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Assessment of dermal toxicity of mustard oil based formulation of *Nerium indicum* Mill.

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Nerium indicum Mill. with red flowers belong to family *Apocynaceae* is considered as botanical source of *Rakta Karavir*a drug of *Upavisha* (semi-poisonous) category of Ayurvedic Pharmacopeia of India. Till date *Rakta Karavira Taila* (mustard oil based formulation of root bark of *Rakta Karavira*) has not been evaluated pharmacologically. In present study, acute and repeated dose 28 day dermal toxicity studies of *Rakta Karavira Taila* was carried out in Charles foster albino rats. Acute dermal toxicity study was assessed at a single dermal application of drug at limit dose of 2000 mg/kg body weight of rats and observed for fourteen consecutive days. The repeated dose 28-day dermal toxicity study was assessed on repeated dermal applications at limit dose of 1000 mg/kg body weight of rats for 28 days consecutive days as per OECD guideline. The test medication was applied equally over a region covering rats body surface area of about 10%. Effects on body weight, haematological, ponderal changes, biochemical, and histological studies were used to evaluate the toxicity of the *Rakta Karavira Taila*. No mortality and toxicity were observed on single dermal application at limit dose level of 2000 mg/kg in acute dermal toxicity study. On repeated dermal applications of *Rakta Karavira Taila* at limit dose of 1000 mg/kg, showed statistically significant decrease in weight of testes, increase in WBC and platelet count while rest of hematological and biochemical parameters are not affected in comparison to control group. Histopathological study showed inflammation and fatty degenerative changes in liver and kidney, degenerative changes in seminiferous tubules and decrease in spermatogenesis in drug administered group. Medicated oil prepared out of root bark of *Nerium indicum* with red flower with mustard oil has potential to produce adverse effect particularly on male reproductive system therefore, should not be used for longer duration at higher dose level.

Keywords: Acute dermal toxicity, *Karavira*, *Nerium indicum*, *Rakta Karavira Taila*, Sub-acute dermal toxicity
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Karavira (*Nerium indicum* Mill.) with red flower belongs to family *Apocynaceae*, a plant from the *Upavisha* (semi-poisonous) category of Ayurvedic Pharmacopeia of India has been bestowed with many therapeutic indications through both internal and external applications[1]. One of the external applications includes the usage of oil prepared out of the root bark of *Shveta Karavira* (*Nerium indicum* with white flower) along with other ingredients in *Sarshapa Taila* (mustard oil) named as *Shveta karaviradya Taila*, through external application, for the treatment of *Kustha* (skin diseases)[2]. *Rakta* and *Shveta* variety of *Karavira* have been botanically identified as *Nerium indicum* Mill.[1] *Karavira* (*Nerium indicum* with red flower) is frequently available plant cultivated in various garden and road side, and regarded as botanical plant source of *Rakta Karavira*[1].

Due to their convenience and ease of administration, Ayurvedic medicines preparing from medicinal plants have gained widespread acceptance in comparison to raw plant components[3]. Some highly potent medicinal plants can cause toxicity if given in high doses over long periods of time. Many traditional medicinal plants are time tested and are possible to have harmful effects when administered in larger doses; hence, phytochemicals from these plants must be tested for safety before efficacy. Even if potential Ayurvedic formulations have proven effective in pharmacological research or clinical trials, their safety must be guaranteed in order to ensure the full therapeutic benefits[4].

In Ayurvedic classics, *Karavira* is highlighted for its wide range of therapeutic indications among which maximum formulations are indicated in case of

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Kushtha (skin disease) and *Shvitra* (leprosy)[5]. Considering the potential effects of *Karavira* (*Nerium indicum* Mill.) a plant of *Upavisha* category it is necessary to conduct safety study of its formulation, *Rakta Karavira Taila* (mustard oil based formulation of root bark of *Rakta Karavira*) which has yet to be scientifically evaluated. Therefore, in accordance with OECD norms, the present acute and repeated dosage 28 day dermal toxicity tests were performed in albino rats.

Materials and Methods

Animals

Male and female Charles Foster albino rats (200–20 g weight) and were nulliparous and not-pregnant were procured from the Institute's animal house. All of the animals were kept in standard husbandry settings, which included 23±3°C temperature, 50–60% relative humidity and 12 h light/dark cycles. The animals were given full access to "VRK" brand standard rat feed pellet feed from Kevel Sales Corporation, Vadodara, as well as water *ad libitum*. The Institutional Animal Ethics Committee provided its approval to experimental protocols (IAEC/22/2017/08) as per CPCSEA, India.

Collection and authentication of drug

The source plant of *Karavira* (*Nerium indicum* Mill.) with red flower were identified by local taxonomist with the help of different flora and its root were collected from local area nearby Jamnagar and were deposited to Pharmacognosy museum, with voucher specimen (No.- PHM 6265-6267/2017-18) for future reference. Root barks were removed manually, washed and shade dried and used for further study.

Test drug

Rakta Karavira Taila (mustard oil based formulation of root bark of *Rakta Karavira*), was used as a test drug in the present study for local application. Raw root bark from *Rakta Karavira* (7 Kg), root bark paste (1.1 Kg) and water (112 L) were used to make the oil. Oil obtained was approx. 6.5 L prepared from this mixture by following the classical guidelines of *Sneha Kalpana*[6].

Acute dermal toxicity study

According to OECD 402 guidelines, the study was carried out[7]. Twenty four hours prior to the test, hair clipper and trimmer machines were used to remove fur from the rat's dorsal portion of the trunk. Approximately 10% of the rats' body surface area was cleaned for the test drug application. For selecting the area to be cleared, the animal's weight was taken into consideration[8]. A uniform application of *Rakta Karavira Taila* (RKT) was made dermally at a limit dose of 2000 mg/kg over a surface area equal to around 10% of the total body surface area. During a 24 h exposure period, the test drug was kept in touch with the skin by wrapping it in porous gauze and non-irritating tape. The remaining test drug was cleaned up by washing with distilled water after the exposure period and then skin thoroughly dried. Each animal was kept under observation for 14 days and checked for toxic symptoms at 30 min, 4 h, and 24 h after the removal of the films. Throughout the experiment, the animals' mortality and morbidity were recorded. Body weight was measured prior to treatment, on days 1 and 7 and at the end of the experiment. Surviving rats were killed on day 15 and gross observations were taken.

Repeated dose 28 day dermal toxicity study

According to OECD 410 guidelines, the study was carried out[9]. Both male and female Charles Foster albino rats were divided into two groups of ten each (five males and five females per group). Group (I)- Conrol group (NC) and Group (II)- *Rakta Karavira Taila* (RKT) at limit dose (1000 mg/kg) treated rats. Twenty four hours prior to the test, hair clipper and trimmer machines were used to remove fur from the rat's dorsal portion of the trunk. Approximately 10% of the rats' body surface area was cleaned for the test drug application. For selecting the area to be cleared, the animal's weight was taken into consideration[8]. A uniform application of *Rakta Karavira Taila* (RKT) was made dermally at a limit dose of 2000 mg/kg over a surface area equal to around 10% of the total body surface area. The drug was applied for 28 consecutive days. During a daily 6 h exposure period, the test drug was kept in touch with the skin by wrapping it in porous gauze and non-irritating tape. The remaining test drug was cleaned with distilled water when the exposure period was over and skin was then thoroughly dried.

Once a week during study period, body weight was noted. The rats were fasted overnight at the end of the experiment, and the following day blood samples were taken from the retro-orbital plexus under light ether anesthesia and collected in a non-vacuum anticoagulant tube (nVAC tube, HXS Tech Co. Ltd., China). A haematological auto-analyzer (Milet Sesloesing Laboratory, MS-4, France) was used to

Table 1 — Effect of *Rakta Karavira taila* on weekly body weight of albino rats in repeated dose dermal toxicity study

Groups	Body weight (g)					
	Initial	7th Day	14th Day	21st Day	28th Day	% change
NC	233.5±6.824	246.3±7.519	246.8±8.055	260.5±9.850	263.9±9.828	13.01 ↑
RCT	246.2±6.243	239.6±8.207	252.9±8.388	265.4±11.644	262.4±11.165	6.58 ↑

Data: Mean±SEM, ↑-increase

test haematology parameters which included platelet count, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), total red blood cells (RBC), total white blood cells (WBC), differential leukocyte percentage, and haemoglobin. A fully automated biochemical random access analyzer (BS-200, Lilac Medicare Pvt. Ltd., Mumbai) was used to test biochemical parameters which includes blood sugar level (BSL)[10], total cholesterol[11], triglycerides[12], HDL-cholesterol[13], VLDL-cholesterol, urea[14], creatinine[15], SGPT[16], SGOT[17], total protein[18], albumin and globulin[19], total bilirubin[20], direct Bilirubin[21], uric acid[22] and calcium[23].

After taking blood samples, the animals were sacrificed at the end of study. The rats were observed for gross lesion and important organs were excised, cleared and wet weight of important organs were noted, afterwards organs such as skin, heart, liver, kidney, spleen, uterus, testis, seminal vesicle, prostate, lungs, stomach, trachea, adrenal gland, ileum, brain and pituitary were transferred to 10% buffered formalin for histopathologicalstudies[24].

Statistical analysis

The data are presented as Mean±SEM. Using Sigma Stat software (Windows version 3.5), the data were analyzed to find significant differences between groups at $p<0.05$ by Student 't' test for unpaired and paired data.

Results and Discussion

The acute and repeated dose 28 day dermal toxicity studies showed no animal deaths in the test drug treated group. Animal's cage-side behaviours, fur, eyes, mucous membranes, respiratory and circulatory systems, autonomic nervous systems, behavioural patterns, and other parameters were unchanged during study period. The *Rakta Karavira Taila* at limit dose level of 2000 mg/kg did not produce any observable toxicity in albino female rats on single dermal application in acute toxicity study.

In a 28-day dermal toxicity study, there was a non-significant rise in body weight in both the control group and the *Rakta Karavira Taila* treated groups at the maximum dose level of 1000 mg/kg. Statistically non-significant changes observed in body weight between control and drug applied groups (Table 1). Ponderal changes were recorded for nine organs of treated albino rats. No significant changes were observed in relative weight of kidney, spleen, heart, liver, seminal vesicles and prostrate in *Rakta Karavira Taila* treated group when compared to control group (Table 2). However, there was statistically significant decrease in weight of testes in *Rakta Karavira Taila* treated group which was corroborative with the observations of histopathological study of testes which showed testicular degeneration and partial and marked necrosis of tubular components with tubules depleted of germ cells in comparison to normal control group (Fig. 1).

Table 2 — Effect of *Rakta Karavira taila* on ponderal changes of albino rats in repeated dose dermal toxicity study

Groups	NC	RKT	% change
Heart (g/100 g)	0.292±0.009	0.297±0.011	1.71 ↑
Liver (g/100 g)	3.281±0.098	3.143±0.113	4.20 ↓
Kidney (g/100 g)	0.644±0.010	0.687±0.033	6.67 ↑
Spleen (g/100 g)	0.214±0.014	0.235±0.017	9.81 ↑
Uterus (g/100 g)	0.262±0.050	0.212±0.027	19.08 ↓
Testis (g/100 g)	0.842±0.023	0.578±0.100*	31.35 ↓
Seminal vesicle (g/100 g)	0.458±0.019	0.432±0.056	5.67 ↓
Prostrate (g/100 g)	0.174±0.010	0.174±0.017	0

Data: Mean±SEM, ↑-increase↓-decrease, *$p<0.05$ when compared to control group (Unpaired 't' test)

Triterpinoids, an active chemical constituent present in *N. oleander* reported for its antifertility activity in male albino rats. The oral administration of methanol extract of the stem of *N. oleander* affected male fertility through its anti-spermatogenic and anti-androgenic action[25]. *Thevetia nerrifolia*, a member of the *Apocyanaceae* family, has been shown to have anti-spermatogenic activity by decreasing total protein and sialic acid content of the testes, epididymides, seminal vesicle, and ventral prostate[26].

When comparing the drug-treated group to the control, a non-significant reduction in uterine weight was seen. The leaves of *Nerium oleander* were found to be lethal to females, inhibiting ovarian development and delaying maturation in the desert locust *Schistocerca gregaria*[27]. Such properties of

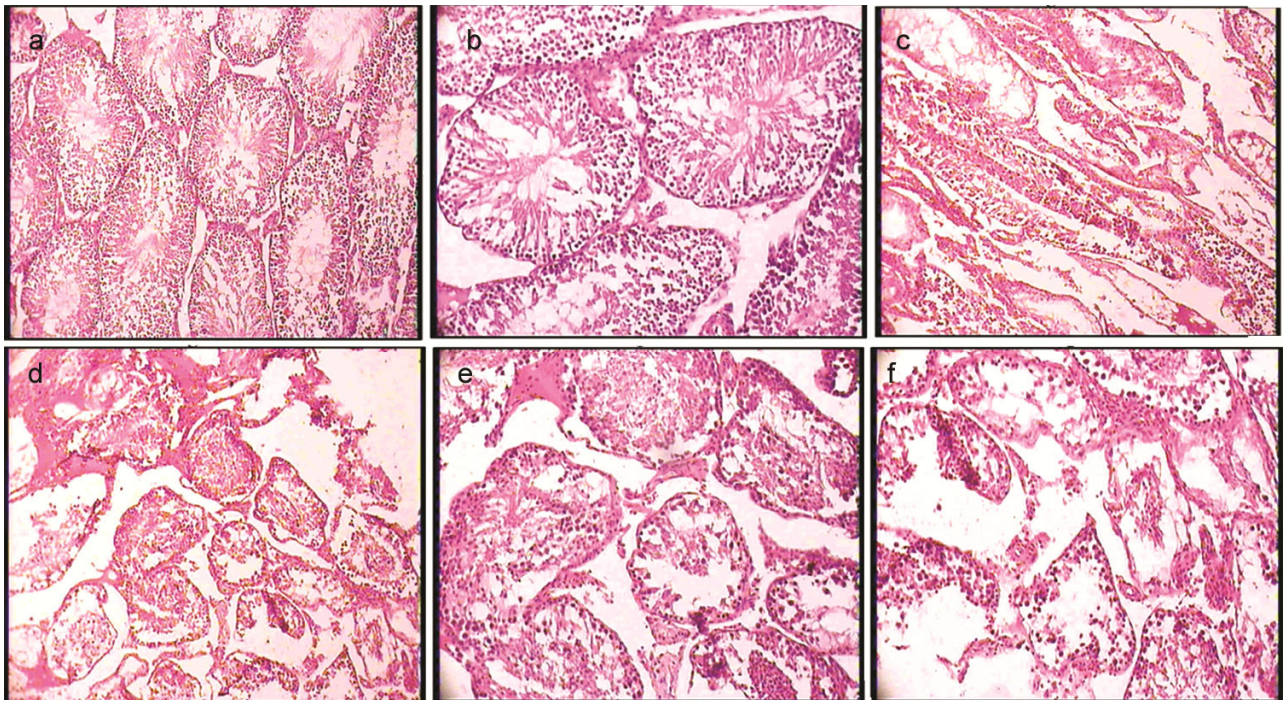

Fig. 1 — Photomicrograph of sections of Testes. (a and b) Normal cytoarchitecture (control group) (x100 and x200 magnifications) respectively; (c and d) (e and f) Testicular degeneration and partial and marked necrosis of tubular components with tubules depleted of germ cells (RKT treated group) (x100 and x200 magnifications) respectively

Nerium oleander might be probable cause of decrease in uterus weight but the histopathological study of uterus did not suggest any adverse changes or show any corroboration with decrease in uterus weight.

There were no significant changes in RBC and other related parameters and in differential counts in drug treated group. On repeated dermal application, rats treated with *Rakta Karavira Taila* showed a significant rise in total WBC and platelet counts when compared to the control group (Table 3). Aqueous extract of fresh leaves of *Nerium oleander* reported to induce haematological changes including increase in the PCV, RBC, hemoglobin, total WBC with neutrophillia and lymphopenia when injected subcutaneously in rabbit.[28] The above activities may be attributed to oleandrin, a similar phytoconstituent present in leaf as well as root bark.[29] The present study suggest the potential role of *Rakta Karavira Taila* on cellular constituents of blood on repeated application on skin of rats may due to absorption and cause systemic toxicity in rats.

Effect on biochemical parameters showed that, there were no significant changes in SGOT, total protein, albumin, globulin, bilirubin, uric acid, creatinine and calcium level in *Rakta Karavira Taila* treated group. All the biochemical parameters values within normal range (Table 4). However, there was statistically significant increase in cholesterol and highly significant increase in case of SGPT level in *Rakta Karavira Taila* treated group on repeated dermal application in rats when compared to control group. SGPT level gets increased in liver damage and in most of disorders of liver which may be consider as one of the considerable markers of liver function and hepatic cells injury or toxicity[30,31]. Further, non-significant increase in HDL-Cholesterol and blood urea levels along with non-significant decrease in

Table 3 — Effect of *Rakta Karavira taila* on hematological parameters of albino rats in repeated dose dermal toxicity study

Groups	NC	RKT	% change
Total WBC (K)	7820±447.909	10250±690.451**	31.07 ↑
Neutrophils (%)	20.2±1.604	25.9±3.784	28.21 ↑
Lymphocytes (%)	75.6±1.551	70.3±3.757	7.01 ↓
Eosinophil (%)	2.3±0.213	1.9±0.180	17.39 ↓
Monocytes (%)	1.9±0.277	1.9±0.233	0
Hb (gm/dL)	14.34±0.176	14.45±0.223	0.77 ↑
PCV (%)	45.45±0.814	45.94±0.652	1.08 ↑
Total RBC (M)	7.888±0.171	8.096±0.149	2.64 ↑
Platelet count (K)	1111.4±47.795	1288.1±45.529*	15.90 ↑
MCV (fL)	57.67±0.474	56.78±0.473	1.54 ↓
MCH (pg)	18.22±0.267	17.87±0.217	1.92 ↓
MCHC (g/dL)	31.57±0.254	31.48±0.223	0.28 ↓

Data: Mean±SEM, ↑-increase, ↓-decrease, *p<0.05, **p<0.01 when compared to control group (unpaired 't' test)

triglyceride and VLDL-cholesterol levels in drug treated group when compared to control group was observed. The above changes may be explained due to damage in liver cell after getting absorbed in systemic circulation on repeated dose of test drug topically which may affect the lipid metabolism[32] and increase the SGPT level in serum of treated rats. Increased level of SGPT in *Rakta Karavira Taila* treated group indicates adverse effects on liver of albino rats.

Table 4 — Effect of *Rakta Karavira Taila* on Biochemical parameters in albino rats during repeated dose dermal toxicity study

Group	NC	RKT	% change
Blood sugar (mg/dL)	118.6± 2.177	108.9±4.023	8.18% ↓
Cholesterol (mg/dL)	52.5±2.592	78.7±8.014*	49.90 ↑
Triglyceride (mg/dL)	115.9±18.432	82.600±8.256	28.73 ↓
HDL (mg/dL)	37.6±1.790	42.2±3.782	12.23 ↑
VLDL (mg/dL)	23.0±3.568	16.6±1.641	27.83 ↓
Blood urea (mg/dL)	46.8±1.444	51.1±2.834	9.19 ↑
Creatinine (mg/dL)	0.89±0.0875	0.78±0.0490	12.35 ↓
S.G.P.T. (IU/L)	67.5±3.215	154.1±10.688**	128.3 ↑
S.G.O.T. (IU/L)	151.6±6.353	150.2±10.515	0.92 ↓
Total protein (g/dL)	7.19±0.115	7.140±0.122	0.69 ↓
Albumin (g/dL)	3.52±0.108	3.38±0.160	3.97 ↓
Globulin (g/dL)	3.67±0.0978	3.81±0.205	3.81 ↑
Total Bilirubin (mg/dL)	0.24±0.016	0.31±0.035	29.16 ↑
Direct Bilirubin (mg/dL)	0.11±0.01	0.11±0.01	0
Uric Acid (mg/dL)	0.74±0.06	0.74±0.087	0
Serum Calcium (mg/dL)	9.98±0.238	9.62±0.169	3.60 ↓

Data: Mean±SEM, ↑-increase↓-decrease, *p<0.05, **p<0.01 when compared to control group (Unpaired 't' test)

The result of present study also corroborates with histopathological study of liver which indicate sinusoidal inflammation and fatty degenerative changes (Fig. 2). Decoction and intramuscular injection of *N. indicum* leaves reported to produce significant iron deposition in sinusoidal space within hepatocyte. This kind of iron buildup in the cells can cause reactive oxygen species to form, which can damage liver cells and tissues[32]. In kidney cytoarchitecture, test drug treated group showed inflammatory changes, mild diffusion with fatty degenerative changes (Fig. 3). Degenerative, deleterious and inflammatory histological changes in renal tissue causing nephrotoxicity could be caused by toxins and chemicals. It may interfering with the internal structure of the glomeruli and renal tubules, causing lysosomal enzyme leakage, oxidative stress and cell death pathway which may responsible for kidney function impairment[33].

Further histopathological studies of other organs such as heart, spleen, adrenal gland, lymph nodes, prostate, seminal vesicle, uterus, ovary, lungs,

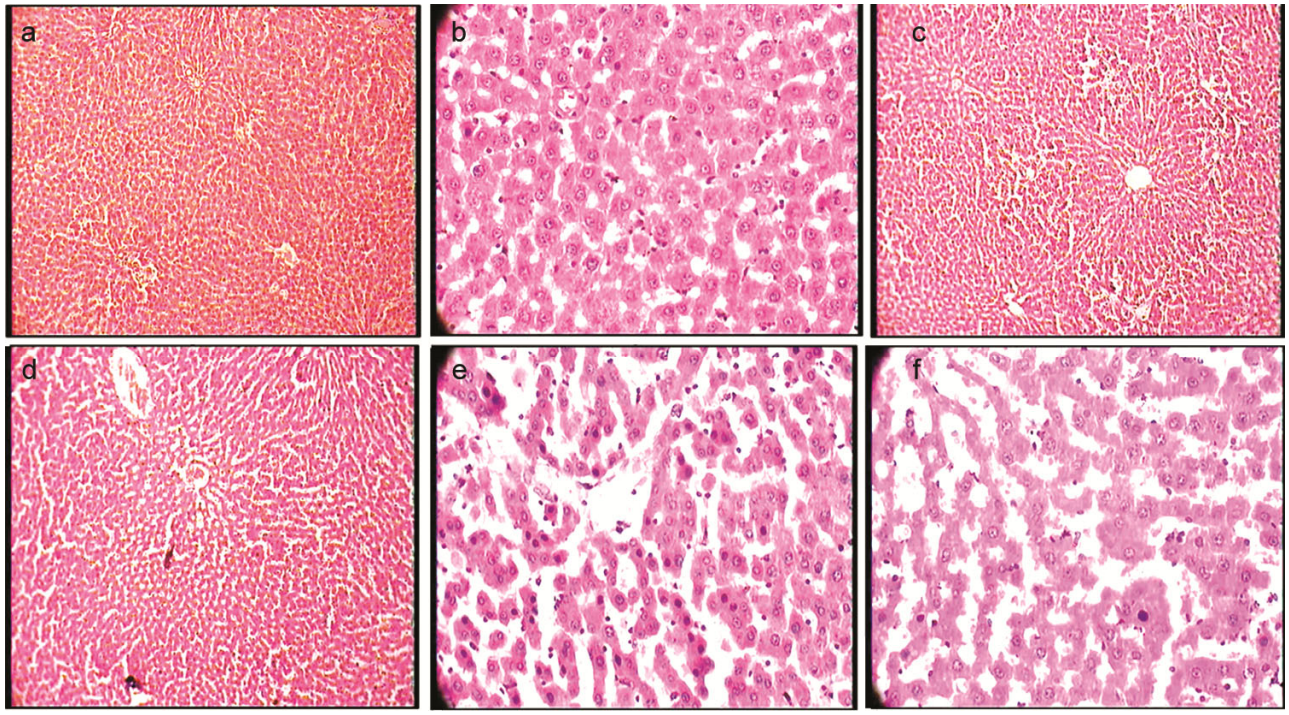

Fig. 2 — Photomicrograph of sections of Liver. (a and b) Normal cytoarchitecture (control group) (x100 and x400 magnifications) respectively; (c) Normal cytoarchitecture (RKT treated group) (x100 magnification); (d) (e and f) Sinusoidal inflammation and fatty degenerative changes (RKT treated group) (x100 and x400 magnifications) respectively

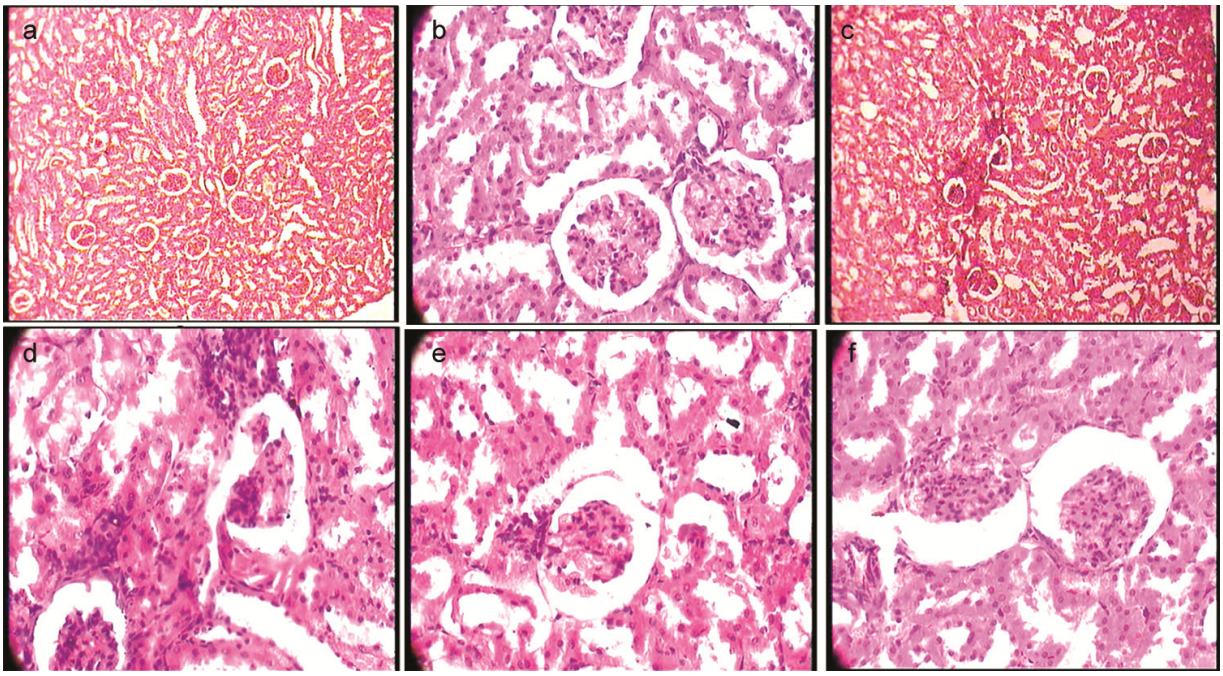

Fig. 3 — Photomicrograph of sections of Kidney. (a and b) Normal cytoarchitecture (control group) (x100 and x400 magnifications) respectively; (c and d) Cell infiltration and fatty degenerative changes (RKT group) (x100 and x400 magnifications) respectively; (e and f) Inflammatory changes (RKT group) (x400 magnification)

trachea, stomach and ileum did not show any significant changes in the cytoarchitecture of in *Rakta Karavira Taila* treated rats when compared to the corresponding sections from control group.

Conclusion

In acute dermal toxicity study, *Rakta Karavira Taila* at limit dose of 2000 mg/kg did not produce any mortality and any observable toxicity in albino female albino rats. The outcomes of repeated dose 28 day dermal toxicity study indicate that, *Rakta Karavira Taila,* on dermal application at the limit dose of 1000 mg/kg has potential to produce adverse effects on liver, kidney and male reproductive system in albino rats.

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Conflicts of Interest

The authors declare no conflicts of interest.

Author's Çontributions

The research was conceptualized by RA and MN. Experimental studies and writing support: VKP, RA, KA and MN. Experimental studies, original draft was prepared by RA and MN; Reviewed and edited by RA and MN while all the authors have read and approved the final manuscript.

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