# Evaluation of Hepatoprotective Activity of *Lauha Bhasma* in D-Galactosamine induced HepG2 cellModel- in Vitro study

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**Abstract**

## Introduction:

The liver is a crucial organ responsible for various metabolic functions, detoxification, and nutrient storage. Liver diseases, such as fatty liver disease, hepatitis, and cirrhosis, are increasingly prevalent worldwide, often linked to lifestyle factors like obesity, excessive alcohol consumption, and viral infections. *Ayurveda*, the ancient Indian system of medicine, emphasizes liver health through holistic approaches, focusing on detoxification and dietary modifications. *Ayurvedic* practices often include herbal and Herbo-mineral remedies. *Bhasma* holds significant importance in managing liver disorders within *Ayurvedic* medicine. By improving digestion, reducing inflammation, and facilitating the elimination of toxins, *Bhasma*-based formulations aim to restore liver vitality and address conditions like fatty liver disease and hepatitis**.** *Lauha Bhasma* known for its hematinic and detoxifying properties, *Lauha Bhasma* helps improve haemoglobin levels and supports overall liver function. It is particularly beneficial in conditions like anaemia and fatty liver disease, where it aids in enhancing digestion and promoting the elimination of toxins. By balancing *Pitta dosha*, *Lauha Bhasma* can help reduce liver inflammation and improve metabolic processes**.** In this study, *Lauha Bhasma* was prepared, analysed and investigated the hepatoprotective activity in D-Galactosamine induced HepG2 cell Model and assessed the results via Viability and proliferation assay, and calculated the LC50, EC50 value of *Lauha Bhasma*. **Aim:** To evaluate Hepatoprotective activity of '*Lauha Bhasma* in D-Galactosamine induced HepG2 cell Model -In Vitro study. **Objectives:** Primary Objectives**:** 1. To prepare *Lauha Bhasma* mentioned in *Rasa Ratna samucchay...* 2. To Evaluate Hepatoprotective effect of *Lauha Bhasma* in D-Galactosamine induced HepG2 cells culture model via Viability and proliferation assay and at morphological level. **Secondary Objectives:**1. To calculate and report the EC 50 and LC 50 value of *Lauha Bhasma.* 2. To analyse *Lauha Bhasma* according to *Ayurvedic* and modern parameters. 3. Development of in-vitro study using HepG2 cell line. **Material And Methods:** *Lauha Bhasma* was prepared according to ref mentioned in *Rasa Ratna Samucchay.* Analytical tests of *Lauha Bhasma* were performed according to methods described in *Ayurvedic* pharmacopoeia of India and Hepatoprotective activity of *Lauha Bhasma* was carried out in vitro study using HepG2 cell line.**Result:** Test item *Lauha Bhasma* showed 47.09, 50.46, 64.53 and 89.91% hepatoprotective activity in D-Galactosamine induced HepG2 cell line at the concentrations of 125, 250, 500 and 1000 µg/mL and EC50 observed at the concentration of 214.7 µg/ml..**Conclusion:-** The study presents *Lauha Bhasma* as a potent Hepatoprotective drug. *Lauha Bhasma* possesses restorative qualities when administered as a hepatoprotective medication.

Keywords: Hepatoprotective activity, LC50 value, EC50 value, D-Galactosamine, In-Vitro Study, HepG2 cell, *Lauha Bhasma*, *Ayurvedic* Pharmacopoeia, *Bhasma*, *Lauha Bhasma*.

## Introduction

*Ayurveda* refers to Ayur = life and Veda = science. *Ayurveda* is concerned with the prevention rather than cure of diseases[1,2] According to the principles of '*Ayurveda*', there is not a single substance in the Universe which does not have a potential to be used as a drug, provided it is used judiciously by the physician where it is required.(3). As per the *Ayurvedic* pharmacopeia more than 80,000 formulations are found for different diseases and clinical conditions and for the wellbeing of the humans.[4] In *Ayurveda*, heavy metals are converted into non-metallic form[5].

*Rasa Shastra* is such a branch of *Ayurveda* which deals with Preaparation of *Ayurvedic* medicine. It is existing since *vedic* period (6) *Rasa Shastra*, an advanced branch of *Ayurveda*, deals with the science of mineral and

metal-based pharmacology, detailing the processing, purification, and therapeutic uses of metallic and mineral substances for medicinal purposes. [7.8] *Rasa Shastra* is dominated by descriptions of mercury processing techniques also mercury" and "*Rasa*" are thought to be synonymous [9] It also describes the uses of mercury, salts, corals, seashells, feathers, and metals including gold, silver, copper, tin, lead, and iron [10.11]. There are various *Ayurvedic kalpa* mentioned in classic *Ayurvedic* texts

Out of all the *Kalpana* mentioned in *Rasa Shastra*, *Bhasma Kalpana* has attained an important position among the *Rasoushadhis* due to its assimilatory organo-metallic constitution and in the *Ayurvedic* world they are highly efficacious. Current time says metals and minerals cause toxicity to biological system but *Ayurveda* proclaims that the *Bhasma* prepared from the same metals and minerals they are not only non-toxic, but also very much beneficial to human body [12-15]

One of the widely used *Bhasma* preparation by *Ayurvedic* Practitioners is *Lauha Bhasma*. *Lauha Bhasma* (LB) is a nano iron particles based medicine used in *Ayurveda* [16].*Lauha Bhasma* is used for the treatment of many diseases such as anaemia, liver disorders, worm infestation, dry skin, irregular fat metabolism, heart disease, pica, rhinitis, etc[17.18]. In modern medicine, Iron plays a vital role. For many years, nutritional interest in iron focused on its role in haemoglobin formation and oxygen transport.[19] ] Iron had early medicinal uses by Egyptians, Hindus, Greeks, and Romans.[20.21] However, it was not until 1932 that the importance of iron was finally settled by the convincing proof that inorganic iron was needed for haemoglobin synthesis.[22] But in India, Iron is used as medicine from the *Vedic* period itself.

*Ayurveda* describes liver disorders and their treatment in fragmented form. Any abnormalities in structure and function of Liver is Known as Liver disorders. Liver disease accounts for approximately two million deaths per year worldwide, 1 million due to complications of cirrhosis and 1million due to viral hepatitis and hepatocellular carcinoma. Cirrhosis is currently the 11th most common cause of death globally and liver cancer is the 16th leading cause of death; combined, they account for 3.5% of all deaths worldwide.[23]

The drug taken for the present study is *Lauha Bhasma* described in *RasaRatnaSamuchhay.*[24] . It is indicated in *Yakrut* and *Pleeha Vikar*. *Chandanadi Lauha* is indicated in Liver and spleen disorders[25] *Navayas Lauha* indicated in Jaundice and liver disorders[26] *Tapyadi Lauha* indicated in Viral hepatitis (250-500mg)[27]. There are 21 *Lauha* kalp mentioned to treat Pandu and Kamala and 11 for Pleeha and yakrut vikar. [28] There are various processes to prepare *Lauha Bhasma* given in *Ayurvedic* texts. In present study we have selected *Triphala* as a maran Dravya. There are various studies showing *Triphala* has great effect on liver health.[29-34]. As per *Ayurvedic* texts, *Lauha kalpas* are used to treat Liver disorders.There is need to study individual hepatoprotective activity of *Lauha Bhasma*. Hence this study is aimed to investigating Hepatoprotective activity of *Lauha Bhasma* in In-vitro model.

## Material & Method:

**Collection and Authentication of Raw Drugs**

All raw drugs were procured from authenticated vendor and further authenticated from experts of *Rasa Shastra* dept. of our institute and botany dept. RTMNU Nagpur.

***Samanya Shodhana* of *Lauha* [35]**

a) Preparation of accessory drugs:
*Takra*[36], *Kanji*[37], and *Kulattha Kwatha* [38] were prepared as per classics for the process of *Samanya Shodhana*.

b) Process of *Shodhana*:
Ingredients
1. Main drug – *Lauha*, 500 g.
2. Accessory drugs – *Til Taila*, 7 L; *Takra*, 7 L; *Gomutra*, 7 L; *Kanji*, 7 L; *Kulattha Kwatha*, 7 L.
Procedure –

- Initially, each media was divided into 7 parts
- Iron fillings were taken on ladle and heated on gas stove until red hot.
- Red hot fillings were quenched into respected media.
- After selfcooling the fillings were removed and dried.
- The above procedure of heating and quenching was repeated 7 In same media.
- This process is done for each respective media mentioned above.
- Every time Fresh media was taken

- The iron fillings were weighed each time before heating and after quenching.
- The change of appearance of the iron fillings and the media was observed after each process.

**Vishesha shodhana of *Lauha*** [39]

a) Preparation of accessory drugs:

*Triphala Kwath*

Ingredients – *Triphala*,- 2 kg; water- 16 L.

**Equipments**: Stainless steel vessel, gas stove, measuring jar, weighing machine, strainer

**Procedure:**

1. *Triphala* and water were boiled in stainless steel vessel until water is reduced to 1/4.
2. The *Kwath*a thus obtained is strained.

b) Process of *Vishesh Shodhan*a:

Ingredients

1. Main drug – *Lauha*, 531 g.
2. Accessory drugs :- *Triphala Kwath*- 7 lit

Procedure –

- Initially, Triphal *Kwath* was divided into 7 parts
- Iron fillings were taken on ladle and heated on gas stove until red hot.
- Red hot fillings were quenched into *Triphala Kwath*.
- After cooling the fillings were removed and dried.
- The above procedure of heating and quenching was repeated 7 times with each media.
- Every time Fresh media was taken
- The iron fillings were weighed each time before heating and after quenching.
- The change of appearance of the iron fillings and the media was observed after each process.

***Marana* of *Lauha*** [40]

a) Preparation of *Triphala Kwath*a in *Gomutra*

Ingredients:

1. *Triphala* coarse powder ½ kg
2. *Gomutra*-4 L

Equipments: Stainless steel vessel, gas stove, measuring jar, weighing machine, strainer

Procedure:

1. *Triphala* and *Gomutra* were boiled in medium flame until *Gomutra* was reduced to1/4 of its volume
2. The *Kwath*a was strained.

b) Process of *Vishesh Shodhan*a:

Ingredients

1. Main drug – *Lauha*, 500 g.
2. Accessory drugs :- *Triphala Kwath*- 7 lit

Procedure

*Bhavana* of *Lauha*

1.*Triphala Kwath*a was prepared in *Gomutra*

2.Shuddha *Lauha* was taken in *Khalva Yantra* and triturated with *Triphala Kwath*a.for 5 hrs

3. *Mardana* was done until the mixture attained paste like semisolid consistency.

4.*Chakrika* (small, round, flat pellets) were made and dried in shadow.

*Puta*

1.The *Chakrika* were placed in between two earthen saucers facing each other

2.*Sharev SamPuta* (sealing with mud smeared cloth) was done and allowed to dry.

3. The *Sharava* were placed in *GajaPuta*(EMF) and incinerated. at 750ºC with acceleration of 40 which take 4 and1/2 hr to reach the temperature of 750 ºC. Maintain the temperature for about 1 and 1/2 hr. then gradually taper the temperature

4. The *Puta* was allowed to cool down on its own

5.Same process was repeated for 25 times until *Lauha Bhasma* was obtained.

6. The weight of *Lauha Bhasma* after triturating, dried pellets and after incinerating was recorded each time

7. The Amount of *Triphala Kwath*a required was also measured each time

8. The temperature of *Puta* were recorded till the *Puta* cools down.

**Dravya for *Samanya Shodhan***


Ashuddha *Lauha*

Til Taila

Takra

Gomutra

Kanji

Kulattha kwath

**Dravya For *Vishesh Shodhan***

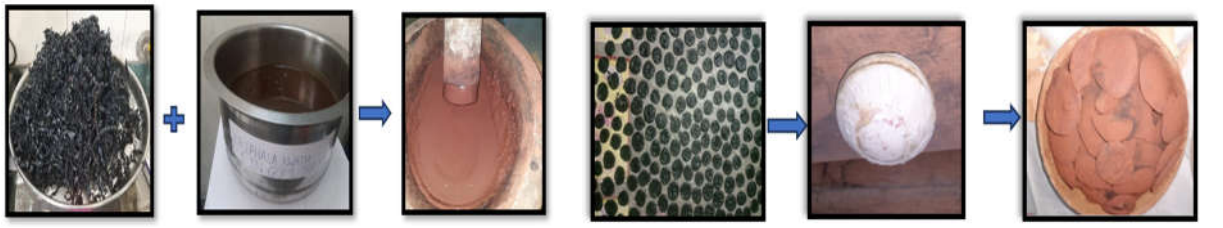

Shodhit


Triphal Kwath

**Samanya and *Vishesh Shodhan* of *Lauha***


Ashuddha *Lauha* → Heating of → Quenching of *Lauha* in liquid → Jala Prakshalan → Shuddha *Lauha*

**Maran of *Lauha***


Vishesh shodhit + Triphala kwath → Bhavana → Chakrika → Sharav samput → After Puta

*Bhasma* Parikshan: -

Unnam | Rekhapunatvam | Varitar

**Analytical Study of Raw Materials** –

In this study, analytical evaluation of *Lauha Bhasma* was carried out: -

**a)** Organoleptic characters[41] : - color, taste, touch and odor and weight
**b)** Physiochemical analysis: -
- Acid insoluble ash [42]
- Total- ash value [43]
- Water soluble ash [44]
- Loss on drying [45]
- pH [46]
- XRD [47]
- SEM [48]

**c)** *Bhasma Parikshan*:-
- *Varitar* [49]
- *Unnam* [50]
- *Rekhapurnatvam* [51]
- *Shlakshan* [52]
- *Sukhshma* [53]

**Experimental study**

**STUDY DESIGN: -**

Assay for hepatoprotective activity was performed on different concentrations of test item dissolved in DMEM media on HepG2 (human liver cancer) cell line. Assay was conducted on 24h timeline.

3 **EXPERIMENTAL PROCEDURES**

3.**1 HepG2 cell line:**

HepG2 human liver cancer cell line generated from human liver tissue, it has adherent type growth mode. The cells are epithelial in morphology, have a modal chromosome number of 55.

3.2 **MTT assay principle:**

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3 (4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such quantified by spectrophotometric means.

**3.3Cell count using trypan blue:**

Cell cultures were removed from culture flasks by enzymatic digestion (trypsin/EDTA) and the cell suspension was centrifuged (1000 RPM, 3 min). The cells were then resuspended in culture medium. 0.1 mL of cell suspension was added to 0.1mL of 0.4% trypan blue solution and loaded to a hemacytometer and cells were

counted immediately. Number of viable cells were counted in four WBC counting chamber of hemocytometer. To calculate the number of viable cells per mL of culture, the formula below was used. Average number of viable cells × 104 × Dilution factor (2) = cells/mL

3.3 **Preparation of media:**

streptomycin solution. Sr. No. as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and DMEM medium was used as media with 10% filtered fetal bovine serum and 1% penicillin-

**Table No.1 Details of media components are given in following table:**

| Sr. No. | Name of component | Make | Batch No. | Expiry date |
|---|---|---|---|---|
| 1. | DMEM | Himedia | 0000647911 | 05-2025 |
| 2. | Fetal bovine serum | Himedia | 0000608853 | 08-2028 |
| 3. | Antimycotic solution | Gibco | 0000216918 | 21-01-2025 |
| 5. | Trypsin EDTA | Himedia | 0000607778 | 30-09-2025 |
| 6 | Thiazolylbluetetrazolium bromide | Merck | MKCS4540 | - |
| 7 | Dimethyl Sulphoxide | Oxford | 4868 | Jul. 2025 |

**Table No. 2 Test item formulation:**

| Weight (mg)/Vol. (mL) | Total Vol. (mL) | Vehicle Vol. (mL) | Conc. In mg/mL | Conc. In µg/mL | Formulation ID |
|---|---|---|---|---|---|
| 20 mg | 2.0 | 2.0 | 10 | 1000 | F1 |
| 0.5 mL of F1 | 1.0 | 0.5 | 5 | 500 | F2 |
| 0.5 mL of F2 | 1.0 | 0.5 | 2.5 | 250 | F3 |
| 0.5 mL of F3 | 1.0 | 0.5 | 1.25 | 125 | F4 |
| 0.5 mL of F4 | 1.0 | 0.5 | 0.625 | 62.5 | F5 |
| 0.5 mL of F5 | 1.0 | 0.5 | 0.3125 | 31.25 | F6 |

**Cytotoxicity Procedure**

1. After receiving of cell line from NCCS cells were passaged for two times before using in the test.
2. Cell cultures were removed from culture flasks by enzymatic digestion (trypsin/EDTA) and the cell suspension was centrifuged (1000 RPM, 3 min). The cells were then resuspended in culture medium and the cell suspension was adjusted at a density of 1 × 105 cells/mL. 100 µL culture medium only (blank) was added into the peripheral wells of a 96-well tissue culture microtitre plate using a multichannel pipette. In the remaining wells, 100 µL of a cell suspension of 1 × 105 cells/mL (1 × 104 cells/well) was dispensed.
3. Culture plates were incubated for 24 h (5 % CO2, 37°C, > 90 % humidity) so that cells could form a half-confluent monolayer. This incubation period ensured cell recovery, and adherence and progression to exponential growth phase.
4. Plates were examined under a phase contrast microscope to ensure that cell growth is relatively even across the microtitre plate.
5. After 24 h incubation, culture medium was removed and 100 µL of treatment medium containing either the appropriate concentration of sample extract, or the negative control, or the positive controlwas added per well in triplicate.
6. Six different concentrations of the test item formulations and four concentrations of positive control (SLS) were used in the assay.
7. Again plates were incubated for 24 h (5 % CO2, 37 °C, > 90 % humidity).
8. After 24 h of incubation treated culture was carefully removed from each well.
9.100 µL of MTT reagent was added and plates were incubated for 3 h.
10. After incubation period MTT reagent and media was removed completely and 100 µL of dimethyl sulphoxide was added to dissolve the crystals of formazon.
11. Absorbance was measured at 570 nm on ELISA plate reader.

3.6 **D-Galactosamine induced HepG2 cell toxicity**

1. HepG2 cells was adjusted to 1 × 105 cells/well in DMEM supplemented with 10% FBS, and 100 µL of the cell suspension will be plated into 96 well culture plate and incubated at 37°C in a humidified atmosphere containing 5% CO2 for 24 h.
2. Post incubation, the cells were treated with different concentrations (125, 250, 500 and 1000 µg/mL) of test substance and incubated for 2 h.
3. Silymarin (12.5 µg/mL) was used as a reference standard.
4. After incubation of 2 h, the cells were treated with 20 mM of D-galactosamine for 24 h.
5. Thereafter, the supernatant was discarded and the cells were washed with phosphate buffer solution, and MTT was added and incubated for 4 h.
6. After incubation the absorbance was measured at 570 nm.
7. Toxicity was calculated considering the viability of the untreated control cells as 100%.
8. Percentage hepatotoxicity was calculated by the following formula.

% Hepatoprotective activity = (Ap- At/Ac-At) *100

- Ac- Absorbance of untreated cells
- At- Absorbance of toxicant i.e. diseased control
- Ap- Absorbance of treated cells

9. The EC50 was calculated based in a nonlinear regression (curve fit) using GraphPad Prism 10.3.1.

**Observation and result**

**Table No. 3 Observation of *Lauha* and Media during Samanya and *Vishesh Shodhan* of *Lauha*: -**

| Sr. No | Media | Observation of *Lauha* | | | | Observation of Media | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Colour | Iron Flakes | Time required to attained red hot stage | other | Colour | Viscosity | Fumes/odour | Other |
| **1** | *Til Taila* | Black | Some flakes converted into coarse powder | After 35 min | Brittleness increased after shodhana | Changed from yellow to blackish brown | Taila become viscous | Pungent smell, Black fumes | Oil caught fire |
| **2** | *Takra* | Bluish Black | Same as above | 20 min | Brittleness increased | Brownish black sediments | --- | Burnt smell and white fumes | *Takra* began to boil |
| **3** | *Gomutra* | Blackish brown | Coarse powder | 20 min | --- | Turned to Brownish Colour | ---- | Strong Pungent smell | ---- |
| **4** | *Kanji* | Brownish | Coarse powder began to dissociate | 25 min | Flakes become more Brittle | Brownish colour | Become viscid | Pungent, Burnt smell | ---- |
| **5** | Kullatha *Kwath* | Brownish | Fine and few coarse powder | 20 min | ---- | Blackish colour and black sediments | ---- | More Fumes were produced | ----- |
| **6** | *Triphala* | Bluish | *Lauha* | After 35 | Heavy | Brownish black | | Burnt smell | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Kwath* | black | turned into fine powder | min | weight fine powder | *Lauha* sediments | | of *Triphala Kwath* | |

**Table No. 5 Results after Samanya and *Vishesh Shodhan* of *Lauha***

| Sr. No. | Media | Wt of *Lauha* before Nirvapana(7 times)(g) | Wt of *Lauha* after Nirvapana(7 times)(g) | Amount of Liquid before Nirvapana(total used for 7 times((ml) | Amount of Liquid after Nirvapana(total used for 7 times((ml) |
|---|---|---|---|---|---|
| **01** | *Til Taila* | 500 | 505 | 7000 | 5600 |
| **02** | *Takra* | 505 | 512 | 7000 | 5075 |
| **03** | *Gomutra* | 512 | 518 | 7000 | 5180 |
| **04** | *Kanji* | 518 | 525 | 7000 | 5075 |
| **05** | Kullath *Kwath* | 525 | 531 | 7000 | 4480 |
| **06** | *Triphala Kwath* | 531 | 537 | 7000 | 4900 |

Initial weight of *Lauha*:- 500 g
Final weight of *Lauha*:- 537 g
Total amount of *Lauha*:- 37 g
% of gain: - 8%

**Observations during *Marana* of *Lauha*:-**
The mean temperature attain during each *Puta* was about 700c.
The highest temperature was maintained for 2-3 hrs and the temperature was reduced to normal nearly after 10 hrs
It took 25 Gaja *Puta* to obtain *LauhaBhasma.* Bluish *Lauha Bhasma* with red hue is obtained

**Table No. 6 Observations during *Marana* of *Lauha*:**

| | Observations | | | | |
|---|---|---|---|---|---|
| | **Temp. of EMF** | **Weight of *Lauha* before *Puta*(g)** | **Weight of *Lauha* after *Puta*** | **Colour of *Bhasma*** | **Other observations** |
| **01** | 750 | 500 | 492 | Black | Surface of *Lauha* fillings become shiny, lustrous. Pellets were fragile |
| **02** | 750 | 491 | 484 | Black | Same as above |
| **03** | 750 | 483 | 477 | Black | Same as above |
| **04** | 750 | 476 | 466 | Black | Same as above |
| **05** | 750 | 465 | 462 | Brownish grey | Same as above |
| **06** | 750 | 461 | 460 | Brownish grey | Pellets become hard and not easily breakable |
| **07** | 750 | 459 | 457 | Brownish grey | Pellets become hard and not easily breakable |
| **08** | 750 | 456 | 454 | Reddish brown | Pellets become soft. *Bhasma* become fine and Lousturless but not fully varitar |
| **09** | 750 | 453 | 451 | Reddish brown | Same as above |

| | | | | | |
|---|---|---|---|---|---|
| **10** | 750 | 450 | 449 | Reddish brown | Same as above |
| **11** | 750 | 448 | 447 | Reddish brown | Pellets become hard |
| **12** | 750 | 446 | 444 | Reddish brown | Pellets exhibited bluish colour on surface, but has reddish brown colour on breaking |
| **13** | 750 | 443 | 442 | Blackish grey | Same as above |
| **14** | 750 | 441 | 438 | Blackish grey | *Bhasma* gradually became more varitar |
| **15** | 750 | 437 | 436 | Blackish Grey | Same as above |
| **16** | 700 | 435 | 432 | Blackish grey | Same as above |
| **17** | 700 | 431 | 429 | Dark purple | Same as above |
| **18** | 700 | 428 | 426 | Dark purple | Same as above |
| **19** | 700 | 425 | 423 | Dark purple | It became more fine and light in weight |
| **20** | 700 | 422 | 421 | Dark purple | Same as Above |
| **21** | 700 | 420 | 418 | Dark purple | Same as Above |
| **22** | 650 | 417 | 414 | Dark purple | *Bhasma* became Varitar |
| **23** | 650 | 413 | 409 | Dark purple | Same as Above |
| **24** | 650 | 408 | 404 | Dark purple | Same as Above |
| **25** | 650 | 403 | 401 | Dark purple | *Bhasma* becomes fine lustureless, Varitar and passed all the tests. |

**Result after *Marana* of *Lauha*:-**
Initial weight of *Lauha*: 500g
Final weight of *Lauha*: 401g
Total loss: 99 g
% of loss: 20%

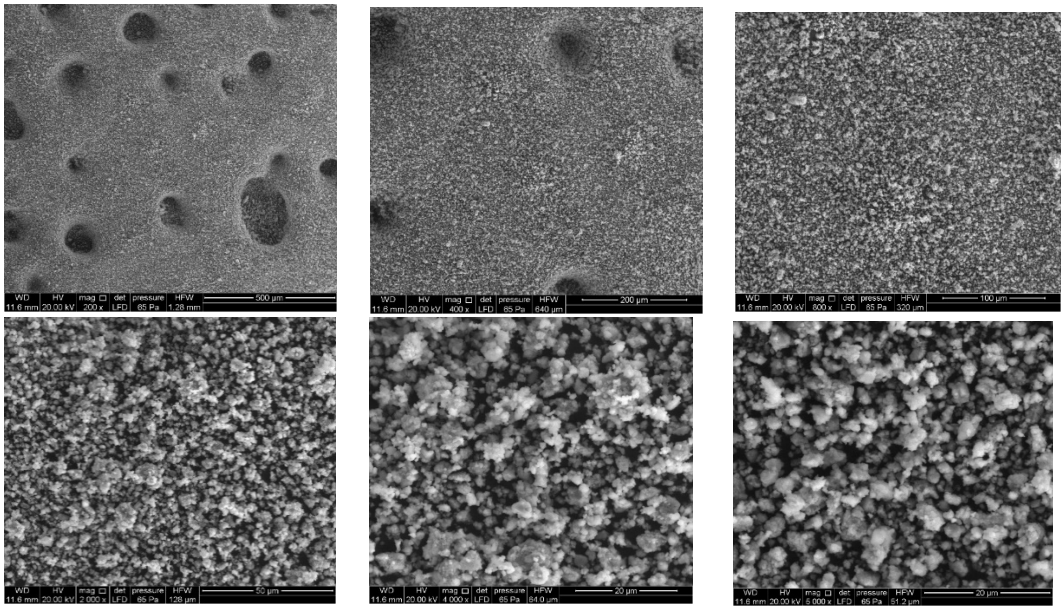
**Table NO.7 Observations and Result of Analytical study:-**

| Sr. No. | Test Name | Results |
|---|---|---|
| **1.** | Loss on drying@105oC | 0.075 |
| **2.** | Acid insoluble ash | 1.25% |
| **3** | pH | 5.5 |
| **4** | Particle Size | 20-50 micron |
| **5** | Water Soluble Ash | 2.21% |
| **6** | Total ash | 7.8% |

**XRD:-**

The XRD study of LB showed peaks of $Fe_2O_3$

**SEM**



Result:- The images were captured at different magnification ranging from 200X to 5000X.*Lauha Bhasma* showed the presence of nano particles of around 20-50 micron. Some of the particles got fused with each other and particles were not of definite shape.

**Experimental study:-**

**Table No. Showing Absorbance, % Viability for vehicle, positive control and test item- *Lauha Bhasma***

| Absorbance at 570 nm- 24 hour | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Formulations** | | **Conc. (µg/mL)** | **Ab. 1** | **Ab. 2** | **Ab. 3** | **Mean** | **Mean Ab. – Blank** | **SD** | **% Viability** |
| Blank | | - | 0.002 | 0.005 | 0.001 | 0.003 | 0.002 | - | - |
| Untreated Control | | - | 0.312 | 0.309 | 0.31 | 0.310 | 0.002 | 0.308 | 100 |
| Vehicle control | | - | 0.297 | 0.317 | 0.277 | 0.297 | 0.020 | 0.294 | 95.67 |
| **Test Item *Lauha Bhasma*** | F6 | 31.25 | 0.269 | 0.279 | 0.285 | 0.278 | 0.008 | 0.275 | 89.38 |
| | F5 | 62.5 | 0.279 | 0.251 | 0.291 | 0.274 | 0.021 | 0.271 | 88.08 |
| | F4 | 125 | 0.273 | 0.295 | 0.254 | 0.274 | 0.021 | 0.271 | 88.19 |
| | F3 | 250 | 0.281 | 0.287 | 0.251 | 0.273 | 0.019 | 0.270 | 87.87 |
| | F2 | 500 | 0.239 | 0.276 | 0.293 | 0.269 | 0.028 | 0.267 | 86.67 |
| | F1 | 1000 | 0.185 | 0.225 | 0.271 | 0.227 | 0.043 | 0.224 | 72.91 |
| | F4 | 0.125 mg/mL | 0.223 | 0.248 | 0.244 | 0.238 | 0.013 | 0.236 | 76.60 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Positive control SLS** | F3 | 0.25 mg/mL | 0.151 | 0.182 | 0.237 | 0.190 | 0.044 | 0.187 | 60.89 |
| | F2 | 0.5 mg/mL | 0.21 | 0.18 | 0.124 | 0.171 | 0.044 | 0.169 | 54.82 |
| | F1 | 1 mg/mL | 0.105 | 0.108 | 0.103 | 0.105 | 0.003 | 0.103 | 33.37 |

The cell line used in the experiment was HepG2 cell line. Exponentially growing HepG2 cells were seeded in 96-well plate at a concentration of 1 x 105 cells/mL. After 24 hrs of incubation the cells were treated with test item, *Lauha Bhasma* at the concentrations of 1000, 500, 250, 125, 62.5 and 31.25 µg/mL, positive control, SLS at the concentrations of 0.125, 0.250, 0.5 and 1.00 mg /mL. Culture plates were incubated for 24 hrs at 37 ± 1 °C and 5% CO2 in the incubator. Thereafter culture plates were subjected to quantitative evaluation. MTT assay was conducted for quantitative analysis of the test item. Cytotoxicity was observed in the group treated with positive control at the concentrations of 0.25, 0.50 and 1.00 mg /mL SLS. Cytotoxicity in terms of cells detachment, changes in cell morphology and cell lysis was observed.

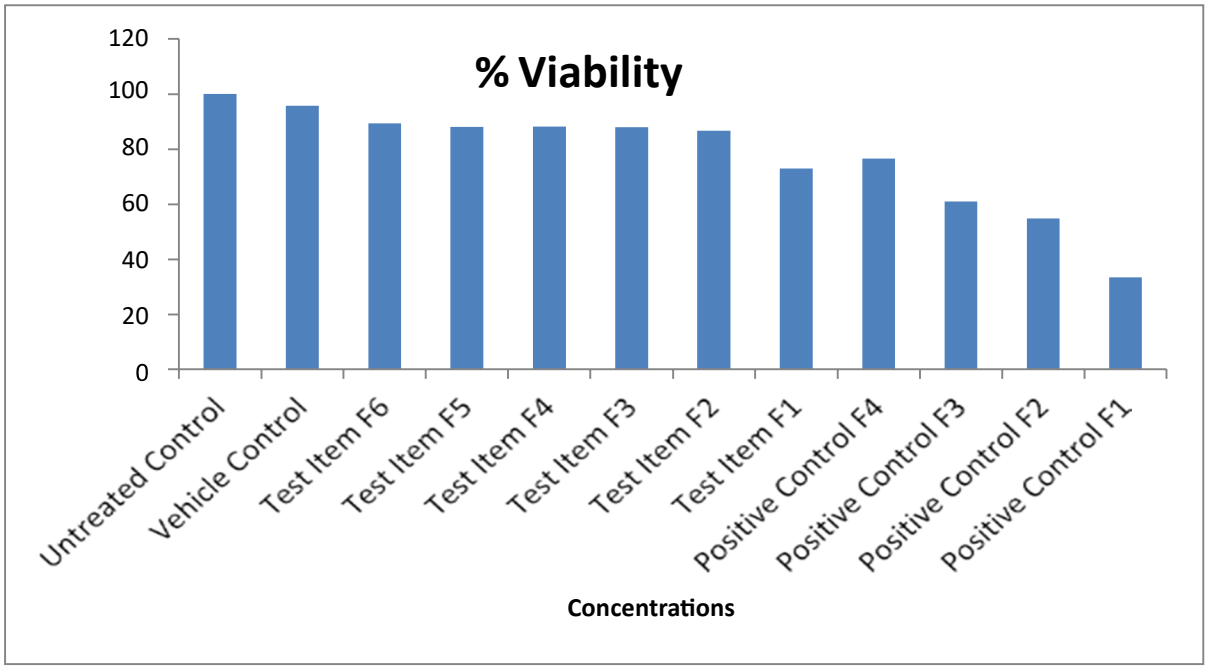
**Table No. Showing Absorbance, % Viability for vehicle, positive control and test item- *Lauha Bhasma* are tabulated below for D-Galactosamine induced HepG2 cell toxicity**

| **Absorbance at 570 nm- 24 hour** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Formulations** | | **Conc. (µg/mL)** | **Ab. 1** | **Ab. 2** | **Ab. 3** | **Mean** | **SD** | **Mean Ab. – Blank** | **% Viability** | **% Hepatoprotec -tive actiity** |
| **Blank** (Media without cells) | | - | 0.035 | 0.031 | 0.035 | 0.034 | 0.002 | - | - | - |
| **Untreated Control** (Untreated cells) | | - | 0.227 | 0.252 | 0.291 | 0.257 | 0.032 | 0.223 | 100.00 | - |
| **Disease Control** (Galactosamine treated cells) | | | 0.169 | 0.178 | 0.096 | 0.148 | 0.045 | 0.114 | 51.12 | 0 |
| **Vehicle control** | | - | 0.119 | 0.204 | 0.23 | 0.184 | 0.058 | 0.151 | 67.56 | 33.64 |
| ***Lauha Bhasma*** | **F4** | **125** | 0.207 | 0.204 | 0.186 | 0.199 | 0.011 | 0.165 | 74.14 | 47.09 |
| | **F3** | **250** | 0.201 | 0.205 | 0.202 | 0.203 | 0.002 | 0.169 | 75.78 | 50.46 |
| | **F2** | **500** | 0.214 | 0.223 | 0.217 | 0.218 | 0.005 | 0.184 | 82.66 | 64.53 |
| | **F1** | **1000** | 0.249 | 0.242 | 0.246 | 0.246 | 0.004 | 0.212 | 95.07 | 89.91 |
| **Reference standard** (Silymarin) | **F1** | **12.5 µg/mL** | 0.245 | 0.238 | 0.246 | 0.243 | 0.004 | 0.209 | 93.87 | 87.46 |

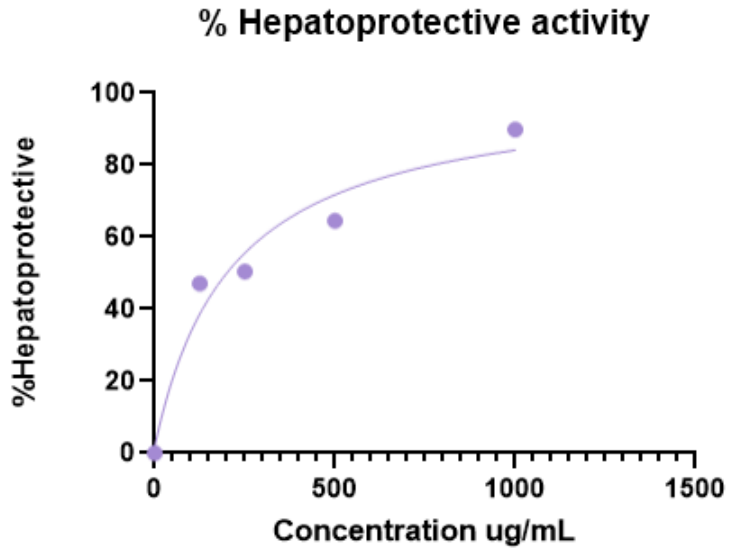
Test item *Lauha Bhasma* tested for 24 hours hepatoprotective activity on D-Galactosamine induced HepG2 cell. Vehicle control treated with DMEM media showed 95.67 % viability. Since the % cell viability showed no cytotoxic effect the hepatoprotective assay was conducted on four concentrations of test item *Lauha Bhasma*. The % hepatoprotective activity was assed at the four concentrations of 1000, 500, 250 and hepatoprotective activity and that of vehicle control was 33.64 %. 125 µg/mL which showed 89.91, 64.53, 50.46 and 47.09 % hepatoprotective activity and that of reference standard silymarin at the concentration of 12.5 µg/mL showed 87.46 %

**Graphical presentation of data**

**Graphical presentation for % cell viability**

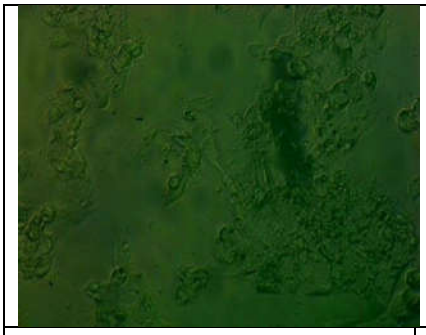
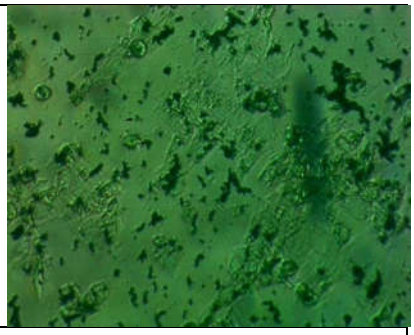
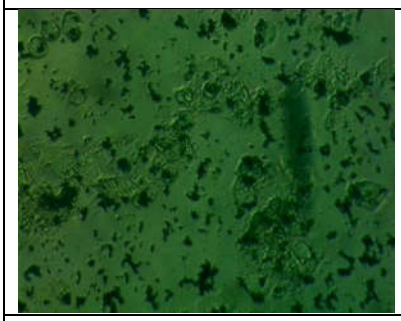
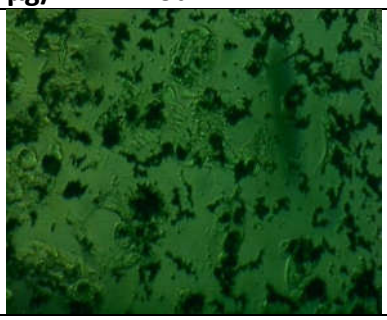
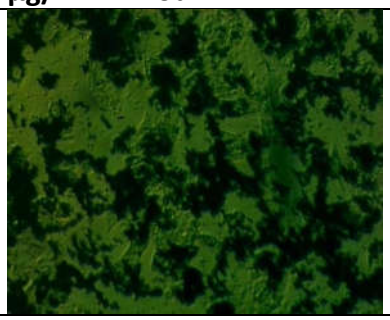
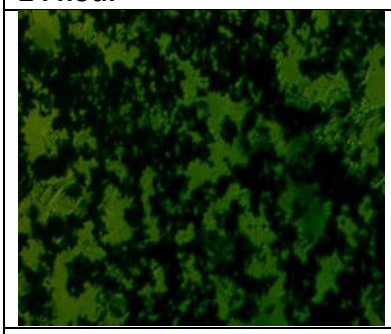
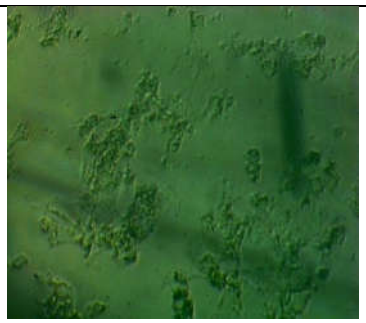


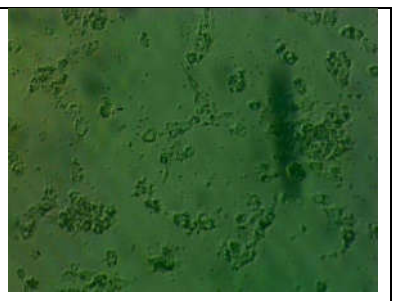

**Graphical presentation for % Viability for vehicle, positive control and test item- Lauha *Bhasma* are tabulated below for D-Galactosamine induced HepG2 cell toxicity 24h timeline.**



**Photographs:**

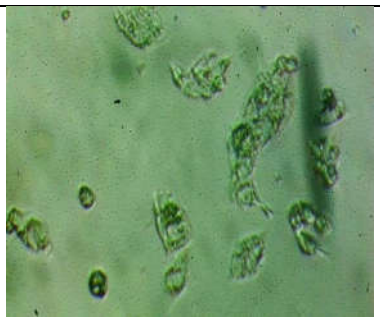
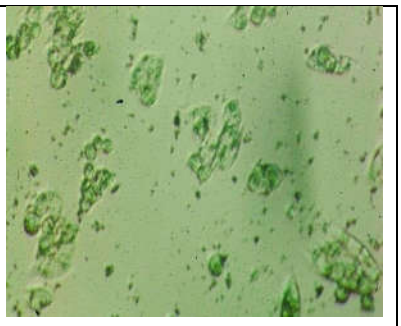
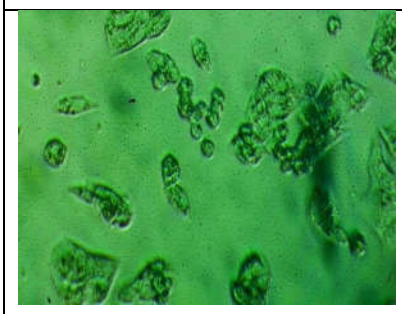
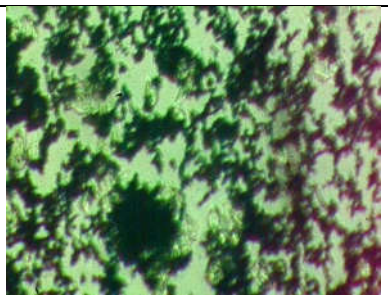
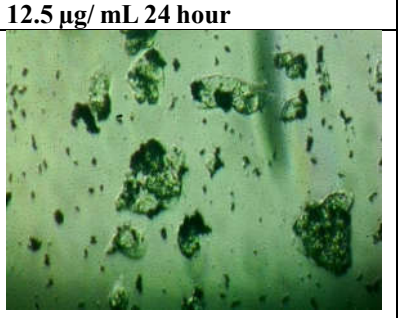
**Cytotoxicity Photographs:**

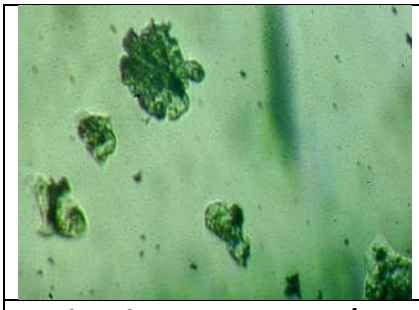
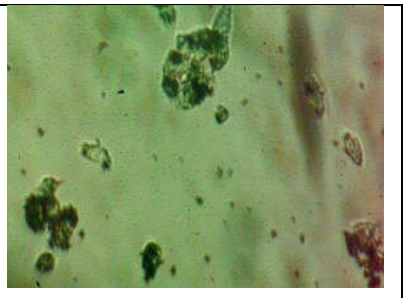
| | | |
|---|---|---|
|  |  |  |
| **Vehicle Control 24 hour** | ***Lauha Bhasma* F6 31.25 µg/mL 24 hour** | ***Lauha Bhasma* F5 62.5 µg/mL 24 hour** |
|  |  |  |
| ***Lauha Bhasma* F4 125 µg/mL 24 hour** | ***Lauha Bhasma* F3 250 µg/mL 24 hour** | ***Lauha Bhasma* F2 500 µg/mL 24 hour** |
|  |  |  |
| ***Lauha Bhasma* F1 1000 µg/mL 24 hour** | **Sodium Lauryl Sulphate-0.125 mg/ mL 24 Hour** | **Sodium Lauryl Sulphate-0.25 mg/ mL 24 Hour** |

| | |
|---|---|
|  |  |

| Sodium Lauryl Sulphate- 0.5mg/ mL 24 Hour | Sodium Lauryl Sulphate- 1.0 mg/ mL 24 Hour |
|---|---|

**D-Galactosamine induced HepG2 cell toxicity Photographs:**

| | | |
|---|---|---|
|  |  |  |
| **Untreated Control 24 hour** | **Diseases Control 20mM D-Galactosamine 24 hour** | **Reference Control Silymarin- 12.5 µg/ mL 24 hour** |
|  |  |  |
| **Vehicle Control 24 hour** | ***Lauha Bhasma* F1 1000 µg/mL 24 hour** | ***Lauha Bhasma* F2 500 µg/mL 24 hour** |

| | |
|---|---|
|  |  |
| ***Lauha Bhasma* F3 250 µg/mL 24 hour** | ***Lauha Bhasma* F4 125 µg/mL 24 hour** |

## Discussion:

*Samanya Shodhan*a was done according to reference of Rasaratnasamucchaya, where Shodhana common to all Dhatu Varga is mentioned. Initially 500 gm of *Lauha* fillings were taken and after Shodhana 537 g of *Lauha* fillings(powder) were obtained. In the process of *Samanya Shodhan*a, *Til Taila* imparted softness, the Amla Rasa of *Takra* imparted brittleness, because of *Gomutra* toxicity was removed and *Kanji* and Kullatha helped to convert the *Lauha* fillings into coarse powder.In *Vishesh Shodhan*, Initially 500g of *Lauha* fillings were taken and finally bluish black coloured Shuddha *Lauha* was obtained. The final weight after both Shodhana was 401g. Heating and cooling immediately in to liquid media leads to decrease in tension and increase in compression

force. Repetitions of this procedure cause destruction in tension and force balance leading to brittleness, reduction in hardness and reduction in particle size. During red hot state of metals and minerals volatile impurities like arsenic are removed completely. *Marana* of *Lauha* involves *Bhavana*, Pellet formation, Sharavsamputikaran,and *Puta*. Process of *Bhavana* known as attrition, which involves the material breaking down due to rubbing action between two surfaces. Flat pellets have a larger surface area and less thickness, they can be heated more evenly and uniformly.

Gaja *Puta* is recommended by RasaRatna Samucchay for the making of *Lauha Bhasma*. EMF is utilized in the current study as *GajaPuta* for *Lauha Bhasma*. Approximately 55 minutes were needed to attain the maximum temperature of 750°C. After one hour of maintenance, the peak temperature self-cooled after ten hours. In this study, 25 *GajaPuta* are given to prepare tha *Lauha Bhasma*.

In Analytical Study, The L.O.D. value of *Lauha Bhasma* was 0.075% of because it contains metallic substances as its main ingredients, traces of organic matter and moisture. The pH value was 5.5 which show acidic nature. *Lauha Bhasma* was evaluated for ash value and it was found 7.8% due to high metal content in the *Lauha Bhasma*. Acid insoluble Ash value was 1.25 % which facilitates the easy absorption of drug.Water soluble ash value is 2.21 %. XRD study of *Lauha Bhasma* suggest that it constitute compounds of $Fe_2O_3$. In SEM study of *Lauha Bhasma* images were captured at different magnification ranging from 200X to 5000X.*Lauha Bhasma* showed the presence of nano particles of around 20-50 micron Hence it can be concluded that, they do not differ chemically. But may differ in their pharmacological actions.

Test item *Lauha Bhasma* tested for 24 hours for cytotoxicity and hepatoprotective activity on D-Galactosamine induced HepG2 cell. The % Viability was assed at the six concentrations of test item *Lauha Bhasma* at **1000, 500, 250, 125 and 62.5 µg/mL** which showed **86.67, 87.87, 88.19, 88.08 and 89.38 % viability**. The positive control SLS was treated at the concentrations of 1.0, 0.5, 0.25 and 0.125 mg/mL, which showed 33.37, 54.82, 60.89 and 76.60 % viability.

Hepatoprotective activity was accessed at the concentrations of 125, 250, 500 and 1000 µg/mL on D-galactoasmine induce HepG2 cells. The EC50 value was calculated using GraphPad Prism 10.3.1. The test item *Lauha Bhasma*, % cell viability observed were 72.91, 86.67, 87.87, 88.19, 88.08 and 89.38 % at the concentrations of 1000, 500, 250, 125, 62.5 and 31.25 µg/mL respectively. Since the **% cell viability showed no cytotoxic effect the LC 50 value cannot be calculated.** The % hepatoprotective activity observed were **89.91, 64.53, 50.46, 47.09 and 33.64 %** at the concentrations of **1000, 500, 250 and 125 µg/mL**. **EC50** value observed at the **concentration of 214.7 µg/ mL.**

**Conclusion:**

After appropriate Shodhana process, all physical, chemical impurities of raw materials were washed out, after *Bhavana* & *Marana*, it was concluded that, all *Bhasma* were properly incinerated as they were passed all classical parameters. Standard Operating Procedure was followed in the preparation and analysis of *Lauha Bhasma*. *Lauha Bhasma*'s hepatoprotective properties were investigated, and an in vitro model of HepG2 cells was created under authentic laboratory settings. *Lauha Bhasma* possesses restorative qualities when administered as a hepatoprotective medication, according to the experimental data above.

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