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Acute and Chronic Toxicity Study of *Vatavidhvamsana Rasa*, an Ayurvedic Herbomineral Formulation

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Abstract

Vatavidhvamsana Rasa (VVR)is a famous herbomineral formulation containing various processed metals, minerals and herbals and is mainly used in treating neurological and muscular diseases. Herbomineral formulations in Ayurveda are always under scrutiny for safety aspects due to the presence of heavy metals. This study is an attempt to evaluate the safety of *Vatavidhvamsana Rasa* through acute toxicity and 90 days repeated dose toxicity. The oral acute toxicity study of VVR was accomplished in Wistar albino rats at a limit dose of 2000 mg/kg. The oral repeated dose toxicity study (90 days) of VVR was carried out by administering VVR with honey at therapeutically equivalent dose (22.5 mg/kg), TEDx5 (112.5 mg/kg) and TEDx10 (225 mg/kg) dose levels. One recovery group (225 mg/kg) was kept for observation for 30 days after the treatment period. *Vatavidhvamsana Rasa* did not produce any observable toxic effects during acute toxicity study. There were also no significant behavioural changes during the entire duration of the acute study and all animals survived during the 14 days of observation. This implicates that the LD50 value of *Vatavidhvamsana Rasa* would be more than 2000 mg/kg by oral route. In chronic toxicity study, *Vatavidhvamsana Rasa* with honey as adjuvant given at different dose levels had not produce any major adverse effects in albino rats during the study period of 90 days along with a recovery period of 30 days. VVR at higher dose showed no significant changes in histopathology, hematological and serum biochemical parameters. At higher dose level of VVR at TEDx10 for 90 days, drug has potential to produce changes in liver and kidney related parameters. From the above data, it can be concluded that VVR with honey administered orally in rats was found to be safe in acute toxicity study and also at therapeutic dose level during chronic toxicity study in albino rats.

Keywords: Acute Toxicity, Chronic Toxicity, Heavy Metal Toxicity, Herbominerals, *Vatavidhvamsana Rasa*

1. Introduction

Metals/minerals such as cinnabar, mercury, copper, tin, iron and arsenic after certain processing is in use as Ayurvedic medicines since thousands of years and are claimed to have certain therapeutic effects[1,2]. Many of these substances are highly toxic in their raw form and their safety and risk are of concern among the scientific community[3,4]. In complementary and traditional medicine, the use of minerals and metals are very often seen as a matter of concern. Especially the mercurial preparations are always under stringent observations for regulating highly toxic

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inorganic elements such as lead, mercury, arsenic and others[5,6]. For the global acceptance of Ayurvedic medicines, proper quality control, standardization of raw drugs and also final products are mandatory. This will help to guarantee that patients get a safe, potent, uncontaminated and effective medication[7]. Acharya Charakahad narrated that even a fatal poison when used with skill and knowledge, will prove to be good medicine, while a medicine used ignorantly may act as a poison[8]. Contrary to Western medicine, which does not promote the use of mercury owing to its toxic effects; Indian traditional practitioners believe that mercury-based formulations have potent therapeutic efficacy sans toxicity, due to the unique, repeated, exclusive and mandatory processes employed during its preparation like *Shodhana* (purification) and *Marana* (incineration process)[9]. Those methods were developed by practitioners of ancient times to detoxify the raw material by chemical transformations and thereby modify its properties by enhancing their therapeutic potential[10–13].

By considering all these facts, it is necessary to validate traditional Ayurvedic medicines especially those containing metals and minerals on the line of safety and efficacy studies. *Anupana* (vehicle) as an adjuvant improves acceptability, palatability and also helps in absorption of the main drug; moreover, it may also act as an early antidote[14]. Many safety studies were conducted in various Ayurvedic herbomineral formulations. *Vatavidhvamsana Rasa* (VVR) is aherbo-mineral Ayurvedic preparation mentioned in the textbook *Yogaratnakara Vatavyadhi chikitsa prakarana*, which has even been quoted in Ayurvedic Formulary of India and is mainly indicated for various neuromuscular diseases[15,16]. VVR contains processed metals, minerals and some herbal ingredients. In the present study, *Vatatvidhvamsana Rasa* was evaluated for safety through acute and chronic toxicity studies.

2. Material and Methods

2.1 Procurement and Authentication of Raw Materials

All the raw materials for the preparation of *Vatavidhvamsana Rasa* (Table 1) were procured from the Pharmacy of the Institute and authenticated as per the standards of Ayurvedic Pharmacopeia of India by the concerned experts. VVR was prepared as per the reference of Ayurvedic Formulary of India Part I.

Purified *Parada* (mercury) was extracted from *Hingula* (Cinnabar) using *Urdhvapatana* (distillation) method. *Gandhaka shodhana* (purification of sulphur) was done in cow's milk by *Dalana*(pouring molten sulphur in to liquid media) method[17,18]. *Sodhana* of *Tankana* was done by heating the *Ashudha Tankana* (raw borax) in to white puffy dehydrated state. *Vatsanabha Sodhana* was carried out using cow's urine. Raw *Vatsanabha* was immersed in cow's urine in a stainless steel tray and kept in sunlight for three days. The cow's urine was replaced every daywith fresh one and finally *Vatsanabha* was washed with hot water[19]. *Samanyasodhana* of *Tamra, Loha, Naga* and *Vanga* was carried out as per the reference of *Rasa Ratna Samuchhaya*. The *Samanyasodhana* procedure was done by dipping red hot/molten metal into five liquid media (seven times in each media)[17.] For the preparation of *Vanga Bhasma*, *Chakrika* (pellets) were made by grinding with *Aloevera* juice and 10 *Puta* (incineration process) was given to prepare *Vanga Bhasma*[20]. *Shuddha Tamra* was mixed with *Samaguna Kajjali* and ground well with *Nimbu Swarasa*. After proper grinding *Chakrikas* were made and which were dried in sunlight and subjected to Puta[17]. *Naga Bhasma* and *Loha Bhasma* previously prepared were procured from Department of Rasasastra and Bhaishajya Kalpana, ITRA, Jamnagar.

Equal quantity of processed mercury and processed sulphur were taken and ground using mortar and pestle, till it attained a lusterless black fine powder form, known as *Kajjali*[17]. After preparing *Kajjali*, all the other ingredients (Table 1) were added and the whole mixture was triturated till a uniform mixture was obtained. It was again triturated three times in each of the ten media (Table 1) and the duration of each *Bhavana* (levigation) was 3 hours. The whole mixture was levigated thirty times with ten different liquid media. Then the final product VVR was dried in shade and stored in an airtight glass container.

2.2 Experimental Animals

Experimental protocols for the toxicity study were approved by Institutional Animal Ethics Committee (IAEC/24/2018/21) as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals(CPCSEA), India. Wistar strain albino rats of both sexes weighing between 180-220g were procured from Animal house attached to the Pharmacology laboratory of the Institute. The experimental animals were given exposure to 12 hour light and dark cycle. The relative humidity was 60±10% and the ambient temperature was 22±3°C. VRK brand rat pellet feed supplied by Keval Sales Corporation, Vadodara was provided throughout the study period and stainless steel sipper tube attached polypropylene bottles were used to give drinking water *ad libitum*.

Table 1. Ingredients and composition of *Vatavidhvamsana Rasa*

S.N.	Ingredients	Botanical name/ English name	Composition	Quantity (g)
1.	*Shodhita Parada*	Purified Mercury	1 Part	20
2.	*Shodhita Gandaka*	Purified Sulphur	1 Part	20
3.	*Vanga Bhasma*	Processed Tin	1 Part	20
4.	*Naga Bhasma*	Processed Lead	1 Part	20
5.	*Tamra Bhasma*	Processed Copper	1 Part	20
6.	*Loha Bhasma*	Processed Iron	1 Part	20
7.	*Abhraka Bhasma*	Processed Mica	1 Part	20
8.	*Shodhita Tankana*	Purified Borax	1 Part	20
9.	*Pippali*	*Piper longum*	2 Parts	40
10.	*Shunthi*	*Zingiber officianalis*	2 Parts	40
11.	*Maricha*	*Piper nigrum*	1 Part	20
12.	*Shodhita Vatsanabha*	Purified Aconitum chasmanthum	4.5 Parts	90
Media used for levigation				
1. Juice of *Nirgundi* (*Vitex negundo* Linn.), 2. Latex of *Arka (Calotropis procera* (Ait.) R.Br.), 3. Juice of *Bhumiamalaki (Phyllanthus niruri* Linn.), 4. Juice of *Bhringaraj (Eclipta alba* Hassk), 5. Decoction of *Kushta (Saussurea lappa* C.B.Clarke), 6. Juice of *Nimbu (Citrus medica* Linn.), 7. Decoction of *Chandrashoora (Lepidium sativum* Linn), 8. Decoction of *Chitrak (Plumbago zeylanica)*, 9. Decoction of *Trikatu (Piper nigrum* Linn., *Piper longum* Linn., *Zingiber officianale* L.) 10. Decoction of Triphala (*Terminalia chebula* Retz., *Terminalia bellirica (Gaertn.) Roxb., Emblica officinalis Gaertn.)*				

2.3 Dose Fixation

The human adult dose of VVR is two *Ratti* (250 mg) with honey as adjuvant as per AFI. FSSAI approved processed honey (Baidyanath) was procured from local market. The therapeutic dose of VVR for the experimental study was calculated by extrapolating the human therapeutic dose to rat based on body surface area ratio (conversion factor 0.018 for rat) by referring to the table of Paget and Barnes (1964)[20]. The Therapeutic Equivalent Dose (TED) of VVR for albino rats was calculated as 22.5 mg/kg. The test drug was mixed with honey as adjuvant and diluted with water as per dose level and prepared suspension orally administered with a cannula in constant dose of 5ml/kg.

2.4 Acute Oral Toxicity Study

The study was carried out as per the guidelines of the Organization for Economic Cooperation and Development (OECD) 42521. Nulliparous and non-pregnant healthy female Wistar strain albino rats were selected and are acclimatized for 7 days before starting the study. Vehicle control group received vehicle as honey in distilled water while test drug treated group consist of five female albino rats received VVR mixed with honey at a limit dose of 2000 mg/kg body weight to female rats in a sequential manner as per OECD 425 guidelines. Food, but not water was withheld overnight before the experiment and further 2 hours after administration of test drugs. Gross behaviour and mortality

were observed throughout the study period for 14 days. The animals were observed continuously for 8 hours after the dosing. All the animals were observed at ½, 1, 2, 3, 4, 5, 6, 24 hours after treatment and daily once for any mortality during the entire period of the study (14 days).

2.5 Repeated Dose 90 days Oral Toxicity Study

Repeated dose 90 days oral toxicity study was carried out as per OECD 408 guidelines[22]. Wistar strain albino rats of both sexes were grouped randomly into five having 6 animals each. The test drug was administered orally once a day for 90 consecutive days. The first group was kept as vehicle control, received the vehicle as honey (5 ml/ kg). Group II, III, IV were administered test drug VVR orally with honey at TED (22.5 mg/kg), TEDx5 (112.5 mg/kg), TEDx10 (225 mg/kg) respectively. Group V (recovery group) was administered VVR with honey at TEDx10 (225 mg/kg) for 90 days and kept for observation for a post-treatment period of 30 days. The body weight of animals of all the groups was noted before starting the experiment and subsequently on every 7th day. The general behavior pattern of rats was also noted by exposing these animals to an open arena. After completing the experiment (on 90th day) rats of all groups except group V were kept for overnight fasting. On the 91st day, for estimation of serum biochemical and hematological values, animals were given mild ether anaesthesia and blood was collected by supra-orbital puncture. The body weight of all animals was recorded before sacrifice. After sacrificed, to record the autopsy changes abdomen was opened through a midline incision, the changes were observed and important organs were dissected. After the post-treatment period of 30 days, rats in the TEDx10 recovery group (Group V) were also sacrificed and organs were dissected.

The automated haematological analyzer was used for testing haematological parameters like total WBC count, differential count (neutrophils, lymphocytes, eosinophils and monocytes), haemoglobin, Packed Cell Volume (PCV), total red blood cell count, platelet count, MCV, MCH and MCHC. The biochemical parameters checked were blood sugar, total cholesterol, serum triglycerides, HDL cholesterol, blood urea, serum creatinine, SGPT, SGOT, total protein, albumin, globulin, alkaline phosphatase, total bilirubin, direct bilirubin, uric acid and serum calcium using fully automated biochemical random access analyzer, BS-200; Lilac Medicare P. Ltd., Mumbai[23–36].

After the experiment, the animals of various groups were sacrificed and record the autopsy changes followed by dissecting out the important organs. All the important organs were carefully dissected cleaned of extraneous tissue and the piece was transferred to 10% buffered formalin solution for fixation and histopathological studies. Brain, liver, heart, spleen, kidney, lung, uterus, prostate, testis, seminal vesicle, stomach, intestine, ovary, adrenal, lymph node, pituitary, trachea and bone marrow were subjected to histopathological study[37]. With the help of Trinocular research Carl–Zeiss microscope (Germany) having different magnifications the histopathological slides were observed and the changes were noted.

2.6 Statistical Analysis

Sigma Stat software was used to find the significant difference between groups ($P<0.05$). Student 't' test for paired and unpaired data and one way analysis of variance was used to compare the mean values of quantitative variables among the groups followed by Dunnett multiple t test.

3. Results

3.1 Acute Toxicity Study

The result of acute study showed normal progressive body weight gain of albino rats throughout study period in vehicle and VVR treated groups. There was no mortality was observed and no any behavioral changes in female albino rats treated with VVR at limit dose of 2000 mg/kg during the experimental study (14 days).

3.2 Chronic Toxicity Study

No mortality was observed in any of the groups of VVR ar TEDx10, TEDx5 and TED dose levels and also in the recovery study (TEDx10 R).A mild increase in food intake was observed in VVR treated groups.Faecal and urine output remained the same in all the treated groups in comparison to the control group.

3.3 Effect of VVR on Body Weight

Progressive weight gain was observed in albino rats of control groups during the study period. The bodyweight of rats in various groups showed a uniform increase at different dose levels of VVR. A significant increase in body weight was noted after 4th, 8th, 12th weeks in VVR at TED group and after 4th, 12th weeks in VVR TEDx5 group. Likewise, a significant increase was observed after 4th, 8th, 10th, 12th weeks in VVR TEDx10 group and after 8th, 14th and 16th weeks in recovery group in comparison to initial body weight (Table 2).

Table 2. Ingredients and composition of *Vatavidhvamsana Rasa*

Weeks	Control	TED	TED×5	TED×10	TED×10R
0 days	218.80±1.77	199.00±4.85	209.80±4.91	216.20±4.25	202.80±5.20
4th Week	238.2±8.67	226.60±7.71*	223.60±6.99*	236.40±7.38*	216.40±6.24
8th Week	247.20±12.06	235.20±6.82*	233.20±8.06	249.60±7.33*	234.8±5.36***
12th Week	248.4±15.66	250.40±8.38*	242.20±7.68*	258.00±7.66***	240.0±7.74*
16th week	-	-	-	-	257.2±9.99***

Data: Mean±SEM, ↑-Increase, *P<0.05, ***P<0.001 when compared to initial values (Paired 't' test)

3.4 Effect of VVR on the Relative Weight of Organs

The data showed an insignificant (P>0.05) change in relative weight of organs like liver, spleen, kidney, uterus, heart, prostate, testis and seminal vesicles in VVR administered groups. At different dose levels, VVR did not show any changes in relative body weight in comparison to the control group. Administration of VVR produced an insignificant decrease in the relative weight of heart, seminal vesicle and uterus at different dose levels in comparison to the vehicle control group. Administration of test drug at TED dose level produced an insignificant increase in the relative weight of testis in comparison to control group while, insignificant effects were seen at the dose level of TEDx5, TEDx10 and TEDx10R in comparison to control group (Table 3).

Table 3. Effect of *Vatavidhvamsana Rasa* on relative weight of organs

Relative weight	Control Group	TED	TED*5	TED*10	TED*10 Recovery
Heart (mg/100 g)	352.37±23.50	301.93±18.85	323.60±16.45	321.34±10.75	306.29±19.55
Liver (g/100 g)	3.24±0.19	3.16±0.12	3.05±0.059	2.98±0.16	2.74±0.28
Spleen (mg/100 g	232.79±18.79	218.35±8.46	214.70±8.13	233.83±32.33	214.94±16.55
Kidney (mg/100 g)	686.75±13.52	666.57±23.63	626.17±14.19	680.03±38.15	634.97±51.79
Testis (mg/100 g)	798.49±65.15	713.20±21.42	821.07±44.60	812.48±79.71	769.55±24.87
Prostate (mg/100 g)	295.30±31.97	279.05±59.41	281.67±12.45	253.67±39.93	245.29±6.48
Seminal vesicles (mg/100g)	439.97±37.30	399.37±32.94	307.98±88.09	282.02±22.66	542.03±22.15
Uterus (mg/100 g)	330.81±48.77	320.01±44.14	307.45±42.49	324.79±40.62	260.24±30.49

@P<0.05, @@P<0.001 when compared to control group (Annova followed by Dunnett's multiple 't' test).

3.5 Effect of VVR on Hematological Parameters

No significant alterations were observed in hematological parameters like WBC, platelets, PCV, eosinophil, neutrophil, haemoglobin, PCV and monocyte count in VVR treated groups at all dose levels in comparison to vehicle control group. A significant increase in lymphocytes was seen in VVR at TEDx10 dose level while same was insignificantly reversed in the recovery group. The total RBC count was significantly decreased in VVR at TEDx5 dose level in comparison to the control group. Administration of VVR produced a significant increase in MCV at the dose level of TEDx5 and TEDx10 in comparison to the control group. After discontinuation of the test drug in the recovery study, the MCV was not affected in comparison to the vehicle control group.Administration of VVR did not produce any significant change on the MCH count at TED, TEDx10 and TEDx10 recovery dose levels in comparison to the control group while at TEDx5 dose level VVR produced significant increase in MCH level. Administration of VVR produced significant decrease in the MCHC count at TEDx5 and

TEDx10 dose level in comparison to control group however after discontinuation of drug in recovery study there was significant increase in MCHC level (Table 4).

Table 4. Effect of *Vatavidhvamsana Rasa* on Heamatological Parameters

Parameters	Control Group	TED	TED*5	TED*10	TED*10 Recovery
TWBC (10^3/mL)	5920.00±615.96	7040.00±607.12	6340.00±540.00	6980.00±854.05	7060.00±211.19
Neutrophil (%)	28.40±1.72	27.40±3.20	22.80±2.35	20.20±3.26	36.60±3.98
Lymphocyte (%)	64.20±2.73	65.80±4.19	71.80±2.52	77.00±4.05*	52.80±4.79
Eosinophil (%)	4.20±1.39	3.80±1.11	2.80±0.37	2.20±0.20	3.00±0.32
Monocyte (%)	3.20±0.20	3.00±0.63	2.80±0.37	2.80±0.37	2.80±0.20
Hb (g/dL)	14.94±0.32	15.22±0.26	14.20±0.35	13.52±0.89	15.52±0.35
PCV (%)	42.82±0.91	43.36±0.68	38.40±2.46	44.40±2.25	43.32±0.65
TRBC (10^6/mL)	8.95±0.29	10.45±1.29	6.91±0.37**	8.28±0.57	9.27±0.34
MCV (fL)	47.92±0.81	46.38±0.90	55.50±0.84@@	53.96±1.27@@	46.60±1.08
MCH (pg/red cell)	16.72±0.29	16.26±0.29	18.50±0.42@@	16.36±0.21	16.68±0.36
MCHC (g/dL)	34.90±0.11	35.08±0.09	33.32±0.59 *	30.42±0.78@@	35.60±0.28 *
Platelets (103/mL)	1114.00±33.39	1183.20±55.84	1103.00±62.94	1075.40±159.31	881.80±128.48

*P<0.05, **P<0.01 when compared to control group (Unpaired't' test), @P<0.05, @@P<0.01 when compared to control group (Annova followed by Dunnett's multiple 't' test)

Table 5. Effect of *Vatavidhvamsana Rasa* on biochemical parameters

Parameters	Control Group	TED	TED*5	TED*10	TED*10 Recovery
FBS (mg/dL)	71.20±0.86	71.80±0.66	80.80±2.27@@	88.20±0.66@@	71.00±1.23
Serum cholesterol (mg/dL)	79.20±10.51	77.00±9.35	62.80±6.57	67.00±9.04	58.60±4.37
Triglycerides (mg/dL)	118.20±22.05	107.20±9.59	72.00±20.75	69.40±10.78	82.00±13.55
HDL (mg/dL)	62.20±6.73	54.60±5.63	48.00±4.57	40.00±5.57*	45.40±2.04*
SGPT (IU/L)	44.60±2.29	58.00±10.62	66.80±9.51	143.40±24.22@@	78.80±21.57
SGOT (IU/L	102.80±4.49	105.60±4.13	98.40±12.05	141.20±12.21@	97.00±8.74
ALP (IU/L)	93.20±3.28	93.60±12.58	85.80±10.81	98.20±3.02	94.60±1.72
Total bilirubin (mg/dL)	0.32±0.020	0.34±0.025	0.38±0.020	0.36±0.025	0.36±0.025
Direct bilirubin (mg/dL	0.22±0.02	0.20±0.00	0.22±0.02	0.22±0.04	0.18±0.04
Blood urea (mg/dL)	40.80±2.18	48.40±5.11	35.20±4.21	34.80±1.66	40.66±4.11
Creatinine (mg/dL)	0.86±0.02	0.92±0.04	0.70±0.07	0.80±0.3	0.86±0.05
Protein (g/dL)	6.88 ± 0.21	6.70 ± 0.16	6.43 ± 0.14	7.05 ± 0.22*	6.43 ± 0.21
Albumin (g/dL)	2.52±0.13	2.56±0.23	2.54±0.26	2.78±0.15	2.98±0.05*
Globulin (g/dL)	4.38±0.09	4.18±0.37	3.90±0.34	4.06±0.19	4.14±0.33
Uric acid (mg/dL)	0.80±0.08	0.74±0.07	0.72±0.14	0.62±0.04	0.98±0.27
Calcium (mg/dL)	11.36±0.17	11.32±0.48	9.90±0.36**	10.44±0.61	10.54±0.57

*P<0.05, **P<0.01 when compared to control group (Unpaired 't' test), @P<0.05, @@<0.01 when compared to control group (Annova followed by Dunnett's multiple 't' test)

3.6 Effect of VVR on Biochemical Parameters

No any significant changes were observed in serum biochemical parameters except blood sugar, HDL-cholesterol, SGPT, SGOT, serum albumin and calcium level in comparison to vehicle control group. A significant increase in blood sugar level was seen in VVRtreated group at TEDx5 and TED×10 dose levels in comparison to control group while same was not affected at TED and TEDx10 recovery groups. Administration of VVR produced significant decrease in the HDL-cholesterol level at TEDx10 dose level and TEDx10 (recovery study)dose levels however, at other dose levels did not produces any significant effect on HDL-cholesterol level in comparison to vehicle control group. Administration of test drug produced highly significant increase in the LDL cholesterol level at TEDx10 (recovery study) and insignificant increase in TEDx10 dose levels however, at other dose levels did not produces any significant change on LDL-cholesterol level in comparison to vehicle control group. A significant increase in SGPT and SGOT levels were seen in VVR at TEDx10 dose level in comparison to vehicle control group. VVR at other dose levels did not produce any significant effects on SGPT and SGOT level in comparison to vehicle control group. Administration of VVR produced significant decrease in the total protein level at TEDx10 dose level in main study while increase after discontinuation of drug in recovery study in comparison to control group. Administration of VVRproduced significant increase in the albumin level at TEDx10 in recovery study in comparison to control group. However, insignificant increase in albumin level was seen at TEDx10 in comparison to control group (Table 5).

3.7 Histopathology Study

The results of histopathological studies of heart, kidney, lungs, trachea, spleen, testis, lymph node, stomach, ileum, ovary, uterus, prostrate, seminal vesicles and adrenal from Vehicle control, *Vatavidhvamsana Rasa* at TEDx10 in main study and recovery study exhibits normal cytoarchitecture. Histopathological studies of the liver sections in the control group showed normal appearance of central vein (CV) and hepatic sinusoids (S) lined by endothelial cells (EC) with normal radiating hepatocytes. There was also normal appearance of the portal triad including hepatic portal vein, interlobular bile duct, and branches of hepatic artery. *Vatavidhvamsana Rasa* treated rats of TED×10 dose level and recovery (TED×10) group showed normal cytoarchitecture with some fatty changes (Figure 1). No adverse effects, major toxicity or behavioral changes were observed in VVR treated groups during course of chronic toxicity study (Figure 2, Figure 3, Figure 4 and Figure 5).

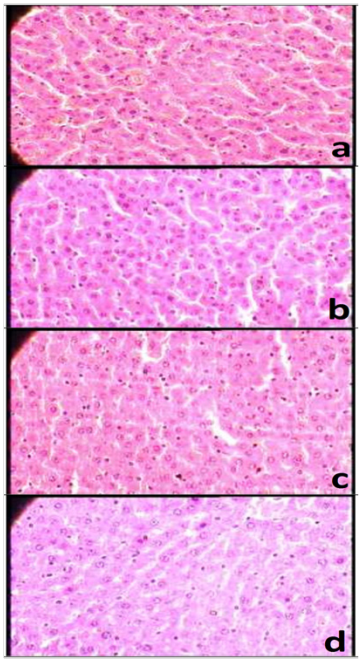


Figure 1. Photomicrographs of sections of the Liver **(a)** normal cytoarchitecture (control group) **(b)** normal cytoarchitecture (VVR, TED) **(c)** normal cytoarchitecture with some fatty changes (VVR, TEDx10) (d) normal cytoarchitecture (VVR, TEDx10 Recovery group) (x400 magnification).

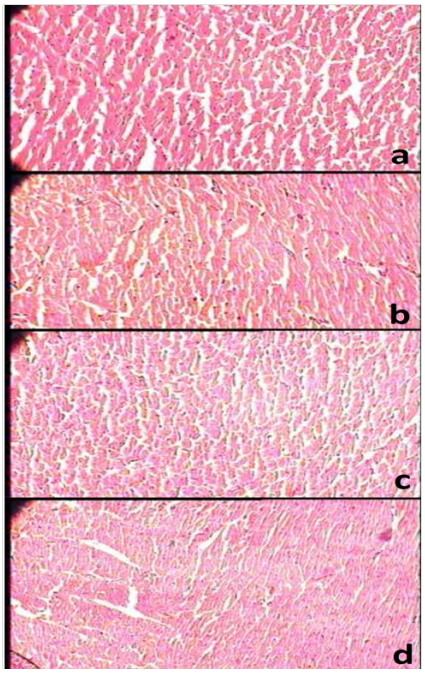


Figure 2. Photomicrographs of sections of the Heart **(a)** normal cytoarchitecture (control group) **(b)** normal cytoarchitecture (VVR, TED) **(c)** normal cytoarchitecture (VVR, TEDx10) **(d)** normal cytoarchitecture (VVR, TEDx10 Recovery group) (x200 magnification).

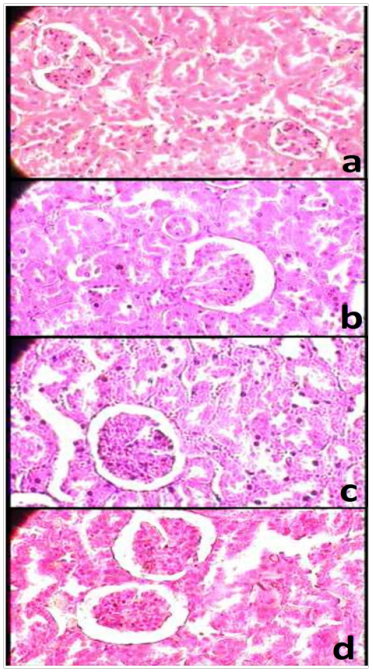


Figure 3. Photomicrographs of sections of the kidney **(a)** normal cytoarchitecture (control group) **(b)** normal cytoarchitecture (VVR, TED) **(c)** normal cytoarchitecture (VVR, TEDx10) **(d)** normal cytoarchitecture (VVR, TEDx10 Recovery group) (x400 magnification).

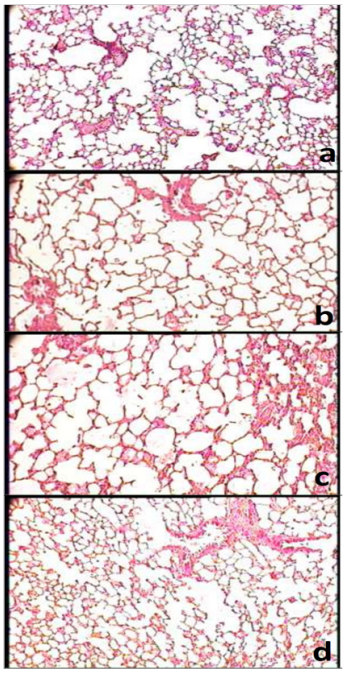


Figure 4. Photomicrographs of sections of the Lungs **(a)** normal cytoarchitecture (control group) **(b)** normal cytoarchitecture (VVR, TED) **(c)** normal cytoarchitecture (VVR, TEDx10) **(d)** normal cytoarchitecture (VVR, TEDx10 Recovery group) (x100 magnification).

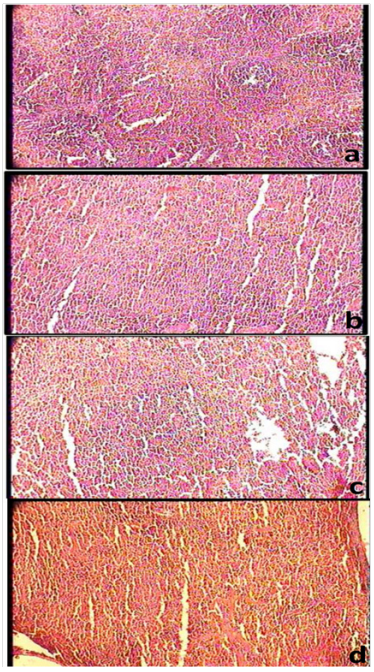


Figure 5. Photomicrographs of sections of the Spleen **(a)** normal cytoarchitecture (control group) **(b)** normal cytoarchitecture (VVR, TED) **(c)** normal cytoarchitecture (VVR, TEDx10) **(d)** normal cytoarchitecture (VVR, TEDx10 Recovery group) (x400 magnification).

4. Discussion

Vatavidhvamsana Rasa is a *KhalveeyaRasayana* medicine which was prepared with all quality control measures and safety.A toxicity study of single ingredients of VVR was conducted and was found safe in previous research works. Safety studies of *Naga Bhasma, Vanga Bhasma, LohaBhasma, Kajjali, TamraBhasma* were found safe in therapeutic dose in animals[38,39]. The acute toxicity study was performed according to the OECD guideline 425. This guideline of OECD recommends preferebly female experimental animals in acute toxicity study to minimize the number and to decrease the variability. Female animals were more sensitive when compared to males and hence acute toxicity study was assessed in females[40]. Bodyweight is considered as an indicator of health status of living beings. Whenever there is a loss in bodyweight by 10% with or without any other toxic symptoms, it is considered as a toxic dose of that test drug[41]. In acute toxicity study, a normal progressive weight gain was observed and which implicates that VVR is devoid of any toxic effects.

VVR at 2000 mg/kg oral dose did not produce any serious toxic symptoms and all animals survived 14 days without

any significant behavior changes. Further, VVR at 2000mg/kg did not produce any mortality and which suggests that the LD50 of VVR is more than 2000mg/kg. According to the recommendations of transport of toxic substances by United Nations, toxic substances are classified in to various divisions. As per UN Division 6.1-PGIII classification substances having oral LD50 of more than 2000 mg/kg is considered as low hazard potential[42]. So that VVRalong with honey can be categorized as a low health hazard potential substance.

In Chronic toxicity study of *Vatavidhvamsana Rasa* (VVR) has been carried out as per OECD 408 guidelines. The main study was of 90 days and recovery study of 120 days (90 days main study followed by a recovery period of 30 days). The toxicity profile observed concerning different types of parameters employed to assess the toxicity potential are summarized in tabular form for easy comparison. A significant increase in body weight was noted among VVR treated groups when compared to vehicle control group and it indicates normal progressive health status among experimental animals. A significant increase in body weight of albino rats was noted in VVR at TEDx10 dose in main and recovery studies in comparison to initial body weight. In TED and TEDx5 groups, there was a significant increase in body weight in comparison to initial body weight of albino rats. Bodyweight is an indicator of health status of any living being and its proper increase indicates a normal progressive health status of the animals[43]. The drug at all dose level shows a progressive and significant increase in body weight compared to the vehicle control group.

In relative organ weight, administration of VVR at TED, TEDx5 and TEDx10 dose levels produced non-significant effects on all important organs in comparison to control group. The drug did not affect the relative weightof heart, liver, spleen, kidney, prostate, seminal vesicles and uterus at the significant level at all dose level in comparison to the control group. The non-significant differences recorded in the organ weight indicating that the chronic oral administration of test drug for 90 days did not cause any deleterious effect on organs.

Analysis of the effects of VVR with adjuvant honey on hematological parameters revealed a non-significant decrease in WBC and platelet count at all dose levels. In haematological parameters, VVR atTEDx10 group showed a significant increase in lymphocyte count. However, these changes could not be classified as adverse effects of test drugs in rats because in recovery group the values are non-significant. All the values were within the normal range and suggesting drug is devoid of any serious hazardous effect.

A study conducted by Nikolic R *et al.* found a significant reduction in the values of erythrocytes, haemoglobin and hematocrit in lead and cadmium intoxication groups compared with the control group. In copper intoxication group there was a significant decrease in hematocrit, thrombocytes, and MCV values[44]. Significant decrease in haemoglobin was observed in VVR at TEDx5 dose level but same was insignificant at TEDx10 and Recovery group indicates the drug is safe to use. There was a significant increase in MCV in VVR treated groups at TEDx5 and TEDx10 dose levels and a significant increase in MCH at TEDx5 dose level. The increased MCV specifies macrocytic anemia (large average RBC size). A significant increase of MCHC was seen in VVR at TEDx5, TEDx10 and TEDx10 recovery dose levels. Elevation in the levels of RBC, MCV, MCH and MCHC was observed in higher dose levels however, these values are still within normal range and recovered after discontinuation of test drug in recovery study. The results of present study suggest the potential role of VVR to activate erythropoiesis without affecting its normal range. Platelet count level was not affected significantly at all the dose levels in comparison to the control group. This showed that even after administration of higher doses of the test drug, it did not produce any serious hematological toxicity.

Some studies carried out in patients suffering from Minamata disease (organic mercury poisoning) exposed that they had elevated levels of glucose in their urine[45]. Mercury exposure can promote DNA-damaging oxidative stress and can alter glucose levels. A significant increase in blood sugar level was observed at TEDx5 and TEDx10 dose level. But this was reversed in TEDx10 Recovery group indicated that the drug was not toxic and the side effects created by the test drug was reversible. So the effects cannot be categorized under deleterious/toxic effects of drugs on the glucose level. However, the drug should be used in diabetic patients with caution as it may cause hyperglycemia at a higher dose on longer administration.

Studies proved that exposure to Cd, Pb, Mn etc. significantly increase the concentration of Liver transaminase activity and bilirubin in male Wistar rats along with oxidative stress in rat liver. Histological injury, lipid peroxidation, glutathione depletion and mitochondrial dysfunction are the main hepatotoxicity observed in various rat models due to various heavy metals. Some studies evaluated combined cytotoxicity of heavy metal mixtures on human liver HL7702 cells and observed synergistic, antagonistic or additive effects of toxicity[46]. Liver contains the highest amount of SGPT in comparison to other tissues. Elevation of Serum SGPT is an indication of liver injury whenever there is any necrosis in hepatic cells[47]. Raised levels of

serum SGPT and SGOT are plausible markers of necrotic lesions in the hepatocytes cells[48,49]. An increase in serum transaminases suggests the drug has the potential to cause liver and heart tissue damage. A significant increase in SGOT and SGPT levels was seen in *Vatavidhvamsana Rasa* treated group at TEDx10 dose level however both are non-significantly altered in the recovery group. In histopathology examination liver shows only fatty changes without any necrotic changes. From this, we can interpret the injury that occurred to liver cells in higher dose is reversible. Further, in the present study test drug exhibited a non-significant change in the serum bilirubin and creatinine levels showing that the drug is devoid of any hepatic and renal toxicity. Also there was no significant change in the levels of blood urea, creatinine, uric acid and calcium in comparison to vehicle control group.

Proteins are the constructive elements of the body and the increase in the level of proteins can be taken as a sign of tissue or organ damage being repaired. Administration of *Vatavidhvamsana Rasa* produced a significant decrease in the total protein level at TEDx10 dose level in the main study while increase after discontinuation of drug in recovery study in comparison to the control group. Administration of *Vatavidhvamsana Rasa* produced a significant increase in the albumin level at TEDx10 in the recovery group in comparison to the control group. Suresh Kumar *et al* studied the level of lead toxicity in automobile workshop workers[50]. It was found out that a significant decrease in total protein level was present compared to control group but was within the reference range of healthy individuals. In the current study also total protein were significantly reduced in TEDx10 dose level but during the recovery period, it increased and were within the normal range.

Serum biochemical analyses helps to find out any hepatic and renal functional alterations while is also useful in screening the toxicity produced by test drugs for proving its safety. From HDL, cholesterol is transferred to VLDL, IDL, LDL and chylomicrons remnants with the aid of Cholesteryl Ester Transfer Protein (CEPT). Thus, cholesterol ends up finally in the liver[51]. A significant decrease in the HDL cholesterol was noted in TEDx10 group and TEDx10 (recovery study) group in comparison to the control group. A significant increase was noted in the LDL cholesterol level at TEDx10 (recovery study) and an insignificant increase in TEDx10 dose levels in comparison to control group. The decrease in HDL-cholesterol level and increase in LDL by test drug at a higher dose level suggests that it is better to use the test drug at therapeutic dose level. This also indicates that at higher dose levels, the test drug has effects on lipid turnover owing to its effect on the liver as observed by an increase in serum transaminase activity. However, the intensity of adverse effects was from mild to moderate grade as there were no significant effects observed on bilirubin and alkaline phosphatase levels which were within normal limits.

5. Conclusion

It is concluded that VVR at the dose level of 2000 mg/kg, orally did not produce any observable toxic effects and mortality in the acute toxicity study. This suggests that LD50 value of VVR may be higher than 2000 mg/kg by oral route. *Vatavidhvamsana Rasa* along with honey as an adjuvant at different dose levels has not produced any major toxic changes in albino rats during chronic toxicity study for 90 days with a recovery period of 30 days. This study demonstrates the safety of *Vatavidhvamsana Rasa* at therapeutic dose level in albino rats.

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7. Conflict of Interest

Nil

8. References

1. Ernst E. Toxic heavy metals and undeclared drugs in Asian herbal medicines. Trends Pharmacol Sci. 2002; 23(3):136–9. PMID: 11879681. https://doi.org/10.1016/S0165-6147(00)01972-6
2. Kumar A, Nair AG, Reddy AV, Garg AN. Bhasmas: unique ayurvedic metallic-herbal preparations, chemical characterization. Biol Trace Elem Res. 2006; 109(3):231–54. PMID: 16632893. https://doi.org/10.1385/BTER:109:3:231
3. Saper RB, Phillips RS, Sehgal A, Khouri N, Davis RB, Paquin J, Thuppil V, Kales SN. Lead, mercury, and arsenic in US and Indian-manufactured Ayurvedic medicines sold via the Internet. JAMA. 2008; 300(8):915–23. Erratum in: JAMA. 2008; 300(14):1652. PMID: 18728265. PMid:18728265 PMCid:PMC2755247. https://doi.org/10.1001/jama.300.8.915
4. Cooper K, Noller B, Connell D, Sadler R, Olszow H, Golding G *et al.* Public health risks from heavy metals and metalloids. Journal of Toxicology

and Environmental Health. 2007;70:1694–9. PMid:17763088. https://doi.org/10.1080/15287390701434885

5. Kumar, A, Garg, AN, Nair AGC, Reddy AVR. Availability of essential elements in bhasmas. Analysis of Ayurvedic metallic preparations by INAA. Hungary. https://doi.org/10.1007/s10967-006-0326-z
6. Chan K. Some aspects of toxic contaminants in herbal medicines. Chemosphere. 2003; 52(9):1361–71. PMID: 12867165. https://doi.org/10.1016/S0045-6535(03)00471-5
7. Mukherjee PK, Harwansh RK, Bahadur S, Banerjee S, Kar A, Chanda J, Biswas S, Ahmmed SM, Katiyar CK. Development of Ayurveda - Tradition to trend. J Ethnopharmacol. 2017; 197:10–24. Epub 2016 Sep 12. PMID: 27633405. https://doi.org/10.1016/j.jep.2016.09.024
8. Kushwaha H., editor. Charaka, Charaka Samhita, Chaukhamba Orientalia, Varanasi; 2010.
9. Kamath SU, Pemiah B, Sekar RK, Krishnaswamy S, Sethuraman S, Krishnan UM. Mercury-based traditional herbo-metallic preparations: A toxicological perspective. Arch Toxicol. 2012; 86(6):831–8. Epub 2012 Mar 23. PMID: 22441626. https://doi.org/10.1007/s00204-012-0826-2
10. Krishnamachary B, Rajendran N, Pemiah B, Krishnaswamy S, Krishnan UM, Sethuraman S, Sekar RK. Scientific validation of the different purification steps involved in the preparation of an Indian Ayurvedic medicine, Lauhabhasma. J Ethnopharmacol. 2012; 142(1):98–104. Epub 2012 Apr 26. PMID: 22561344. https://doi.org/10.1016/j.jep.2012.04.021
11. Kohli KR. Ayurvedic medicines and heavy metals issue. Ayurveda Herit 2005; 1:5–6.
12. Chauhan P. Ayurvedic Metallic Medicines are NOT FATAL (Part 1 to 3). Ayurveda for you. 2019. http://ayurveda-foryou.com/heavy_metals/ heavy_metals1.html
13. Chandra S. Ayurvedic research, wellness and consumer rights. J Ayurveda Integr Med. 2016; 7(1):6–10. Epub 2016 May 25. PMID: 27297503; PMCID: PMC4910573. https://doi.org/10.1016/j.jaim.2016.05.002
14. Dwivedi V, Anandan EM, Mony RS, Muraleedharan TS, Valiathan MS, Mutsuddi M, Lakhotia SC. *In Vivo* effects of traditional Ayurvedic formulations in *Drosophila melanogaster* Model Relate with Therapeutic Applications. Journal. PLOS. 2012; 7(5):1–14. PMid:22606337 PMCid:PMC3351451. https://doi.org/10.1371/journal.pone.0037113
15. Tripathi I, Sastri VL. Yogaratnakara. Varanasi: Chaukamba Krishnadas Academy; 1998.
16. Anonymous. The Ayurvedic Formulary of India. 1st ed. The Ministry of Health and Family Welfare, Dept. of Indian systems of Medicine and Homoeopathy, Part II. New Delhi: 2000.
17. Vaghabhata. Rasaratna Samuchaya. Varanasi: Chaukhambha Sanskrit Sansthan; 2012.
18. Krishnarama B, Manimala S. Varanasi: Chaukhamba Krishndas Academy; 2008.
19. Shastri K (Ed.), Sharma S, Rasatarangini. Delhi: Motilal Banarasidas Publication; 2009.
20. Paget GE, Branes JM. Toxicity tests. Laurence DR, Bocharach AL, Eds. Evaluation of drug activities: Pharmacometrics. New York: Academic Press; 1964. https://doi.org/10.1016/B978-1-4832-2845-7.50012-8
21. OECD (2008), Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264071049-en
22. OECD (2018), Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264070707-en
23. Pennock CA, Murphy D, Sellers J, Longdon KJ. A comparison of autoanalyser methods for the estimation of glucose in blood. Clin Chim Acta. 1973; 48(2):193–201. PMID: 4758882. https://doi.org/10.1016/0009-8981(73)90365-3
24. Roeschlau P, Bernt E, Gruber W. Enzymatic determination of total cholesterol in serum. Z Klin Chem KlinBiochem. 1974; 12(5):226. PMID: 4440114.
25. McGowan MW, Artiss JD, Strandbergh DR, Zak B. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. Clin Chem. 1983; 29(3):538–42. PMID: 6825269. https://doi.org/10.1093/clinchem/29.3.538
26. Tiffany TO, Jansen JM, Burtis CA, Overton JB, Scott CD. Enzymatic kinetic rate and end-point analyses of substrate, by use of a GeMSAEC fast analyzer. Clin Chem. 1972; 18(8):829–40. PMID: 5043271. https://doi.org/10.1093/clinchem/18.8.829
27. Slot C. Plasma creatinine determination. A new and specific Jaffe reaction method. Scand J Clin Lab Invest. 1965; 17(4):381–7. PMID: 583827 https://doi.org/10.3109/00365516509077065

28. Bradley DW, Maynard JE, Emery G, Webster H. Transaminase activities in serum of long-term hemodialysis patients. Clin Chem. 1972;18(11):1442. PMID: 4652847. https://doi.org/10.1093/clinchem/18.11.1442b
29. Gowda S, Desai PB, Hull VV, Math AA, Vernekar SN, Kulkarni SS. A review on laboratory liver function tests. Pan Afr Med J. 2009; 3:17. PMID: 21532726; PMCID: PMC2984286.
30. Tietz NW. editor. Text book of Clinical Chemistry. Philadelphia: WB Saunders and Company. 1986; 579. https://doi.org/10.1016/0307-4412(86)90182-2
31. Doumas BT, Arends RL, Pinto PC in standard methods of clinical chemistry. Academic Press Chicago. 1972; 7:175–89. https://doi.org/10.1016/B978-0-12-609107-6.50022-2
32. Wilkinson JH, Boutwell JH, Winsten S. Evaluation of a new system for the kinetic measurement of serum alkaline phosphatase. Clin Chem. 1969; 15(6):487–95. PMID: 5786804. https://doi.org/10.1093/clinchem/15.6.487
33. Pearlman FC, Lee RT. Detection and measurement of total bilirubin in serum, with use of surfactants as solubilizing agents. Clin Chem. 1974; 20(4):447–53. PMID: 4818198. https://doi.org/10.1093/clinchem/20.4.447
34. Burtis CA, Ashwood ER, Eds. Tietz Textbook of Clinical Chemistry, 3rd ed. Philadelphia, PA: WB Saunders. 1999; 1136.
35. Kabasakalian P, Kalliney S, Westcott A. Determination of uric acid in serum, with use of uricase and a tribromophenol-aminoantipyrine chromogen. Clin Chem. 1973; 19(5):522–4. PMID: 4703662. https://doi.org/10.1093/clinchem/19.5.522
36. Moorehead WR, Biggs HG. 2-Amino-2-methyl-1-propanol as the alkalizing agent in an improved continuous-flow cresolphthalein complexone procedure for calcium in serum. Clin Chem. 1974; 20(11):1458–60. PMID: 4421368. https://doi.org/10.1093/clinchem/20.11.1458
37. Raghuramulu N, Nair KM, Kalyanasundaram S. (Ed). A Manual of Laboratory Techniques, National Institute of Nutrition (NIN) Hyderabad India; 1983. p. 246–53.
38. Mahesh S, Patgiri BJ, Praveen Kumar KS. A review on *Vatavidhvamsana Rasa*, An Ayurvedic Herbo-mineral Preparation. Int. J. Res. Ayurveda Pharm. 2020; 11(4):143–6 http://dx.doi.org/10.7897/2277-4343.1104105
39. Mahesh S, Patgiri BJ. Praveen Kumar KS. Pharmaceutico-analytical study of *Vatavidhvamsana Rasa*. International Journal of Ayurvedic Medicine. 2020; 11(2):235–40. https://doi.org/10.47552/ijam.v11i2.1446
40. Lipnick RL, Cotruvo JA, Hill RN, Bruce RD, Stitzel KA, Walker AP, Chu I, Goddard M, Segal L, Springer JA, *et al.* Comparison of the up-and-down, conventional LD50, and fixed-dose acute toxicity procedures. Food Chem Toxicol. 1995; 33(3):223–31. PMID: 7896233. https://doi.org/10.1016/0278-6915(94)00136-C
41. Timbrell JA. Principles of Biochemical toxicology. Taylor and Francis Ltd., London; 1982.
42. Anonymous. Class 6-toxic and infectious substances. UN recommendations on the transport of dangerous goods-model regulations. Part-2. 12th revised ed. Ch. 2.6. Geneva: United Nations Publications; 2001.
43. Heywood, "Long term toxicity," in Animals and Alternatives in Toxicity Testing, M. Balls, R. J. Riddell, and A. N. Worden, Eds., Academic Press, London, UK;1983.
44. Nikolic R, Krstic N, Jovanovic J, Kocic G, Cvetkovic TP, Radosavljevic-Stevanovic N. Monitoring the toxic effects of Pb, Cd and Cu on hematological parameters of Wistar rats and potential protective role of lipoic acid and glutathione. Toxicol Ind Health. 2015; 31(3):239–46. Epub 2013 Jan 4. PMID: 23293128. https://doi.org/10.1177/0748233712469652
45. Khan AR, Awan FR. Metals in the pathogenesis of type 2 diabetes. J Diabetes MetabDisord. 2014;13(1):16. PMID: 24401367; PMCID: PMC3916582. https://doi.org/10.1186/2251-6581-13-16
46. Rice KM, Walker EM Jr, Wu M, Gillette C, Blough ER. Environmental mercury and its toxic effects. J Prev Med Public Health. 2014; 47(2):74–83. PMid:24744824 PMCid:PMC3988285. https://doi.org/10.3961/jpmph.2014.47.2.74
47. Philips CA, Paramaguru R, Augustine P. Ayurveda metallic-mineral 'Bhasma'-associated severe liver injury. BMJ Case Rep. 2018. PMid:29960971 PMCid:PMC6040517. https://doi.org/10.1136/bcr-2018-225590
48. Satyanarayana U, Chakrapani U. Biochemistry. 4th ed. Elsevier: New Delhi; 2013.
49. Amin A, Hamza AA. Oxidative stress mediates drug-induced hepatotoxicity in rats: A possible role of DNA fragmentation. Toxicology. 2005; 208(3):367–75. PMID: 15695022. https://doi.org/10.1016/j.tox.2004.11.039

50. Pachathundikandi SK, Varghese ET. Blood zinc protoporphyrin, serum total protein and total cholesterol levels in automobile workshop workers in relation to lead toxicity: Our experience. Indian J Clin Biochem. 2006; 21(2):114–7. PMid:23105626 PMCid:PMC3453998. https://doi.org/10.1007/BF02912924

51. Gad SC. The rat: Pathology. Gad SC, Chengellis CP.Eds. Animal Models in Toxicology. New York: Marcel Dekker; 1992. p.478. https://doi.org/10.1002/jat.2550130517