CHAPTER 4 – IN VIVO TOXICITY EVALUATION OF LAUHA BHASMA

4.1 INTRODUCTION

The usage of Ayurvedic herbo-metallic preparations has been in vogue over several centuries in clinical practice [1, 2]. There is a global perception that Ayurvedic medicines are toxic, based on several reports that indicate the presence of heavy metals like mercury and lead above the permissible limits in these preparations [2–4]. The toxic effects of metals in case of improper bhasma preparations have also been portrayed in the literature [5]. Iron is also known for its toxic nature, apart from its essential functions in the biological systems. Iron can produce free radicals, which ultimately result in alteration of metabolic pathways and alteration in the biological systems [6]. Due to these toxicity concerns, several questions have been raised about the safety of these preparations. Some of them are

- Do Ayurvedic purification and calcination procedures convert iron into medicine?
- Whether the prepared Lauha bhasma is safe or toxic?

To address these questions, attempts have been made here to study the in vivo toxicity profile of Lauha bhasma in Wistar rats. Acute oral toxicity (single dose) and sub acute toxicity studies (repeated dose) were carried out by oral administration of Lauha bhasma. All the experiments were carried as per the WHO guidelines.
4.2 MATERIALS AND METHODS

4.2.1 Study design

Wistar rats of both sexes in the age group of 4-6 weeks (Acute oral toxicity) and 6-8 weeks (Sub acute oral toxicity) were used for the study. The rats were housed in polypropylene cages and kept in a 12 h light/dark cycle. The room temperature was maintained at 21±2°C and they were fed with standard rat feed pellet and deionized water ad libitum throughout the entire study. The animals were acclimatized for 5 days before the initiation of the study. The procedures used were reviewed and approved by the Institutional Animal ethics committee (120/SASTRA/IAEC/RPP, SASTRA University).

4.2.2 Acute oral toxicity study (14-days single dose toxicity study)

A total of 12 rats were divided into two groups (I & II) with equal number of male and female. The body weight of male ranged from 105 - 125 g and female from 85 - 100 g. Group I rats were treated as normal control where they received honey with distilled water in the ratio of 2:3 through oral route. Group II animals were administered with single dose of Lauha bhasma (2000 mg/ kg) as a suspension in honey with distilled water in the ratio of 2:3 through oral route. Body weight was measured on Day 0, Day 7 and Day 14. The fecal samples were collected for 14 days to determine the elimination profile of iron. On the 14th day, animals were sacrificed and major organs including liver, spleen, intestine, brain and kidney were collected for bio-distribution studies.
4.2.3  Bio-distribution studies and Fecal analysis

The organs and fecal samples were subjected to microwave digestion using ultra-pure nitric acid (Merck) and 50 % Hydrogen peroxide(Merck) in the ratio of (4:1) using microwave digestor (Anton-Paar, Graz, Austria). The digested samples were then quantitatively estimated for the iron content using atomic absorption spectroscopy (AAnalyst 400, Perkin Elmer, USA).

4.2.4  Sub acute oral toxicity study (28-days repeated dose toxicity study and 14 days recovery study)

A total of 60 rats (n=10) were divided into six groups (Group I to Group VI) with equal number of male and female. The therapeutic dose of Lauha bhasma for humans is 250mgs [7]. This dose was converted to animal dose [8] as follows:

\[
\text{Human equivalent dose (mg/kg)} = \frac{\text{Rat dose (mg/kg)} \times \text{Rat Km (6)}}{\text{Human Km (37)}}
\]

The human equivalent dose was found to be 25 mg/kg. By fixing 25mg/kg as normal dose, the medium and high doses were calculated as 100 mg/kg and 400 mg/kg respectively. Group I was treated as normal control and received honey with distilled water in the ratio of 2:3 for 28 days. Lauha bhasma was administered to Group II to Group IV animals at different doses and was given as a suspension in honey and distilled water (2:3) for 28 days.
Group II received the therapeutic dose (25mg/kg body weight) of *Lauha bhasma*, Group III received the medium dose (100 mg/kg) and Group IV received the high dose (400 mg/kg). Group V and Group VI were treated as satellite groups in which group V was treated as satellite control and received honey with distilled water in the ratio of 2:3. Group VI received the high dose (400 mg/kg body weight). Weekly body weight was measured. On the 28\textsuperscript{th} day animals from Group I to Group IV were sacrificed. Blood was collected by retro orbitary puncture for biochemical and hematological analysis. Major organs were collected for histopathological studies. Group V and Group VI were treated with standard rat feed pellet and deionized water *ad libitum* for the next 14 days. On the 42\textsuperscript{nd} day the satellite group animals were sacrificed as mentioned above.

### 4.2.5 Biochemical and Hematological analysis

Biochemical analysis was performed using Auto Analyzer (A15 Biosystems, Spain) and the kits were procured from Bio Systems. Hematological parameters were analyzed using Mythic 22 Hematology Analyzer (Orphee SA, Switzerland)

### 4.2.6 Histopathology

Tissues were collected and preserved in 10 % buffered formalin. All tissues required for histopathological evaluation were embedded in paraffin wax, sectioned at a thickness of approximately 3-5 microns, stained with hematoxylin-eosin and examined by light microscopy.
4.2.7 Statistical analysis:

All the values were expressed as Mean±SD. A level of $p < 0.05$ was considered statistically significant and accordingly data were interpreted. Two way anova was performed to find the significant difference between the control and *Lauha bhasma* treated groups in bodyweight and iron excretion pattern. Bonferroni post test was done in iron excretion pattern followed by two way anova. One way anova was performed to find the significant difference between the control and *Lauha bhasma* treated groups in biochemical and hematological parameters.

4.3 RESULTS AND DISCUSSION

4.3.1 Effects on body weight

Oral administration of *Lauha bhasma* did not cause any mortality or adverse effects throughout the study period. No significant difference was observed in body weight between the control and the *Lauha bhasma* treated groups in both the acute oral toxicity study and sub acute oral toxicity study.
**Figure 4.1** Body weight of control and *Lauha bhasma* treated animals in acute oral toxicity study. The values are expressed as Mean ± SD for 3 animals.

**Figure 4.5** Weekly body weight in control and different doses of *Lauha bhasma* treated male rats in sub acute oral toxicity study. The values are expressed as Mean± SD for 5 animals.
Figure 4.6 Weekly body weight in control and different doses of Lauha bhasma treated female rats in sub acute oral toxicity study. The values are expressed as Mean± SD for 5 animals.

4.3.2 Bio-distribution studies

In acute oral toxicity studies there was no deposition of iron in major organs including liver, kidney, brain intestine and spleen when compared to control group in both male and female rats. From Figure 4.4 it was apparent that there was no significant difference between the control and Lauha bhasma treated groups in the concentration of iron in the organs. This also correlated reasonably very well with the iron excretion pattern (Figure 4.5) as high amount of iron excretion was seen on day 1 in Lauha bhasma treated group.

Machado et al. [9] reported that lower deposition of iron was observed in liver when administered as iron peptide complex (30mg/kg and 60 mg/kg body weight). However when administered as iron sulfate at same doses, excess deposition of iron was observed in liver and spleen [9]. Papanastu et al. [10] reported that under normal circumstances, the
concentration of iron was found to be higher in spleen when compared to liver. After administration with high dose of iron, the concentration was found to be higher in liver due to excess deposition when compared to spleen [10].

**Figure 4.7** Bio distribution studies of control and *Lauha bhasma* treated animals in acute oral toxicity study. The values are expressed as Mean± SD for 3 animals in each group of
both male and female rats.

Figure 4.8 Fecal sample analysis of control and Lauha bhasma treated animals in acute oral toxicity study. The values are expressed as Mean± SD for 3 animals in each group of both male and female rats (* p<0.05).

However in the present study there were no deposition of iron in major organs including liver, kidney, brain, intestine and spleen in Lauha bhasma treated groups when compared to control group which may be attributed to the addition of triphala.

4.3.3 Effect on Biochemical & Hematological parameters:

The Lauha bhasma treated groups of three different doses did not exhibit any significant changes in the biochemical parameters including glucose, cholesterol, protein, urea, creatinine, SGOT, SGPT when compared to the control group (Figure 4.6- Figure 4.8)
Figure 4.9 Biochemical parameters of control and different doses of *Lauha bhasma* treated groups, (A) Glucose, (B) Protein. The values are expressed as Mean± SD for 5 animals.
Figure 4.10 Biochemical parameters of control and different doses of *Lauha bhasma* treated groups, (C) Cholesterol,(D) Creatinine. The values are expressed as Mean± SD for 5 animals.
Figure 4.11 Biochemical parameters of control and different doses of *Lauha bhasma* treated groups, (E) ALT, (F) AST. The values are expressed as Mean± SD for 5 animals.
No significant changes were observed in major hematological parameters including RBC, hemoglobin and hematocrit in *Lauha bhasma* treated groups when compared to control group. Lymphocytes count was also found to be normal between the groups.

**Figure 4.12** Hematological parameters of control and different doses of *Lauha bhasma* treated groups (A) RBC, (B) Hemoglobin. The values are expressed as Mean± SD for 5 animals.
Sarkar et al. [11] performed investigations on toxicity of *Lauha bhasma* in experimental animals, followed by recovery studies. *Lauha bhasma* was administered to experimental animals (Albino rats) at a dosage of five times the therapeutic effective dose. The drug was administered for 60 days and 45-day recovery period was observed. Hyperglycemia was observed in *Lauha bhasma* treated group along with elevated levels of serum liver enzymes and the levels were restored to normalcy in the recovery period [11].

In another study iron administered as iron dextran injection in mice resulted in iron overload and caused liver injury that resulted in significant elevation of serum enzymes including ALT, AST, ALP and bilirubin. Further an increase in liver iron content was also observed. However when the iron overloaded mice was treated orally with *Terminalia chebula* extract there was a decline in the level of serum enzymes and the liver iron content

**Figure 4.13** Hematological parameters of control and different doses of *Lauha bhasma* treated groups (C) Hematocrit. The values are expressed as Mean± SD for 5 animals.
also got lowered. *Terminalia chebula* scavenges free radicals and inhibits the free radical formation by chelating excess iron with its active components [12]. Hence it is evident that iron should not be administered alone.

It has been reported that iron supplementation when administered continuously to Sprague-Dawley rats resulted in increased serum cholesterol and triglycerides level. This is due to the decreased antioxidant levels which resulted in increased lipid peroxidation in liver and leads to leakage of hepatic enzymes in the circulation [13].

However, in the present study, *Lauha bhasma* even at high doses did not cause any adverse effects as evidenced from the biochemical and hematological parameters.

### 4.3.4 Histopathological Studies

Liver and spleen play a major role in iron metabolism[7,15]. Normal cyto architecture of liver and spleen were observed in the high dose treated group. Liver exhibited normal hepatocytes and sinusoids. No signs of iron deposition in the form of hemosiderin as brown patches, were observed in liver and spleen in the high dose treated group (Figure 4.11)


Meltem *et al.* [15] studied the effect of chronic iron toxicity in Sprague-Dawley rat liver by administering iron-sorbitol for two days per week for a period of eight weeks. Iron overload in hepatocytes was observed by appearance of characteristic yellowish brown depositions when stained with hematoxylin-eosin. The iron overload in liver was further
confirmed by Perls prussain blue stain, Massons trichrome stain and periodic acid Schiff reagent (PAS) [15].

**Figure 4.14** Representative histopathology images of liver: [A] - Control; [B] - High dose of 400 mg/kg of *Lauha bhasma*. Hepatocytes (H) and sinusoids (S) are shown. Representative histopathology images of spleen: [C] - Control; [D] - High dose of 400 mg/kg of *Lauha bhasma*. Peri-arteriolar lymphoid sheath (PALS) and central splenic arteriole (A) are shown. Representative histopathology images of kidney: [E] - Control; [F] - High dose of 400 mg/kg of *Lauha bhasma*. Glomerulus (G) and tubules (T) are shown.
In another study, attempts were made to study the role of iron supplementation in treatment of anemia as well as when administered during normal conditions. Iron was administered to rabbits in fumarate form for a period of three weeks. Signs of anemia characterized by decrease in animal size and activeness, decolorized organs (pale) were observed in starved group. After iron supplementation, the starved animals showed moderate recovery from the anemia during the first week. During the third week, the cyto architecture of liver and kidney were restored along with increased iron deposition with time. In case of well-fed groups, excess iron deposition with disruption of liver and kidney cyto architecture was observed. Liver sections of such animals showed dark stained iron accumulation with ruptured hepatic cell membrane. Iron deposition in the form of granules scattered in the cytoplasm of kidney was noticed, which increased in volume and number by time. The renal tubules appeared irregular, dilated with flattened epithelial lining and shrinkage of the glomerolus leading to dilated urinary space [16].

It has been reported in a 28-day study that the iron deposition in liver and spleen was reduced when supplemented as iron-peptide complex rather than iron sulphate [9]. Aggressive behavior was observed in ferrous sulfate treated group [9].

Feeding rats with iron rich diets led to the accumulation of iron in periportal region of the liver, mostly in the cytoplasmic region of the hepatocytes. In spleen, splenic atrophy was observed by loss of cells in white pulp region and deposition of iron as hemosiderin in sinusoidal macrophages[17]. Iron administration either as diet or intravenous or intraperitoneal administration led to deposition of iron as hemosiderin in hepatocytes and kuppfer cells surrounding the entire hepatic lobule of liver of Wistar male rats. Hemosiderin laden macrophages were observed in the spleen in iron overloaded conditions
However, the results in the present study showed that the rats administered even with high dose of *Lauha Bhasma* did not show any signs of toxicity in liver, spleen and kidney. Representative histopathology images of thymus, oesophagus and pancreas are shown in Figure 4.12

**Figure 4.15** Representative histopathology images of thymus: [A] – Control; [B] - High dose of 400 mg/kg of *Lauha bhasma*. Cellular cortex (C) is shown. Representative histopathology images of oesophagus: [C] – Control; [D] - High dose of 400 mg/kg of *Lauha bhasma*. (SS) Stratified squamous epithelium (SS), stratum corneum (SC) and submucosa (SM) are shown. Representative histopathology images of pancreas: [E] – Control; [F] - High dose of 400 mg/kg of *Lauha bhasma*. Exocrine pancreas (EX) and endocrine pancreas (EN) are shown.
In an earlier study, porta caval shunt was surgically created in male Sprague-Dawley rats to study iron accumulation in pancreas [18]. The rats were fed with carbonyl iron diet for 17 weeks. Histopathological section revealed chronic pancreatitis characterized by acinar atrophy, proliferation of intercalated ducts, mononuclear cell infiltration and fibrosis. Oxidative stress was also confirmed with the elevated levels of glutathione disulfide [18]. However, the results in the present study showed that the rats administered even with high dose of *Lauha Bhasma* did not show any signs of toxicity in pancreas.

Representative histopathology images of intestine, brain and ovary are shown in Figure 4.13.

Mohammed *et al.* [19] studied the effect of changes in brain neuro transmitters of rats due to administration of iron fortified diet. The levels of serotonin and dopamine were found to be reduced significantly. Increase in lipid peroxidation in brain as a result of excess iron deposition led to oxidative stress that ultimately resulted in neuro degenerative disorders. Histopathology studies revealed the conditions of meningeal haemorrhage, congestion and edema due to excessive iron overload. Further degenerated neurons, satellitosis and neuronophagia were also observed in cerebral cortex [19].

Sobotka *et al.* [20] investigated toxicity and neuro behavioral changes in rodents fed with different concentrations of dietary iron. Dose dependent toxicity and behavioral changes were observed [20]. Another study revealed behavioral impairments combined with brain iron accumulation in rats injected with ferrous sulphate for five days [21].
Figure 4.16 Representative histopathology images of intestine: [A] – Control; [B] - High dose of 400 mg/kg of *Lauha bhasma*. Intestinal crypts (C) are shown. Representative histopathology images of brain: [C] – Control; [D] - High dose of 400 mg/kg of *Lauha bhasma*. Neurons (N), eosinophilic cytoplasmic processes (C) and glial cells (G) are shown. Representative histopathology images of ovary: [E] – Control; [F] - High dose of 400 mg/kg of *Lauha bhasma*. Ovarian stroma (OS) and ovarian follicle (OF) are shown.
In vitro studies were carried out using PC 12 cells incubated with ferrocene and dopamine. The addition of Fe2+ (in the form of ferrocene) increased the oxidation of monoamines. The oxidation products were bound covalently to sulfhydryl groups of serotonin binding proteins leading to apoptosis in PC-12 cells [22].

The effect of ferrous fumarate at low dose on dextran sulfate sodium (DSS) induced colitis in rats has been reported [23]. The signs of intestinal inflammation and plasma redox status were observed in DSS-administered groups with respect to control groups. A significant increase in colitis score was observed in DSS-administered groups. Colitis score in DSS-administered groups was further increased due to administration of ferrous fumarate. Rats that received ferrous fumarate only showed normal cytoarchitecture with no excess iron deposition. No changes were observed in plasma redox status in DSS induced colitis rats when compared to control groups [23].

In another study, the effect of oral iron supplementation at two different doses was investigated in DSS induced colitis rats. Iron with doses of 0.3 % and 3 % showed higher histological scores of colitis accompanied by heavier rectal bleeding and shortening of the colon. The levels of antioxidant vitamins were also decreased with increased lipid peroxidation [24]. The free radical generating capacity and lipid peroxidation of the intestine in rats were assessed through administration of iron enriched diets for six months [25]. Though the chronic administration of iron did not increase crypt cell proliferation, increased free radical generation in colon and lipid peroxidation in caecum were observed [25]. However, the results in the present study showed that the rats administered even with high dose of Lauha Bhasma did not show any signs of toxicity in intestine and brain.
Histopathology of other organs were also carried out and normal cytoarchitecture was observed in high dose Lauha bhasma treated group, when compared to control group. Representative histopathology images of thyroid, lungs and heart are shown in Figure 4.14.

**Figure 4.17** Representative histopathology images of thyroid: [A] – Control; [B] - High dose of 400 mg/kg of Lauha bhasma. Cuboidal epithelium (CE) and homogenous colloid (C) are shown. Representative histopathology images of lungs: [C] – Control; [D] - High dose of 400 mg/kg of Lauha bhasma. Alveolar wall (Arrow mark) and alveolar lumen (AL) are shown. Representative histopathology images of heart: [E] – Control; [F] - High dose of 400 mg/kg of Lauha bhasma. Cardiac myocytes (CM) and cytoplasm (C) are shown.
Representative histopathology images of adrenal gland, pituitary gland and bone marrow are shown in Figure 4.15.

**Figure 4.18** Representative histopathology images of adrenal gland: [A] – Control; [B] - High dose of 400 mg/kg of *Lauha bhasma*. Cortex (C) and medulla (M) are shown. Representative histopathology images of pituitary gland: [C] – Control; [D] - High dose of 400 mg/kg of *Lauha bhasma*. Pars distalis, (D) pars intermedia (I) and pars nervosa (N) are shown. Representative histopathology images of bone marrow: [E] – Control; [F] - High dose of 400 mg/kg of *Lauha bhasma*. Erythroid and myeloid precursors (P) and megakaryocytes (M) are shown.
4.4 CONCLUSION

Iron, despite its essential role in biological system can cause adverse toxic effects ranging from male sterility to tumorogenesis. *Lauha bhasma* even when administered at a high dose was not toxic to the vital organs of the rats. Histopathological studies showed that the cytoarchitecture of rats treated with high dose of *Lauha bhasma* was comparable to normal control. This was further supported by the biochemical and hematological parameters which showed that *Lauha bhasma* did not cause any significant changes in the levels of those parameters.

4.5 REFERENCES


