# TOXICITY STUDY OF TWO IRON CONTAINING BHASMAS, SWARNA MAKSHIKA BHASMA AND KASIS BHASMA

*Dissertation submitted to*

**The Tamil Nadu Dr. M.G.R. Medical University, Chennai.**

*In partial fulfillment for the award of the degree of*

**MASTER OF PHARMACY**

**IN**

**PHARMACOLOGY**

**Submitted by**

**REGISTRATION No. 26096173**

**Under the Guidance of**

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**APRIL – 2012**

**DEPARTMENT OF PHARMACOLOGY**

**J.K.K. NATTRAJA COLLEGE OF PHARMACY**

**Komarapalayam – 638183**

**Tamilnadu.**

# *CERTIFICATES*

# EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"Toxicity Study of Two Iron Containing Bhasmas, Swarna Makshika Bhasma and Kasis Bhasma"** submitted by the student bearing **Reg. No: 26096173** to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment for the award of **Master of Pharmacy** in **Pharmacology** was evaluated by us during the examination held on ……

**Internal Examiner** **External Examiner**

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# DECLARATION CERTIFICATE

I here by declare that the dissertation work entitled **"Toxicity Study of Two Iron Containing Bhasmas, Swarna Makshika Bhasma and Kasis Bhasma"**submitted to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment to the requirement for the award of degree of **Master of Pharmacy** in **Pharmacology**, is a bonafide research work carried out by me during the academic year 2011-2012, under the guidance and supervision of **Dr. P. ASHOKKUMAR, M.Pharm. PhD,** Professor Department of Pharmacology, in the Department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam Erode.

I further declare that, this work is original and this dissertation has not been submitted previously for the award of any other degree, or any other University. The information furnished in this dissertation is genuine to the best of my knowledge and belief.

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**Date:** **Reg. No.26096173**

# *ACKNOWLEDGEMENT*

I acknowledge, first of all, the Almighty God for his goodness and grace, which have brought me this far successfully. Without him, I could not have made this far.

I take this opportunity with pride and enormous gratification to express the deeply embedded feeling of thanks and gratefulness to all the persons who backed me directly or indirectly thoroughout the materialization of this research work at this magnitude.

I express whole hearted gratitude to my guide **Dr. P. ASHOKKUMAR, M.Pharm. Ph .D,** Professor Department of Pharmacology, for suggesting solution to problems faced by me and providing indispensable guidance, tremendous encouragement at each and every step of this dissertation work. Without his critical advice and deep-rooted knowledge, this work would not have been a reality.

I am proud to dedicate my deep sense of gratitude to the founder, (Late) Thiru **J.K.K. Nattrajah Chettiar,** providing us the historical institution to study. I wish my sincere thanks and respectful regards to our reverent Chairperson **Smt. N. Sendamaraai, B.Com.,** Managing Director **Mr. S. Omm Sharravana, B.Com., LLB.,** and Executive Director **Mr. S. Ommsingarravel, B.E., M.S.,** J.K.K. Nattraja Educational Institutions, Komarapalayam for their blessings, encouragement and support at all times.

It is most pleasant duty to thank for our beloved Principal **Dr. P. Perumal, M.Pharm., Ph.D., AIC.** J.K.K. Nattraja College of Pharmacy, Komarapalayam for ensuring all the facilities were made available to me for the smooth running of this project.

I express my sincere thanks to **Mr. Rajesh V, M.Pharm.** Assistant Professor & Head, Department of Pharmacology, **Mrs. M. Sudha, M.Pharm.** Lecturer, Department of pharmacology, **Mr. senthil kumar M.Pharm.** Lecturer, Department of pharmacology for excellent and valuable guidance with constant support to me for completing this research work in a very successful manner.

Our glorious acknowledgement to our administrative officer **Dr. K. Sengodan, M.B.B.S.**, for encouraging us in a kind and generous manner to complete this work.

My sincere thanks to **N. Venkateswara murthy, M.Pharm.,** Professor and Head of the Department, Department of Pharmacy Practice. **Dr. L. Panayappan, M.Pharm., Ph.D.,** Assistant Professor, **Mr. Raja Rajan** Lecturer, Department of Pharmacy Practice, **Miss. S. Thangamani M.Pharm.,** Lecturer, Department of Pharmacy Practice, for their valuable suggestions during my project work.

My sincere thanks to Professor**., Dr. R. Sambath kumar. M.Pharm., Ph.D**., Head of the Department, Department of Pharmaceutics, **Mrs. S. Bhama, M.Pharm.,** Assistant Professor, **Mr. M. Senthilkumr, M.Pharm.,** Assistant Professor, **Mr. R. Kanagasabai, B. Pharm. M.Tech.,** Assistant Professor, **Mr. K. Jaganathan, M.Pharm.,** Lecturer, Department of Pharmaceutics for their valuable help during my project.

It is my privilege to express deepest sense of gratitude toward **Mr. P. Sivakumar, M.Pharm., Ph.D** Professor & Vice Principal, **Mr. M. Vijayabaskaran**, **M.Pharm,** Assistant Professor, **Mrs.P.Vijayanthimala, M.Pharm,** Lecturer, **Mrs. K. Mahalakshmi M.Pharm.,** Lecturer, Department of Pharmaceutical chemistry, for their valuable suggestions and inspiration.

My sincere thanks to **Mr. V. Sekar, M.Pharm.,** Head of the Department, Department of Analysis,**.,** ,**Mr. Senthilraja, M.Pharm.,** Assistant Professor, **Mr. D. Boopathy, M.Pharm.,** Assistant Professor and **Mr. S. Jayaseelan, M.Pharm.,** Assistant Professor, Department of Pharmaceutical Analysis for their valuable suggestions.

My sincere thanks to **Dr. S. Sureshkumar, M.Pharm., Ph.D.,** Head of the Department, Department of Pharmacognosy and **Mr. M. K. Senthil Kumar, M.Pharm.** Assistant Professor, Department of Pharmacognosy for their valuable suggestions during my project work.

I greatly acknowledge the help rendered by **Mrs. K. Rani,** Office Superintendent, **Mr. K. Sakthivel,** Clerical Assistant, **Miss. Prabha,** Librarian **Mrs. V. Gandhimathi, M.A., M.L.I.S.,** for their co-operation.

My sincere thanks to **Mr Sameer Deshmukh, Mr. Alok Srivastava** Dept. of pharmacology and **Mr. Prince joseph, Mr. Yasir k.p** Dept. of Pharmacy practice for their valuable help during my project.

I would like to acknowledge my sincere thanks to **Miss. Indu T.H M.Pharm** Dept of Pharmacy practice J.S.S College of pharmacy ooty, **Mr. Jomy C John M.Pharm** Asst. Professor, Nehru College of pharmacy Thrissur, **Mr. Bijo Mathew M.Pharm** Asst. professor, Grace College of pharmacy, **Mr. Jacob Mathew M.Pharm** for their valuable suggestions during my project work.

I owe my thanks to all the technical and non-technical staff members of the institute for their precious assistance and help.

Last, but nevertheless, i am thankful to my colleagues and all my friends for their co-operation, encouragement and help extended to me throughout my project work.

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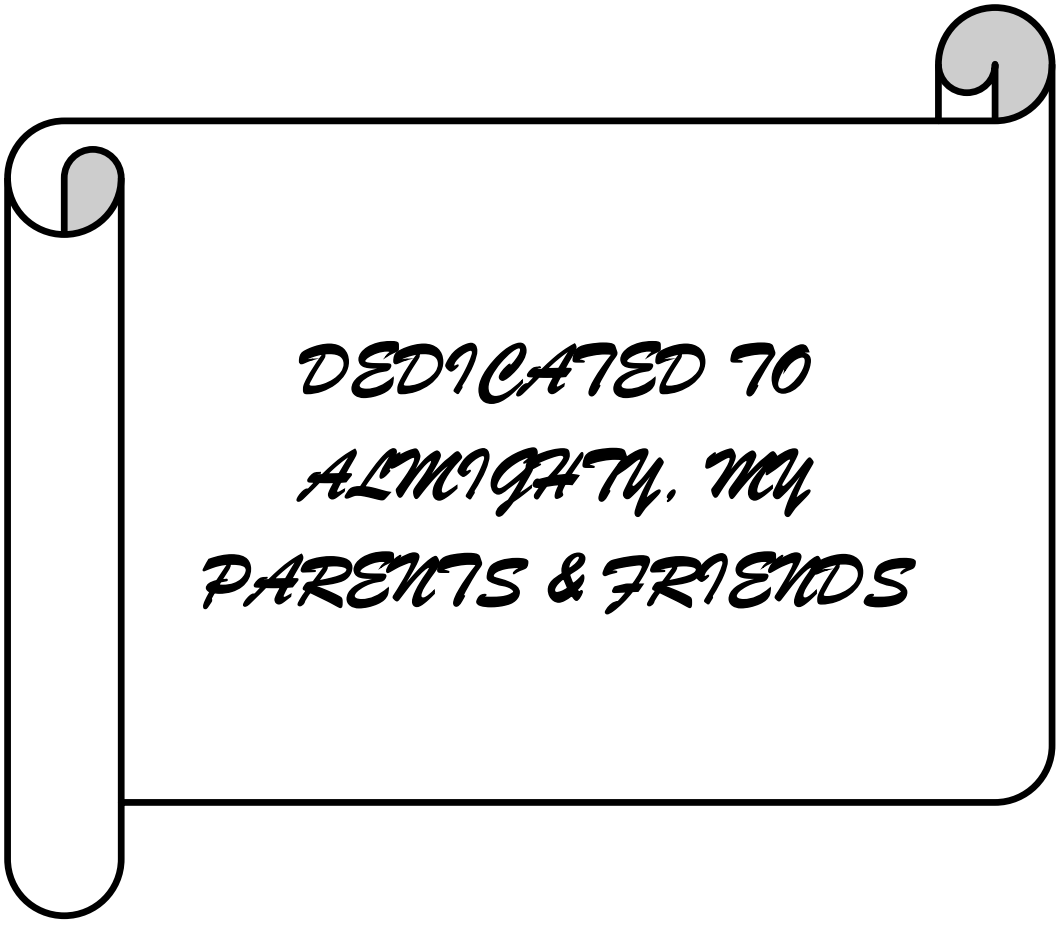
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ALMIGHTY, MY
PARENTS & FRIENDS

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# CHAPTER - 1

# INTRODUCTION

# CHAPTER 1

# INTRODUCTION

*"Everything should be tested and proved and then it becomes a part of scientific medicine- old and new."* - Pandit Jawaharlal Nehru

*"India can benefit enormously if it can build a golden triangle between traditional medicine, modern medicine and modern science."* -Dr R.A Mashelkar Director CSIR

## 1.1 TOXICOLOGY

Toxicology is "the study of poisons". Toxicology involves knowledge of physiological, biochemical, and morphological effects of a chemical. This approach of the physiological and biochemical understanding of toxicity was first used by the famous French physiologist, Claud Bernard, more than 150 years ago. Toxicology reflects the development of society a progression from simplicity to sophistication, from crude to cultured from elemental to elegant and from taking lives to saving lives.[1]

Toxic was originally *tekw*, a word meaning to run or flee, later becoming *toxsa* in Persian and *toxon* in greek, meaning bow. The toxin meaning may have come from the poison used to tip of the arrows, or as Robert graves suggested ,from the yew tree(*taxus*) from which arrows were best made and whose berries where long known to be poisonous. From this concept we can say that toxicology and plant system has a distant relationship.

"Toxicology is defined as study of the adverse effects of chemical, physical and biological agents on living organisms and the ecosystems, including the prevention amelioration of side effects".[1]

### 1.1.1 RELATIONSHIP BETWEEN DOSE AND TOXICITY

Theophrastus Phillipus Auroleus Bombastus von Hohenheim (also referred to as Paracelsus) is also considered "the father" of toxicology. He is credited with the classic toxicology statement, "Alle Dinge sind Gift und nichts istohne Gift; allein die Dosis macht, dass ein Ding kein Gift ist." which translates as, "All things are poison and nothing is without poison; only the dose makes a thing not a poison." This is often condensed that: "The dose makes the poison".[2]

The relationship between the dose of the chemical and the effects produced in the organism is called the Dose- Response- Relationship. The relationship between dose and its effects on the exposed organism is of great significance in toxicology. The major criterion regarding the toxicity of a chemical is the dose, i.e. the amount of exposure to the subject. Almost all substances are toxic under the right conditions. The term LD50 refers to the dose of a toxic substance that kills 50 percent of a test population. As the dose of a chemical is increased, the response (proportion of a population showing a specified change) or effect (actual magnitude of the change in the parameter) also increases.[2]

### 1.1.2 FACTORS AFFECTING TOXICITY

Toxicity of a substance can be affected by different factors,

- Pathway of administration (whether the toxin is applied to the skin, ingested, inhaled, injected)
- Time of exposure (a brief encounter or long term),
- Number of exposures (a single dose or multiple doses over time),
- Physical form of the toxin (solid, liquid, gas),
- Genetic makeup of an individual,
- Individual's overall health and many others. [1]

### 1.1.3 SUB DISCIPLINES OF TOXICOLOGY

**Type of toxicology**

- Analytical toxicology
- Behavioral toxicology
- Carcinogenesis
- Clinical toxicology
- Information toxicology
- Developmental toxicology
- Drug toxicology
- Mutagenesis
- Pesticide toxicology
- Food toxicology
- Pre Clinical Toxicology
- Toxicogenomics
- Aquatic Toxicology
- Environmental Toxicology
- Forensic Toxicology
- Systemic Toxicology

**Type of organ specific toxicology**

- Dermatoxicology
- Hepatotoxicology
- Neurotoxicology
- Occupational toxicology.
- Ocular toxicology
- Renal Toxicology
- Pulmonary toxicology
- Cardiac Toxicology
- Reproductive toxicology
- Ototoxicology
- Immunotoxicology

- Genetic toxicology[2]

### 1.1.4 TOXICOLOGY IS OFTEN SUB DIVIDED ON THE BASIS OF EXPOSURE DURATION

**Acute toxicity**

In acute toxicity Effects are observed within a short time of exposure to the chemical. This exposure may be a single dose, or a short continuous exposure, or multiple doses administered over 24 hours or less. Acute toxicity looks at lethal effects following oral, dermal or inhalation exposure.

**Sub acute (sub chronic) toxicity**

In sub acute toxicity Adverse effects are observed following repeated daily exposure to a chemical, or exposure for a significant part of an organism's lifespan (usually not exceeding 10%)

**Chronic toxicity**

Adverse effects are observed following repeated exposure to a chemical during a substantial fraction of an organism's lifespan (usually more than 50%) or some time up to the full span of the animal life.

## 1.2 TOXICITY TESTING

Toxicity can be measured by observing its effects on the target (organism, organ, tissue or cell). Because individuals typically have different levels of responses to the same dose of a toxin, a population-level measure of toxicity is often used which relates the probabilities of an outcome for a given individual in a population. One such measure is the LD50 (lethal dose 50). This means dose that kill 50% of mortality in a population. When such data does not exist, estimates are made by comparison to known similar toxic things, or to similar exposures in similar organisms. Then "safety factors" are added to account for uncertainties in data and evaluation processes. For example, if a dose of toxin is safe for a laboratory rat, one might assume that one tenth that dose would be safe for a human, allowing a safety factor of 10 to allow for interspecies differences between two mammals. If the data are from fish, one might

use a factor of 100 to account for the greater difference between two chordate classes (fish and mammals). Similarly, an extra protection factor may be used for individuals believed to be more susceptible to toxic effects such as in pregnancy or with certain diseases. Or, a newly synthesized and previously unstudied chemical that is believed to be very similar in effect to another compound could be assigned an additional protection factor of 10 to account for possible differences in effects that are probably much smaller. Obviously, this approach is very approximate; but such protection factors are deliberately very conservative and the method has been found to be useful in a deep variety of applications.

It is more difficult to determine the toxicity of chemical mixtures than a pure chemical, because each component displays its own toxicity, and components may interact to produce enhanced or diminished effects. Common mixtures include gasoline, tobacco smoking, and industrial waste. Even more complex are situations with more than one type of toxic entity, such as the discharge from a malfunctioning sewage treatment plant, with both chemical and biological agents[1]

**LD50 (median lethal oral dose)**

It is a statistically derived single dose of a substance that can be expected to cause death in 50 per cent of animals when administered by the oral route. The LD50 value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

**TYPES OF TOXICITY STUDIES**

- Acute toxicity studies
- Sub acute toxicity studies
- Sub chronic toxicity studies
- Chronic toxicity studies
- Neurotoxicity studies
- Carcenogencity studies
- Genotoxicity studies
- Developmental and reproductive toxicity studies.

**1.2.1 ACUTE TOXICITY STUDIES**

**Objectives of acute toxicity studies**

1. The objectives of acute toxicity testing are to define the "Intrinsic toxicity of the chemical or drugs" to assess the susceptible, to identify target organs, to provide information for risk assessment after exposure to chemical.

2. To provide information for the design and selection of dose level for prolonged studies.

3. The acute toxicity data are essential in the classification, labeling and transportation of chemical.

4. Acute toxicity studies are frequently designed to express the potency of toxicant in terms of median lethal dose (MLD50)

5. Many acute toxicity studies have been conducted to determine the LD50 of the chemical or drug.

6. A well designed acute toxicity studies should include consideration of both lethal and non lethal parameters.

7. To discover and report any adverse health effect that could be related to the chemical under study.

8. Acute toxicity studies are carried out by different manners like

    - **Oral acute testing**
    - **Dermal acute toxicity testing**
    - **Inhalation toxicity studies**

**Single Dose Oral Toxicity in rats/LD50 in rats**

Animal model studied:
mice, n=10
Study duration : 14 days

**Data obtained from acute dose studies that are useful in the design of subsequent studies**

1. LD 50
2. Mortality
3. All animals survived the 2000mg/kg oral dose.
4. Body Weights
5. Body weight changes were normal in 7/10 animals. Three females lost weight during the second week of the study.
6. Systemic Observations.
7. Physical signs included diarrhea, soiling of the anogenital area and wetness of the mouth and anogenital area.
8. Necropsy Findings
9. Necropsy results were normal in all animals.[3]

### 1.2.2 SUB – ACUTE TOXICITY STUDIES

Repeated dose toxicity studies are conducted to screen for potential adverse effects of a chemical using laboratory animals as surrogates for a target species, most often the human. Repeated – dose studies may be of varying duration, generally 1 – 4 weeks for short term studies, 3 months for sub chronic studies and 6 – 12 months for chronic studies. Procedures are done under OECD 407 guidelines

**Sub-Acute Oral Toxicity Studies**

Sub-acute oral toxicity study was designed and conducted to determine the toxicity profile of Forslean when administered daily for 28 days in Sprague Dawley rats. Test material suspended in 0.1% aqueous CMC was administered to the animals at various dose levels. Hematological and biochemical analyses were carried out at the end of experimentation.[4]

**Objectives of sub acute studies**

- Determine adverse effects of the test compound at dose low enough to allow survival of most animals as opposed to acutely toxic doses.
- Determine adverse effect over a longer exposure period than used in acute studies.
- Determine dose – response for adverse effects following repeated dosing and identify the NOAEL.
- Identify organs affected by exposure to test material (organ)
- Provide initial data for comparative risk assessment.

**Data obtained from sub – acute dose studies that are useful in the design of subsequent studies**

- Palatability of test material
- Body weight response
- Physical observation
- Behavioral changes
- Clinical pathology
- Toxic kinetics
- Gross necropsy
- Histopathology
- Identification of target organs
- Dose response
- Isolated organ weight

**1.2.3 SUB-CHRONIC TOXICITY STUDIES**

Sub chronic studies are designed to examine the adverse effect resulting from respected exposure over a portion of the average life span of an experimental animal. Properly designed gives valuable information on the cumulative toxicity of a substance target organs and physiological, metabolic tolerance of a compound at low dose (relatively to acute toxicity testing dose level) prolonged exposure. With the help of this method various parameters, including histopathologic evaluation a wide variety of ADR are detected. Result of this study provides information for selecting dose level for chronic, reproductive and carcinogenicity. Sub chronic studies data sufficient to for diet the benzoid long term, low dose, exposure of a particular compound. Even though acute data indicated that the compound is practically non-toxic but it may toxic from prolonged exposure even at low dose level. This toxic effect may due to cumulation, changes in enzyme level and disruption of physiological and biochemical hemostasis. Therefore sub chronic studies are essential for all new chemical. The exposure period in sub chronic studies may vary, depending upon the object of study, the species selected for study and the route of administration employed. A general sub chronic study doesn't exceed 10% of the animal life span.

Sub chronic toxicity studies are 13 to 26 weeks in duration. The animals are dosed daily by the same route that the substance would normally be administered to humans. They are then observed for any toxicity, as well as changes in body weight or food consumption. At the end of the dosing regimen, the animals are euthanized and their tissues evaluated for evidence of toxicity.[5]

### 1.2.4 CHRONIC TOXICITY STUDIES

Chronic studies are designed for long term toxicity of toxicants or toxins. Duration of study will be 6 months to one year.

**Chronic toxicity studies are designed to**

- Examine, biological nature of toxic effects, from low dosages, at the cellular level, measuring parameters that cannot usually be obtained in acute studies because of the high dosage administered and rapidity of onset of toxic signs and symptoms.
- Ascertain variations in species responses to repeated exposure to the agent.

- Assess possible cumulative effects of the repeated exposure as body burdens of agent and or bio transformation products are acquired with time.
- Determine nature of macroscopic and microscopic organs or tissue damage as it develops hopefully I relation to the level and duration of exposure.
- Identify the approximate dosage at which the altered physiological, biochemical and morphological changes might occur.
- Predict long range adverse health effects in the species arising from repeated or chronic exposure to the particular agent.[5]

**Parameters measured during studies**

1. All animals committed to study must be daily observed.
2. One measurement of particular use in extended chronic studies, is the survival time of the control and toxicant treated animals, presenting the data in a plot of % survival time against time
3. Simple and most revealing end points are the body weight of animal, measured every 2 or 3 days (twice weekly) throughout the duration of the study.
4. Weigh isolated organs from animals. Their weight should relate to the body weight of the animal.
5. Assessment of animal behavior, neurological function, hepatic and renal function etc can be carried out periodically without the destruction of valuable animals.
6. In chronic studies, routine clinical examinations should be conducted periodically on control animal to provide a baseline of changes with age and on the treated animals to ascertain whether or not exposure to the toxicant can elicit any age related and dose dependent changes in organ function.

**Duration of studies**

Chronic studies regardless of the species used were of 2 years duration, this time interval being based on the approximate life span of rodent. Chronic studies on non rodent are of 1 year duration.[5]

### 1.2.5 NEUROTOXICITY STUDIES

Neuro or behavioral toxicities are complex having multiple dimensions and behavioral domains. These are motoric, sensory and cognitive domains, each of which can range from simple to highly complex levels of function. First clues of toxicity to humans may be subjective disturbances such as nervousness or personality changes other signs such as tremor or akenasi etc.

In past the neurotoxicity was thought to be neuropathy involving neuropathological lesions or neurological dysfunction. Now it was clear that along with neuropathy there are many other signs of nervous system toxicity (e.g. loss of motor co-ordination, sensory deficits, learning and memory dysfunctions) that may not be reflected in neuropathy or other types of studies.[6]

**Neurotoxicity studies are designed to**

- To identify whether the nervous system is permanently or reversibly affected by the chemical tested;
- Contribute to the characterization of the nervous system alterations associated with exposure to the chemical, and to understanding the underlying mechanism.
- Determine dose-and time-response relationships in order to estimate a no-observed adverse-effect level (which can be used to establish safety criteria for the chemical).[6]

**Data obtained from neurotoxicity studies**

- Body weight/body weight changes including body weight at kill
- Food consumption and water consumption,
- Toxic response data by sex and dose level, including signs of toxicity or mortality;
- Nature, severity and duration (time of onset and subsequent course) of the detailed clinical observations (whether reversible or not)
- A detailed description of all functional test results for sensory reactivity to stimuli of different modalities (e.g., auditory, visual and proprioceptive); for assessment of limb grip strength; for motor activity assessment (including details of automated devices for detecting activity);
- Necropsy findings;
- A detailed description of all neurobehavioral, neuropathological, and neurochemical or electrophysiological findings.
- Absorption and metabolism data, if available.
- Statistical treatment of results, where appropriate.

**Duration of study**

In neurotoxicity studies the animals are dosed with the test substance daily, seven days each week, for a period of at least 28 days. Neurotoxicity studies are combined with other toxicity studies also[6].

## 1.3 TOXICANTS AND TOXINS

Toxicology deals with toxins and toxicants. A toxicant is any chemical that can injure or kill humans, animals, or plants; a poison. The term **"toxicant"** is used when talking about toxic substances that are produced by or are a by-product of human-made activities. Where as **toxin** is any poisonous substance of microbial (bacteria or other tiny plants or animals), vegetable, or synthetic chemical origin that reacts with specific cellular components to kill cells, alter growth or kill the organisms.[2]

Various toxicants and toxins are:

- Food bourne toxicants
- solvents and industrial toxicants
- crop protection chemicals
- metals
- ionizing radiations
- plant and animal toxins[1]

From these we are dealing with metal toxicants.

### 1.3.1 METAL TOXICANTS

Metals are elements generally characterized by ductility, luster, electropositive nature and having a property of conducting heat and electricity. But more than that metal posses a variety of physiological effects. . Scarcity of some metal ions can lead to disease. Well-known examples include pernicious anemia resulting from iron deficiency, growth retardation arising from insufficient dietary zinc, and heart disease in infants owing to copper deficiency.[1]

Metals also played an important role in medicinal system also. The use of metals in medicine traces back to antiquity with various elements, such as arsenic, gold and iron, being used to treat different ailments. In more modern times, perhaps the first metal-based drug to find wide use was an arsenical, Salvarsan, developed by Paul Ehrlich, used in the treatment of Syphilis. Diseases such as anemia (iron), asthma (gold and magnesium), bipolar disorder (lithium), diabetes (vanadium), rheumatoid arthritis (gold), stroke (magnesium), tropical diseases (antimony and rhodium) and ulcers (bismuth) can all be treated by metal-based drugs. Most successful metal containing drug is cisplatin having a platinum center used in cancer chemotherapy. In most of the complimentary and alternative medicines like ayurveda and siddha also uses metal containing drugs for various diseases.[7]

The main problem involved in metal based drug therapy is toxicity. Clearly there is a subtle balance between therapeutic benefit on the one hand and deleterious side effects on the other. Essential elements may be toxic at a dose that overwhelms homeostatic controls on absorption and excretion. The variety of physiological effects

that metals can have is also the reason that adverse effect can often demonstrated in most organs: neurotoxicity, hepatotoxicity reproductive toxicity and nephrotoxicity. In this study iron containing ayurvedic medicines is taken as a metal toxicants.

## 1.4 IRON

Iron is silver-white solid metal found mainly in combination with other elements as oxides, carbonates, sulfides, and silicates. Iron represents approximately 35 and 45 mg/kg of body weight in adult women and men, respectively. The majority of total body iron, about 60±70%, is present in hemoglobin in circulating erythrocytes. Another 10% of essential body iron is present in the forms of myoglobins, cyto-chromes, and iron-containing enzymes, amounting to no more than 4±8 mg of iron. In healthy individual, the remaining 20±30% of surplus iron is stored as ferritins and hemosiderins in hepatocytes and reticuloendothelial macrophages. Being one of the most abundant metals in the human body, iron plays important roles in cellular processes such as the synthesis of DNA, RNA, and proteins; electron transport; cellular respiration; cell proliferation and differentiation; and regulation of gene expression.[8]

Iron is also the key component of many cellular enzymes, such as oxidases, catalases, peroxidases, cytochromes, ribonucleotide reductases, aconitases, and nitric oxide synthases. Most importantly, iron plays a crucial role in maintaining cellular iron homeostasis by regulating gene expression at the posttranscriptional level.

Globally iron deficiency is the most common cause of anemia, there was a decrease in mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. Various iron salts are used for the treatment iron deficiency anemia.[8]

**Uses of iron**

- Treatment of iron deficiency anemia
- Supplemental intake of iron during pregnancy
- Multivitamin preparations

### 1.4.1 ABSORPTION OF IRON

Absorption of both heme and non heme iron occur predominantly in the proximal small intestine, specifically in the crypt cells of the duodenum and jejunum

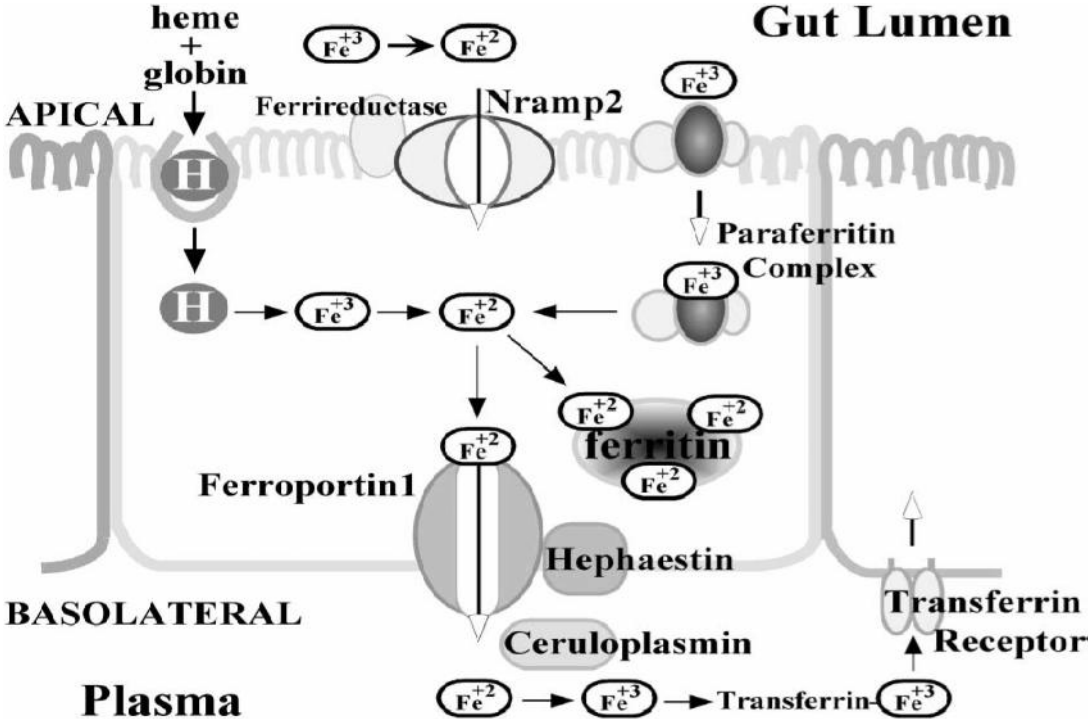



Fig .Pathways of heme and non heme iron uptake and transport in the intestine[8]

it was well known that under normal circumstances only a small proportion of any iron compound given by mouth is absorbed. As an explanation of this small absorption McCANCE and WIDDOWSEN have suggested that the absorption of iron is conditioned mainly by the relative concentration of free ionized iron in the lumen of the intestine It was reported in a study of absorption of iron compounds from the small intestine of rats as absorption was found to be increased when the iron salts were added either an organic reducing agent (ascorbicacid, cystein, glutathione, formaldehyde sulphoxylate) or some organic acid. Especially plant acids (citric, tartaric, succinic, malic acid), lactic acid, pyruvic acid and some amino acids (glutamic, aspartic acid) were active in this respect. From these studies and explanations we can elaborate some statements.[8-9]

- Iron is absorbed as ferrous ion only.

- Iron can persist in the ionized ferrous form only in acid medium.
- The normal PH of the small intestine is about 6. At this PH, almost all the iron is transformed in the complex or ferric form, neither of which is absorbed to a measurable degree. This explains the poor absorption of iron from a simple solution introduced into the gut.
- The intestine tends to reestablish its neutral reaction if acids are introduced together with the iron salt. Hydrochloric and phosphoric acid are very quickly absorbed. For this reason these acids cannot keep the PH inside the gut down for a long time and therefore do not promote iron absorption to a measurable degree.
- The organic acids and reducing agents (all acid in character) that promote iron absorption appear to do so by keeping the PH of the intestine down for a longer time. As soon as the intestinal content becomes neutral, all the iron is transformed into the complex or ferric form and the absorption stops.[9]

### 1.4.2 TOXICITY OF IRON

Iron levels must be well maintained within cells, because excess iron is highly toxic. The deleterious effect of iron is due to the generation of reactive oxygen species by Fenton reaction.

$$Fe^{+3} + {}^{\cdot}O_2^{-} \longrightarrow Fe^{+2} + O2; \quad Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + {}^{\cdot}OH + OH^{-}$$

Fenton reaction have the potency to generate highly reactive oxygen species, such as hydroxyl($OH^{-}$) and superoxide radicals(${}^{\cdot}O_2$) which are highly toxic and they react rapidly with high affinity with every molecule found in living cells. Due to this reaction of free radicals there will be DNA damage; impaired synthesis of proteins, membrane lipids and carbohydrates; induction of proteases; and altered cell proliferation.

Free iron can also react directly with unsaturated fatty acid and induce lipid hydro peroxides to form alkoxyl or peroxyl radicals thus impair cellular integrity and cause

cell death. This deleterious potential of iron might play a role in neurodegenerative disorders such as Parkinson's or Alzheimer's diseases; hepatic and nephritic disorders and carcinogenesis.[8]

- Toxic dose: 10-20 mg elemental iron
- Fatal dose: 40-1600 mg/kg of elemental iron

### 1.4.3 NEUROTOXICITY BY IRON

As in other organs, the brain needs iron for metabolic processes and iron deficiency or excess result into pathological states. Some physiological roles of iron within the brain are in embryonic neuronal development, in myelin formation, in synthesis and metabolism of neurotransmitters, in oxidative phosphorylation and ATP synthesis. Brain contains the highest quantity of iron other than that of liver 60 mg of iron is distributed through out the brain.[1]

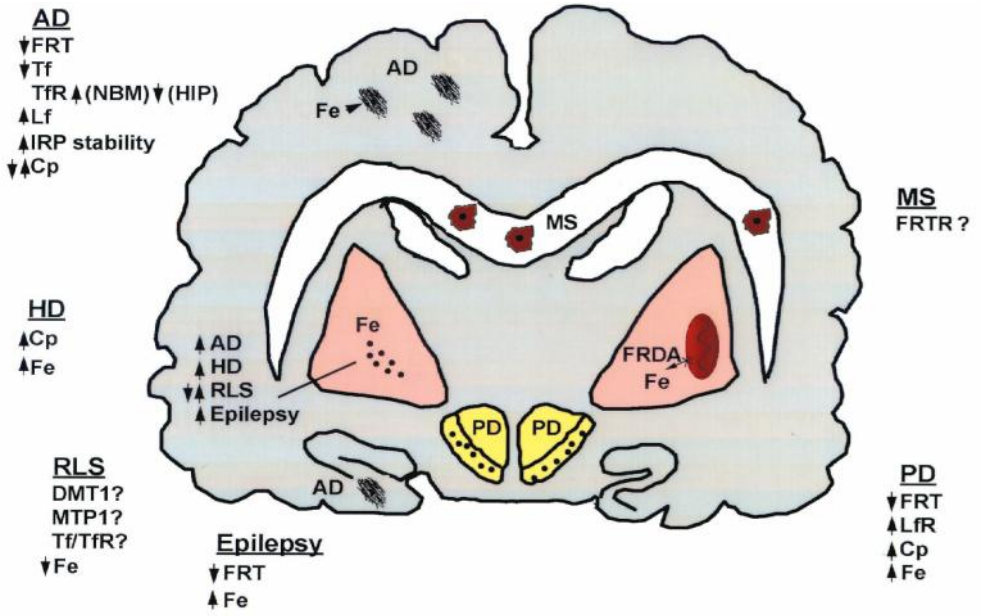



**Fig: 1.1** A schematic diagram of the changes in iron and iron management proteins in neurodegenerative disorders. [10]

Disruption of iron regulatory mechanisms and iron accumulation may contribute to development and progression of neurodegenerative disorders such as AD and PD. In Alzheimer's diseased humans brain is characterized by the accumulation of iron within senile plaques and neurofibrillary tangles, and also by lowered expression of transferring receptor. As a consequence, these brains are subject to high levels of oxidative stress.[11,12]

As for PD, dopaminergic cell loss and disease progression are accompanied by the accumulation of high iron concentrations, that are particularly associated with aggregation of _-synuclein (especially the mutated form found in familial PD) within Lewy bodies.

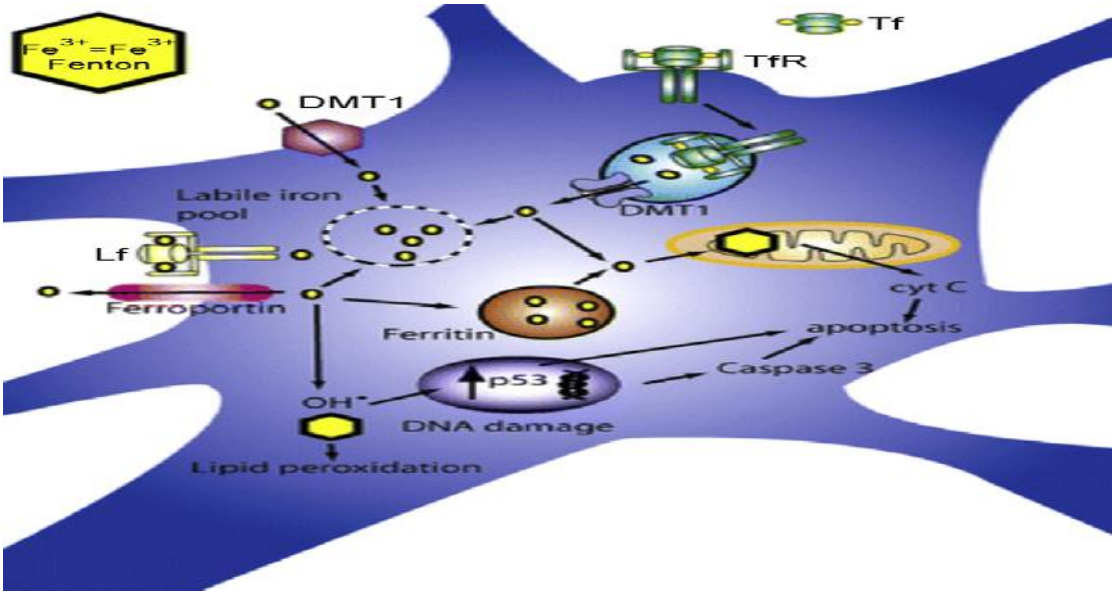



Fig: 1.2 Representation of iron-induced cell death in neuronal cells[10]

Many other neurodegenerative diseases, such as Huntington's disease, Frederic's ataxia, amyotrophic lateral sclerosis, and prion disease shows increased accumulation of iron in brain. So proper neurotoxicity studies should be conducted for iron based preparations.[10]

### 1.4.4 HEPATOTOXICITY OF IRON

Iron accumulates in liver at large quantities, Due to the generation of destructive free radicals via fenton reaction there will be detritions of liver cells occurs. Free iron is delivered by way of the portal vein to the liver causing damage and iron deposition in these areas. The result in severe cases is periportal liver damage (opposed to the centrilobular necrosis seen with APAP).[8]

In the liver, hereditary hemochromatosis has been found associated with an increased risk of hepatocellular carcinoma. Several mechanisms have been implicated in promoting carcinogenesis. Iron enhances cellular proliferation genes. Moreover, ROS formation may induce chromosome strand breaks and mutations. Not to be neglected, iron may suppress the immune system also.[13]

In a study it was reported as iron overload reduced body growth with the increase of liver weight. This might be why marked deposition of iron in the liver injured the hepatic cells and disturbed liver function, i.e. levels of activity of hepatic enzymes such as LD and ALP were lowered in iron-overloaded animals. Hepatocellular injury due to the superoxide radical, H2O2, and the hydroxyl radical produced with the deposited iron might stimulate the hepatocellular development and increase the liver weight. The reduced function of the liver could reduce body growth. Iron overload in the liver is known to reduce bone mass with liver cirrhotic change in liver iron overload syndromes. So liver cells are highly suspicious for toxicity by iron containing preparation.[8,13]

### 1.4.5 NEPHROTOXICITY OF IRON

Several diseases are associated with accumulation of iron in the kidney, and this is generally thought to be deleterious to renal function. In nephrotic kidney diseases that are associated with increased permeability of the glomerular filter, increased Tf(trans ferrin) reabsorption causes iron accumulation in PT lysosomes and this is associated with kidney injury. Iron-induced ROS formation in PT cells could well contribute to the progression of protein uric chronic kidney diseases.[14]

In a study it was reported that Iron overload into the kidney lowered renal function, i.e. the plasma levels of BUN and CRE were elevated, the plasma levels of Ca and iP were reduced, and vacuolization, swelling, desquamation, and necrosis in the proximal tubular epithelial cells of the kidney were observed in iron-overloaded rats. The urinary discharge of Ca might be increased by the disturbance in reabsorption of urinary Ca from the proximal tubular epithelial cells affected by the hydroxyl radical produced with iron overload.[13]

Another study shows that an iron-chelate of nitrilotriacetate, Fe-NTA induces acute and sub-acute renal injury in animals.Repeated intraperitoneal administration of ferric nitrilotriacetate (Fe-NTA) was reported to cause high degree of renal adeno-carcinoma in rats and mice. So iron overload of the kidney may well be associated with an increased risk of renal carcinoma.[15]

## 1.5 METAL CONTAINING AYURVEDIC MEDICINES

Ayurvedic medicine (also called Ayurveda) is one of the world's oldest medical systems. It originated in India and has evolved there over thousands of years. The term "Ayurveda" combines the Sanskrit words ayur (life) and veda (science or knowledge). Thus, Ayurveda means "the science of life". Ayurvedic medicines have developed into highly sophisticated systems of diagnosis and treatment over the centuries. Ayurvedic treatment goals include eliminating impurities, reducing symptoms, increasing resistance to disease, and reducing worry and increasing harmony in the patient's life. In recent times public interest was shifted towards ayurvedic medicines due to its less side effects.[16]

Ayurvedic medicine holds the following beliefs about the three doshas: vata, pitta, and kapha.

The ***vata dosha*** combines the elements ether and air. It is considered the most powerful *dosha* because it controls very basic body processes such as cell division, the heart, breathing, discharge of waste, and the mind .

The ***pitta dosha*** represents the elements fire and water. *Pitta* controls hormones and the digestive system. A person with a *pitta* imbalance may experience negative emotions such as anger and may have physical symptoms such as heartburn within 2 or 3 hours of eating .

The ***kapha dosha*** combines the elements water and earth. *Kapha* helps to maintain strength and immunity and to control growth. An imbalance of the *kapha dosha* may cause nausea immediately after eating [16]

Ayurvedic medicines are divided into 2 major types: herbal-only and rasa shastra.siddha nagarjuna considered to be the father of Indian alchemy or 'vedic chemistry' and rasa sastra.

### 1.5.1 RASA SHASTRA

Rasa-shastra is a branch of Ayurveda, which deals with Rasa, Uparasa, Lohas (I.e. Minerals & Metals), Yantras, Methods of Shodhana, process of Satvapatana, Druti, Process of Bhasma preparation etc."[17]

Rasa sastra includes the extraction of metals from their mineral,their purification and conversion in to digestible metallic bhasmas.the word rasa denote numerous things including mercury. At the time of $15^{th}$ century the technology for the preparation of bhasmas has been developed and use of other metals like zinc, tin and antimony also begun.

The process of manufacturing metal containing bhasmas include

1) Satvapatna(metal extraction)

2) Bhasmikarana (conversion to non toxic chemical form)

3) Other Processes (conversion to metallic chemicals by using sublimation and distillation)

The processing of minerals, metals and gems can be classified in to the following steps

1. Selection and control of raw materials
2. Sodhana or purification(grinding with various drugs to remove their toxins)
3. Marana or conversion to non toxic fine powdered form
4. Mardan or bhavana (preparation of intermediate paste)
5. Jarana or putapak(medicines are properly made by incineration in putas).[17,18]

The various types of bhasmas in Indian ayurvedic system include Abhraka Bhasma, Vaikrant Bhasma, Swarnamakshika Bhasma, KasisBhasma, Svarna Bhasma, Rajat Bhasma, Tamra Bhasma, Loha Bhasma, Mandur Bhasma, Naga Bhasma, Vanga Bhasma,Yashad Bhasma, Trivanga Bhasma, Pittal,Kamsya and Varthaloha Bhasma, Shankha Bhasma, Shukti Bhasma, KapardikaBhasma, Godanti Bhasma, Praval Bhasma, Mrigashringa Bhasma, MayurpicchaBhasma, Kukkutand twaka Bhasma, Hiraka Bhasma, Manikya Bhasma.

Ayurvedic medications have the potential to be toxic. Many materials used in them have not been thoroughly studied in either Western or Indian research. According to a new study, one-fifth of Ayurvedic medicines made in USA and India and sold in USA through the Internet contain more than permissible levels of toxic metals includes mercury, zinc ,lead and arsenic. The study recently appeared in the Journal of American Medical Association (JAMA).[19]

Some cases are also reported about the lead and mercury poisoning from Indian folk remedies. A study reported 3 cases of lead poisoning in India by ayurvedic metal containing medicines Also a study reported that Iron overload occurs in 1/200 to 1/400 of people. In the average primary care practice, this figure translates to about 4 to 8 patients with iron overload.[16] Toxicological profile for most of metal containing ayurvedic medicines are sorely missing so proper toxicological studies should be conducted with special emphasis on neurotoxicity ,nephrotoxicty and hepatotoxicity.[5]

In this investigation we are dealing with the toxicity of two iron containing bhasmas they are swarna makshika bhasma and kasisa bhasma although number of iron bearing Ayurvedic medicines are available but we restrict to these *bhasma* only because

- No toxicological profile was available for these bhasmas

- Large quantities of these bhasmas are imported to western countries like U.S.A and U.K

- They are not only effective in treatment of anemia but also used in therapy of multiple diseases,

- These bhasmas are readily available in market and cost effective.

# 1.6 DRUG PROFILE

## 1.6.1 SWARNA MAKSHIKA BHASMA (S.M.B)

### Chemical Nature

X-ray crystallography shows that Raw Swarna makshika bhasma contains $CuFeS_2$ also known as chelco pyrite. Final product of S.M.B contains $Fe_2O_3$, $FeS_2$, CuS and $SiO_2$.

**Particle size**: 1-2 microns.

### PREPRATION

### Sodhana

At first raw swarna makshika was powdered with help of iron mortar and pestle. Powdered swarna makshika was subjected to intense heat in a dry iron pan with frequent addition of lemon juice till the liberation of sulphur fumes stopped and it turned red. The process was completed in 3 days and the final product called purified swarna makshika.

### Marana

Equal amounts of purified swarna makshika and purified sulphur were triturated with lemon juice till a homogeneous paste was formed .after triturating ,small pellets of uniform size and thickness were prepared and dried in sunlight .pellets were kept inside a sharava (shallow earthen disc) and another sharava was inserted over it. The joint between the two disc was sealed.the properly sealed and dried samputa was subjected to puta system of heating with 3.5 kg cow dung cakes the process is repeated using purified sulphur in equal proportion. Bhasma of desired quantity was obtained in 13 putas.

**Dosage:** 125 mg-250mg twice daily (9mg/kg).

**Uses:** When there is severe deficiency of iron in the body this drug is sometimes preferred. It is also used for anemia, insomnia, convulsions, poor digestion and skin diseases.[20]

### 1.6.2 KASIS BHASMA (K.B)

Kaseesa (Green vitriol), is the ancient drug widely used in therapeutics of Ayurveda.

Fig: 1.3 Kasis bhasma

It is brownish black, odorless and tasteless

**Chemical nature**

Raw kasis bhasma is $FeSO_4\ 7H_2O$. but final product consist of ferrous sulphate , ferric oxide and various metals like copper, nickel cadmium, lead etc.

**PREPRATION**

**Sodhana**

Raw kasis was taken in an end runner and a fine powder is made. Bhringaraja swarasa was added little by little till the whole powder becomes wet. It is grinded for 3 hours till the liquid added dries up and this is considered as one bhavana. The above process was repeated for 3 times,the obtained kasis is dried and used. Duration for sodhana is 9 hours.

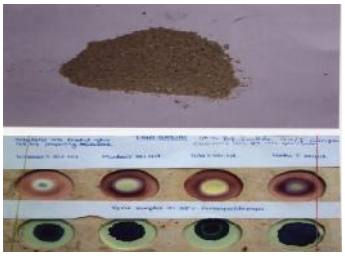


Fig: 1.4 Purified kasis

**Marana**

*Shuddha Kaseesa* was made in to fine powder with the help of an end runner, Nimbu swarasa was added little by little till the powdered drug gets fully immersed and grinded. This is made in to fine paste and pellets (chakrika) were prepared and dried. After drying these chakrika was kept in sarava, sarava samputa was prepared by sealing the edges of sarava with the help of a cloth smeared with multani mitti and dried. After drying, it was subjected to puta with abovesaid upalas. After swangasitala (self cooling) the sarava samputa were taken out and product of *Kaseesa* was collected and weighed. The same procedure was repeated for four times and before each puta Nimbu swarasa Bhavana (trituration) was given.

**Dosage:** 125mg- 250 mg daily (4.5mg/kg).

**Uses:** Haematinic & alterrative. Used in anemia, enlargement of liver and spleen, abdominal diseases, cough & general weakness.[21]

# CHAPTER - 2

## *Literature Review*

# LITERATURE REVIEW

Scientific literature review is the main basis for the planning of any scientific work and due to the same reasons here the review of literature regarding "Toxicity study of two iron containing bhasmas swarnamakshika bhasma and kasisa bhasma"

- **R.B Saper *et al*. *JAMA*. 2008 August.** reported that Lead, Mercury, and Arsenic in US- and Indian-Manufactured Ayurvedic Medicines Sold via the Internet

The author reported that from One hundred ninety-three of the 230 requested medicines 20.7% contains detectable amount of heavy metal. Lead was the most commonly found metal, followed by mercury and arsenic. The prevalence of metal-containing products did not differ significantly between US- and Indian-manufactured products. The median lead concentration in Indian manufactured vs US-manufactured lead-containing products was similar. Mercury was present in greater concentrations in Indian-manufactured products. Rasa shastra compared with non– rasa shastra medicines were more than twice as likely to contain metals. Rasa shastra metalcontaining medicines had higher lead and mercury median concentrations than non–rasa shastra metal-containing medicines.

- **P. K Sarkar *et al.* Indian journal of experimental biology, 2009,** reported toxicity and recovery study of two iron containg bhasmas

The author reported that toxicity and recovery study of two iron containing bhasmas ,lauha bhasma and mandura bhasma(55mg/kg) for 60 days in Charles foster rats shows an increase in serum alkaline phosphtase level in mandura bhasma recovery group, where as the elevation is non significant in case of lauha bhasma recovery group.in liver autopsy moderate fatty degenerative changes, diffused necrosis, periportal necrosis, central vein congestion and sinusoidal dilatation is seen lauha bhasma treated group while in case of mandura bhasma treated group also shows mild fatty changes and sinusoidal dilatations.in case of kidney a mild decrease in serum urea level in both the test drug treated group. A mild increase in the serum

creatinine level also shown in both the treated group when compared with control but histopathological observation of control and drug treated rats indicate absence of any gross pathological lesion in kidney

- **A.V singh *et al*, Indian Journal of Clinical Biochemistry, 2003,** reported toxicology and free radicals scavenging property of tamra bhasma

The author reported that tamra bhasma at doses of 20mg/100g showed necrosis and inflammation of kidney but serum urea and creatinine does not shows significant changes. liver showed some histopathological changes at the dose of 20mg/100g body weight. But biochemical analysis did not showed any change in the activity of serum GOT, GPT,alkaline phosphatase.

- **Singh *et al.* Journal of Herbal Medicine and Toxicology 4 (1), 2010,** reported acute and subchronic toxicity study of calcium based ayurvedic 'bhasmas' and a 'pishti' prepared from marine animals.

The author highlights experimental results on toxicity study of calcium basedfour ayurvedic bhasmas and a pishti prepared from marine -sourced animals viz.Shankha bhasma, Kapardika bhasma, Muktashukti bhasma, Pravala bhasma, andPravala pishti respectively. Mice administered above mentioned drugs did not show any abnormalbehaviour and no mortality was observed during 72 hrs in any experimental group.The levels of glucose, cholesterol, alkaline phosphatase, glutamate oxaloacetate transeminase, glutamate pyruvate transeminase, blood urea nitrogen, creatinine, total protein and albumin were not altered significantly in four bhasmas and a pishti treated rats compared to control rats. Serum electrolyte concentration viz. sodium, potassium, chloride and calcium showed no significant change, when compared to the control rats. The levels of haemoglobin and WBC count were not changed significantly. There was no sign of haemopoetic, hepatic or renal toxicity. Histopathological observation of control and drug treated rats indicate absence of any gross pathological lesion in liver, stomach and kidney. All animals were found healthy during entire study period and devoid of deleterious effects. All

four bhasmas and a pishti taken for the study did not showed any sign(s) of toxic manifestation.

- **NAGARAJU.V *et al.* Ancient Science of Life, Vol. IV No.1, July 1984**, reported **Toxicity studies on vanga bhasma**

The author reported dose-effect relation of Vanga bhasma on Digestive system (G.I. T., Liver and Pancreas) has been presented. But for local irritation, no significant toxicity attributable to Vanga bhasma has been observed even in eight time's higher dose than therapeutic dose, on exposure to the drug for ten days.

- **DAWOOD SHARIEF, S.*, et al. Journal of Cell and Tissue Research Vol. 9(2),2009,*** reported histopathological studies of the effect of nagaparpam, a zinc based drug of siddha medicine, in rats

The author reported that in 15 day treatment, liver and kidney had normal histology with no marked changes in the tissue architecture. in 30 day study, the kidney tissues remained normal while the liver tissues exhibited a few apoptotic cells with mild focal and lobular inflammation. On 60 day treatment, liver showed mild lobular inflammation and apoptotic cells were found in all doses. However brain, kidney and testis remained normal even in higher doses. The results suggest that NP does not show any toxicity in short term administration which is advocated in siddha literature for all metal based drugs.

- **P.V Balendu *et al.* International Journal of Toxicological and Pharmacological Research 2010**, reported Acute and Sub acute Toxicity Study of Ayurvedic Formulation (AYFs) Used for Migraine Treatment

The author highlights Acute and Sub acute Toxicity of five classical ayurvedic formulations (Narikela Lavana, Sootashekhara Rasa, SitopaladChurna, Rason Vati and Godanti Mishran) has been employed as prophylactic remedy for migraine was administrated in various doses ranging from 1.47 –6.48g/kg for mice and 0.7 – 7.45 g/kg for rats Animals from high dose treatment group and satellite

group of both the species showed decreased motor activity (reduced alertness, reduced exploratory behavior). The effect lasted for approximately two hours post dose administration and they responded comparably to control group in the functional test on rotarod. It may be assumed that the AYFs at high dose may have mild CNS depressant activity but pathological study of brain does not showed any signs of toxicity. And it does not shows any biochemical and histopathological changes in kidney and liver.

- **K.J Thompson *et al.* Brain Research Bulletin, Vol. 55, 2001**, reported Iron and neurodegenerative disorders

The author reported that As a result of a loss in iron homeostasis, the brain becomes vulnerable to iron-induced oxidative stress. Oxidative stress is a confounding variable in understanding the cell death that may result directly from a specific disease and is a contributing factor to the disease process. The underlying pathogenic event in oxidative stress is cellular iron mismanagement.

- **M.P Horowitz *et al.* J Alzheimer's Dis. 2010**, reported Mitochondrial Iron Metabolism and Its Role in Neurodegeneration.

The author reported about the physiological role that mitochondria play in cellular iron homeostasis and, in so doing, attempts to clarify how mitochondrial dysfunction may initiate and/or contribute to iron dysregulation in the context of neurodegenerative disease. And the ways in which iron is utilized therein, and how mitochondria are integrated into the system of iron homeostasis in mammalian cells. Author discuss about the dysregulation of iron in neurodegenerative diseases and effect of iron chelation in such diseases.

- **LIU *ET AL*. the Society for Experimental Biology and Medicine 2008**, reported Mercury in Traditional Medicines: Is Cinnabar Toxicologically Similar to Common Mercurial's?

The author comments on natural mineral cinnabar used in traditional medicines. Cinnabar is insoluble, has very low bioavailability and thus is poorly absorbed from the gastrointestinal tract. Once absorbed into the blood, the mercury disposition from cinnabar follows the pattern for inorganic mercury salts and preferentially distributed to the kidneys, with a small portion to the brain. The heating, overdose and the long-term use of cinnabar are major causes of mercury intoxication, but at the therapeutic doses, the adverse effects cinnabar-containing traditional medicines seem to be tolerable and reversible.

- **Uchida *et al.*/FEBS Letters 357 (1995)** , *reported* Acute nephrotoxicity of a carcinogenic iron chelate Selective inhibition of a proteolytic conversion of e2u-globulin to the kidney fatty acid-binding protein

The author reported the mechanism of acute nephrotoxicity of an ironchelate in vivo. Administration of a renal carcinogen ferric nitrilotriacetate (Fe-NTA) (15 mg Fe/kg body weight, intraperitoneally) led to selective loss of a renal protein with an apparent molecular mass of 17 kDa An immunochemical study using anti-a2u-globulin polyclonal antibodies confirmed that a single injection of Fe-NTA led to a decrease in k-FABP levels. However, a 19-kDa protein identical to the C~zuglobulin progressively appeared in the kidney, suggesting that the proteolytic processing of azu-globnlin in the renal proximal tubules was suppressed by the treatment with Fe-NTA. By monitoring k-FABP and its precursor Ot2u-globulin , it was determined that repeated exposure to Fe-NTA caused suppression of both proteolytic and endocytotic activity of the kidney.

- **Chandramouli R *et al* /J. Pharm. Sci. & Res. Vol.2 (12), 2010** reported Designing Toxicological Evaluation of Ayurveda and Siddha Products to Cater to Global Compliance – Current Practical and Regulatory Perspectives.

The author discusses on viable means to design, conduct and document studies of ayurvedic products for all possible toxicological manifestations in GLP conditions that can withstand global scrutiny and audit. It explains how to plan the protocol, choose the test systems, chalking out the observation routine, and conduct, acute, sub-acute, sub-chronic, and chronic toxicity studies, with an additional emphasis on

carcinogenicity, neurotoxicity, genotoxicity and developmental & reproductive toxicity studies. Determining scientifically consistent toxicological profile of ayurvedic drugs complying with current regulatory practices can be a fillip to their prospects of wide spread acceptance.

- **Tripathi *et al.* / International Journal of Engineering, Science and Technology, Vol. 2, No. 8, 2010,** reported Chemical phases of some of the Ayurvedic heamatinic medicines

The author reported that Chemical phases of iron in some of the Ayurvedic medicines are investigated through Mössbauer Spectroscopy and FTIR. In all the four medicines studied, iron is found in oxide form, either Fe2O3 or Fe3O4 or both. The size distribution of iron particles is different for different medicines. FTIR spectra are characteristically different for different drugs. These oxide particles are dispersed in a base probably consisting of ash of herbs or compound used as the medium for the preparation of these medicines which might control the efficacy and concentration of iron in these drugs.

- **Dr. Devanathan.R *et al.* / Journal of Pharmacy Research 2011**, reported Standardization of Kaseesa Bhasma: An Ayurvedic Medicine

The author reported that in analytical study of kasisa bhasma by the help of AAS and flame photometer shows a marked improvement in the concentration of Iron, in the final product as compared to the raw drug. Especially the concentration of iron in the raw drug was 243.97 ppm, while in the purified sample it was 394.40 ppm and in the final product it was increased to 885.40 ppm. An increase in number of *putas* proportionately decreases the *doshas* (impurities) and subsequently increases the therapeutic value of *Kaseesa Bhasma*.

- **s. mohapatra et al.international journal of ayurvedic research vol 1, 2010**, reported physicochemical characterization of ayurvedic bhasma (swarna makshika bhasma): an approach to standardization

The author reported that the analytical study of S.M bhasma by the help of X ray diffraction revealed that SM raw material contains CuFeS2 and SM bhama contains Fe2)3, FeS2, CuS and SiO2.scanning electron microscope showed that SM bhasma were uniformly arrange in agglomerates of size 1-2 microns compared to the raw.

- **Devanathan R *et al*. international journal of research in ayurveda and pharmacy 2(1), 2011**, reported concept of bhasmikarana

The author reported that preparation of bhasma is an elaborate process involving sodhana and bhasmikaran, bhasmas are prepared by the process of marana and putapaka method, the metals and minerals are converted in to micro fine form.

# CHAPTER - 3

## *Aim & Scope of Work*

# CHAPTER 3

# AIM AND SCOPE OF WORK

For centuries detoxified digestible metals (bhasmas) are used for the treatment in ayurveda, this comes under rasasastra a branch of ayurveda. Rasasastra includes the extraction of metals from their mineral, their purification and conversion in to digestible metallic bhasmas. By this processes the metals are detoxified and converted in to wonder substances.

Nowadays information's about ayurvedic medicines are widely available in internet and are exported largely to western countries. The prevalence of metals in ayurvedic medicines sold via the Internet and exported from India is under scanner. One-fifth of ayurvedic medicines made in India and sold in western countries through the Internet contain more than permissible levels of toxic metals includes mercury, zinc ,lead and arsenic. Although ayurvedic medicines are time tested for its efficacy proper toxicological studies are sorely missing so at fundamental level the bhasma as a medicine needs detailed scientific scrutiny about its toxicological profile and physicochemical characteristics.

In this study we are dealing with Swarna makshika and kasisa bhasma .These are two iron containing bhasmas widely used as haematinic and for iron deficiency anemia.

- The aim of present study was to evaluate the toxicity profile of two iron containing bhasmas swarna makshika and kasis bhasma with special emphasis on Neurotoxicity, Nephrotoxicity and Hepatotoxicity.

Although number of iron bearing Ayurvedic medicines are available but we restrict to these bhasma only because:

- No toxicological profile was available for these bhasmas.

- Large quantities of these bhasmas are imported to western countries like U.S.A and U.K.

- They are not only effective in treatment of anemia but also used in therapy of multiple diseases.

- These bhasmas are readily available in market and cost effective.

# CHAPTER – 4

# CHAPTER 4

## PLAN OF WORK

## PART 1: ACUTE TOXICOLOGICAL STUDIES

- Selection of animal species
- Housing and feeding conditions
- Preparation of animals
- Preparation of doses
- Administration of doses
- Observations
- Data collection and reporting.

## PART 2: NEUROTOXICITY STUDY IN COMBINATION WITH REPEATED DOSE SYSTEMIC TOXICITY (HEPATOTOXICITY AND NEPHROTOXICITY) STUDY

- Selection of animal species
- Housing and feeding conditions
- Preparation of animals
- Preparation of doses
- Administration of doses
- Observations
- Data collection and reporting

# CHAPTER –5

# CHAPTER 5

# MATERIALS AND METHODS

## 5.1 ACUTE TOXICOLOGICAL STUDIES

Acute toxicological studies are carried out according to OECD Guidelines 425. Description of method and materials which are done in these studies are mentioned below.

### 5.1.1 SELECTION OF ANIMAL SPECIES

The preferred rodent species which are used in this study is swiss albino mice. Normally female mice are used. This is because literature surveys of conventional $LD_{50}$ tests show that usually there is little difference in sensitivity between sexes, but in those cases where differences are observed, females are generally slightly more sensitive. Hence in this study, 17 female mice are used for both bhasmas.

Healthy young adult mice's of commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. At the commencement of its dosing, each animal should be between 4 and 8 weeks old and its weight should fall in an interval within ± 20 % of the mean initial weight of any previously dosed animals.

### 5.1.2 HOUSING AND FEEDING CONDITIONS

The temperature in the experimental animal room should be 22°C (± 3°C). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light and 12 hours dark. The animals are housed individually. For feeding, food pellets are purchased from Kerala Agricultural University, College of Veterinary and Animal Sciences Mannuthy, Thrissur used with an unlimited supply of drinking water.

### 5.1.3 PREPARATION OF ANIMALS

The animals are randomly selected, marked to permit individual identification of both bhasma treating groups, and kept in their cages for at least 5 days prior to dosing to allow for acclimatization to the laboratory conditions. As with other sequential test designs, care must be taken to ensure that animals are available in the appropriate size and age range for the entire study.

### 5.1.4 PREPARATION OF DOSES

In general, test substances should be administered in a constant volume over the range of doses to be tested by varying the concentration of the dosing preparation. i.e., at a constant concentration, may be more relevant to the subsequent risk assessment of that substance, and is a requirement of some regulatory authorities. In either case, the maximum dose volume for administration must not be exceeded. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not normally exceed 1 ml/100g of body weight; however in the case of aqueous solutions, 2 ml/100g body weight can be considered. With respect to the formulation of the dosing preparations, the use of an aqueous solution/suspension/emulsion is recommended wherever possible, followed in order of preference by a solution/suspension/emulsion in oil (e.g. corn oil) and then possibly solution in other vehicles. For vehicles other than water the toxicological characteristics of the vehicle should be known. Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

In this study, vehicle used is 3% gum acacia (3g in 100ml).was prepared prior to the preparation of drug suspension, for 2000mg/kg dose, 2000mg of swarna makshika bhasma and kasis bhasma was suspended in 10ml of 3% acacia separately and for 5000mg/kg dose; 5000mg of swarna makshika bhasma and kasis bhasma was suspended in 10 ml of 3% acacia separately shortly prior to the administration. Doses are prepared in mortar and pestle.[3]

### 5.1.5 PROCEDURE

**Administration of Doses**

The test substance is administered in a single dose by gavages using a stomach tube or a suitable intubation cannula. In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. In these studies all the doses are given as a single dose.

Animals were fasted prior to dosing. With the mice, food but not water should be withheld overnight. Following the period of fasting, the animals should be weighed and the test substance administered. The fasted body weight of each animal is determined and the dose is calculated according to the body weight. After the substance has been administered, food may be withheld for 1-2 hours in mice. Where a dose is administered in fractions over a period of time, it may be necessary to provide the animals with food and water depending on the length of the period. [3]

**Limit test and Main Test**

The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity below regulatory limit doses. Information about the toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance. In those situations where there is little or no information about its toxicity, or in which the test material is expected to be toxic, the main test should be performed. [22]

**Limit Test**

**Limit Test at 2000 mg/kg**

Dose one animal each for the test dose of both test drugs. If the animal dies, conduct the main test to determine the $LD_{50}$. If both animals survive for each test drug, dose four additional animals sequentially for both drugs so that a total of five animals are tested for both test drugs. However, if three animals die, the limit test is terminated and the main test is performed. The $LD_{50}$ is greater than 2000 mg/kg if

three or more animals survive. If an animal unexpectedly dies late in the study, and there are other survivors, it is appropriate to stop dosing and observe all animals to see if other animals will also die during a similar observation period. Late deaths should be counted the same as other deaths. The results are evaluated as follows (O=survival, X=death).

The $LD_{50}$ is less than the test dose (200 mg/kg) when three or more animals die.

O X O X X

O O X X X

O X X O X

O X X X X

If a third animal dies, conduct the main test.

Test five animals. The LD50 is greater than the test dose (2000 mg/kg) when three or more animals survive.[3]

O O O O O

O O O X O

O O O O X

O O O X X

O X O X O

O X O O O/X

O O X X O

O O X O O/X

O X X O O

**Limit Test at 5000 mg/kg**

Exceptionally, and only when justified by specific regulatory needs, the use of a dose at 5000 mg/kg may be considered. For reasons of animal welfare concern, testing of animals in GHS Category 5 ranges (2000-5000mg/kg) is discouraged and should only be considered when there is a strong likelihood that results of such a test have a direct relevance for protecting human or animal health or the environment.

Dose one animal each for the test dose of both test drugs. If the animal dies, conduct the main test to determine the LD50. If the animal survives, dose two additional animals each for both test drugs. If both animals survive, the LD50 is greater than the limit dose and the test is terminated (i.e. carried to full 14-day observation without dosing of further animals).

If one or both animals die for each drug, then dose an additional two animals for each test drugs, one at a time. If an animal unexpectedly dies late in the study, and there are other survivors, it is appropriate to stop dosing and observe all animals to see if other animals will also die during a similar observation period. Late deaths should be counted the same as other deaths. The results are evaluated as follows (O=survival, X=death, and U=Unnecessary).

The LD50 is less than the test dose (5000 mg/kg) when three or more animals die.

O X O X X

O O X X X

O X X O X

O X X X

The LD50 is greater than the test dose (5000 mg/kg) when three or more animals survive.

O O O

O X O X O

O X O O

O O X X O

O O X O

O X X O O

**5.1.6 OBSERVATIONS**

Animals are observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions and time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed. All observations are systematically recorded with individual records being maintained for each animal.

Additional observations will be necessary if the animals continue to display signs of toxicity. Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. The principles and criteria summarized in the Humane Endpoints Guidance Document should be taken into consideration. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be humanely

killed. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.[3]

**Bodyweight**

Individual weights of animals should be determined shortly before the test substance is administered and at least weekly thereafter for both test drug groups. Weight changes should be calculated and recorded. At the end of the test surviving animals are weighed and then humanely killed.

### 5.1.7 PATHOLOGY

All animals (including those which die during the test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy. All gross pathological changes should be recorded for each animal. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours after the initial dosing may also be considered because it may yield useful information.[23]

### 5.1.8 DATA COLLECTION AND REPORTING

**Data**

Individual animal data should be provided. Additionally, all data should be summarized in tabular form, showing for each test dose the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and the time course of toxic effects and reversibility, and necropsy findings. A rationale for the starting dose and the dose progression and any data used to support this choice should be provided.[3]

## 5.2 NEUROTOXICITY STUDY IN COMBINATION WITH A REPEATED DOSE SYSTEMIC TOXICITY (HEPATOTOXICITY AND NEPHROTOXICITY) STUDY

The neurotoxicity study was carried out in combination with a repeated dose systemic toxicity (hepatotoxicity and nephrotoxicity) study was done according to the OECD 424 and 407 Guidelines. The description of method and materials used in these studies are mentioned below.

### 5.2.1 SELECTION OF ANIMAL SPECIES

The preferred rodent species is the rat, although other rodent species, with justification, may be used. Commonly used laboratory strains of young adult healthy animals should be employed. In this study, both male and female wistar rats are used. The females should be nulliparous and non-pregnant. About 140 rats (70 male and 70 female) are purchased from Kerala Agricultural University, College of Veterinary and Animal Sciences Mannuthy, Thrissur, Kerala with the registered number 40/072/CPCSEA for both test drugs. Dosing should normally begin as soon as possible after weaning, preferably not later than when the animals are six weeks, and, in any case, before the animals are nine weeks of age.. However, when this study is combined with other studies this age requirement may need adjustment. At the commencement of the study the weight variation of animals used should not exceed ± 20% of the mean weight of each sex. Where a repeated dose study of short duration is conducted as a preliminary to a long term study, animals from the same strain and source should be used in both studies.[6]

### 5.2.2 HOUSING AND FEEDING CONDITIONS

The temperature in the experimental animal room should be 22°C (± 3°C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. Loud intermittent noise should be kept to a minimum. For feeding, food pellets are purchased from Kerala Agricultural University, College of Veterinary and Animal Sciences Mannuthy, Thrissur used with an unlimited supply of drinking water. The choice of diet may be

influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals may be housed individually, or be caged in small groups of the same sex.[2]

### 5.2.3 PREPARATION OF ANIMALS

Healthy young animals are randomly assigned to the treatment and control groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are identified uniquely and kept in their cages for at least five days prior to the start of the study to allow for acclimatization to the laboratory conditions.[6]

### 5.2.4 ROUTE OF ADMINISTRATION AND PREPARATION OF DOSES

**Route of administration –** orally by gavage using a stomach tube or a suitable intubation cannula.

**Vehicle –** 3% gum acacia suspension

**Preparation of vehicle -** 3% gum acacia was prepared prior to the preparation of test dose by triturating 3g gum acacia in 100 ml distilled water.

**Test drugs –** swarna makshika bhasma and kasis bhasma was procured from a renowned ayurvedic pharmaceutical firm having branches abroad also.

**Preparation of doses –**

The test substances were suspended in 3% gum acacia solution shortly prior to the administration .The toxic characteristics of vehicle should be known. In addition, consideration should be given to the following characteristics of the vehicle: effects of the vehicle on absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.[6]

### 5.2.5 PROCEDURE

**Number and sex of animals**

The study was conducted as a combination study; at least 20 animals (10 females and 10 males) should be used in each dose and control group for the evaluation of detailed clinical and functional observations. At least five males and five females, selected from these 10 males and 10 females, should be perfused *in situ* and used for detailed neuro histopathology at the end of the study. In cases where only a limited number of animals in a given dose group are observed for signs of neurotoxic effects, consideration should be given to the inclusion of these animals in those selected for perfusion. When the study is conducted in combination with a repeated dose toxicity study, adequate numbers of animals should be used to meet the objectives of both studies. If interim kills or recovery groups for observation of reversibility, persistence or delayed occurrence of toxic effects post treatment are planned or when supplemental observations are considered, then the number of animals should be increased to ensure that the number of animals required for observation and histopathology are available.[6]

**Treatment and control groups**

In this study three dose groups and a control group was used for each test drug. But if from the assessment of other data, no effects would be expected at a repeated dose of 1000 mg/kg body weight/day, a limit test may be performed. If there are no suitable data available, a range finding study may be performed to aid in the determination of the doses to be used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used in administering the test substance, the control group should receive the vehicle at the highest volume used.[6]

**Reliability check**

The laboratory performing the study should present data demonstrating its capability to carry out the study and the sensitivity of the procedures used. Such data should provide evidence of the ability to detect and quantify, as appropriate, changes in the different end points recommended. Historical data may be used if the essential

aspects of the experimental procedures remain the same. Periodic updating of historical data is recommended. New data that demonstrate the continuing sensitivity of the procedures should be developed when some essential element of the conduct of the test or procedures has been changed by the performing laboratory.[6]

**Dose selection**

Dose levels should be selected by taking into account any previously observed toxicity and kinetic data available for the test compound or related materials. The highest dose level should be chosen with the aim of inducing neurotoxic, hepatotoxic and nephrotoxic effects or clear systemic toxic effects. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dose-related response and a no-observed-adverse effect (NOAEL) at the lowest dose level. The groups and dose selected for this study was

| | | |
|---|---|---|
| **Swarna makshika bhasma** | - | Group 1 - 250 mg/kg |
| | | Group 2 - 500 mg/kg |
| | | Group 3 - 1000 mg/kg |
| **Kasis bhasma** | - | Group 1 - 250 mg/kg |
| | | Group 2 - 500 mg/kg |
| | | Group 3 - 1000 mg/kg |
| **Control (3% gum acacia suspension)** | - | Group 4 |

**Limit test**

If a study at one dose level of at least 1000 mg/kg body weight/day, using the procedures described, produces no observable neurotoxic, hepatotoxic and nephrotoxic effects and if toxicity would not be expected based upon data from structurally related compounds, then a full study using three dose levels may not be considered necessary. Expected human exposure may indicate the need for a higher oral dose level to be used in the limit test. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test substance

often may dictate the maximum attainable level of exposure. For the conduct of an oral acute study, the dose for a limit test should be at least 2000 mg/kg.[6]

**Administration of doses**

The animals are dosed with the test substance daily, seven days each week, for a period of at least 28 days. The test substance was administered by gavage, this should be done in a single dose using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume test drug administered was 1 ml/100 g body weight. For a substance administered by gavage, the dose should be given at similar times each day, and adjusted as necessary to maintain a constant dose level in terms of animal body weight.Where a repeated dose study is used as a preliminary to a long term study, a similar diet should be used in both studies.[6]

**5.2.6 OBSERVATIONS**

**Frequency of observations and tests**

In repeated dose studies, the observation period should cover the dosage period. Observations should be made with sufficient frequency to maximize the probability of detection of any behavioral and/or neurological abnormalities. Observations should be made preferably at the same times each day with consideration given to the peak period of anticipated effects after dosing.

**Observations of general health condition and mortality/morbidity**

All animals should be carefully observed at least once daily with respect to their health condition as well as at least twice daily for morbidity and mortality.

**5.2.7 DETAILED CLINICAL OBSERVATIONS**

Detailed clinical observation was done on both test drug treated groups once before the first exposure (to allow for within-subject comparisons) and at different intervals thereafter, for 28 days study. Detailed clinical observations should be made outside the home cage in a standard arena. In this study standard arena used was open field arena.

**Open field test**

The behaviour of animals was studied by means of open field test (ambulation, grooming, rearing, object exploration, defecation score) , four times: prior to exposure, after one week, two weeks, and four weeks of the experiment. Parallelly, a group of unexposed animals kept in the same condition was tested as a control. The open field arena was circular, 85 cm in diameter, with a white floor and a 50 cm high white wall. The floor had three concentric black circles, and the two outer circles were divided into segments by six radiating lines. Six additional short radiating lines subdivided all of the segments in the outermost circle into two, resulting in a total of 19 sections of equal area in the floor. The light level was 310 lux. For testing, each animal was placed on a starting point in the centre of one of the middle circle segments and then observed for 2 min. The number of the floor sections visited by the rat with all four feet, the number of rearing to the hind paws, and the number of faeces boluses were recorded.[25]

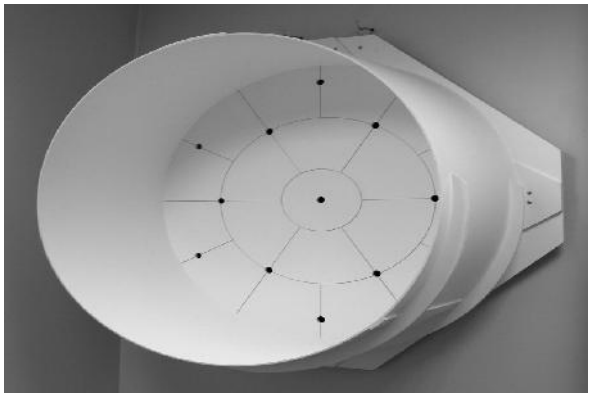


Fig: 5.1 circular open field apparatus

Effort was made to ensure that variations in the test conditions are minimal (not systematically related to treatment) and that observations are conducted by trained observers unaware of the actual treatment. Clinical observations should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern and/or mouth breathing, any unusual signs of urination or defecation, and discolored urine). Any unusual responses with respect to body position, activity level (e.g., decreased or increased exploration of the standard arena) and co-ordination of movement should also be noted. Changes

in gait (e.g., waddling, ataxia), posture (e.g., hunched-back) and reactivity to handling, placing or other environmental stimuli, as well as the presence of clonic or tonic movements, convulsions or tremors, stereotypes (e.g., excessive grooming, unusual head movements, repetitive circling) or bizarre behavior (e.g., biting or excessive licking, self-mutilation, walking backwards, vocalization) or aggression should be recorded.[25]

### 5.2.8 FUNCTIONAL TESTS

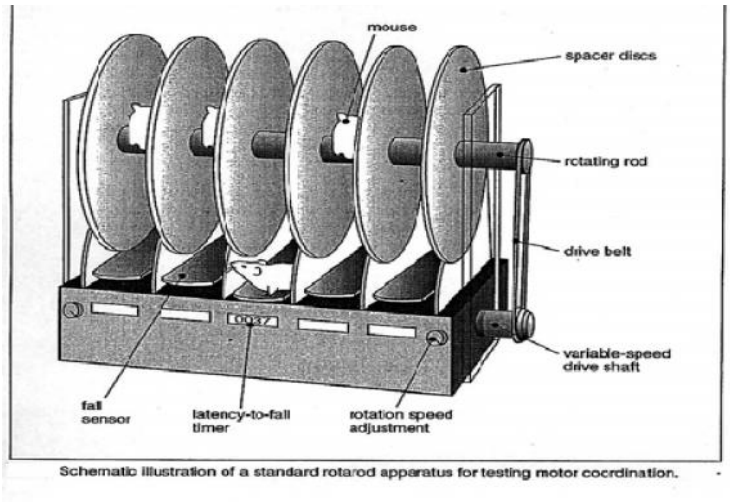
Functional tests for 28 days study was conducted once prior to exposure and during the fourth week of treatment as close as possible to the end of the exposure period. Functional tests should include sensory reactivity to stimuli of different modalities [e.g., auditory, visual and proprioceptive stimuli assessment of limb grip strength and assessment of motor activity.

**Assessment of limb grip strength**

Assessment of limb grip strength was done by rote rod apparatus.

**Rota rod test**

The apparatus consist of a Rotating rod diameter is ca. 5cm made of hard plastic material covered by grey rubber foam lanes width is ca. 5cm. The apparatus must allow an accelerating speed from 4rpm to 40rpm in 300 sec.




Fig; 5.2 Rotarod apparatus.

The limb grip strength was assessed by means of rota rod testing, two times; prior to exposure and fourth week of treatment for both test drugs. parallely a group of un exposed animals also assessed for limb grip strength. On the day of testing, mice had been kept in their home cages and acclimate to the testing room for at least 15 min. Three trials separated by 15 min inter-trial intervals (ITI) were conducted for all groups. For testing rota rod was turned on at an appropriate speed (20-25) .animals was placed one by one on the rotating rod. 'Fall of time' was recorded when the rat falls from the rotating rod. Similarly fall of time was recorded at the fourth week of treatment also. At the end of each test, the rotorod is cleaned with 70% alcohol solution, and dried with paper toweling. Fall off time before and after treatment was compared and % decrease in time was calculated for both test drugs.[26]

**Assessment of motor activity**

Assessment of motor activity was done using actophotometer.

**Actophotometer**

It consist of six built in photo electric cells which are connected in circuit with a counter .when the beam of light falling on the photocell was cut off by the animal , a count is recorded. The locomotor activity was assessed by means of actophotometer testing, two times; prior to exposure and fourth week of treatment for both test drugs. parallely a group of un exposed animals also assessed for motor activity.

Fig: 5.3 Actophotometer**.**

**Testing**

Actophotometer was turned on (check and make sure that all the photocells are working for accurate recording) and each rat was placed individually in the activity cage for 10min.Basal activity score of all the animals was recorded. Similarly basal activity score at fourth week of exposure was also recorded. Difference in the activity before and after treatment of both the test drugs was compared and % decrease in activity was calculated.[26]

### 5.2.8 BODY WEIGHT AND FOOD/WATER CONSUMPTION

For studies of 28 days duration, all animals should be weighed at least once a week and measurements should be made of food consumption (water consumption, when the test substance is administered by that medium) at least weekly.[6]

### 5.2.9 CLINICAL BIOCHEMISTRY

When the neurotoxicity study is carried out in combination with a repeated dose systemic toxicity (hepatotoxicity and nephrotoxicity) study clinical biochemistry determinations should be carried out as set out in the respective Guideline of the systemic toxicity study. Collection of samples should be carried out in such a way that any potential effects on neurobehaviour are minimized.

Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained of all animals just prior to or as part of the procedure for killing the animals (apart from those found moribund and/or intercurrently killed). All surviving animals fasted overnight and were anesthetized afterwards for blood collection from retro-orbital. Blood samples were collected into dry non-heparinized centrifuge tubes. The non-heparinized blood was allowed to coagulate before being centrifuged and the serum was separated. The serum was assayed for creatinine, blood urea nitrogen (BUN), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatases (Alk-P), total bilirubim and direct bilirubin. [27]

### 5.2.10 PATHOLOGY

**Gross necropsy**

When the neurotoxicity study is carried out in combination with a repeated dose systemic toxicity (hepatotoxicity and nephrotoxicity). Tissues from at least 10 animals (5 males and 5 females)/group were subjected to detailed gross necropsy. The brain, kidney and liver of all animals selected should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying. The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination.

**Histopathology**

The neuropathological examination should be designed to complement and extend the observations made during the *in vivo* phase of the study. Tissues from at least 10 animals (5 males and 5 females)/group should be fixed *in situ*, using generally recognized perfusion and fixation techniques. A stepwise examination of tissue samples is recommended in which sections from the high dose group are first compared with those of the control group. If no neuropathological alterations are observed in the samples from these groups, subsequent analysis is not required. If neuropathological alterations are observed in the high dose group, samples from each of the potentially affected tissues from the intermediate and low dose groups should then be coded and examined sequentially. The areas examined for brain should normally include: the forebrain, the centre of the cerebrum, including a section through the hippocampus, the midbrain, the cerebellum, the pons and the medulla oblongata. The neuropathological findings should be evaluated in the context of behavioural observations and measurements, as well as other data from preceding and concurrent systemic toxicity studies of the test substance.

For hepatotoxicity and nephrotoxicity histopathology should be carried out on the preserved liver and kidney respectively of all animals in the control and high dose groups. These examinations should be extended to animals of all other dosage groups, if treatment-related changes are observed in the high dose group. All gross lesions shall be examined.[6, 4]

### 5.2.11 STATISTICAL ANALYSIS

Results were expressed as mean ± standard deviation. (S.D.) In acute and sub acute toxicity, statistical significance was determined by one-way analysis of variance (ANOVA) and Dunnett test. P values less than 0.05 were considered significant.

### 5.2.12 DATA

When the neurotoxicity study is carried out in combination with a repeated dose systemic toxicity (hepatotoxicity and nephrotoxicity). Individual data should be provided. Additionally, all data should be summarized in tabular form showing for each test or control group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, type and severity of any toxic effects, the number of animals showing lesions, including the type and severity of the lesions. When possible, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods should be selected during the design of the study.[6,4]

# CHAPTER - 6

## *Results & Discussion*

# CHAPTER 6

# RESULTS AND DISCUSSION

## 6.1 ACUTE TOXICOLOGICAL STUDIES

### 6.1.1 PRELIMINARY OBSERVATION OF ANIMALS ADMINISTERED SWARNA MAKSHIKA BHASMA AND KASIS BHASMA (OECD GUIDELINES 425)

Animals are observed individually during the first 30 minutes after dosing and with special attention given during the first 4 hours. The animals show different behavior changes after administration of both test drugs. The following table's shows sign of toxicity produced by animals administered swarna makshika bhama and kasis bhasma.[3]

### 6.1.2 LIMIT TEST AT 2000mg/kg

In this limit test, 5 swiss albino mice's are used for each test drugs. Procedure for the limit test was discussed in chapter 5. The results of the observation are tabulated in table 6.1. Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic, somatomotor activity and behavior pattern. Attention also is directed to observations in tremors, convulsions, salivation, diarrhoea, lethargy and sleep. No animal found dead during experiment.

**OBSERVATION AT LIMIT TEST 2000mg/kg**

| **TEST DRUGS** | **SWARNA MAKSHIKA BHASMA** | **KASIS BHASMA** |
|---|---|---|
| **RESPIRATORY BLOCKAGE IN NOSTRIL** | | |
| DYSPNOEA | - | - |
| APNOEA | - | - |
| TACHYPNEA | - | - |
| NOSTRIL DISCHARGE | + | + |
| **MOTOR ACTIVITIES** | | |

| | | |
|---|---|---|
| LOCOMOTION | - | + |
| SOMNOLENCE | - | + |
| LOSS OF RIGHTING REFLEX | - | - |
| ANAESTHESIA | - | - |
| CATALEPSY | - | - |
| ATAXIA | - | - |
| TOE WALKING | - | - |
| PROSTRATION | - | + |
| FASCICULATION | - | + |
| TREMOR | - | - |
| HIND LIMB PARESIS | - | - |
| **CONVULSION (IN VOLUNTARY CONTRACTION)** | | |
| CLONIC/TONIC/TONIC-CLONIC CONVULSION | - | - |
| ASPHYXIAL CONVULSION | - | - |
| OPISTOTONES(TITANIC SPASM) | - | - |
| **REFLEXES** | | |
| CORNEAL | - | - |
| EYELID CLOSURE | - | - |
| RIGHTING | - | - |
| LIGHT | - | - |
| AUDITORY AND SENSORY | - | - |
| **OCULAR SIGNS** | | |
| LACRIMATION | - | - |
| MIOSIS | - | - |
| MYDRIASIS | - | - |
| PTOSIS | - | - |
| CHROMODACRYORRHEA | - | - |
| IRITIS | - | - |
| CONJUNCTIVITIS | - | - |
| **CVS SIGN** | | |

| | | |
|---|---|---|
| HEART RATE | - | - |
| VASODILATION | - | - |
| VASOCONSTRICTION | - | - |
| ARRHYTHMIA | - | - |
| **SALIVATION** | | |
| SALIVA SECRETION | + | + |
| **PILOERECTION** | | |
| CONTRACTION OF ERECTILE TISSUE | - | - |
| **ANALGESIA** | | |
| DECREASE IN REDUCTION TO INDUCE PAIN | - | + |
| **MUSCLE TONE** | | |
| HYPO OR HYPERTONIA | - | - |
| **GIT SIGN** | | |
| WATERY STOOL | - | - |
| EMESIS | - | - |
| RED URINE | - | - |
| RED STOOLS | + | + |
| **SKIN** | | |
| DISCOLORATION OF SKIN | - | - |
| ERYTHEMA | - | - |
| **DEATH** | - | - |

**Table 6.1 Observation at limit test 2000mg/kg**

### 6.1.3 LIMIT TEST AT 5000mg

In this limit test, 3 Swiss albino mice's are used for testing of both bhasmas. Procedure for the limit test was discussed in chapter 5. The results of the observation are tabulated in the following table 6.2. Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic,

somatomotor activity and behavior pattern. Attention also is directed to observations in tremors, convulsions, salivation, diarrhoea, lethargy and chocking. All animals died within 10 minutes of Swarna makshika and kasis bhasma administrations.[3]

**OBSERVATION AT LIMIT TEST 5000mg/kg**

| **TEST DRUGS** | **SWARNA MAKSHIKA BHASMA** | **KASIS BHASMA** |
|---|---|---|
| **RESPIRATORY BLOCKAGE IN NOSTRIL** | | |
| DYSPNOEA | - | - |
| APNOEA | - | - |
| TACHYPNEA | - | - |
| NOSTRIL DISCHARGE | + | + |
| **MOTOR ACTIVITIES** | | |
| LOCOMOTION | + | + |
| SOMNOLENCE | - | + |
| LOSS OF RIGHTING REFLEX | - | - |
| ANAESTHESIA | - | + |
| CATALEPSY | - | - |
| ATAXIA | - | - |
| TOE WALKING | - | - |
| PROSTRATION | + | + |
| FASCICULATION | + | + |
| TREMOR | - | - |
| HIND LIMB PARESIS | + | + |
| **CONVULSION (IN VOLUNTARY CONTRACTION)** | | |
| CLONIC/TONIC/TONIC-CLONIC CONVULSION | - | - |
| ASPHYXIAL CONVULSION | - | - |
| OPISTOTONES(TITANIC SPASM) | - | - |
| **REFLEXES** | | |
| CORNEAL | + | + |
| EYELID CLOSURE | + | + |

| | | |
|---|---|---|
| RIGHTING | - | - |
| LIGHT | - | - |
| AUDITORY AND SENSORY | - | - |
| **OCULAR SIGNS** | | |
| LACRIMATION | - | - |
| MIOSIS | - | - |
| MYDRIASIS | - | - |
| PTOSIS | - | - |
| CHROMODACRYORRHEA | - | - |
| IRITIS | - | - |
| CONJUNCTIVITIS | - | - |
| **CVS SIGN** | | |
| HEART RATE | - | - |
| VASODILATION | - | - |
| VASOCONSTRICTION | - | - |
| ARRHYTHMIA | - | - |
| **SALIVATION** | | |
| SALIVA SECRETION | + | + |
| **PILOERECTION** | | |
| CONTRACTION OF ERECTILE TISSUE | - | - |
| **ANALGESIA** | | |
| DECREASE IN REDUCTION TO INDUCE PAIN | + | + |
| **MUSCLE TONE** | | |
| HYPO OR HYPERTONIA | - | - |
| **GIT SIGN** | | |
| WATERY STOOL | - | - |
| EMESIS | + | + |
| RED URINE | - | - |

| | | |
|---|---|---|
| RED STOOLS | + | + |
| **SKIN** | | |
| DISCOLORATION OF SKIN | - | - |
| ERYTHEMA | - | - |
| **DEATH** | + | + |

**Table 6.2 Observation at limit test 5000mg/kg**

### 6.1.4 RESULT

From the above observations, it's clear that the swarna makshika and kais bhasma shows some toxic reaction in animals. Especially kasis bhasma shows neurotoxicity symptoms like decreased locomotor activity, hind limb paresis etc. At limit test 2000mg/kg, no death is observed. Hence the $LD_{50}$ of swarna makshika and kasis bhasma was found to be greater than 2000mg/kg. But at limit test 5000mg/kg, all animals died during experiment. So the $LD_{50}$ is below 5000mg/kg. Hence from the above observations we can conclude that the $LD_{50}$ of swarna makshika and kasis bhasma was between 2000mg/kg and 5000mg/kg.

## 6.2 OBSERVATIONS OF NEUROTOXICITY STUDY IN COMBINATION WITH A REPEATED DOSE SYSTEMIC TOXICITY (HEPATOTOXICITY AND NEPHROTOXICITY) STUDY (GUIDELINES 424,407) OF SWARNA MAKSHIKA BHASMA AND KASIS BHASMAS.

In this test, 140 wistar rats are used. 20 rats each are used in four different groups such as control, 250mg/kg, 500mg/kg, and 1000mg/kg for both swarna makshika and kasis bhasma. In this, each group contain 10 male and 10 female rats. Procedure for the limit test was discussed in chapter 5. The results of the observation are tabulated in the following table 6.3. Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic, somatomotor activity and behavior pattern. Attention also is directed to observations in tremors, convulsions, salivation, diarrhoea, lethargy and sleep.

### 6.2.1 BODY WEIGHT ANALYSIS

Weights of all animals are noted everyday. Difference in weight in different doses of animal and noted and recorded. The below table shows the body weight analysis of rats in the sub acute toxicity studies of yellow cow dung powder.

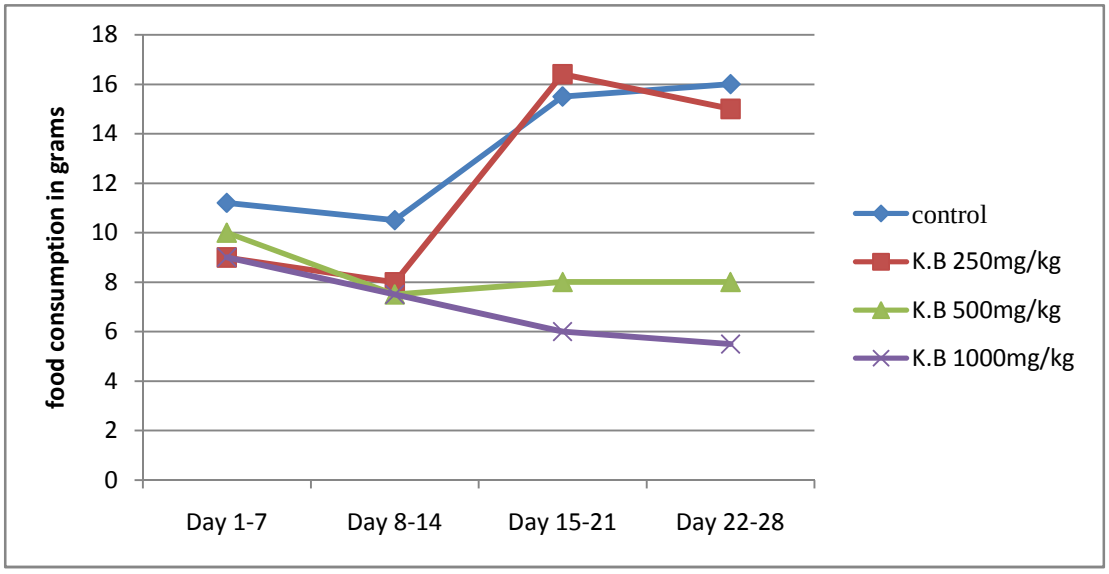
| DRUG USED | GROUPS | DAY 0 gm | DAY 7 gm | DAY 14 gm | DAY 28 gm | Total weight gain gm |
|---|---|---|---|---|---|---|
| | CONTROL | 125±10.42 | 155±11.2 | 165±12.5 | 180±10 | 55±1.667 |
| SWARNA MAKSHIKA BHASMA | 250mg/kg | 123.7±2.44 | 130.5±3.91 | 160±5.09 | 165.5±5.244 | 42±2.87 |
| | 500mg/kg | 121.5±2.37 | 146.5±4.848 | 153.75±6.09 | 160.7±10.41 | 39±8.04 |
| | 1000mg/kg | 148.7±7.31 | 160.2±5.543 | 166.2±2.5 | 170.2±5.00 | 22±2.31 |
| KASIS BHAMA | 250mg/kg | 141.2±3.5 | 153.7±5.5 | 165±4.6 | 182±9.4 | 40.8±5.9 |
| | 500mg/kg | 148.7±4.19 | 158.7±2.3 | 148.7±8.22 | 165.7±5.8 | 17±1.61 |
| | 1000mg/kg | 158.7±5.2 | 160.7±11.5 | 170±4.7 | 170.5±2.4 | 11.8±2.8 |

**Table 6.3 body weight analysis**

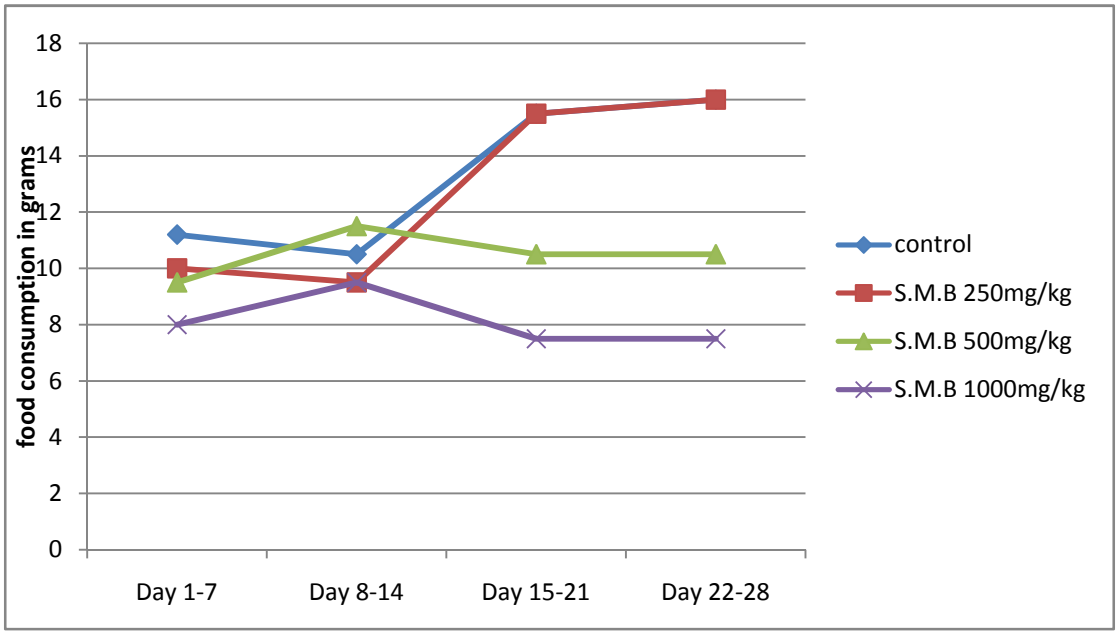
Results are expressed in mean: n=20, * significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$.

### 6.2.2 FOOD CONSUMPTION GRAPH

During the experiment, the mean daily food consumption of individual rats is recorded. The following graphs show the daily food consumption of rats in different groups like control, 250mg/kg, 500mg/kg, and 1000mg/kg of swarna makshika bhasma and kasis bhasma.



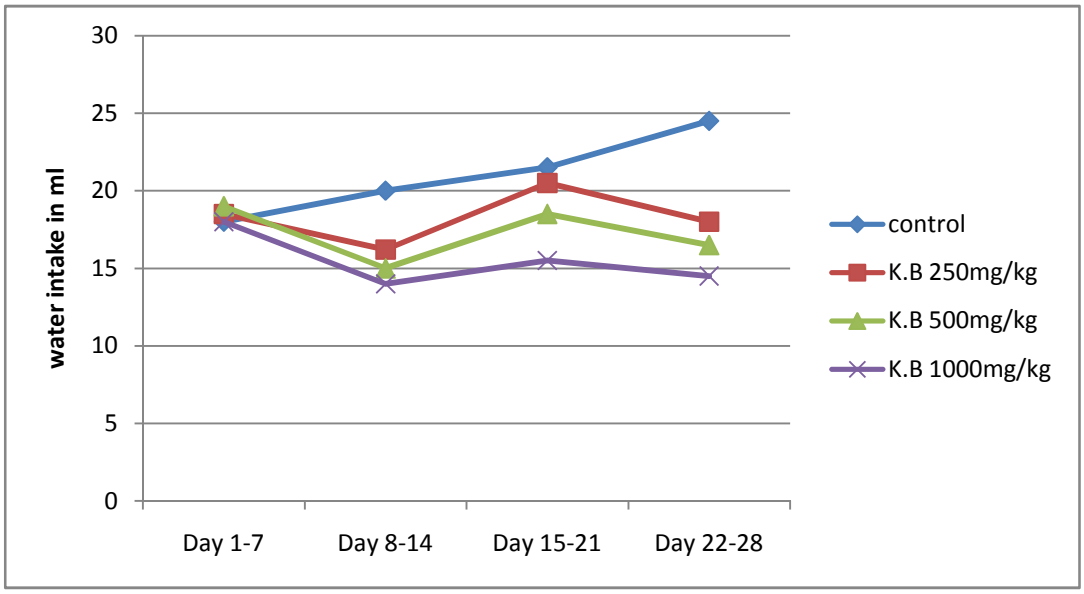

**Graph 6.1** days v/s food consumption in gm for Kasis bhasma treated groups



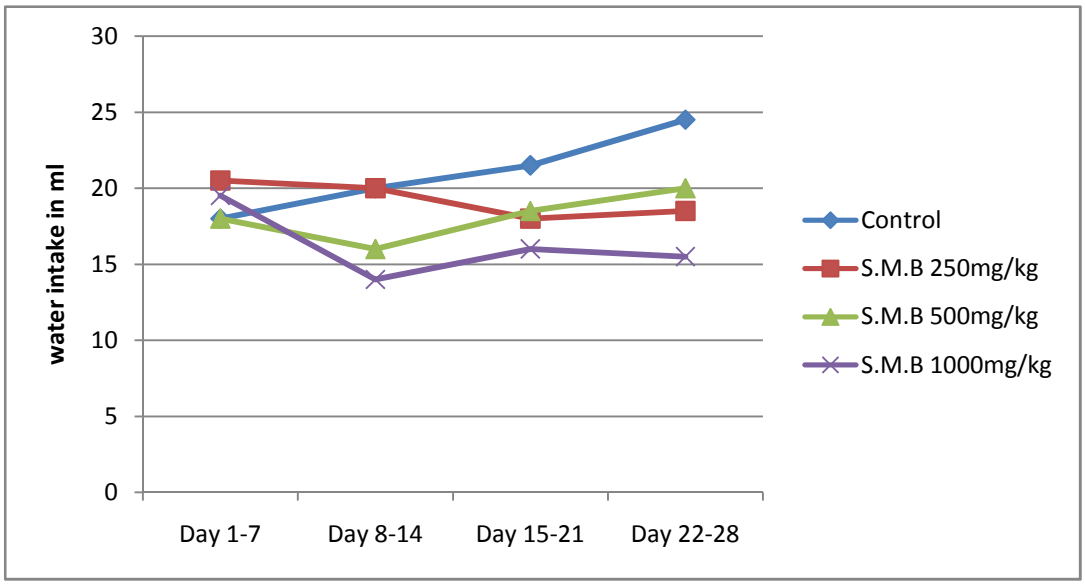

**Graph 6.2** days v/s food consumption in gm for Swarna makshika bhasma treated groups**.**

### 6.2.3 WATER CONSUMPTION GRAPH

During the experiment, the mean daily water intake of individual rats was recorded. The following graphs show the daily water intake of rats in different groups like control, 250mg/kg, 500mg/kg, and 1000mg/kg of both the test drugs.




**Graph 6.3** days v/s water intake in ml for kasis bhasma treated groups




**Graph 6.2** days v/s water intake in ml for swarna makshika treated group

## 6.2.4 DETAILED CLINICAL OBSEVATIONS

### Open field test

The behavior of animals was studied by means of open field test (ambulation, grooming, rearing, object exploration, defecation score) , four times: prior to exposure, after one week, two weeks, and four weeks of the experiment. Parallelly, a group of unexposed animals kept in the same condition was tested as a control.

| Examined Groups | | CONTROL | KASIS BHASMA | | | SWARNA MAKSHIKA BHASMA | | |
|---|---|---|---|---|---|---|---|---|
| | | | 250 mg/kg | 500 mg/kg | 1000 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg\kg |
| Prior to Exposure | A | 38.3±11.05 | 36.2±8.04 | 40±6.82 | 51.4±12.42 | 44.4±8.43 | 48.2±7.25 | 47.8±10.349 |
| | B | 2.00±0.57 | 3.00±0.62 | 2.00±0.50 | 1.9±0.20 | 3.2±0.67 | 2.8±0.68 | 2.4±0.56 |
| | C | 18.20±3.12 | 16..4±2.40 | 16.2±2.2 | 13.2±2.2 | 19.4±2.1 | 19.8±3.4 | 18.2±2.8 |
| | D | 2.40±0.87 | 2.50±0.54 | 2.8±0.62 | 3.00±0.24 | 2.8±0.64 | 2.5±0.45 | 2.4±0.54 |
| After 1 Week Exposure | A | 38±8.71 | 30.2±6.4 | 21.4±9.42 | 24.4±14.2 | 38±11.4 | 31±12.04 | 26.4±5.02 |
| | B | 2.00±0.91 | 2.4±0.45 | 2.8±0.64 | 2.2±0.54 | 1.5±0.62 | 2.6±0.24 | 2.00±0.68 |
| | C | 6.00±2.54 | 10.4±4.62 | 11.2±6.31 | 8.62±3.2 | 15.2±2.84 | 13.2±4.2 | 14.2±3.4 |
| | D | 3.20±1.39 | 2.8±0.94 | 4.2±1.50 | 4.8±2.2 | 3.2±1.1 | 5.2±0.67 | 2.2±1.2 |
| After 2 Week Exposure | A | 40±8.05 | 25.2±9.4 | 17.2±6.23 | 24.4±14.45 | 37±8.4 | 31±6.04 | 23.8±11.2 |
| | B | 1.40±0.45 | 1.2±0.20 | 2.2±0.89 | 2.4±0.62 | 3.2±1.2 | 2.1±0.022 | 1.9±0.69 |
| | C | 9.60±1.80 | 8.2±1.4 | 4.2±0.43 | 5.4±1.72 | 12.2±1.8 | 14.2±0.56 | 15.8±2.8 |
| | D | 1.40±0.93 | 1.9±0.89 | 3.2±0.52 | 3.9±2.48 | 2.9±0.68 | 1.9±0.56 | 1.5±.97 |
| After 4 Week Exposure | A | 40±12.95 | 24.1±11.2 | 14.4±8.42 | 19.2±8.45 | 34±11.23 | 28±9.42 | 22±6.29 |
| | B | 1.20±0.29 | 1.2±0.52 | 1.8±0.89 | 2.1±0.67 | 1.9±1.2 | 1.6±0.41 | 1.9±0.24 |
| | C | 4.70±1.20 | 6.4±1.9 | 8.9±0.81 | 12.2±0.92 | 4.2±0.21 | 6.2±1.8 | 6.9±1.6 |
| | D | 1.90±0.86 | 1.2±0.20 | 1.5±0.68 | 2.9±0.92 | 1.8±0.54 | 2.00±1.7 | 1.9±0.65 |

**A -ambulation; B - grooming; C - rearing; D – defecation**

**Table 6.4 open field test**

Results are expressed in mean: n=20, * significant at $p < 0.05$, ** significant at $p < 0.01$.

### 6.2.5 FUNCTIONAL TESTS OBSERVATION

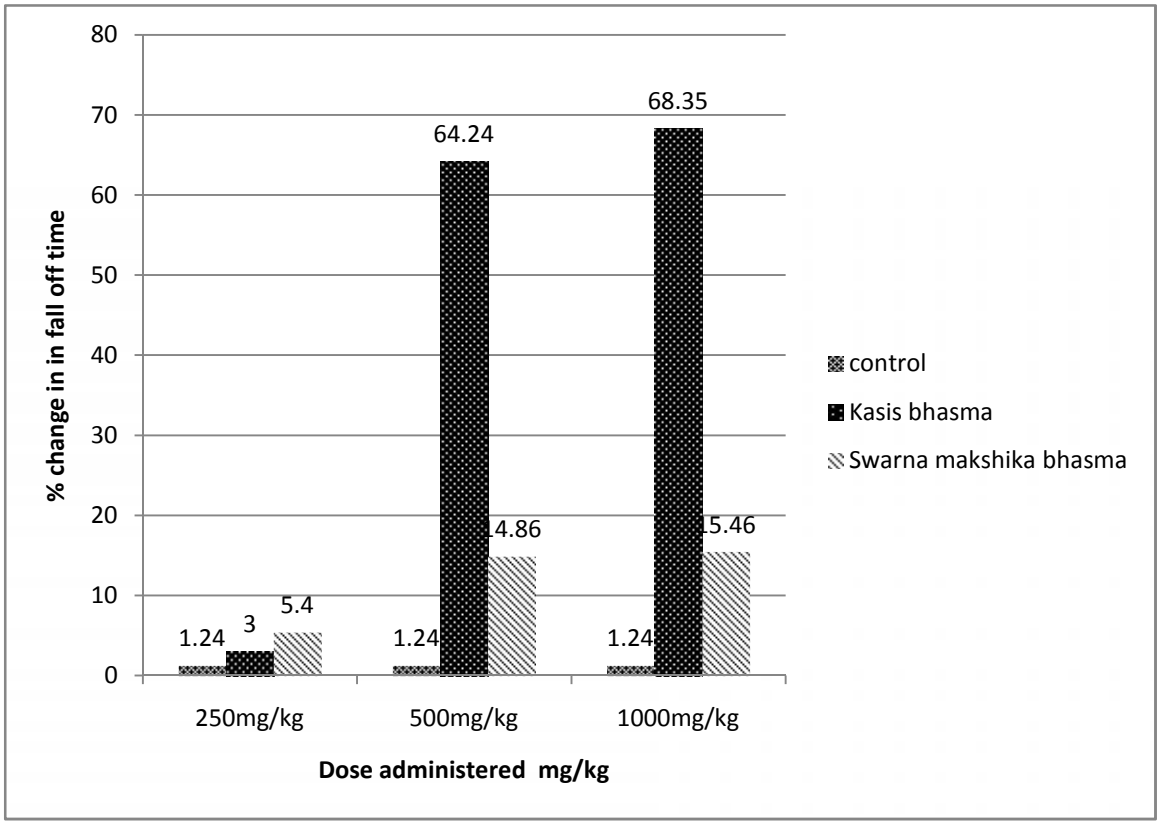
**Assessment of limb grip strength (rota rod test)**

The limb grip strength was assessed by means of rota rod testing, two times; prior to exposure and fourth week of treatment for both test drugs. parallely a group of un exposed animals also assessed for limb grip strength. Fall off time before and after treatment was compared and % decrease in time was calculated for both test drugs.

| DRUGS USED | GROUPS | FALL OFF TIME(SEC) | | %CHANGE IN FALL OFF TIME |
|---|---|---|---|---|
| | | BEFORE DRUG EXPOSURE | AT 4th WEEK OF EXPOSURE | |
| KASIS BHASMA | control | 101.8±5.12 | 100.53 ±2.3 | 1.24% ↓ |
| | 250mg/kg | 85.416±6.43 | 82.858±7.45 | 3% ↓ |
| | 500mg/kg | 111.86±8.2 | 39.4±3.2 | 64.24% ↓ |
| | 1000mg/kg | 173.8±6.2 | 55±7.2 | 68.35% ↓ |
| SWARNA MAKSHIKA BHASMA | 250mg/kg | 78.686±2.52 | 74.38±4.2 | 5.4% ↓ |
| | 500mg/kg | 73.46±2.2 | 62.54±7.8 | 14.86% ↓ |
| | 1000mg/kg | 89.812±5.62 | 75.92±8.42 | 15.46% ↓ |

**Table 6.5 rota rod test**

Results are expressed in mean: n=20, * significant at $p < 0.05$, ** significant at $p < 0.01$




Graph 6.5 % change in fall of time vs. dose administered by both bhasmas

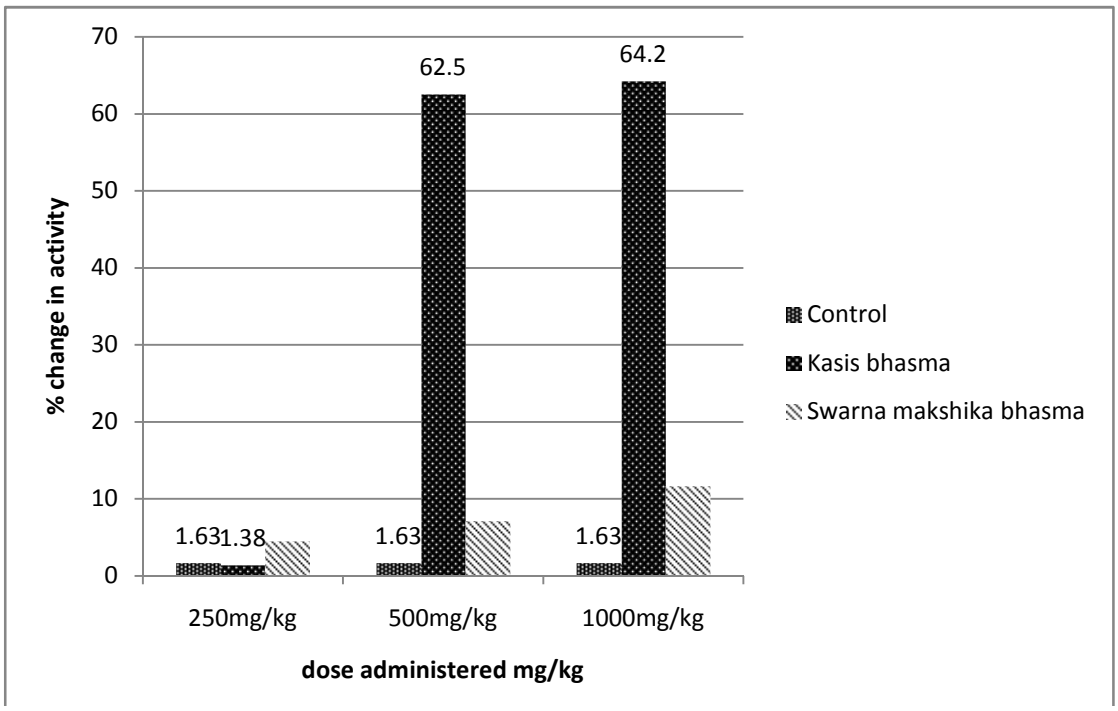
**Assessment of locomotor activity**

The locomotor activity was assessed by means of actophotometer testing, two times; prior to exposure and fourth week of treatment for both test drugs. Parallely a group of unexposed animals also assessed for motor activity. Difference in the activity before and after treatment of both the test drugs was compared and % decrease in activity was calculated.

| DRUGS USED | GROUPS | LOCOMOTOR ACTIVITY SCORES IN 10 MIN | | %CHANGE IN LOCOMOTOR ACTIVITY |
|---|---|---|---|---|
| | | BEFORE DRUG EXPOSURE | AT 4th WEEK OF EXPOSURE | |
| KASIS BHASMA | control | 64±4.3 | 61 ±5.2 | 1.63% ↓ |
| | 250mg/kg | 72.2±8.2 | 71.2±4.2 | 1.38% ↓ |
| | 500mg/kg | 82.6±4.3 | 31±6.4 | 62.5% ↓ |
| | 1000mg/kg | 182±9.2 | 65±5.4 | 64.2% ↓ |
| SWARNA MAKSHIKA BHASMA | 250mg/kg | 57.2±3.2 | 55.2±4.2 | 4.49% ↓ |
| | 500mg/kg | 56.2±7.2 | 52.2±5.3 | 7.1% ↓ |
| | 1000mg/kg | 77.2±3.5 | 68.2±4.4 | 11.6% ↓ |

**Table 6.6 assessment of locomotor activity**

Results are expressed in mean: n=20, * significant at $p < 0.05$, ** significant at $p < 0.01$




Graph 6.6 % change in locomotor activity vs. dose administered for both bhasmas

### 6.2.6 CLINICAL BIOCHEMISTRY

When the neurotoxicity study is carried out in combination with a repeated dose systemic toxicity (hepatotoxicity and nephrotoxicity) study clinical biochemistry determinations should be carried out as set out in the respective Guideline of the systemic toxicity study. Blood samples are collected from eye orbital of rats before dosing commenced, at the end of 28 day administration period. Biochemical studies are performed at laboratory in Giri micro labortary Gandhi nagar, Erode,Tamil Nadu. Clinical biochemical analyses of blood included creatinine, blood urea nitrogen (BUN), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatases (Alk-P), total bilirubin,direct bilirubin.

## 6.2.7 BIOCHEMICAL EXAMINATION

| TESTS | CONTROL | KASIS BHASMA | | | SWARNA MAKSHIKA BHASMA | | |
|---|---|---|---|---|---|---|---|
| | | 250 mg/kg | 500 mg/kg | 1000 mg/kg | 250 mg/kg | 500 mg/kg | 1000 Mg/kg |
| UREA (mgs/dl) | 51±1 | 50±2 | 51±5 | 52±3 | 55±6 | 50±3 | 46± 2 |
| CREATININE (mgs/dl) | 1.7±0.10 | 1.5±0.40 | 1.1±0.25 | 0.8±0.90 | 1.2±0.31 | 0.9±0.22 | 0.6±0.41 |
| S.G.O.T (IU/L) | 130.9±0.50 | 135±0.60 | 138±0.80 | 195±0.40*** | 140±0.67** | 160±0.92** | 188±0.21*** |
| S.G.P.T (IU/L) | 61.8±1.00 | 59±2.5 | 58±1.43 | 60±1.58 | 56±2.3 | 59±3.1 | 66±1.2 |
| ALKALINE PHOSPHATASE (IU/L) | 150.6±1.00 | 170±1.52 | 180±2.24 | 198±2.14*** | 210±1.3*** | 290±0.87** | 353±0.94*** |
| DIRECT BILIRUBIN (mg/dl) | 0.32±0.005 | 0.4±0.021 | 0.6±0.041 | 0.8±1.2 | 0.5±0.002 | 0.6±0.05 | 0.9±0.002 |
| TOTAL BILIRUBIN (mg/dl) | 0.53±0.005 | 0.6±0.009 | 0.8±0.010 | 0.82±0.12 | 0.7±0.008 | 0.9±0.009** | 1.1±0.006*** |

**Table 6.7 bio chemical examinations**

Results are expressed in mean: n=20, * significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $P<0.001$.

### 6.2.8 ORGAN WEIGHT DATA

Tissues from at least 10 animals (5 males and 5 females)/group was subjected to a detailed gross necropsy. The liver, kidneys and brain of all animals are subjected to be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying. The following table shows the net organ weight of treated and control animal

| ORGANS | CONTROL gm | KASIS BHASMA | | | SWARNA MAKSHIKA BHASMA | | |
|---|---|---|---|---|---|---|---|
| | | 250 mg/kg gm | 500 mg/kg gm | 1000 mg/kg gm | 250 mg/kg gm | 500 mg/kg gm | 1000 mg/kg gm |
| LIVER | 6.01±0.0114 | 5.04±0.01241 | 5.62±0.0154 | 4.76±.01424 | 5.66±.0084 | 6.08±0.01042 | 7.458±0.0223** |
| KIDNEY | 0.58±0.01025 | 0.60±0.0146 | 0.50±0.01051 | 0.56±.01402 | 0.67±0.0145 | 0.53±0.0102 | 0.61±0.0104 |
| BRAIN | 1.531±0.0142 | 1.45±0.01256 | 1.53±0.01247 | 1.65±.0042 | 1.65±0.0152 | 1.21±0.0024 | 1.53±0.0042 |

**Table 6.8 organ weight data**

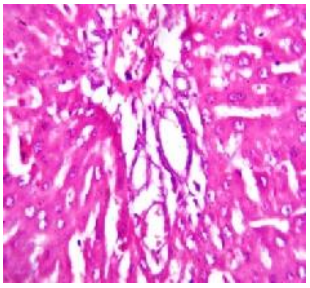
Results are expressed in mean: n=10, * significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$

### 6.2.9 HISTOPATHOLOGICAL STUDIES

Tissues from at least 10 animals (5 males and 5 females)/group was subjected to detailed gross necropsy. The brain, kidney and liver of all animals selected should be trimmed of any adherent tissues. The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination.

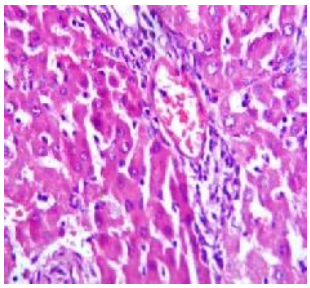
LIVER

A. Control:

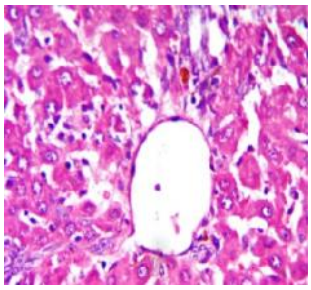


SWARNA MAKSHIKA BHASMA

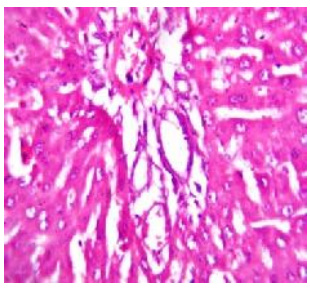
A1.1000mg/kg



A3.500mg/kg

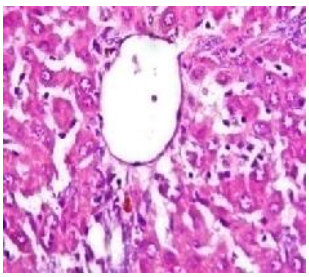


A5. 250mg/ kg

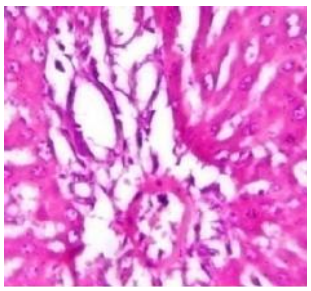


KASIS BHASMA

A2.1000mg/kg



A4.500mg/kg



A6.250mg/kg

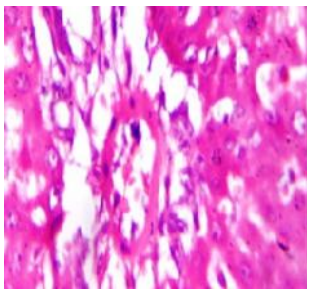
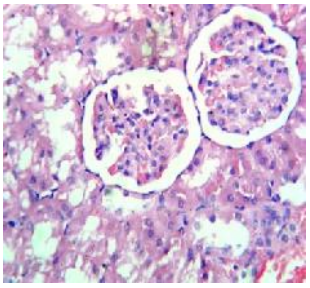


Fig 6.1(A-A6)-Histopathology of liver

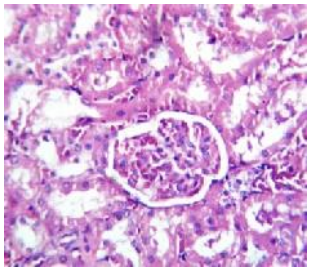
KIDNEY

B. Control:

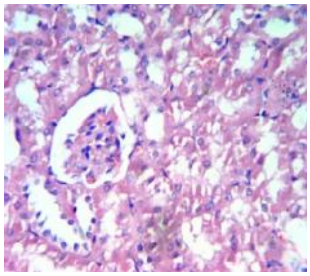


SWARNA MAKSHIKA BHASMA

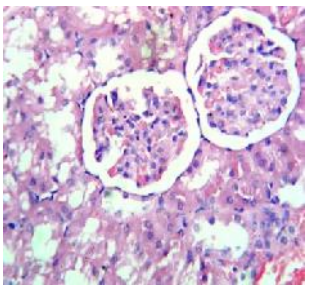
B1.1000mg/kg



B3.500mg/kg

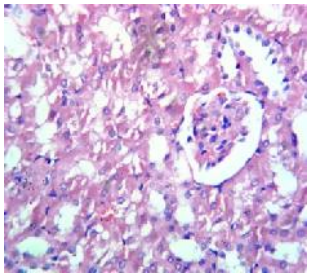


B5. 250mg/kg

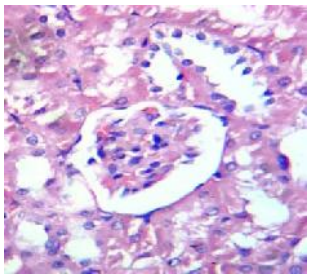


KASIS BHASMA

B2.1000mg/kg



B4.500mg/kg



B6.250mg/kg

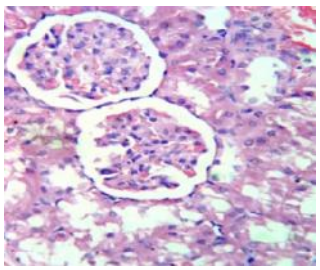
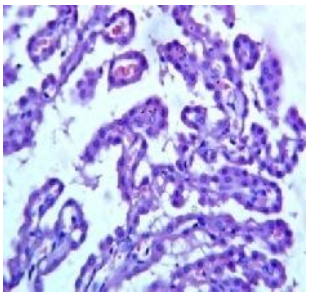


Fig 6.2(B-B6)-histopathology of kidney

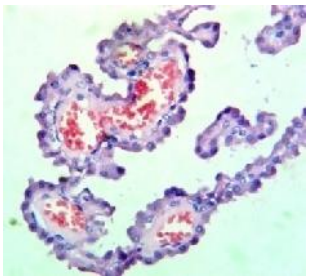
BRAIN

C. Control:



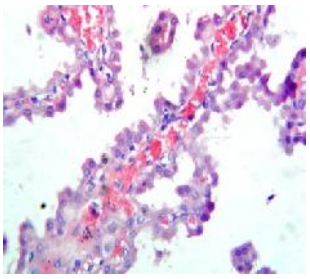
SWARNA MAKSHIKA BHASMA

C1.1000mg/kg

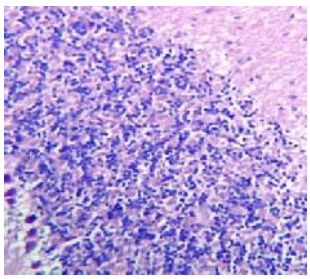


KASIS BHASMA

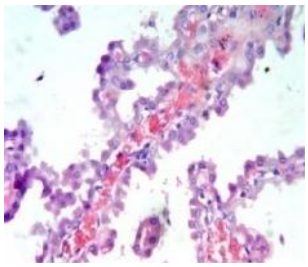
C2.1000mg/kg



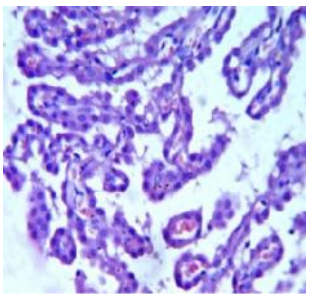
C3.500mg/kg



C4 .500mg/kg



C5.250mg/kg



C6.250mg/kg

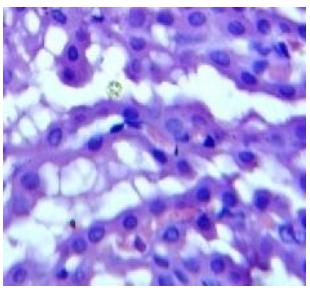


Fig 6.3(C-C6)-histopathology of brain

# DISCUSSION

## ACUTE ORAL TOXICITY STUDIES (guidelines 425)

### LIMIT TEST AT 2000mg/kg

In this limit test, 5 Swiss albino mice's are used. Observations include (table 6.1) changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic, somatomotor activity and behavior pattern are observed. Kasis bhasma when compared with swarna makshika bhasma and control shows little changes in motor activity. No animal found dead during experiment.

### LIMIT TEST AT 5000mg/kg

In this limit test, 2 Swiss albino mice's are used. Observations (table 6.2) include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic, somatomotor activity and behavior pattern. Attention also is directed to observations in tremors, convulsions, salivation, diarrhea, hind limb paresis, lethargy and chocking. In both bhasmas sign of neurotoxicity was observed. Both test drugs shows hind limb paresis, changes in motor activity and red stools. Both animals died within 10 minutes of drug administration.

From the above observations, it's clear that both kasis bhasma and swarna makshika bhasma shows toxic reaction in animals. At limit test 2000mg/kg, no death is observed. Hence the $LD_{50}$ of both test drugs was greater than 2000mg/kg. But at limit test 5000mg/kg, all animals died during experiment. So the $LD_{50}$ is below 5000mg/kg. Hence from the above observations we can conclude that the $LD_{50}$ of kasis bhasma and swarna makshika bhasma is between 2000mg/kg and 5000mg/kg.

# NEUROTOXICITY STUDY IN COMBINATION WITH A REPEATED DOSE SYSTEMIC TOXICITY (HEPATOTOXICITY AND NEPHROTOXICITY) STUDY

## OBSERVATIONS

## BODY WEIGHT ANALYSIS

Body weight indicates the health status of any living being. Here weights of all animals are noted everyday. Difference in weight in different doses of animal were noted and recorded for both test drugs. In the neurotoxicity study combined with hepatotoxicity and nephrotoxicity average body weight gain in swarna makshika and kasis bhasma treated groups of rats showed a slight decrease in high( 1000mg/kg) doses when compared to control, but the decrease in weight is non significant. So body weight analysis of this study could suggest that there are no or less harmful effects of test drugs on body function as a whole.[28]

## FOOD/WATER CONSUMPTION

Food/ water consumption of all rats are noted everyday. In the neurotoxicity study in combination with hepato and nephrotoxicity study, the food/water consumption of swarna makshika and kasis bhasma treated groups of rats showed a slight decrease but was not significantly different from those of the control group. Analysis of food and water consumption by treated rats doesn't shows any signs of toxicity.

## DETAILED CLINICAL OBSEVATIONS

### Open Field Test

The behavior of animals was studied by means of open field test (ambulation, grooming, rearing, defecation score) , four times: prior to exposure, after one week, two weeks, and four weeks of the experiment. Parallelly, a group of unexposed animals kept in the same condition was tested as a control.

The results of the present study (table 6.4) showed that both kasis and swarna makshika bhasma does not produce any significant differences in the open field test

statistically. However kasis bhasma showed a slight variation in the observed ambulation of tested rats when compared with other parameters. From this above observations it's clear that there were no differences in the measures of anxiety and emotionality statistically. So there was no significant influence for both this bhasmas on the behavior of animals in open field test was noted.[29]

**Functional tests observation**

**Assessment of Limb Grip Strength**

The limb grip strength was assessed by means of rota rod testing, two times; prior to exposure and fourth week of treatment for both test drugs. parallely a group of un exposed animals also assessed for limb grip strength. Fall off time before and after treatment was compared and % decrease in time was calculated for both test drugs. Fall off time before and after treatment was compared and % decrease in time was calculated for both test drugs.

The results of present study (table 6.5) showed that animal from high dose treatment groups 500mg/kg and 1000mg/kg of kasis bhasma shows a significant decrease in % fall off time that is 64.24% and 68.35% respectively. However there were no differences in the limb grip strength for swarna makshika treated groups when compared with control by rota rod test.

**Assessment of Locomotor Activity**

The locomotor activity was assessed by means of actophotometer testing, two times; prior to exposure and fourth week of treatment for both test drugs. Parallely a group of unexposed animals also assessed for motor activity. Difference in the activity before and after treatment of both the test drugs was compared and % decrease in activity was calculated.

The results of present study (table 6.6) showed that animal from high dose treatment groups 500mg/kg and 1000mg/kg of kasis bhasma shows a significant decrease in the % change of locomotor activity that is 62.5% and 64.2% respectively. However there were no differences in the motor activity for swarna makshika treated groups when compared with control by rota rod test.

## CLINICAL BIOCHEMISTRY

The liver and kidney are one of the major internal organs in the body and have several important functions. Symptoms of disorder in those organs appear only in serious diseases. To test whether the substance destroys and impairs liver and kidney functions, clinical blood chemistry examination was performed in the female and male rats.

All surviving animals fasted overnight and were anesthetized afterwards for blood collection from retro-orbital. Blood samples were collected into dry non-heparinized centrifuge tubes.The separated serum was assayed for creatinine, blood urea nitrogen (BUN), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatases (Alk-P), total bilirubin, direct bilirubin .

### Effect on kidney

There was no adverse effect on renal function after the administration of two different bhasmas(swarna makshika and kasis bhasma). Biochemical parameters revealed that excretory function of kidney was well maintained, as there was no rise in the values of blood urea nitrogen (BUN), and serum creatinine levels in plasma of rats treated with above-mentioned drugs.

### Effect on liver

Two different bhasmas used in this study showed little disturbance in hepatic functions as compared to control group rats. Table 6.7 represents the changes in the activities of SGOT, SGPT, ALP, TBL and direct bilirubin. In the assessment of hepatotoxicity the determination of enzyme levels such as aspartate transaminase and alanine transaminase is largely used. The results of present study showed that all the dose range of swarna makshika bhasma treated group had a significant increase in the S.G.O.T levels when compared with control($P<0.001$).while kasis bhasma high dose treatment group(1000mg/kg) only shows a significant increase in SGOT value. The SGOT value of control 130.9±0.50 IU/L whereas S.G.O.T value of kasis and swarna makshika bhasma treated groups significantly increased up to 195±0.40 IU/L and

188±0.21 IU/L respectively. Elevated levels of serum enzymes were indicative of cellular leakage and loss of functional integrity of cell membrane in liver.[30]

Alkaline phosphatase concentration is related to the functioning of hepatocytes, high level of alkaline phosphatase in the blood serum was related to the increased synthesis of it by cells lining bile canaliculi usually in response to cholestasis and increased biliary pressure.[31] The results of clinical biochemistry study showed that all the dose range of swarna makshika bhasma treated group had a significant increase in the alkaline phosphatase levels when compared with control($P<0.001$).While kasis bhasma high dose treatment group(1000mg/kg) only shows a significant increase in alkaline phosphatase. The alkaline phosphatase value of control group was 150.6±1.00 IU/L whereas ALP value of swarna makshika bhasma increased up to 353±0.94 and for kasis bhasma high dose treated group shows an ALP value of 198±2.14.

Serum bilirubin(total bilirubin, direct bilirubin) is one of the most sensitive tests employed in the diagnosis of hepatic diseases. Hyperbilirubinemia was observed due to excessive heme destruction and blockage of biliary tract. As a result of blockage of the biliary tract there was a mass inhibition of the conjugation reaction and release of unconjugated bilirubin from damaged and dead hepatocytes.[32] The results of clinical biochemistry study showed that high dose range (500mg/kg, 1000mg/kg) of swarna makshika bhasma treated group had a significant increase in the total bilirubin levels when compared with control ($P<0.001$).the total bilirubin value for control was 0.53±0.005. Whereas TBL value for swarna makshika treated group increased significantly up to 1.1±0.006.However all dose range of kasis bhasma treated group doesn't shows any rise in value of total protein.

**ORGAN WEIGHT DATA ANALYSIS**

The liver, kidneys and brain of all animals are subjected to be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying. Table 6.8 represents the changes in weight of different organs for different doses of both bhasmas. The organs such as liver, kidney, brain of swarna makshika and kasis bhasma treated group doesn't showed any significant increase in organ weight when compared to control, except the rats receiving the dose

of 1000mg/kg of swarna makshika bhasma shows a significant increase in the liver weight ($P<0.01$).

**HISTOPATHOLOGICAL STUDIES**

The representative microscopic findings in the organs of the rats for neurotoxicity study in combination with a repeated dose systemic toxicity (hepatotoxicity and nephrotoxicity) study, oral treatments of swarna makshika and kasis bhasma are shown in Figure 6.1 to Figure 6. 3.

**Effect on liver**

Liver biopsies of two different bhasma treated rats are shown in figure. The results of pathological study of liver when compared with the control goup showed that swarna makshika bhasma high dose(1000mg/kg) treatment groups had significant changes in the liver pathology, includes focal periportal inflammation by chronic inflammatory cells composed chiefly of lymphocytes .the hepatiocytes, hepatic sinusoids and central veins appear normal. Whereas all the dose range kasis bhasma treated groups and low dose (250 ,500mg/kg) swarna makshika bhasma treated rats show normal lobular architecture of liver with normal central portal vein, radiating plates of hepatocytes and peripheral portal tracts composed of hepatic artery, bile ductile and distal portal vein. No focal or diffuse foci of necrosis of hepatocytes, and infiltration of chronic inflammatory cells were observed.

**Effect on kidney**

Kidney biopsy of all the dose range of kasis and swarna makshika bhasma treated rats when compared with control shows normal architecture composed of normal renal glomeruli, collecting tubules, interstitial tissue and blood vessels. There were no foci of necrosis, degeneration or fibrosis in the interstitium.

**Effect on brain**

Brain biopsy of all the dose range of kasis and swarna makshika bhasma treated rats when compared with untreated group shows normal architecture composed of normal cell bodies of neurons and the glial cells with normal fibrillary cytoplasm and normal choroid plexus.

The results of detailed clinical studies and functional tests (rotarod test and locomotor activity test) revealed that kasis bhasma given in high doses are susceptible to neurotoxicity or CNS depressant action, kasis bhasma high dose treatment groups (500mg/kg and 1000mg/kg) showed decrease in motor activity, muscle strength and exploratory behavior. This may be due to over dose and excessive absorption of iron in to mucosal cells of the duodenum and jejnum results in an increased free iron concentration in blood.[8] Excessive absorption of kasis bhasma may be due to the presence of organic acids in its preparation process. The organic acids and reducing agents (all acid in character) that promote iron absorption appear to do so by keeping the PH of the intestine down for a longer time.[9]

Increased free iron concentration in blood may lead to the dysregulation of brain iron homeostasis .dysregulation of iron homeostasis may results in the accumulation of iron in the brain cells. Excessive cellular iron is harmful to brain cells and promotes ROS formation through Fenton chemistry. Which may results in the development of neurodegenerative disorders like AD, PD and ALS. However, neither the mechanisms underlying iron accumulation nor its complete role in the pathogenesis of the diseases are clear.[12]

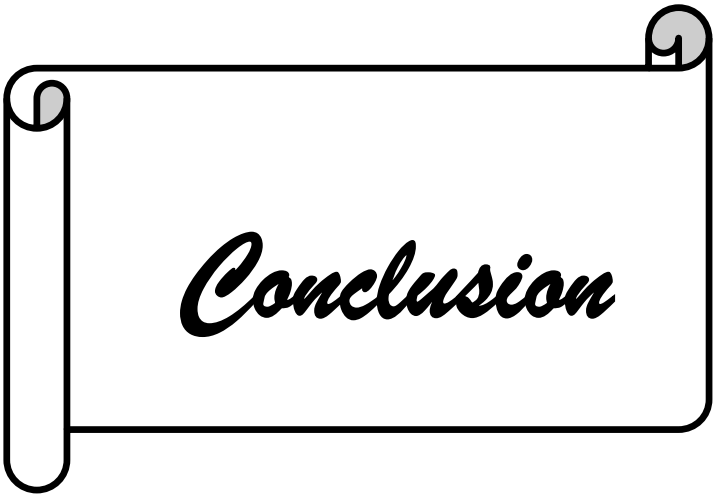
Whereas all the dose range tested for swarna makshika bhasma doesn't produce any significant difference in the detailed clinical and functional tests when compared with the control. No signs of neurotoxicity were observed for swarna makshika bhasma treated groups.

Results of clinical biochemistry, organ weight analysis and histopathological studies revealed that swarna makshika bhasma at high doses are highly susceptible to hepatotoxicity. Impairment in the hepatic functions in toxicity study can be explained as ferrous iron is absorbed in to mucosal cells of the duodenum and jejunum and is oxidized to ferric iron and bound to ferritin, it is then slowly released in to the plasma where it is bound to transferrin, a specific iron binding globulin, and transported to tissues. Iron bound to transferrin is non toxic with over dosage free iron that exceeds the iron binding capacity of transferrin, and high ferritin levels cause tissue damage .free iron injures hepatic cells and cause hepatotoxicity.[28]

Normally high dose iron preparations produce nephrotoxicity. However no sign of nephrotoxicty was observed in clinical biochemistry, organ weight analysis and histopathological studies for all dose range of both swarna makshika and kasis bhasma treated groups when compared with control group.[33]

Mortality was not observed for all the dose range of kasis bhasma and swarna makshika bhasma treated groups.

# CHAPTER - 7



# CHAPTER 7

## CONCLUSION

Ayurvedic is largely practiced using ancient protocols and parameters. Though there is a need of scientific scrutiny of its principles of treatment, very few attempts have been made for its scientific and systemic validation. In the present investigation we tried to assess the toxicity profile of two iron containing bhasmas, swarna makshika and kasis bhasma. No significant Adverse Effect was observed for low dose range of swarna makshika and kasis bhasma tested animal. The higher dose range tested was well above and at an adequate safety distance of the recommended dose in humans. Though the animals treated with high dose of swarna makshika bhasma showed hepatotoxicity and for kasis bhasma high dose treated groups shows neurotoxicity or CNS depressant action.

Thus in conclusion , present study indicates that there are chances of hepatotoxicity and neurotoxicity along with the clinical administration of higher doses of swarna makshika and kasis bhasma respectively .while low doses of both bhasmas was found to be safe. So both bhasmas are considered to be safe at clinically administered doses (9mg/kg for S.M.B and 4.5mg/kg for K.B). However, further detailed studies are required to know the long term chronic toxicity of swarna makshika and kasis bhasma.

# CHAPTER - 8

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