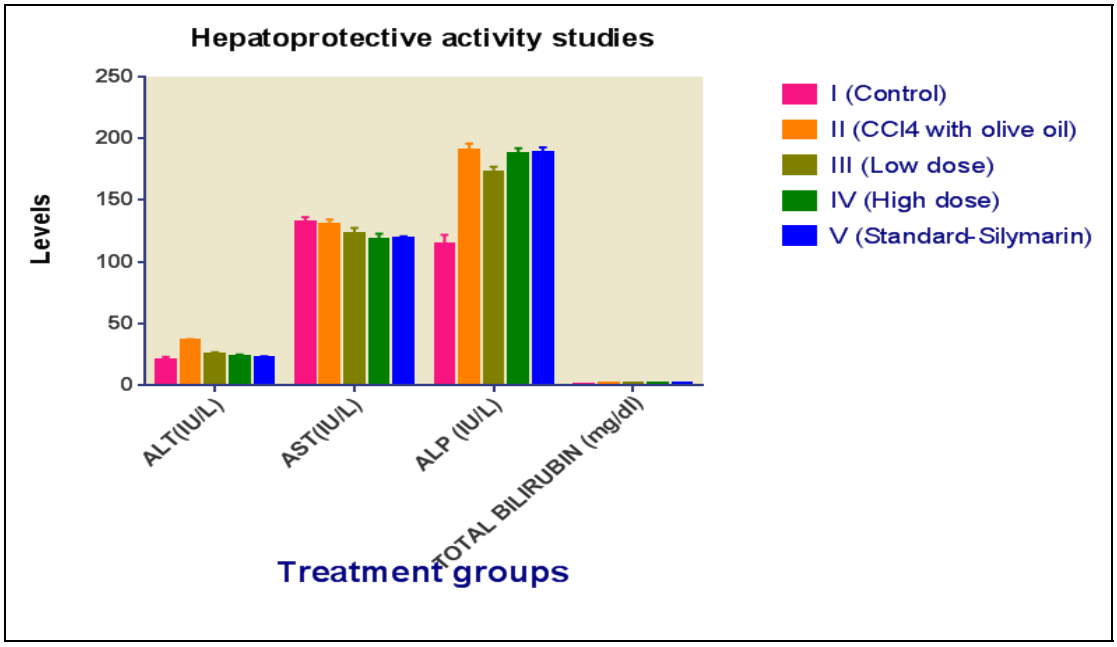

Fig. 6.6: Hepato protective activity studies

6.5.2. Erythropoietic activity

Preclinical assessment erythropoietic activity of the test drug involved 3 groups of Wistar rats (n=6 in each group) of either gender, with an initial normal body weight. No statistically significant difference (F=3.13, p=0.33) was observed between the groups, among both the parameters assessed such as, RBC count (x10^6 Cu /mm) and haemoglobin (g/dl) levels.

The mean findings with standard deviations are summarized in the Table 6.9. and depicted graphically in the Fig 6.7. These results confirmed the presence of hematopoietic activity in the test groups (Group II & III) given with the test drug formulations.

Table 6.9: Erythropoietic Activity (n=6 in each group)*

Parameters#(units)	**I (CONTROL)**	**II (LOW dose)**	**III (HIGH dose)**
RBC (10^6 Cu /mm)	4.38 ± 0.04	4.30±0.05	4.67±0.05
Hemoglobin (g/dl)	14.17 ± 0.20	14.24± 0.04	15.37 ± 0.29
***Values are expressed as mean ± SD # Data analyzed using One way ANOVA; p<0.01 considered as significant.**			

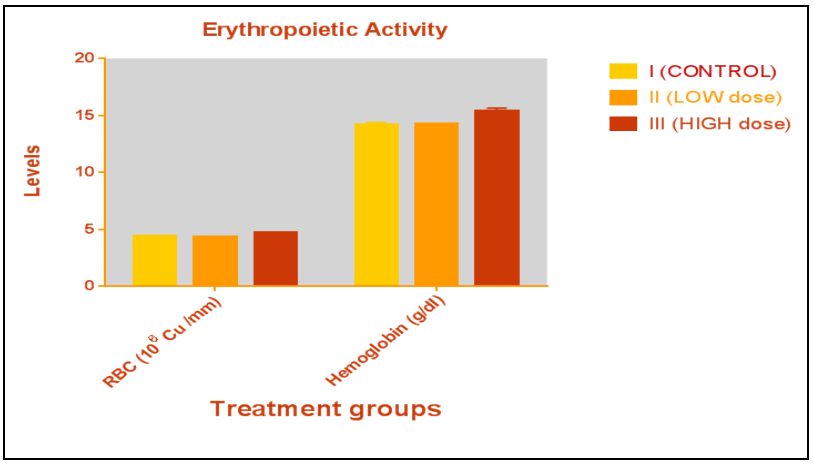

Fig. 6.7: Erythropoietic Activity studies

6.6. CLINICAL EVALUATION OF TRIAL DRUG *ABP*

6.6.1. Patient Recruitment

The study protocol was designed for effective conduction of the clinical trial, which was approved by the Institutional Ethics Committee (IEC), NIS, Chennai. **IEC approval No.NIS/IEC/2011/1/18** (See: Annexure I)**.** The trial upon IEC approval was registered in Clinical Trial Registry of India (CTRI); Trial Registration No.: **CTRI/2014/07/004802** (See: Annexure II)

Specific case record forms (CRF), which are simple, clear, was designed in such a way that it contains all the information related to the study were used to record the clinical data. Following the approval of the IEC and CTRI registration, patients with anaemia were recruited as per inclusion and exclusion criteria. Ambulatory patients of either sex, in the age group of 18-60 years, all economic classes, with blood parameters Hb: 6 -10 g/dl, MCV < 80 fl, MCHC < 34 g/dl, Serum iron concentration < 50 µg/dl, Serum Ferritin < 30 ng/ml and those willing to participate were recruited.

The trial centres National Institute of Siddha, Chennai and Siddha Regional Research Institute, Puducherry were chosen to conduct the clinical trial since they had a good patient census. Patients visiting the out-patient departments of the selected trial centres were screened for IDA using clinical examination and related diagnostic parameters as per Screening Proforma.

A Pilot study on ten patients was commenced to assess the feasibility of the proposed trial. The patients were recruited as per inclusion and exclusion criteria with their consent. Based on the analysis sought from the pilot study, the trial was continued at two centres as described above.

A total of 192 patients were screened out of which 92 were recruited after obtaining their informed consent. The selected patients were issued an Information sheet explaining the purpose and procedure of the clinical trial. Informed Consent forms in

English or Tamil duly signed by the patient was obtained before start of treatment on Day 0. After recruitment the baseline diagnostic parameters were recorded before prescribing the trial drug *ABP* as per the protocol. The detailed history with respect to the patient was recorded in Case History Proforma. The Periodical Clinical assessment and assessment of Diagnostic parameters was done in regular intervals as per protocol. There were 10 drop-outs from the treatment schedule and a total of 82 patients completed the trial successfully.

The diagnostic parameters – Physical examination and Laboratory investigations were done on 0 day (before treatment with ABP) and on 45th day (before treatment with ABP). The prevalence of *Veluppu Noi* / IDA was observed among all completed cases with respect to their age, sex, height, weight, food habits, *kaalam* (Seasonal variations), *thinai* (Land tracts), *udaliyal* (Body constitution), occupation, etiological factors etc. Among the trial participants the incidence of prominent clinical feature was observed. The eight fold diagnostic tools, *Enn vagai thervu – Naadi, Sparisam, Naa, Niram, Mozhi, Vizhi, Malam and Moothiram*, unique to Siddha system were recorded for all the patients at 0 day, 15th day, 30th day and 45th day of treatment with *ABP*.

6.6.2. Age, Gender distribution

As per inclusion criteria only the patients in the age group of 18-60 years were recruited for the trial. The frequency distribution of age groups is summarized in the Table 6.10 and Fig. 6.8 (a) and (b). The distribution was found to be normal with around 38% and 30.5% of the participants in the age group 31-40 and 41-50 years, respectively. From the baseline demographic data it was observed that the Male:Female ratio was 8:74, with a mean age of 38.7±9.88 years and BMI of 25.39±4.52.

Table 6.10: Frequency distribution of age groups		
Age group (in yrs)	**%**	**N**
18-30	20.73	17
31-40	37.80	31
41-50	30.49	25
51-60	10.98	9

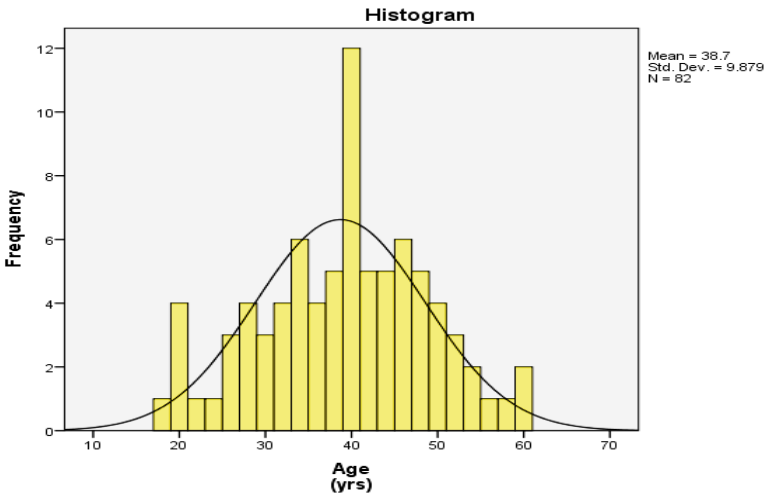

Fig. 6.8 (a): Frequency Distribution of age groups of recruited patients

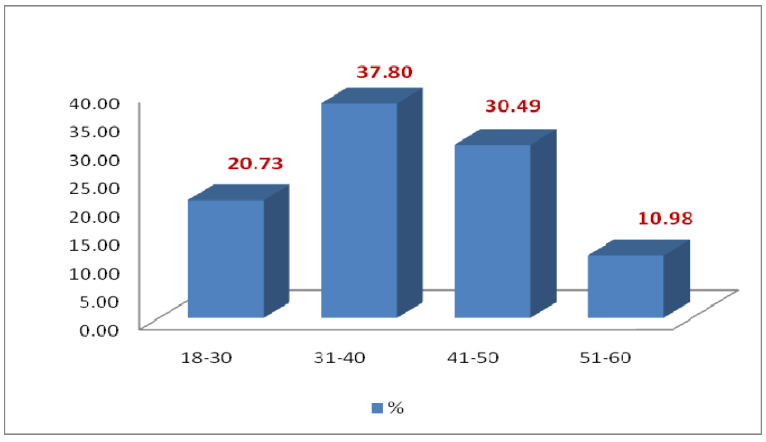

Fig. 6.8 (b): Age Distribution in percentages

The complete baseline demography of both the centres (Centre I: NIS, Chennai; Centre II: SRRI, Puducherry) is described in the Table 6.11.

Table. 6.11: Baseline Demography

Characteristics	**Pilot study**	**Centre I**	**Centre II**	**Both**
No. of patients screened	30	56	106	192
No. of patients recruited	10	34	48	92
No. of patients completed follow up	10	32	40	82
No. of patients lost to follow up	0	2	8	10
Male: Female	0:10	5:27	3:37	8:74
Age (years)	39.60 (10.53)	39.28 (8.9)	38.0 (10.3)	38.7 (9.88)
Height (cm)	157.50 (5.37)	154.30 (9.32)	159 (4.92)	156.98 (7.35)
Weight (kg)	60.15 (7.40)	62.51 (10.14)	62.7 (9.1)	62.29 (9.35)
BMI	24.21 (2.33)	26.53 (5.59)	24.8 (3.63)	25.39 (4.52)

**Data expressed as mean (SD)*
Centre I: National Institute of Siddha (NIS), Chennai
Centre II:Siddha Regional Research Institute for (SRRI), Puducherry
Pilot study: At SRRI, Puducherry

6.6.3. Dietary habits

Among the recruited patients, the food habits were found to be in the ratio of Veg:Non-veg as 19:63. Most of the patients (76%) were non vegetarians.

6.6.4. Relation with *Kaalam, Thinai* and *Udaliyal* (See:Table 6.12; Fig.6.9; 6.10& 6.11)

Table. 6.12: Baseline Demography w.r.t. Thinai and Naadi

Characteristics	Sub Groups	Pilot study	Centre I	Centre II	Both
No. of patients		10	32	40	82
Male: Female		0:10	5:27	3:37	8:74
Mean Age ± SD (years)		39.60±10.53	39.28±8.9	38.0±10.3	38.7±9.88
Food Habits: Veg/Non-Veg		3/7	8/24	8/32	19/63
Recruitment Season (Kaalam)‡	Ilavenil	10	-	5	15(18.3)
	Mudhuvenil	-	31	30	61(74.4)
	Kaar	-	1	5	6(7.3)
Residing Land (Thinai)‡	Marutham	6	26	23	55(67.1)
	Mullai	-	-	2	2(2.4)
	Neithal	4	6	15	25(30.5)
Udaliyal (Body constitution)‡	Kaphapitham	-	6	2	8(9.8)
	Kaphavatham	2	4	3	9(11.0)
	Pithakapham	1	5	7	13(15.9)
	Pithavatham	1	3	9	13(15.9)
	Vathakapham	2	4	3	9(11.0)
	Vathapitham	4	7	14	25(30.5)
	Vatham	-	3	2	5(6.1)
Naadinadai (Pulse)‡	Kaphapitham	2	2	4	8(9.8)
	Kaphavatham	-	3	7	10(12.2)
	Pithakapham	1	6	9	16(19.5)
	Pithavatham	5	6	5	16(19.5)
	Vathakapham	-	3	4	7(8.5)
	Vathapitham	2	12	11	25(30.5)

**Data expressed as mean (SD);* ‡No. of subjects (%)
Centre I: National Institute of Siddha (NIS), Chennai
Centre II: Siddha Regional Research Institute for (SRRI), Puducherry
Pilot study: At SRRI, Puducherry

When observing the baseline demographic data, it was found that 74.4 % of the patients were recruited during *Mudhuvenil kaalam* (Mid June to Mid August), 18.3% of patients were recruited during *Ilavenil kaalam* (Mid April to Mid June) and 7.3% were recruited during *Kaar kaalam* (Mid August to Mid October)**.**

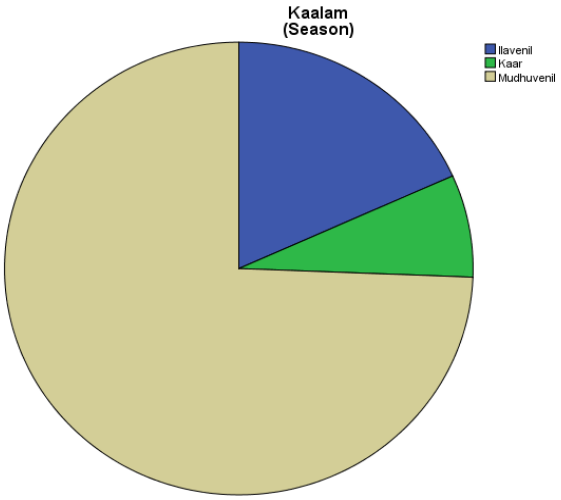

Fig. 6.9: Distribution of season (kaalam) during which the patient was recruited

The baseline data shows that 67% of patients belong to the land tract of *Marutham,* nearly 31% were from that of *Neithal* and 2% were from *Mullai thinai*.

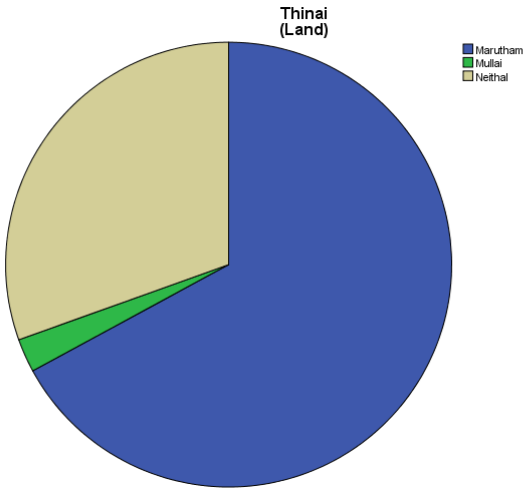

Fig. 6.10: Distribution of land tract (Thinai) from which the patient was recruited

The body constitution (*Udaliyal*) of all the patients was assessed using a simple questionnaire. It was observed that most of patients had *Thontham udaliyal*, with nearly 31% of the patients were of *Vathapitha udaliyal,* 10% of *Kaphapitham*, 11% each of *Kaphavatham* and *Vathakapham*, 16% each of *Pithakapham* and *Pithavatham*; 5 patients had *Vatha udaliyal*.

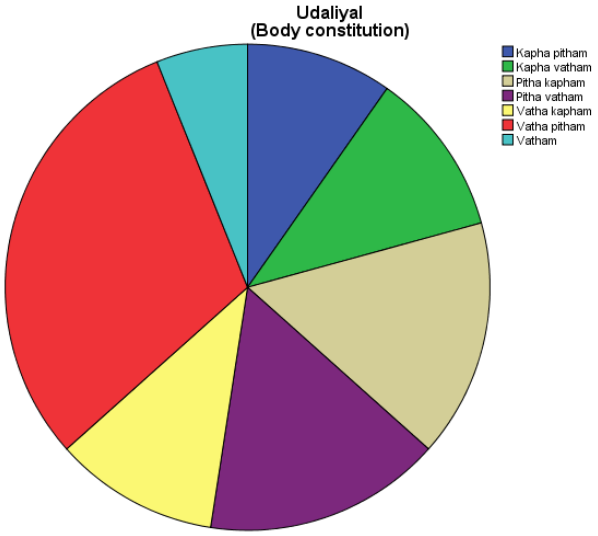

Fig.6.11: Body constitution (Udaliyal) of recruited patients

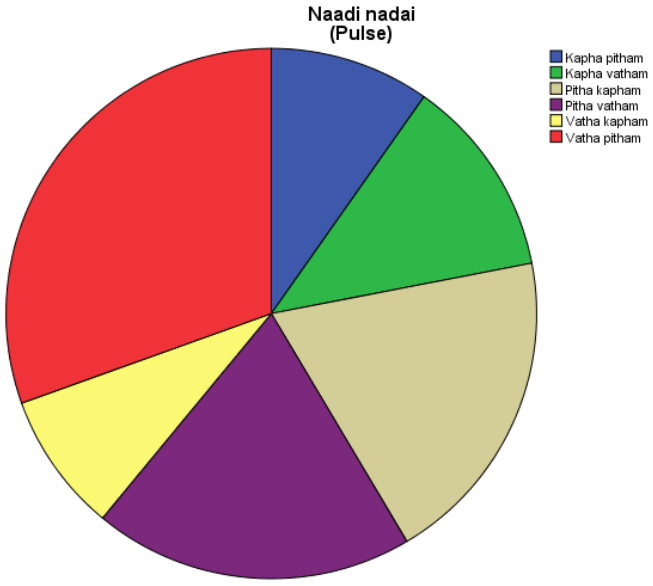

Fig.6.12: Traditional pulse reading (Naadi nadai) in the recruited patients

6.6.5. Predominance of *Naadi nadai*

The eight fold examination (*Enn vagai thervu*) was recorded for all the patients. In particular, the *Naadi nadai* was observed in all patients in which Vathapitha naadi was recorded in 25 patients (30%), Pithakapham and Pithavatham in 20% of patients each, Kaphapitham in nearly 12% and Vathakapham in 9% of patients. (Fig.6.12)

6.6.6. Haematological parameters

From the baseline data it was found that the haematological parameters viz., Haemoglobin, total count, MCHC, MCV were low and within the inclusion criteria. (Table 6.13)

Table 6.13: Baseline Haematological Characteristics*

Parameters (units)	Centres I & II	Centre I	Centre II	Pilot study
No. of subjects (n)	82	32	40	10
TC (Cells /Cu.mm)	6732.20 (1799.50)	6451.25 (1688.84)	7080.00 (1893.44)	6240.00 (1335.1)
DC:P (%)	60.49 (6.23)	60.91(6.14)	60.18 (6.67)	60.40 (3.8)
DC:L (%)	34.43 (6.33)	33.06 (6.54)	35.00 (6.36)	36.50 (3.75)
DC:E (%)	2.7 (2.5)	2.25 (2.74)	3.23 (2.32)	1.80 (1.17)
DC:M (%)	2.1 (2.21)	3.00 (2.65)	1.55 (1.75)	1.30 (0.64)
DC:B (%)	0	0	0	0
ESR (mm/hr)	18.65 (7.71)	25.29 (10.96)	18.28 (6.68)	15.50 (5.35)
Hb (g%)	8.38 (1.15)	8.20 (1.24)	8.50 (1.12)	8.43 (0.71)
M.C.H.C (%)	28.00 (3.32)	27.76 (2.46)	28.80 (3.48)	25.78 (3.73)
M.C.V (fl)	67.28 (11.77)	64.48 (13.73)	69.76 (9.82)	66.50 (9.12)
Serum Ferritin (ng/ml)	6.61 (7.07)	7.24 (8.58)	6.24 (6.00)	6.48 (4.70)
Serum Iron (µg/dl)	20.34 (8.96)	19.16 (6.97)	21.03 (10.64)	21.40 (5.89)
Total RBC (million/cumm)	4.00 (0.73)	-	3.98 (0.68)	4.05 (0.34)

**Data expressed as mean (SD)*;
Centre I: National Institute of Siddha (NIS), Chennai
Centre II: Siddha Regional Research Institute for (SRRI), Puducherry
Pilot study: At SRRI, Puducherry

Table 6.14: Haematological Characteristics-Before & After treatment (n=82)[*]

Parameters (units)	Before treatment	After treatment	Mean Difference (95% CI)	SEM	P value
TC (Cells /Cu.mm)	6732.20 (1799.50)	6915.38 (1660.79)	-162.694 (-328.808;3.420)	83.456	.055
DC:P (%)	60.49 (6.23)	60.92 (6.37)	-0.418 (-2.107;1.271)	0.848	.623
DC:L (%)	34.43 (6.33)	35.05 (6.27)	-0.444 (-2.066;1.179)	0.815	.588
DC:E (%)	2.7 (2.5)	2.53 (2.19)	0.262 (-0.319;0.842)	0.292	.372
DC:M (%)	2.1 (2.21)	1.37 (2.05)	0.703 (0.278;1.129)	0.214	**.001**
ESR (mm/hr)	18.65 (7.71)	19.98 (6.55)	-1.459 (-3.734;0.816)	1.135	.204
Hb (g%)	8.38 (1.15)	9.17 (1.46)	-0.781 (-1.001;-0.562)	0.110	**.001**
M.C.H.C (%)	28.00 (3.32)	30.24 (3.47)	-2.242 (-2.892;-1.592)	0.326	**.001**
M.C.V (fl)	67.28(11.77)	72.37 (12.34)	-5.084 (-6.873;-3.295)	0.899	**.001**
Serum Ferritin (ng/ml)	6.61 (7.07)	8.72 (7.83)	-1.961 (-2.794;-1.127)	0.419	**.001**
Serum Iron (µg/dl)	20.34 (8.96)	26.36 (13.24)	-5.979 (-7.942;-4.017)	0.986	**.001**
Total RBC (million/cumm)	4.00 (0.73)	4.06 (0.53)	-0.038 (-0.251;0.175)	0.106	.723

**Data expressed as mean (SD)*
Analyzed using paired t test with 2 tailed significance, considering p<0.01 as significant

The mean Hb before treatment was 8.38 and the same was 9.17 after treatment with *ABP* with a significant p-value<0.001(Fig. 6.13 & 6.14; Table. 6.14).

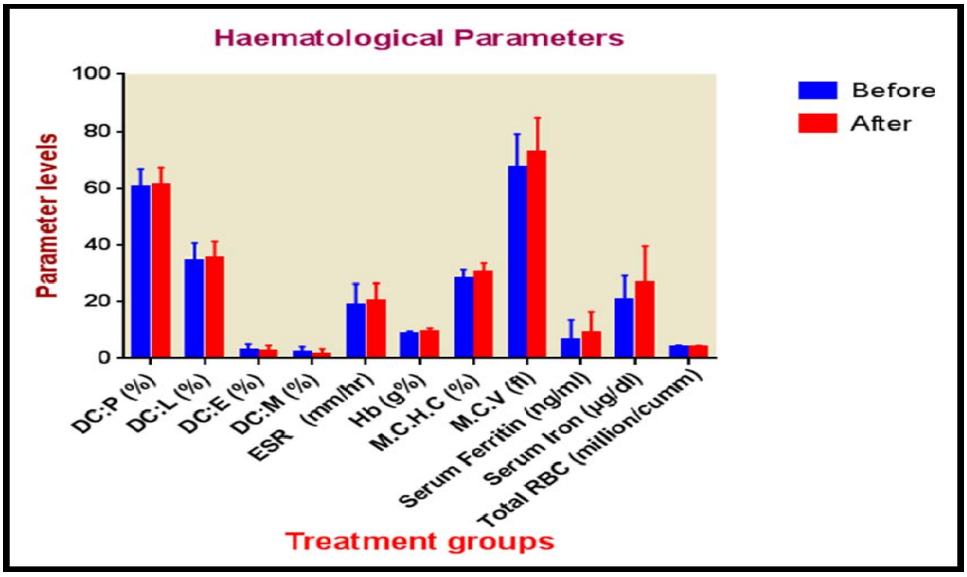

Fig. 6.13: Shows the comparison of haematological parameters before and after treatment with *ABP*

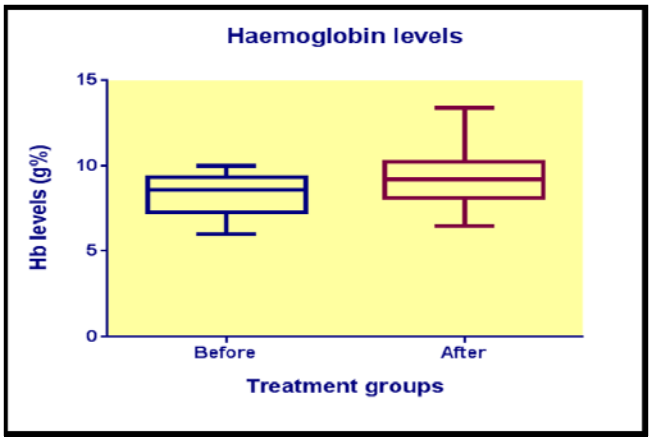

Fig. 6.14: Comparison of Haemoglobin before and after treatment with *ABP* before and after treatment with *ABP*

Serum Iron and Ferritin levels

For all recruited patients the serum iron and ferritin levels were investigated apart from routine haematological parameters. The mean serum ferritin before treatment was 6.61 and after treatment it was 8.72. The serum iron level before and after treatment with *ABP* was 20.34 and 26.36, respectively. (Fig. 6.15, 6.16)

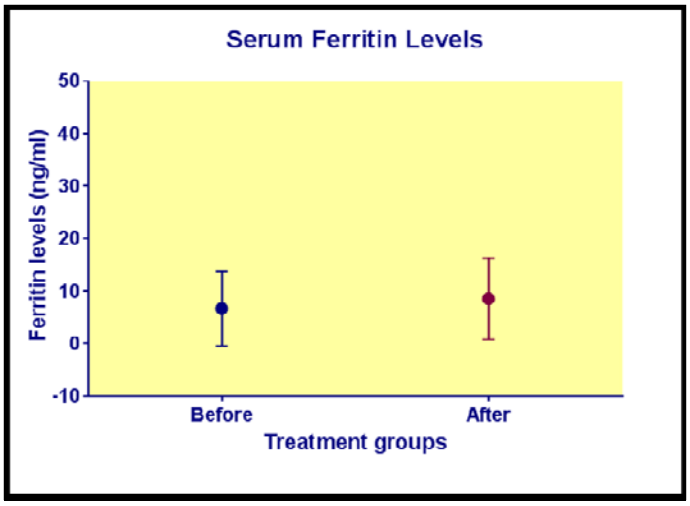

Fig. 6.15: Shows the comparison of Serum Ferritin levels before and after treatment with *ABP*

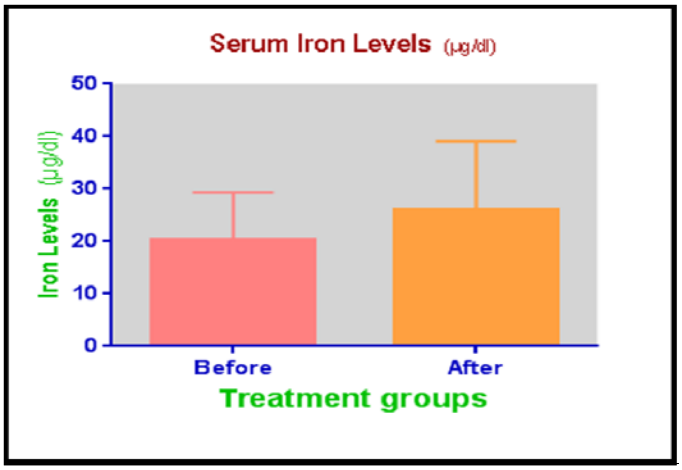

Fig. 6.16: Shows the comparison of Serum Iron level before and after treatment with *ABP*

6.6.7. Liver function and Renal function tests (LFT and RFT)

To evaluate the safety of the trial drug, *ABP,* the liver and renal function tests were performed before and after the treatment period. From the results, it is found that there is no significant changes in the parameters. The mean with SD for all the related parameters of LFT and RFT are shown in the Table.6.17 and Table.6.18, respectively. (Fig. 6.17a,617b & 6.18)

Table 6.15: Baseline Liver Function Tests*

Parameters (units)	Centres I & II	Centre I	Centre II	Pilot study
No. of subjects (n)	82	32	40	10
Total Serum Bilirubin (mg%)	0.98 (0.38)	0.72 (0.17)	0.94 (0.32)	1.03 (0.27)
Direct Serum Bilirubin (mg%)	0.26 (0.15)	-	0.24 (0.15)	0.35 (0.13)
Serum S.G.O.T (U/l)	21.30 (3.37)	21.67 (3.68)	21.40 (3.48)	20.80 (2.52)
Serum S.G.P.T (U/l)	25.06 (7.63)	18.67 (4.50)	26.49 (7.04)	21.60 (8.16)
Serum Alk. Phosphate (IU/l)	114.59 (41.9)	87.52 (13.57)	135.56 (42.9)	114.71(45.85)
Serum Total Protein (g%)	6.59 (0.54)	-	6.73 (0.36)	6.67 (0.46)
Serum Albumin (g%)	4.00 (0.40)	-	4.12 (0.37)	4.01(0.34)
Serum Globulin (g%)	2.84 (0.42)	-	2.86 (0.28)	2.71(0.20)

**Data expressed as mean (SD)*
Centre I: National Institute of Siddha (NIS), Chennai
Centre II: Siddha Regional Research Institute for (SRRI), Puducherry
Pilot study: At SRRI, Puducherry

Table 6.16: Baseline Renal Function Tests*

Parameters (units)	Centres I & II	Centre I	Centre II	Pilot study
No. of subjects (n)	82	32	40	10
Blood Urea (mg%)	25.15 (4.94)	25.16 (6.08)	24.75 (3.82)	26.70 (4.27)
Serum Creatinine mg%)	0.81 (0.11)	0.78 (0.14)	0.83 (0.08)	0.83 (0.08)
Urine Pus cells (no./HPF)†	2 to 3	1 to 2	0 to 2	0 to 2
Urine Epithelial cells (no./HPF) †	1 to 2	1 to 2	2 to 3	1 to 2

**Data expressed as mean (SD);* †Expressed in median
Centre I: National Institute of Siddha (NIS), Chennai
Centre II: Siddha Regional Research Institute for (SRRI), Puducherry
Pilot study: At SRRI, Puducherry

Table 6.17: Liver Function Tests – Before & After treatment period (n=82)*

Parameters (units)	Before treatment	After treatment	Mean Difference	SEM	P value
Total Serum Bilirubin (mg%)	0.98 (0.38)	0.86 (0.22)	0.040 (-0.054;0.133)	0.047	0.398
Direct Serum Bilirubin (mg%)	0.26 (0.15)	0.25 (0.11)	0.001 (-0.001;0.002)	0.001	0.240
Serum S.G.O.T (U/l)	21.30 (3.37)	23.15 (4.97)	-1.768 (-3.074; -0.462)	0.651	0.009
Serum S.G.P.T (U/l)	25.06 (7.63)	27.84 (6.57)	-3.162 (-5.047; -1.276)	0.940	0.001
Serum Alk. Phosphate (IU/l)	114.59 (41.9)	117.38 (36.56)	0.346 (-5.986;6.679)	3.179	0.914
Serum Total Protein (g%)	6.59 (0.54)	6.72 (0.35)	-0.115 (-0.295;0.065)	0.090	0.206
Serum Albumin (g%)	4.00 (0.40)	4.08 (0.44)	-0.136 (-0.282;0.011)	0.073	0.068
Serum Globulin (g%)	2.84 (0.42)	2.78 (0.33)	0.058 (-0.111,0.227)	0.084	0.497

**Data expressed as mean (SD)*
Analyzed using paired t test with 2 tailed significance, considering p<0.01 as significant

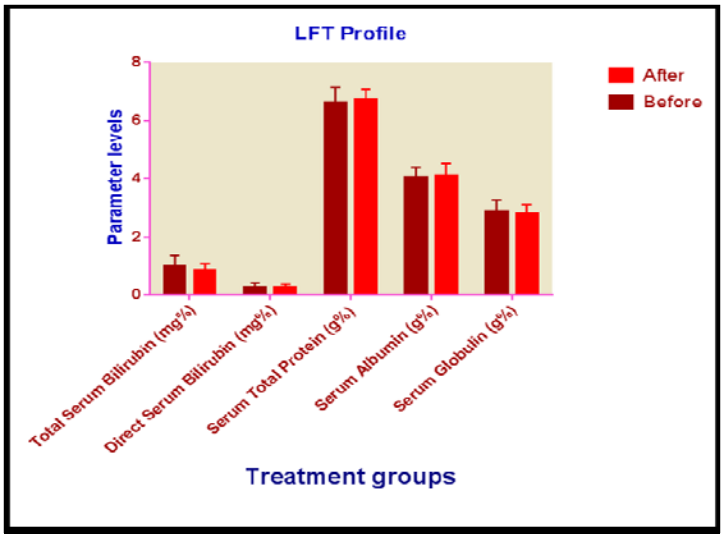

Fig. 6.17(a): Shows the comparison of LFT parameters before and after treatment with *ABP*

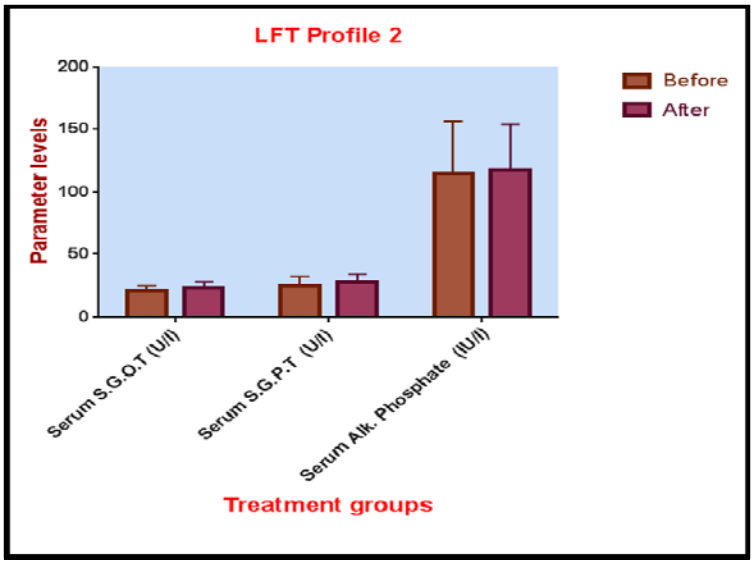

Fig. 6.17(b): Shows the comparison of LFT parameters before and after treatment with *ABP*

Table 6.18: Renal Function Tests – Before & After treatment period (n=82)

Parameters (units)	Before treatment	After treatment	Mean Difference	SEM	P value
Blood Urea (mg%)*	25.15 (4.94)	26.79 (5.54)	-1.575 (-3.022; -0.128)	0.727	0.033
Serum Creatinine (mg%)*	0.81 (0.11)	0.83 (0.10)	0.000 (-0.001;0.000)	0.000	0.507
Urine Pus cells (no./HPF)†	2 to 3	1 to 2	-	-	-
Urine Epithelial cells (no./HPF) †	1 to 2	1 to 2	-	-	-

**Data expressed as mean (SD); †Expressed in median*
Analyzed using paired t test with 2 tailed significance, considering p<0.01 as significant

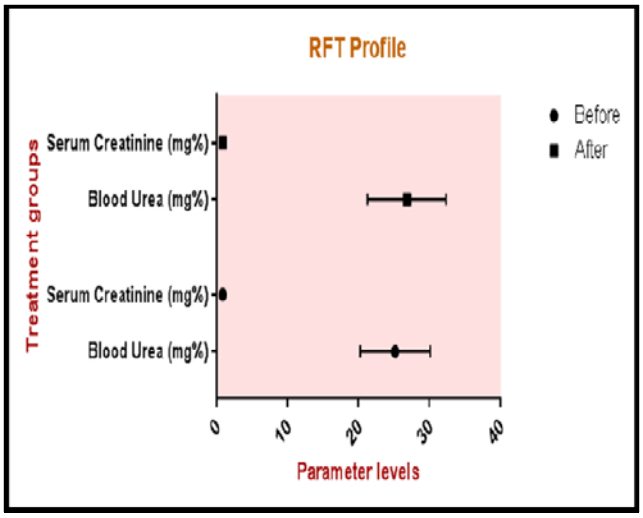

Fig. 6.18: Shows the comparison of RFT parameters before and after treatment with *ABP*

6.6.8. Clinical signs and symptoms

Assessment of clinical signs and symptoms were recorded in the clinical assessment form and analysed for prognosis. More than 90% of the recruited patients had pallor and weakness; over 70% of patients had dyspnea and around 60% had dyspnea on exertion.

7. DISCUSSION

Indian traditional systems of medicine (AYUSH) have stood the test of time for their safety, efficacy and cultural acceptability for many centuries. Amongst these, Siddha medicine includes an array of mineral and metal based formulations proven to be safe even in current clinical use. As per Siddha literature, there are certain metal based formulations indicated specifically for many health ailments such as anaemia, diabetes, arthritis, gastric ulcers, etc. Iron based siddha formulations are found to be clinically effective and safe in the management of anaemia from routine clinical observations. In allopathic system, synthetic drugs containing elemental metallic nutrients are used as iron supplements in treatment of anaemia. Though these are clinically efficacious with widespread use, they are also well known for their side effects. Especially in anaemia, elemental iron based formulations produce adverse reactions like gastric irritation, nausea, vomiting, constipation, etc.

Based on extensive literature review and current knowledge of management of anaemia in SSM, *Aya Bringaraja Paanidham*, the formulation for the treatment of *veluppu noi* (anaemia) was selected in the present study. There are many siddha formulations showing clinically significant response rate as observed from routine treatment regimen prescribed for anaemia patients. Eventhen only a few are explored scientifically in completed and ongoing clinical trials.

Aya Bringaraja Paanidham (ABP) is one of the scientifically unexplored traditional siddha formulation to the best of our knowledge till date. ABP is a unique combination of medicinal plants and iron, unlike other formulations and also contains a balanced amount of ingredients that has hematinic, laxative, digestive and carminative properties. This makes the drug multi-functional and supposed to reduce the need of other drugs to stimulate digestion, prevent constipation and improve blood parameters. Moreover, the formulation includes honey and palm jaggery which are proven for their

beneficial medicinal properties. These unique features of ABP were prioritized for its selection as the test drug in the present prospective cohort study.

Hence this study was planned to evaluate scientifically and systematically a natural iron based siddha formulation used in anaemia and to establish a public health remedy through siddha medicine for the benefit of population with anaemia.

For the preparation of ABP the raw materials should be pure, authenticated and standardized. The botanical raw materials and iron used in this formulation were collected, identified and authenticated. They were subjected to organoleptic, microscopical and physical studies. The raw materials used for the preparation of ABP were identified and characterized as per pharmacognostical and geological standards considering both of their macroscopic and microscopic descriptions. These raw materials are the herbal ingredients such as *Wedelia chinensis* (Karisalai), *Emblica officinalis* (Nellikkai), *Terminalia chebula* (Kadukkai), *Zingiber officinale* (Chukku) etc., and iron. The formulation was prepared as per Siddha scriptures, in strict adherence to GMP guidelines, under direct supervision using the infrastructure of a private Siddha Medicine manufacturing company (GMP certified).

In the present study both the physicochemical and pharmacological properties of ABP were analysed initially. Following this, upon confirmation of the safety and efficacy parameters of ABP, the prospective clinical trial was initiated. As per the protocol, ninety two patients were recruited and followed up for a treatment period of forty-five days. Among them eighty two patients completed the treatment schedule. The haematological, LFT, RFT parameters were analysed pre and post treatment period. During the treatment period prognosis and the patient's adherence was verified through clinical examination during their scheduled visits for every fifteen days.

Medicinal plant materials should be completely free from visible signs of contamination, by moulds or insects, including animal excreta, soil, etc. No obscured odour, discolouration or signs of detioration should be detected. When analysed by the

macroscopic examination the foreign matter present in the formulation was found to be within normal limits and did not show presence of any insect or moulds. Hence it could be used for animal and human studies.

According to the Drug Testing Protocol for AYUSH drugs, it is mandatory to standardize the formulation and to determine the quality and purity of the formulation. The parameters like total ash, acid insoluble ash and water soluble ash were determined for ABP. The total ash and acid insoluble ash values were 7.62% and 1.56% respectively.The low ash values of the formulation indicates that extraneous matter were controlled to minimum in the preparation of ABP.

The Physicochemical analysis of ABP was performed and the results were found within normal limits. Excess of water in formulations will encourage microbial growth and detioration of quality of the formulation. To determine this, Loss on drying was determined by gravimetric method which showed that the value is 29.62%. This is because the formulation contains juices of medicinal plants and the preparation process of manapagu does not eliminate moisture completely. The formulation should be stored in proper room conditions to avoid detioration.

The results of Microbiological studies on the test drug ABP have shown that microbes like *E.coli, Salmonella* were absent/ 25g and it did not show any visible growth of bacteria or fungi and their total count were found to be 10^3 CFU/g and < 10 CFU/g, respectively. These values are well within permissible limits of 10^5 CFU/g for bacteria and 10^3 CFU/g for fungi. These results confirm that the selected formulation ABP passes the microbial standardization.

The chromatographic techniques are helpful to determine the quality of herbs and formulations of herbal/ herbo-mineral origin. In order to minimise batch to batch variations in the quality of these formulations, it is necessary to analyse and verify the quality and quantity of medicinal plants added in the formulation using proper quality control techniques. In the present study, the trial drug prepared was subjected to HPTLC,

HPLC and GC-MS analysis and a preliminary qualitative chromatographic fingerprinting were done. Ethyl acetate extract (EA) of ABP was subjected to HPTLC, HPLC and GC analysis and standard peaks were developed for its unknown phytocomponents. HPTLC fingerprinting of the formulation exhibits maximum of seventeen peaks of which five main peaks with R_f values 0.57, 0.61, 0.71, 0.78 and 0.97 were observed. Almost similar peaks were observed when the sample was subjected to densitometric scanning at 254 and 366 nm. GC-MS chromatogram of EA extract of the trial drug *ABP* showed ten major peaks indicating the presence of ten unknown phytocomponents. These peaks were relatively compared with NIST database entities based on its structural similarities by the inbuilt software of the instrument. The chromatographic analyses have confirmed that the formulation contains many active components which shall be evaluated for further studies.

The AAS analysis has shown that the heavy metal content in the trial drug are below detetction limits and hence within permissible limits as per WHO guidelines. The concentration of iron in *ABP* was found to be 2.79 mg/kg using Atomic absorption spectroscopy (AAS) method. Though there is no established limit of iron in medicinal plants, WHO has set a limit of 20 ppm of iron in edible plants. This confirms that the formulation contains iron less than 3 ppm and is well within the permissible limit.(70)

The preparation of ABP involved a specific processing the iron ore, *Bhavana Ayam,* the samples of which were subjected for a non-destructive analysis technique, ESCA. The ESCA survey has shown the presence of C and O in both raw iron ore and the bhavana ayam. But C-O was more predominantly seen with good base line, indicating enrichment of both elements in the bhavana ayam sample. The process has changed the form of carbon, oxygen and Fe in to a crystal of Penta valent Iron for better activity. No iron in 0 and 2 oxidation states were seen as detectable peaks. The status of C and O have also been improved.

The pharmacological studies including Acute toxicity, Sub-acute toxicity, Hepatoprotective and Erythropoietic activity were done as relevant to OECD guidelines.

Acute toxicity studies in albino mice confirmed that a high dose of ABP even upto 2600 mg/kg body weight was not found to be a lethal dose as no mortality was observed. The Sub acute toxicity studies were carried out in normal Wistar albino rats considering biochemical, haematological and physiological parameters. The repeated dose Sub-acute toxicity study on ABP has shown that different doses of the formulation were well tolerated and there was no cumulative toxicity as evidenced from biochemical data. The results of all the investigations showed no significant difference between the control and test groups subjected to various dosages of ABP. Thus the absence of acute and sub acute toxicity induced by the trial drug, ABP, confirmed its significant safety in experimental animal models. Generally the conventional iron formulations cause iron overload in the body and hence damage the liver. Whereas the test drug, ABP under activity studies showed no such iron overload or hepatic damage. In the present study, the test drug, ABP has significant protective effect against hepatotoxicity induced by CCl_4 which may be attributed to the individual or combined action of phytoconstituents present in it.

In the Open labeled Clinical Trial, the diagnostic parameters – Physical examination and Laboratory investigations were done on 0 day (beforetreatment with ABP) and on 45th day (before treatment with ABP). The prevalence of *Veluppu Noi* / IDA was observed among 82 completed cases with respect to their age, sex, height, weight, food habits, *Kaalam* (Seasonal variations), *Thinai* (Land tracts), *Udaliyal* (Body constitution), occupation, etiological factors etc. Among the trial participants the incidence of prominent clinical feature was observed. The eight fold diagnostic tools – *Naadi, Sparisam, Naa, Niram, Mozhi, Vizhi, Malam and Moothiram*, unique to Siddha system were recorded for all the 82 patients at 0 day, 15th day, 30th day and 45th day of treatment with ABP.

Among the 82 completed cases, the incidence of *Veluppu Noi* was found to be higher in the age group of 31-40 years. It was also observed that incidence of IDA in females (95%) was higher compared to males. The age distribution among the recruited patients for the trial was found to be normal with around 38% and 30.5% of the

participants in the age group 31-40 and 41-50 years, respectively. As per siddha text, *veluppu noi* is considered to be predominant both in *Pitham* (34-66 years) and *Vatham* (0-33 years) period of age groups. The age distribution data of the present study was found to be consistent with the relative age group as mentioned in the text. Similarly, as already mentioned in the review, a recent survey on North Indian population reported a higher prevalence rate of anaemia in the age group 20-50 years. (54)

In the present study, the dietary habits among the recruited patients were found to be disproportionate (Veg:Non veg – 19:63) and thus the available evidence relating dietary habit with IDA was found to be inconclusive. Eventhen it can be postulated that the IDA incidence rate in the study population may be due to malnourishment. Since, > 90% of the study population were from mid and low income group, the above postulation can be emphasized further.

As per Siddha literatures, this disease is said to be prevalent in *Kurunchi* (Hilly tract) but during this study it was observed that the patients were from *Neidhal* and *Marutham* (Sea and Agricultural tract). This may be due to the proximity of the trial centres at Chennai and Puducherry, which are close to sea and surrounding regions.

Over 90% of the study population was screened and recruited during the period of mid April to mid August. As per the text, the incidence of *veluppu noi* is said to be predominant during the *Ilavenil* and *Muthuvenil Kaalam*, which are confined to the seasons as mentioned above. Thus the present study also substantiates the evidence as given in the siddha literatures.

The predominance of *udaliyal* and *naadi nadai* in majority of this study population were confined to *Vathapitham* (31% and 30%, respectively). Involvement of *Pitham* plays the major role in *veluppu noi*. The findings of the present study are in line with the siddha concept.

A significant mean increase of the haematological parameters such as Hb, MCV and MCHC levels was observed after the treatment period. The main clinical parameter,

Hb level increased from 8.38±1.15 (pre treatment mean±SD) to 9.17±1.46 (post treatment mean±SD), showing a significant difference ($p < 0.001$). The same trend was also observed for the other major parameters such as MCV and MCHC levels.

A significant mean increase of the add on parameters such as Serum iron and ferritin levels was also observed after the treatment period. The serum iron level increased from 20.34±8.96 (pre treatment mean±SD) to 26.36±13.24 (post treatment mean±SD) and serum ferritin levels increased from 6.61±7.07 (pre treatment mean±SD) to 8.72±7.83 (post treatment mean±SD) showing a significant difference ($p < 0.001$). This significant increase in the above parameters was found to be within normal range and implies that the trial drug, ABP has not imposed iron overload in blood.

Among the LFT and RFT parameters investigated pre and post treatment, no significant difference was observed except for SGPT levels. Though a mild but statistically significant increase was noted in its levels from 25.06±7.63 (pre treatment mean±SD) to 27.84±6.57 (post treatment mean±SD), it was found to be within the normal range. These findings ensure that there was no observable impairment in the liver and renal functions imposed exclusively by the trial drug, ABP. Thus it can be stated that ABP is neither hepatotoxic nor nephrotoxic, both clinically and statistically as per the above observations. This shall be attributed to the presence of herbal ingredients in ABP like *Wedelia chinensis* (Karisalai), *Emblica officinalis* (Nellikkai), *Terminalia chebula* (Kadukkai), *Zingiber officinale* (Chukku) etc that are proven for hepato-protective activity.

Assessment of clinical signs and symptoms were recorded in the clinical assessment form and analysed for prognosis. More than 90% of the recruited patients had pallor and weakness which shoed a significant clinical improvement during the treatment period; prognosis in improvement of breathlessness which was found in more than 70% was moderate and around; over 60% of the patients reported that their quality of life has improved after the treatment with ABP.

CCl_4 induces hepatotoxicity by metabolic activation through maintaining semi-normal metabolic function. CCl_4 in the endoplasmic reticulum produces trichloromethyl free radical (CCl_3) which in turn combines with cellular lipids and proteins in the presence of oxygen to form trichloromethylperoxyl radical, which may attack lipids leading to elicit lipid peroxidation, the destruction of Ca++ homeostasis, and finally, cell death. In an animal study it was noted that administration of CCl_4 increased the levels of SGOT, SGPT, ALP and bilirubin (total and direct) in rats. A significant reduction was observed in LFT parameters in the groups treated with silymarin and both alcoholic and aqueous extract of *Wedelia chinensis*. Liver necrosis and vacuolization were observed only in toxicant treated group, showing the sign of protection against these toxicants to considerable extent rendered by silymarin and test extracts. Silymarin is the composite name given to three flavanoids isolated from milk thistle, *Silybum marinum* and is used as hepatoprotective against experimental hepatotoxicity of various chemicals including CCl_4. This study revealed that among the two extracts tested, alcoholic extract at a dose level of 500 mg/kg was found to possess significant protective effect against hepatotoxicity induced by CCl_4 which may be attributed to the individual or combined action of phytoconstituents present in it (71). In this present study the test drug, ABP was evaluated preclinically for its hepatoprotective and erythropoietic activities, the results of which has confirmed its hepatoprotective activity against CCl_4 induced liver damage.

The test drug includes certain herbs like *Emblica officinalis, T.chebula and T.bellerica* that are rich antioxidants and well known for their gallic acid, ascorbic acid contents. The enhancing property of ascorbic acid on iron absorption are well known but the magnitude of these effects varies based on the population. Iron absorption rate from regular daily diet among Indian women was assessed in a trial, consisting of both IDA and control groups (n=40; Age: 18-35 years). Though the sample size was small, the above said properties were well documented in this study, with the finding that ascorbic acid significantly increased the iron absorption rate both the groups ($p<0.001$). (72)

In a study comparing the efficacy of iron supplementation (commercially available) with and without ginger extract as an add-on supplementation, in anaemia patients (n=62; Age: 18-55 years), the response in terms of haemotological parameters was assessed. Significant increase ($p<0.05$) in the endpoints was observed in the group supplemented with both iron and ginger extract compared to the other. The authors concluded stating that as ginger contains ascorbic acid, reducing sugars and amino acids it might aid in absorption of iron, since these components are already proven for facilitating non-haeme iron (73). In a similar way, the test drug, ABP contains *chukku* (*Zingiber officinale*) along with processed iron, which may enhance iron absorption in anemic patients.

Siddha literatures suggest intake of *thriphala chooranam* (fine powder of the above mentioned myrobalans) along with iron based formulations to avoid constipation. This formulation contains a balanced ratio of *thriphala* along with *trhikatugu*. This may enhance the iron absorption in the blood without inducing any adverse effects like gastric irritation or constipation.

Though there are many iron based formulations used in the treatment of anaemia, siddha formulations are unique in their nature and activity. The selected formulation in the present study, *Aya bringaraja paanidham*, is one such formulation with a balanced combination of medicinal plants with *bhavana ayam* (traditionally processed iron), honey and palm jaggery. Survey of literatures show that these ingredients are supposed to posses activities like hematinic, digestive, carminative, laxative etc. Unlike other siddha formulations, ABP does not require a adjuvant or vehicle for its better absorption in the body. Moreover, the presence of honey and palm jaggery makes it palatable and shall be used for all age groups.

Helminthic infections play a major role in inducing anaemia, especially in children and adolescents. Conventionally separate doses of anthelmintic drugs are administered which cause eventful side effects. But ABP contains herbs like *Zingiber officinale, Terminalia chebula,* etc. which additionally exhibit anthelmintic properties.

Hence it may be promising drug in the management of anaemia due to helminthic infections also. In the present study, only three patients had helminthic infection as reported in their stool examination and this data was not adequate to substantiate the anthelminthic activity of ABP.

The physicochemical characterization of ABP was done using chromatographic techniques but the fingerprinting was not quantified in this study. The clinical trial was planned as an open, non-randomised study with a small sample size to do a preliminary validation of the efficacy and safety of ABP. Anyhow there were no adverse drug reactions in the recruited patients due to administration of ABP. Randomised controlled clinical studies with a larger sample size may throw more light on its validation.

The work flow of the present study starting from SOP based preparation of drug followed by physicochemical, pharmacological till clinical studies, is unique to be in siddha medicine research to the best of our knowledge.

8. SUMMARY AND CONCLUSION

SUMMARY

Anaemia being a global public health issue has made a major impact on socio-economic development in both developing and developed countries. Since the management of anaemia is a decisive issue worldwide, WHO encourages traditional medicines to establish a safe, effective and economic drug for the same. Siddha system includes many herbal and herbo-mineral formulations which are therapeutically used in anaemia. Among these, iron based formulations play an important role. *Aya Bringaraja Paanidham* (ABP) is one such formulation which has a unique combination of herbs and processed iron.

The aim of this study was to evaluate the safety and efficacy of a Siddha herbo-mineral drug, *Aya Bringaraja Paanidham* (ABP) in the management of *Veluppu noi* (Anaemia). The primary objective to achieve this aim was to evaluate the pharmacological and clinical efficacy of ABP. In addition, secondary objective was to determine the toxicity of ABP in experimental animal models and human participants. To reach these objectives, the basic objective was to prepare the trial formulation, ABP and to standardize the same using modern techniques.

The study was done in three parts: first part involving identification and authentication of botanical and other raw materials, followed by preparation of *ABP*; second, the preclinical studies including Physicochemical analysis Pharmacological safety and efficacy studies; third, the clinical trial to evaluate the efficacy and safety of *ABP*.

The botanical and other raw materials used for the preparation of *ABP* were collected, identified and authenticated. After confirming their quality, the formulation was prepared as per Siddha scriptures, in strict adherence to GMP guidelines, under

direct supervision using the infrastructure of a private Siddha Medicine manufacturing company (GMP certified).

The physicochemical analysis including organoleptic characters, ash values, extractive values, Microbial content, High performance thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC), Gas chromatography (GC-MS) and Atomic absorption spectrometry (AAS) were done at the Central Research Facility, Sri Ramachandra University, Chennai. The physicochemical standards for ABP was established using these techniques.

In addition, to know the status of iron in *ABP*, a non destructive analytical method, Electron spectroscopic chemical analysis (ESCA) was done at Indian Institute of Chemical Technology (CSIR), Hyderabad. The ionic state of iron in the sample was determined. The physicochemical and microbiological studies have confirmed that the trial drug was free from microbial contamination and heavy metals. All the parameters were found to be within normal or permissible limits.

The Pharmacological studies were approved by the Institutional Animal Ethics Committee (IAEC), S.C.R.I, Chennai during 2011. The pharmacological studies, including toxicity (Acute and Sub-acute) and biological activity (Hepatoprotective and Erythropoietic activities) of the test drug *ABP* were done at Department of Pharmacology, Siddha Central Research Institute, Chennai and C.L.Baid Metha College of Pharmacy, Chennai. The results have shown that the trial drug, ABP is effective and safe in experimental animal models.

The Clinical trial on the trial drug *ABP* was duely approved by the Institutional Ethics Committee (IEC), National Institute of Siddha, Chennai, during the year 2011(**IEC approval No.NIS/IEC/2011/1/18**). The trial was registered retrospectively in CTRI (**CTRI/2014/07/004802**). The Clinical trial to evaluate the efficacy and safety of *ABP* was done at two centres: National Institute of Siddha, Chennai and Siddha Regional Research Institute (under Central Council for Research in Siddha, Chennai), Puducherry.

The patients were selected as per the Inclusion, Exclusion criteria and recruited for the trial after receiving their informed consent. The baseline diagnostic parameters were recorded before administering the trial drug ABP as per the protocol.The detailed history with respect to the patient was recorded in Case History Proforma. The Periodical Clinical assessment and assessment of diagnostic parameters was done in regular intervals as per protocol. A total no. of 192 patients were screened out for the trial including pilot study, out of which 92 were recruited with their consent. There were 10 drop-outs due to missed out treatment regimen or withdrawl and a total of 82 patients completed the trial successfully. From this prospective clinical trial, the trial drug was validated for its efficacy and safety in human participants.

CONCLUSION

The Physio-chemical analysis of the trial drug *Aya Bhringaraja Paanidham* has revealed its safety through the results observed found to be with in normal limits as per WHO standards. The results of Toxicity studies (Acute and Sub-Acute) were found safe thereby ensuring the safety of trial drug in experimental animal models. The Pharmacological screening has shown that the trial drug has Erythropoeitic and Hepato-protective activity in experimental animal models. The Clinical Trial has shown that ABP is effective, safe and cost effective in treating IDA patients.

The improvements in the diagnostic features, both clinical and hematological were significant with no adverse alterations in Liver and Renal function tests.

This study has validated the traditional formulation ABP for its safety and efficacy in human participants. The trial drug ABP is found to be economical and safe in the management of *Veluppu Noi* / Anaemia.

9. FUTURE RECOMMENDATIONS

- This study forms the basic platform for standardization procedures that can be carried out for any iron based formulations prescribed for any ailment used in Indian traditional medical system.

- The unknown phytocomponents identified using chromatographic techniques in the present study can be analysed for the structural similarities of the known compounds, leading to their identification and characterization.

- The molecular mechanisms and biochemical pathways behind the erythropoietic and hepatoprotective activities of ABP can be explored through *in vitro* cell line based studies.

- The clinical trial of the present study can be replicated as a multi centered trial with larger sample size involving different groups of population with respect to age, gender, socio-economic status, etc.

- Moreover, the above work can also include evaluation of parameters such as *Thrithodam*, *Naadi nadai, Neikuri*, etc. based on the siddha concepts.

- As per the present clinical trial, the incidence of anemia was found to be high among patients from *Neithal thinai* (coastal tract) rather than *Kurinchi* (hilly tract) as given in siddha literatures. Though this finding is not concludable due to the proximity of trial centres to *Neithal thinai* , this can be used as a base work to plan a prevalence study considering the incidence rate of IDA with respect to *thinai*.

- Randomised controlled trials (RCT) comparing the efficacy and non-inferiority of ABP with other conventional iron supplements can be designed in a parallel arm.

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