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Research Article

IN-VIVO TOXICITY EVALUATION OF *SHILA SINDOOR*

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ABSTRACT

Ayurveda advocates the use of *Rasoushadhis* (metallic preparations) along with herbal preparations. Unprocessed metals and minerals like mercury and arsenic are highly toxic. *Shila Sindoor* is one of the *Rasoushadhi* being possessed with mercury, sulphur and arsenic disulphide as ingredients. In this study 12 rats were selected randomly from stock colony and divided into 2 equal groups of 6 rats each of group-I (vehicle control) and group-II (treatment group). Group-I rats were treated orally with 0.5ml of compound consisting of 3 parts of de-ionized water and 2 parts of honey. Group-II rats were administered with 0.5ml of compound similar to group-I, vortexed with test compound *Shila Sindoor* (250mg/Kg body weight) for 14 consecutive days to evaluate the toxic effects of *Shila Sindoor*. There was no morbidity or mortality during the study. Liver and kidney are the two major vital organs to maintain metabolism and protect human body by eliminating the toxins or deposit in these organs to defend other organs of body from toxicity. The results suggested that body weight, 13 different blood parameters like WBC, RBC, platelet count etc., 8 types of biochemical parameters like SGOT, SGPT, creatinine, urea etc., and lipid-peroxidation of group-II were not statistically significant in comparison with group-I. The histopathological study of kidney and liver of both groups revealed normal histology. In this paper genuine effort is put forth to appraise the safety of *Rasoushadhis* after meticulous process as described in Ayurveda. The result evinced that drug *Shila Sindoor* is safe for consumption at treatment dose as prescribed in classics.

KEYWORDS: *Shila Sindoor*, Toxicity, SGOT, SGPT, Creatinine, Lipid-peroxidation.

INTRODUCTION

Rasoushadhis surpassed all drugs in Ayurveda for its efficiency. *Rasashastra* is a specialised branch that deals with preparation of medicines using metals, minerals and poisonous herbs. *Kupipakwa Rasayana* are the most popular and advance preparations of *Parada* which involves the *Jarana* process. In this process, *Parada* is heated steadily along with *Gandhaka* and other metals and minerals resulting in a very intimate bondage which may help to exhibit superior qualities compared to other formulations with same ingredients. *Shila Sindoor*[1] is one of the *Rasoushadhis* prepared by *Kupipakwa* method; it is *Sagandha* (with sulphur), *Sagni* (drug processed by heating) and *Kantastha* (final product accumulates at neck of bottle) *Kupipakwa Rasayana*. *Shila Sindoor* was prepared by processing *Shodhita Parada* (purified mercury), *Shodhita Gandhaka* (sulphur) and *Shodhita Manashila* (arsenic disulphide) in equal quantity by weight as its ingredients. According to Goodman-Gillman pharmacology mercury readily forms covalent linkages to sulphur. When sulphur is in the form of sulphydryl group, divalent mercury replaces the hydrogen atom to form mercaptides. The affinity of mercury for thiols provides the basis for treatment of mercury poisoning. Minerals and metals when processed with addition of sulphur or sulphur containing minerals, they immediately get converted into sulphides and most of these are non-toxic. Classically, processed preparations had been proved for its safety and clinical efficacy. Processed drugs acquire the therapeutic properties due to transformation of chemical bonding that differentiates it from the raw compound. To assess and analyse the perfection of processing, many tests and methods were explained in the literature. Till date the literature survey suggest not many references on research conducted with this preparation called *Shila Sindoor.* Hence this study was undertaken to clear the myths about toxicity of mercury and arsenic formulations and establish evidence based facts by evaluating the toxic effects of the processed drug, *Shila Sindoor*.

MATERIALS AND METHODS

1. *Shodhana* (purification) of ingredients
2. Preparation of *Shila Sindoor*
3. Acute toxicity study

1. *Shodhana* (purification) of ingredients

All ingredients were collected according to *Grahya Lakshanas* (physical characteristics) of raw drugs as described in Ayurvedic classics and subjected to *Shodhana* (Table:1). *Parada Shodhana*[2] (purification of mercury) was done by triturating with *Haridra churna* (powder of *curcuma longa* L.) and *Kumari Swarasa* (leaf pulp of *Aloe vera* L.). This paste was made into *Chakrikas* (small flat cake like pieces) dried in shade and subjected to *Urdhwapatana* (sublimation). On *Svangasheetata* (self-cooling), *Shodhita Parada* was collected from the inner surface of upper pot in *Urdhwapatana* process. *Gandhaka Shodhana*[3] (Sulphur purification) was adapted with *Godugdha* (cow's milk) by *Bhudhara* method. Later *Shodhita Gandhaka* was washed with warm water and collected. *Manashila Shodhana*[3] (arsenic disulphide purification) was done by triturating with *Ardraka Swarasa* (juice of *Zingiber officinale* Rosc.) for seven times [Figure-1].

2. Preparation of *Shila Sindoor*

Samaguna Kajjali[3] was prepared by triturating equal quantity by weight of *Shuddha Parada* (purified mercury) and *Shudhha Gandhaka* (purified sulphur) till *Samyak Siddha Kajjali Lakshanas*[6] (signs of complete formation of Kajjali). This was followed by trituration of *Shuddha Manashila* with above prepared *Samaguna Kajjali* for duration of 6 hours.[4,5] Then *Kumari Swarasa*[6] of quantity sufficient was added for the above combination and triturated. The entire product after drying was filled in *Kachakupi* (glass bottle) which was covered with 7 consecutive layers of cloth smeared with multani mud up to mouth of *kupi*[7,8]. This *Kachakupi* was filled with *Kajjali* and it was placed in *Valukayantra* (iron vessel filled with sand). After setting the entire apparatus, fire wood was ignited with help of camphor. This process was conducted by following *Kramagni* (gradual heating) pattern. Pyrometer was used for recording temperature every hour at neck of *Kachakupi* and *Valuka*. The temperature near the base of *Kachakupi* was maintained between 150°C – 250°C for *Mrudvagni* (mild heat), raised to 350°C – 600°C for *Madhyamagni* (moderate heat) and followed to *Teevragni* (intense heat) with raise in temperature to 750°C. Gradual heating process to prepare *Kupipakwa* was confirmed with stage of fumes and flames. The bottom of the bottle appeared like *Udayabhaskaravarna* (rising Sun) i.e. red in colour. After confirmation with copper coin test, bottle was corked and *Teevragni* was continued for next 2 hours. After *Svangasheetata*, bottle was removed from *Valukayantra* and the cloth with multani was scrapped off [Figure-2]. Bottle was broken at the level of 2 inches below the collected portion of the *Shila Sindoor* and drug was collected by tapping over the outer surface of glass bottle[7,8] (Table:2).

Table: 1. Materials required for preparation of *Shila Sindoor* and Toxicology study

S.No	Materials used for preparation of *Shila Sindoor*	Materials used for toxicity study
1.	Mortar & pestle	Metal cages for individual rat
2.	*Valukayantra* (name of the apparatus prepared of sand used for processing)	Centrifuge tubes (plastic graduated 15 ml)
3.	Pyrometer	Vortex mixer
4.	Thick cloth	Micropipette
5.	Multani mitti	Incubator
6.	*Kachakupi* (glass bottle with seven consecutive layers of cloth smeared with multani)	Eppendorf tips (20-200µl) (100-1000µl)
7.	Camphor	Disposable syringe (5 ml)
8.	Match box	Aspirator needles (16/18 no's) 6' balled
9.	Firewood	Centrifuge
10.	*Shalaka* (thin iron rod)	Schimatzu model UV 1601
11.	Torch	Spectrophotometer
12.	Copper coin	Butter paper (small pieces)
13.	*Tula Yantra* (weighing balance)	Honey
14.	*Kajjali* for *Kupi* (45g of Hg+45g of S+45g of As_2S_2)	De–ionized water
15.	---	Test compound (*Shila Sindoor*)

Table: 2. Pharmaceutical preparation of *Shila Sindoor*

S.No	Drug used for process	Duration of process	Quantity before process	Quantity after process	Loss/ Gain	% of yield
1.	*Parada Shodhana*	6 hr	250 g	238 g	12 g	95.2%
2.	*Gandhaka Shodhana*	½ hr	250 g	246 g	04 g	98.4%
3.	*Manashila Shodhana*					
4.	*Samaguna Kajjali*	36 hr	260 g	257 g	03 g	98.8%
5.	*Kajjali* (*Manashil Yukta*)	06 hr	375 g	373 g	02 g	99.4%
6.	*Shila Sindura*	19:45 hr	135 g	83 g	52 g	59.28%

ACUTE TOXICITY STUDY

Ethical Clearance for the Study: Animal studies were conducted according to the Institute Animal Ethical Committee regulations approved by the Committee for the Purpose of the Control and Supervision of Experiments on Animals (CPCSEA). Clearance of experimental design by the Institutional Ethical Committee on rats was taken at DFRL, Mysore. The study was approved and conducted in DFRL, Mysore.

Procurement and selection of animals: Healthy male rats of Wistar Strain were used for study, reared in the Defence Food Research Laboratory animal facility, Mysore. A total of 12 Wistar Strain male albino rats weighing between 140-160 g (4 – 5 weeks old) were selected randomly from the stock colony.

Treatment of experimental rats: The rats were housed in an acrylic fiber cages in thermo regulated room with temperature of 25 ± 2°C and were maintained in a 12 hours light/dark cycle. Rats were fed with commercial pellet diet of 15-20 gm/day (Sri Venkateswara Enterprises, Bangalore, India) and drinking water ad libitum.

Experimental design: Twelve male Wistar rats were randomly divided into the following two experimental groups with six rats each: control group and treatment group. Control group (Group-I) rats were orally administered with 0.5ml of compound consisting of 3 parts of de-ionized water and 2 parts of honey for 2 weeks. The treatment group (Group-II) rats were administered orally with 0.5ml of compound vortexed with test compound *Shila Sindoor* (250mg/Kg body weight) in 3 parts of de-ionized water and 2 parts of honey [Figure3].

Dosage schedule: The required dose for rat was calculated by using the standard dose calculation procedure from recommended clinical dose.

Conversion formula as per SOP: The highest possible human dose of *Shila Sindoor* is 250 mg/ human/ day was converted to the dose in rats as mg/kg was administered to Wister Albino rats orally for 14 days (Gosh, 1984)

- Human dose is 125 mg, BD, i.e. 250 mg
- Total clinical dose (a) x conversion factor (b) 0.018 = (c) per 200 gm of rat

125 mg x 2(a) x 0.018 (b) = 4.5 mg (c) / 200 g of rat

4.5 x 1000/200 = 22.5 mg / kg.

Sacrifice of animals: Animals were sacrificed after 14 days by administering mild anaesthesia. Blood was collected from heart using heparinized syringe and plasma was separated which was stored at 80°C for further use. Liver and kidney were stored in 10% formalin solution for histopathology studies. The tissue samples (liver and kidney) were processed for histo-pathological procedure which was conducted by staining sections in haematoxylin Eosin (Lesher & Poole, 1989, (a)) and TBARS assays as per the prescribed procedures.

OBSERVATIONS AND RESULTS

Table 3: Observations before and after the experimental study

Observation	Before administration	After 14 days of administration	
		Group-I	Group-II
Food intake	Normal	Normal	Normal
Water intake	Normal	Normal	Normal
Activity	Normal	Normal	Normal
Colour of eyes	Red	Red	Red
Colour of fur	White	White	White
Stool colour	Black	Black	Black

Table 4: Blood analysis of both groups

Group	WBC (*103)	RBC (*106)	Hb gm/dl	HCT	MCV	MCH	MCHC
Group-I	5.10 ± 1.28	7.78 ± 0.68	12.75 ± 4.18	45.32±3.14	58.30±1.13	17.83±0.82	31.17±0.59
Group-II	4.22 ± 0.93	7.72 ± 0.51	14.40 ± 1.32	45.90±4.07	59.38±2.15	18.60±0.73	31.38±0.32

Table 5: Other blood parameters of both groups

Group	PLT (*103)	LYM %	LYM (*103)	RDW	PDW	MPV
Group-I	461.67 ±89.59	86.17±3.95	4.87 ±0.65	28.78 ±0.31	9.33 ±0.56	7.63 ±0.23
Group-II	709.50 ±93.96	75.97±4.67	3.10 ±0.90	31.53 ±1.79	9.00 ±0.68	7.33 ±0.40

Table-6: Serum analysis of both groups

GROUP	SGOT	SGPT	Creatinine	ALP	Urea	Uric acid	Total Bilirubin	Direct Bilirubin
Group-I	22.88 ± 2.65	6.60 ± 0.89	0.40 ± 0.05	1.03 ± 0.08	35.83 ±3.37	1.17± 0.19	0.14 ± 0.18	0.06 ± 0.02
Group-II	28.62 ± 2.77	7.55 ± 1.08	0.40 ± 0.02	1.02 ± 0.49	31.00 ± 3.90	0.98 ± 0.15	0.07 ± 0.04	0.05 ± 0.02

Serum Study of Group-I (Vehicle Control) and Group-II (*Shila Sindoor)* values are mean ± SD of 6 rats. Values bearing different superscripts in the same column are significantly different if $p < 0.05$. There was no significant difference in SGOT, SGPT and creatinine, urea, uric acid, total bilirubin and direct bilirubin in both the rat groups.

Determination of TBRAS (Thiobarbituric acid-reactive substances) assay

TBARS as malon di aldehyde (MDA mmol/cm/g tissue) was analyzed by Buege and Aust (1978). Liver and kidney tissues (100mg) were homogenized in 2ml of phosphate buffer (pH 7.0). TCA (10%), 0.5ml and 2ml of TBA mixture were added to tissue homogenate (0.5ml).[9] The TBA mixture contained TBA (0.35%), SDS (0.2%), FeCl3 (0.05mM) and BHT in glycine-HCl buffer (100mm, pH 3.6). The above reaction mixture was boiled at 100^{o}C for 30 minutes and then allowed to cool. The mixture was centrifuged at 8000 rpm for10 min and the absorbance was measured at 532 nm[10].

Histopathology reports of the tissue

Micro section of liver showed normal architecture with hepatic lobules and hepatocytes arranged in sheets and cords with central veins normal for all rats. Micro section of kidney of all rats also showed normal cortex and medulla with normal glomeruli and collecting tubules.

DISCUSSION

Shodhana is an essential step while processing a drug. Purification of mercury was performed with *Haridra* and *Kumari* which possess *Vishahara, Naga*, *Vanga Doshahara* and, *Malahara* properties that facilitate detoxification of mercury. *Bhudaraputa* procedure separates chemical impurities and thus detoxifies sulphur. *Bhudaraputa* process is similar to sublimation followed by granulation. After *Shodhana*, hot water wash may help to remove the remnants of milk and limpidity. The *Ushna* and *Teekshna* qualities of *Gandhaka* may reduce by *Mrudu* (soft), *Snigdha Guna* (unctuous property) of milk. *Gandhaka* obtained after *Shodhana* was brittle and shiny which may be due to the change in its crystalline structure (from monoclinic to rhombic), on melting. Consumption of *Ashodhita Manashila* (impure arsenic disulphide) was considered toxic, its symptoms and management were explained in detail by Acharyas. Trituration with *Gingiber officinale* may facilitate interaction between As_2S_2 and gingerol or shogaol. The weight gain after *Shodhana* was due to absorbance of these organic molecules into As_2S_2.

Mercury and arsenic are metalloid and sulphur is non-metal. Mercury belongs to IIB group, it can give away 1 or 2 electrons to form a bond, Arsenic belongs to VA group and can share by giving away 3 electrons forming As^{+3}. Sulphur belongs to VIA group and can share by taking 2 to 6 electrons forming S^{-2} to S^{-6} valence. Both arsenic and mercury have more affinity towards sulphur to form sulphide compound. Trituration of mercury, sulphur and arsenic disulphide for 6 hours and further continuation of triturating with liquid media (*Aloe vera* pulp) plays a vital role in binding the ingredients into a single molecular form. *Kajjali* is black sulphide of mercury prepared from classically treated and detoxified mercury and sulphur. *Kajjali* should pass the tests like *Rekhapurnata*, *Nischandrata* and *Tamra Pareeksha*. All these tests signify the fineness, subtleness that strike out the chances of free mercury.

The objective of *Mrith lepana* for *Kachakupi* is to strengthen it for sustained heat as it is more pyro-sensitive. *Kajjali* should be filled 1/3rd of the Kupi, which may facilitate more space for the free movement of gases and boiling of *Kajjali* during the process. *Valuka Yantra* is specially designed for uniform and indirect heating through sand (*Valuka*) and it is inert and may prevent the sudden fluctuations of temperatures and also may render resistance to the apparatus from atmospheric temperature variations. Appearance of Sulphur fumes after 2 hours of heating indicates melting of *Kajjali* which was confirmed by cold *Shalaka* test. Appearance of dense and profuse fumes indicates melting of *Kajjali* and processing of *Parada* with *Gandhaka*. Stage of flames suggests burning of extra sulphur and organic matter. Copper coin test helps to confirm absence of free sulphur and escaping of white mercury fumes. Copper coin test, demonstrated the turning into greyish white which was placed over the mouth of the *Kachakupi*. This may be due to reaction of mercury with copper, in copper coin test. These tests indicate complete bonding of *Kajjali* and suitable for corking of *Kachakupi* for further process of sublimation and collection of final product *Shila Sindoor*. Final product *Shila Sindoor* is up to 50-60% of *Kajjali* taken due to the loss of major proportions of sulphur, minimum quantity of arsenic and traces of mercury. Cessation of fumes might indicate complete escape of excess free sulphur.

As per the present day scenario, intake of mercury, arsenic compound and its products are claimed to be highly toxicity. According to Ayurvedic science consumption of any processed drug at prescribed dose is considered to be safe. Healthy male albino rats of 140 g – 160 g of 6 animals in each group, were selected for the study, for 14 days to evaluate toxicity and drug's effect on metabolism of these animals before clinical trials. The method of preparation of compound for administration was followed as per CCRAS rules. As drug was in powder form, for administration of *Shila Sindoor* a solvent media was required. So honey and de-ionized water was used. Honey is used as an *Anupana* (adjuvant) for administration of *Shila Sindoor* according to Ayurvedic classics too. Regular examination of rats was observed outside the cage to evaluate their general physical health and activity. General physical changes like colour and consistency of fur, colour of eye, nature and consistency of stool, secretions from eyes, ears, nostrils and, body weight of each animal were observed to elicit gross systemic toxicity, if any. Intake of food and water were monitored. Around 15-20 g of pellet diet was consumed. Food and water intake monitoring would help to assess the physical health of animal and to ensure non-injury of oral cavity or oropharynx during force feeding apart from toxic effects.

The data is expressed as mean standard deviation of the mean (SD). Data was analyzed using Student's t-test. Differences at $p<0.05$ were considered to be significant. There was no significant change in the parameters like weight gain or food and water intake of the rats posted in group-II with comparison to the rats of

vehicle control group. The weight gain of all animals of both groups were similar i.e., statistically not significant. The blood parameters like RBC, WBC, platelet count etc., were evaluated to assess any adverse effects of drug that enters the blood circulation crossing the body filter system. Toxic substances are filtered by body to protect functioning of vital organs. All the 13 parameters were assessed statistically for both groups and were not statistically significant. The values of all 7 tests of serum biochemical parameters were not statistically significant. The TBRAS Assay was conducted to liver and kidney tissues of rats included in both the groups. Optic density is the marker value of this test. The value of optic density is specific to the type of organ. For example, optic density of brain tissue is 1.9-2.1, that of liver is 1.7-1.9. If the presentation of normal limits in final optic density, it suggests non-lysis of tissue and no remarkable pathological changes that arise due to toxic effect. Micro-section of liver and kidney suggested normal architecture. All the above described tests of blood, serum biochemical parameters, TBRAS Assay and histo-pathological tests were conducted to evaluate the liver and kidney function as they are the two major vital organs to maintain metabolism and protect human body by eliminating the toxins or deposit in these organs to defend other organs of body to function normally. Further evaluation in other animals is warrenteed before conducting human trails.

CONCLUSION

Kupipakwa Rasayana is one of *Murchita Parada yoga* and a highly evolved pharmaceutical process. *Shila Sindoor* is *Sagandha*, *Sagni*, *Kantastha Kupipakwa Rasayana*. The study of albino rats did not show any significant physical changes to assess gross pathological changes. The evaluated values of blood and serum parameters were in normal limits in comparison with vehicle control group suggesting *Shila Sindoor* did not pass the body filtering system and entered the blood stream. The critical evaluation of hepatic and renal functions demonstrated no evident changes in these tissues on microscopic examination. So drug *Shila Sindoor* is safe for administration, at a treatment dose of 2 ratti (250mg), as prescribed by *Acharyas*.

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Figure-1: Shodhana of ingredients

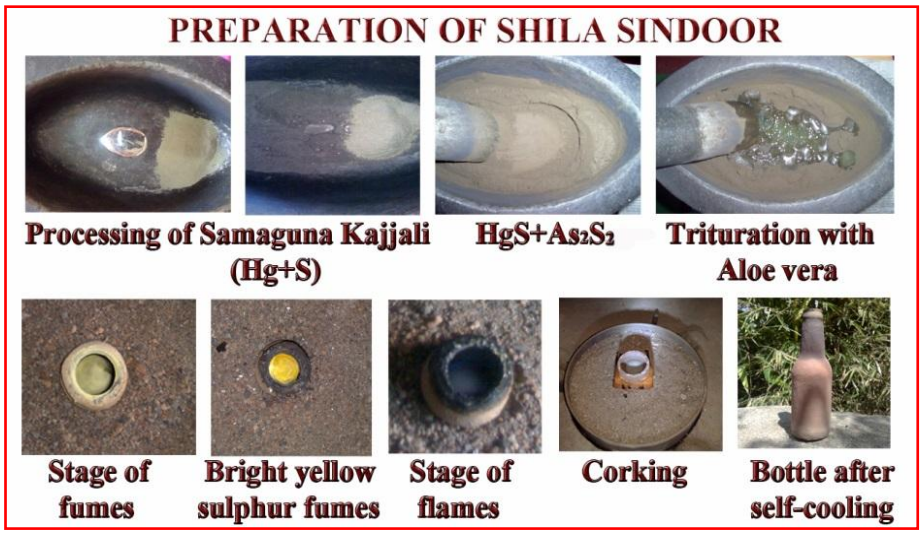


Figure-2: Preparation of *Shila Sindoor*

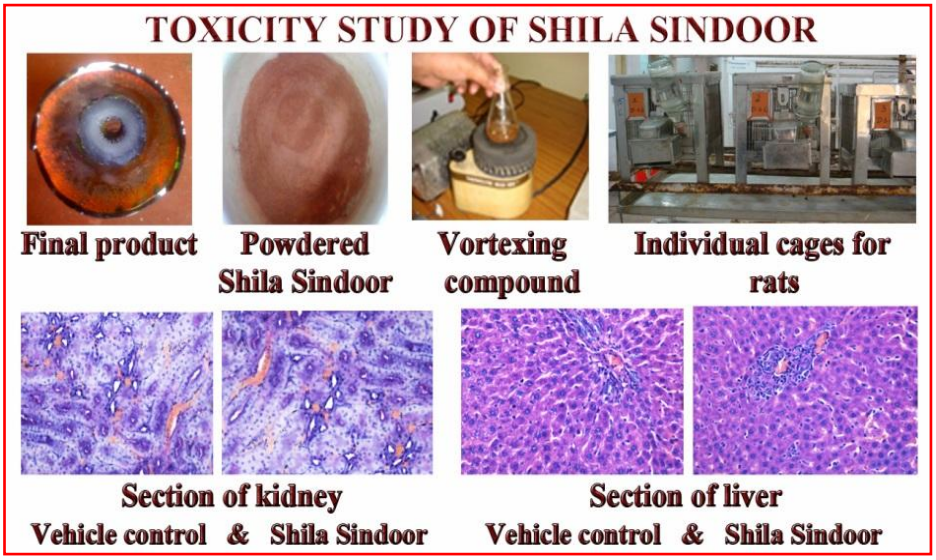
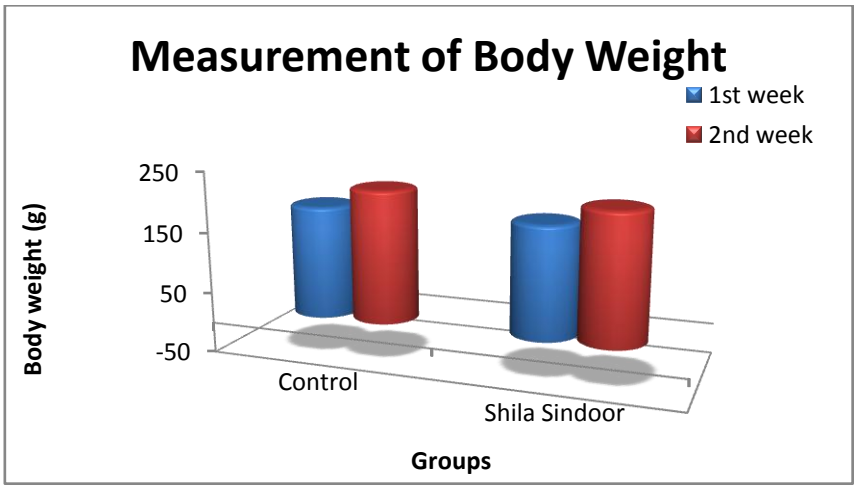
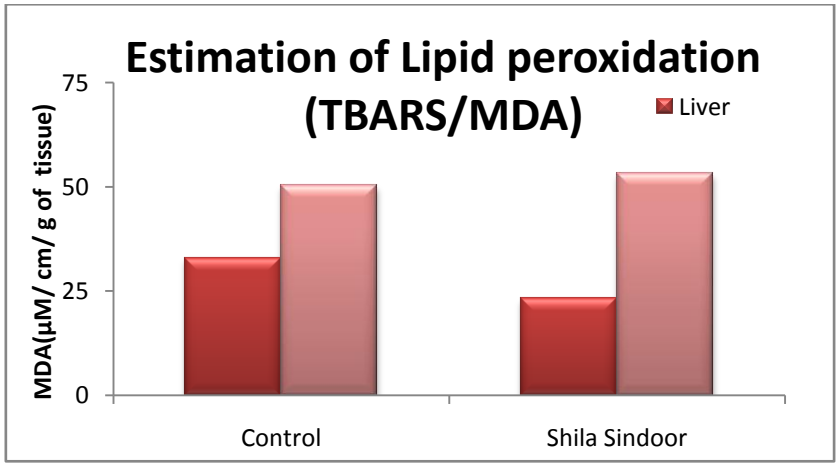


Figure-3: Toxicity study



Graph-1: Body weight gain (%) of both control vehicle and group treated with *Shila Sindoor* for 14 days. Data express the mean SD for six rats on every week.



Graph-2: Estimation of lipid peroxidation