

# Assessment of the systemic toxicity of *Laghu vishagarbha taila*, an Ayurvedic medicated oil formulation after dermal exposure

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*Laghu vishagarbha taila* (LVT) is a medicated oil preparation used in the Ayurvedic system of medicine and applied topically for the treatment of painful musculoskeletal and inflammatory disorders. It contains some mildly poisonous phytoconstituents which may show untoward effects upon application. The present study evaluated the toxicity of LVT in the acute, subacute, and subchronic dermal toxicity study in Wistar rats. LVT was tested for its compliance using physicochemical and analytical parameters as per standard methods prescribed in Ayurvedic Pharmacopoeia of India, while acute, subacute, and subchronic toxicity studies were carried out as per OECD 402, 410, and 411 guidelines, respectively. In the acute dermal toxicity study, a single dose of LVT (2000 mg/kg) was applied topically to rats, while in subacute and subchronic dermal toxicity study, the rats were topically applied LVT (1000 mg/kg) up to 28 and 90 days, respectively. LVT did not cause any alterations in clinical signs and no mortality or moribund stage was observed. The change in weekly body weight was insignificant compared with the vehicle control group. In subacute and subchronic dermal toxicity study, there were no significant changes in behavior, body weight, feed consumption, biochemical and hematological parameters, organ weight, and histological parameters compared with vehicle control rats. Topical application of single and repeated doses of LVT in rats did not exhibit adverse effects and suggests that the LD<sub>50</sub> of LVT is more than 2000 mg/kg in the acute dose and NOAEL is more than 1000 mg/kg/day in repeated dose application.

**Key words:** *Laghu vishagarbha taila*; *Datura metel*; *Aconitum ferox*; Ayurvedic formulation; dermal toxicity.

## Introduction

The topical application of drugs to the skin is an effective and popular route of administration, which causes local as well as systemic effects in the treatment of various disorders including local painful conditions. The topical route is additionally useful because it avoids first-pass effects and gastrointestinal irritation, and provides a sustained effect. Percutaneous absorption of drugs from topical formulations involves the permeation through the skin to reach the target tissue. In Ayurveda, a variety of medicines has been advocated for the treatment of painful musculoskeletal disorders. Medicated oil or *taila* is a traditional dosage form for topical application in the treatment of such disorders.

An Ayurvedic medicated oil, LVT is an official formulation mentioned in Ayurvedic Formulary of India [1]. LVT is useful in the treatment of “Vataroga” (inflammatory disorder), “Pakshaghata” (paralysis/hemiplegia), “Hanustambha” (lockjaw), “Manyastambha” (neck rigidity/torticollis), “Katigraha” (stiffness in the lumbo-sacral region), “Sarvangagraha” (stiffness and tightness in all limbs), and “Shirahkampa” (tremor/shaking of the head) [1, 2]. LVT contains about 10 herbal ingredients viz. *Sesamum indicum* L., seed oil; *Datura metel* L. Leaf juice; *Oryza sativa* L., husk decoction; *Saussurea lappa* (Decne.) Sch. Bip., roots; *Acorus calamus* L., rhizome; *Smilax china* L., Root; *Piper nigrum* L., fruits; *Aconitum ferox* Wall. ex Ser., roots, *D. metel* L., seeds; and sodium chloride (Table S1,

supplementary material). The ingredients, such as *D. metel* L. (leaf juice and seeds) and *A. ferox* Wall. ex Ser. (roots), are considered to be poisonous substances [3–6] and categorized as “upavisha” (mild poison) as per Ayurvedic Toxicology—“Agadantantra” [7–9]. In Ayurvedic traditional system of medicine, normally these medicinal plants are used in formulation only after their purification [10–12]. However, these substances, if not processed properly, may exhibit toxic or untoward reactions on long-term use [13–15]. Though there is a lack of scientific studies of the toxicity and adverse effects on LVT, it was thought very imperative to investigate the toxicological profile of this Ayurvedic medicated oil formulation on long-term dermal application. Furthermore, there are still no studies that have evaluated dose descriptors for LVT viz. LD<sub>50</sub>, No Observed Effect Level (NOEL), and No Observable Adverse Effect Level (NOAEL) which are required as per regulatory guidelines. The NOEL is the highest dose level at which and below which no effects of the test compound are observed among the evaluated parameters. A refinement of this concept is the use of the NOAEL which only takes into account effects that are regarded as adverse. Both these terms are sometimes used interchangeably. However, NOAEL is a more precise term to be used while communicating findings of the toxicity study as the “toxicity refers to examining ‘adverse effect’” as endpoint such as clinical or pathological findings [16, 17]. Thus, toxicity is aimed to derive the highest dose of test substance which causes detectable adverse alteration of morphology, functional capacity, growth, development, or lifespan of the target organism under defined conditions of exposure [18]. Higher LD<sub>50</sub> (for acute dose study) or NOAEL (for repeated dose toxicity studies) indicates lower systemic toxicity or lower chronic toxicity.

Hence, the present study was conducted to assess the toxicological profile of LVT by performing acute, subacute (28 days repeated dose), and subchronic (90 days repeated dose) dermal toxicity studies in rats according to Organization for Economic Cooperation and Development (OECD) guidelines 402, 410, and 411, respectively [19–21].

## Material and Methods

### Drugs and chemicals

LVT was procured from Indian Medicines Pharmaceutical Corporation Ltd., Mohan, Distt. Almora (Batch No. ATA66) and sesame oil was procured from the local market. Hematological reagents (Sysmex Corporation, Japan) such as cell pack, stromatolyser, sulfolyser and cell clean, and biochemical kits for estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), albumin, total bilirubin, total protein, urea, glucose, triglycerides, total cholesterol, phosphorus, and chloride were procured from Transasia Biomedicals Pvt. Ltd, Mumbai, India,

whereas biochemical kits for estimation of gamma-glutamyl transpeptidase (GGT), creatinine, calcium, sodium, and potassium were procured from Proton Biological India Pvt. Ltd, Bengaluru, India. All other common chemicals and reagents were procured from local scientific suppliers and of the highest purity grade available.

### Standardization of test drug—LVT

Before proceeding with the toxicity evaluation, the test drug—LVT was evaluated for various physicochemical parameters (weight/ml, acid value, saponification value, iodine value, and peroxide value) and safety parameters (heavy metals, pesticide residue, aflatoxins (B1, B2, G1, G2), and microbial load) as per standard methods [22].

### Experimental animals and ethical approval

Wistar rats (200–300 g) of both sexes were selected for the assessment of dermal toxicity. The animals were acclimatized to standard laboratory conditions of temperature 25 ± 2°C with relative humidity 55 ± 5% under 12-h light:12-h dark cycle. They were provided with regular rodent feed (Ashirwad brand, Chandigarh) and drinking water *ad libitum*. All the protocols and experiments were approved by the Institutional Animal Ethics Committee (Approval no. NRIASHRD-GWL/IAEC/2014/03) and conducted according to ethical guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India.

### Drug preparation

Both the test drug and sesame oil were in liquid form, they were applied as such, and no vehicle was required for any solubilization or dilution. The base oil of the LVT preparation is sesame oil, and hence, sesame oil was used as vehicle control.

### Topical application of test items

Approximately 24 h before the test, fur was removed from the dorsal area of the trunk of the test animals by clipping or shaving. Care was taken to avoid abrading the skin, which could alter the permeability of the test drug. The sesame oil and LVT were applied topically once in acute toxicity study and once daily up to 28 and 90 days in repeated dose study to individual animals of control and test group, respectively. The oils were applied using a soft painting brush uniformly over an area that is ~10% of the total body surface area. As much as the area was covered with thin and uniform film as possible. Oil was held in contact with the skin with a porous gauze dressing and nonirritating tape throughout a 24-h exposure period. The test site was further covered properly to retain the gauze dressing and oil and ensured that the animals did not ingest/lick the test substance. At the end of the exposure period, residual oil was removed.

### Acute dermal toxicity study

Acute dermal toxicity study of LVT was carried out in rats as per Organization for Economic Co-operation and Development (OECD) guidelines 402 [19]. A limit test at one dose level of 2000 mg/kg bodyweight was carried out in the control and test group as per guideline. A total of 20 rats were selected based on their body weight and randomly divided into two groups. Each group consisted of 10 animals (5 males and 5 females). Females were nulliparous and nonpregnant. Group I served as the vehicle control group which received sesame oil, while group II served as the test group that received LVT (2000 mg/kg), once topically. The animals were observed continuously for behavioral, neurological, and autonomic profiles for 2 h and after a period of 24, 48, 72 h and thereafter up to 14 days for any lethality, moribund state, or death. Cage side observations included changes in fur, eyes, and mucous membranes, and also respiratory, circulatory, autonomic, and central nervous systems, somatomotor activity, and behavior patterns. All animals were observed for morbidity and mortality twice daily. Particular attention was directed to the observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma. Body weights of each animal were recorded at the start of the study and thereafter at weekly intervals. Animals were sacrificed with an overdose of diethyl ether on the 15th day of the study and subjected to a detailed postmortem examination. The experimental design is mentioned in Fig. S1 (Supplementary material).

### Subacute (28 days repeated dose) and subchronic (90 days repeated dose) toxicity study

Subacute dermal toxicity study of LVT was carried out in rats as per OECD guidelines 410 [20]. A limit test at the dose level of 1000-mg/kg bodyweight was carried out in the control and test group as per the guideline. A total of 20 rats were selected in the subacute toxicity study based on the body weight and randomly divided into two groups. Each group consisted of 10 animals (5 males and 5 females). Females were nulliparous and nonpregnant. Group I (vehicle control group) received sesame oil, while group II (test group) received LVT topically up to 28 days.

Subchronic dermal toxicity study of LVT was carried out in rats as per OECD guidelines 411 [21]. A limit test at the dose level of 1000-mg/kg bodyweight was carried out in the control and test group as per the guideline. A total of 40 rats were selected in the subchronic toxicity study based on the body weight and randomly divided into two groups. Each group consisted of 20 animals (10 males and 10 females). Females were nulliparous and nonpregnant. Group I (vehicle control group) received sesame oil, while group II (test group) received LVT topically up to 90 days.

The following observations were made during study up to 28 days in subacute and 90 days in subchronic dermal toxicity study.

### Cage side observations

Rats were examined for clinical signs and mortality after application of the drug, at 5–10 min, 30–45 min, 1, 2, 4, 6, and 24 h followed by once daily throughout the study period. Cage side observations included changes in fur, eyes, and mucous membranes, and also respiratory, circulatory, autonomic, and central nervous systems, somatomotor activity, and behavior patterns [23]. All animals were observed for morbidity and mortality twice daily. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma.

### Body weight

The body weights of each animal were recorded at the start of the study and thereafter at weekly intervals.

### Feed consumption

The weekly feed consumption of rats was recorded by measuring the difference between feed offered and feed leftover in subsequent weeks.

### Blood collection

Blood collection was carried out to assess the changes in hematological and biochemical parameters in rats on day 29 in subacute and day 91 in subchronic dermal toxicity study. After overnight fasting, the rats were anesthetized under diethyl ether and blood samples were collected from the retro-orbital plexus. For hematology, blood was collected in K<sub>3</sub>EDTA (Potassium Ethylene Diamine Tetra Acetate) vacutainer, whereas for biochemical analysis, blood was collected in heparinized microcentrifuge tubes. The heparinized blood was allowed centrifugation at 4000 rpm for 15 min to obtain plasma. The plasma was used to assess the biochemical parameters.

### Hematological parameters

The hematological parameters such as white blood cells (WBC), red blood cells (RBC), hemoglobin (HBG), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelets (PLT), red cell distribution width (RDW), platelet distribution width (PDW), mean platelet volume (MPV), platelet large cell ratio (P-LCR), plateletcrit (PCT), neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO), and basophils (BASO) were evaluated by using fully automated hematology analyzer (Sysmex XT-2000iV™).

### Biochemical parameters

The plasma was analyzed for biochemical parameters such as Aspartate transaminase (AST), Alanine transaminase (ALT), alkaline phosphatase (ALP), GGT, urea, creatinine, albumin, total bilirubin, total protein, fasting glucose, triglycerides, cholesterol, calcium, phosphorus, chloride, sodium, and potassium by using semiautomated clinical chemistry analyzer-Macrolab 300 (Vital Scientific).

### Necropsy and histopathology

On the 29th day of the subacute study and the 91st day of the subchronic study, after the collection of blood, the animals were sacrificed with an overdose of diethyl ether and subjected to a detailed postmortem examination. The organs viz. brain, heart, lungs with trachea, kidneys, adrenals, liver, pancreas, spleen, testes/ovaries, epididymis, thymus, gastrointestinal tract (GIT), and skin were weighed and collected in 10% neutral buffered formalin. The fixed tissues were embedded in paraffin and cut into a 5- $\mu$ m-thin section and stained with hematoxylin–eosin dye and examined microscopically.

### Statistical analysis

The results were expressed as mean  $\pm$  S.E.M. All the data were analyzed by Student “t” test using Graph Pad Prism ver 4 software (GraphPad Software, San Diego, CA, USA). A value of  $P < 0.05$  was considered significant in all cases.

## Results

### Quality compliance evaluation of LVT

The results of all the physicochemical and analytical parameters are presented in Tables S2–S7 (Supplementary material). There was no detection of any heavy/toxic metals (Pb, As, Hg, and Cd), aflatoxins (B1, B2, G1, and G2), and pesticide residues observed in LVT. The total bacterial and fungal count in LVT was less than 10 cfu/g, while Enterobacteriaceae were found absent. All the results were within the standard limits prescribed in ASU (Ayurveda, Siddha, and Unani) guidelines [24].

### Clinical signs and mortality

The cage side observation showed no signs of alterations in any of the parameters of rats upon acute, subacute, and subchronic topical application of LVT compared with the vehicle control group. No mortality or moribund stage was observed throughout the study period.

### Effect on body weights

In the acute toxicity study, there was no significant ( $P > 0.05$ ) difference observed in the weekly body weight of both male and female rats of the test and vehicle control group (Table 1). In both subacute and subchronic dermal toxicity studies, the LVT treatment did not cause any significant ( $P > 0.05$ ) change in body weight of male and female rats compared with vehicle control rats (Table 2).

### Effect on feed consumption

The LVT-treated rats gained weight with age, and there was no significant change ( $P > 0.05$ ) in their body weight compared with vehicle control rats. It was observed that 28 and 90 days repeated application of LVT showed a nonsignificant ( $P > 0.05$ ) change in feed consumption in both male and female rats compared with vehicle control rats (Table 2). The feed intake was insignificantly ( $P > 0.05$ ) affected compared with vehicle control rats.

### Effect on hematological and biochemical parameters

The hematological parameters such as hematocrit, hemoglobin, erythrocyte count, total and differential leucocyte count and percent, platelet count, and other related corpuscular parameters remained nonsignificantly ( $P > 0.05$ ) altered by subacute and subchronic topical application of LVT compared with vehicle control rats (Table 3). In subacute and subchronic toxicity studies, LVT topical application did not cause any significant ( $P > 0.05$ ) change in biochemical parameters such as AST, ALT, ALP, GGT, urea, creatinine, albumin, total bilirubin, total protein, fasting glucose, triglycerides, cholesterol, calcium, phosphorus, chloride, sodium, and potassium compared with vehicle control rats (Table 4).

### Effect on gross morphological changes during necropsy

During necropsy of acute, subacute, and subchronic toxicity studies, no gross morphological changes were observed in skin and internal organs from LVT-treated test groups compared with vehicle control rats.

### Effect on weights and histopathology of organs

The repeated dose (28 and 90 days) dermal application of LVT did not cause any treatment-related effect on organ weights of male and female rats compared with the vehicle control group (Table 5). Histopathology evaluation of various organs/tissues viz. brain, heart, lungs with trachea, kidneys, adrenals, liver, pancreas, spleen, testes/ovaries, epididymis, thymus, and GIT of the vehicle control group and LVT-treated group did not reveal any treatment-related effect during 28 days repeated dose (Fig. 1) and 90 days repeated dose (Fig. 2) dermal toxicity study. The skin of control, as well as LVT-treated group revealed mild-to-moderate hyperkeratosis, which can be attributed to frequent rubbing due to application of oil for dermal application during the study.

## Discussion

There are different formulations available as Vishagrabha taila and used in clinical practice. Out of these, “laghu” (small) and “maha” (large) Vishagrabha taila are a classical Ayurvedic formulation. The prefix “laghu” or “maha” indicates the number of constituents present in the oil and also the therapeutic profile of the oil formulation. Other vishagarbha taila are proprietary products prepared by commercial firms and contain some similar ingredients. The name Visha in the formulation means “Poison” due to the presence of poisonous plant substances such as *Aconitum* and *Datura*. Aconitine and related alkaloids found in *Aconitum* species cause cardiotoxicity, neurotoxicity, and gastrointestinal toxicity. Cardiac manifestation includes feeble and irregular pulse, hypotension, and cardiac arrhythmia. Gastrointestinal manifestation includes nausea, vomiting, salivation, pain in the abdomen,

**Table 1.** Effect of acute dose of LVT on body weight (g) of rats

Days	Weekly body weight (g)			
	Male		Female	
	Vehicle Control	LVT	Vehicle Control	LVT
1	243.40 ± 2.785	242.60 ± 2.675	210.20 ± 4.872	206.20 ± 3.469
7	267.60 ± 3.749	268.20 ± 2.870	224.20 ± 7.445	211.20 ± 6.981
14	290.0 ± 3.619	288.00 ± 7.681	228.20 ± 7.958	218.40 ± 6.786

Values are expressed as mean ± SEM, n = 5. No significant differences were observed among the groups.

**Table 2.** Effect of 28 and 90 days repeated dose application of LVT on body weight and feed consumption of rats

Toxicity Study and Duration	Week	Weekly body weight (g)				Weekly feed consumption (g)			
		Male		Female		Male		Female	
		Vehicle Control	LVT	Vehicle Control	LVT	Vehicle Control	LVT	Vehicle Control	LVT
Subacute (28 Days repeated dose)	1st	263.4 ± 5.51	268.2 ± 6.55	210.0 ± 17.28	216.2 ± 13.37	168.6 ± 10.96	163.2 ± 17.10	213.8 ± 3.38	211.6 ± 3.27
	2nd	275.8 ± 6.58	285.4 ± 9.88	203.0 ± 5.03	216.4 ± 11.10	148.0 ± 5.64	159.0 ± 6.61	216.4 ± 3.68	215.4 ± 3.72
	3rd	283.0 ± 6.39	297.8 ± 7.14	217.0 ± 13.58	232.0 ± 11.77	166.2 ± 9.62	151.0 ± 10.62	220.4 ± 4.16	216.4 ± 4.26
	4th	293.6 ± 8.04	312.4 ± 9.88	176.0 ± 9.88	184.8 ± 6.53	124.8 ± 10.24	120.8 ± 12.06	223.8 ± 3.26	218.6 ± 6.06
Subchronic (90 Days repeated dose)	1st	267.5 ± 7.72	264.5 ± 7.82	222.9 ± 5.56	220.4 ± 5.40	187.0 ± 7.82	177.2 ± 6.80	135.4 ± 6.07	163.0 ± 7.54
	2nd	278.1 ± 7.45	269.5 ± 7.59	223.2 ± 5.61	222.9 ± 5.82	197.4 ± 11.11	170.9 ± 8.52	132.0 ± 8.63	161.1 ± 9.14
	3rd	285.8 ± 7.25	274.1 ± 7.64	223.8 ± 5.81	226.4 ± 6.25	202.0 ± 11.35	170.8 ± 9.55	139.2 ± 8.06	140.8 ± 9.52
	4th	296.9 ± 6.35	280.2 ± 8.06	227.3 ± 6.22	227.6 ± 6.34	194.2 ± 11.23	160.4 ± 11.03	134.7 ± 8.81	146.4 ± 8.09
	5th	306.0 ± 6.56	284.0 ± 7.80	229.4 ± 6.34	230.3 ± 6.59	189.6 ± 6.78	159.2 ± 9.48	134.5 ± 8.63	146.4 ± 8.63
	6th	316.5 ± 7.11	286.9 ± 9.65	231.3 ± 6.37	230.0 ± 7.61	177.2 ± 7.92	158.3 ± 11.53	129.6 ± 7.54	136.3 ± 8.81
	7th	323.2 ± 7.19	288.6 ± 9.66	231.9 ± 6.80	229.7 ± 7.37	172.0 ± 8.67	147.9 ± 10.70	124.1 ± 6.86	131.4 ± 6.99
	8th	329.5 ± 7.17	291.7 ± 10.24	231.4 ± 6.70	232.7 ± 7.66	162.2 ± 8.28	141.5 ± 8.29	117.8 ± 6.29	130.0 ± 6.27
	9th	334.7 ± 7.52	290.6 ± 10.57	231.6 ± 6.22	230.5 ± 7.30	160.8 ± 3.12	135.4 ± 7.65	107.2 ± 12.78	119.8 ± 7.02
	10th	341.4 ± 6.30	295.7 ± 11.29	233.2 ± 6.36	230.7 ± 7.32	162.3 ± 5.41	140.6 ± 9.13	116.9 ± 5.43	122.4 ± 8.52
	11th	345.6 ± 5.60	299.2 ± 10.07	235.2 ± 6.32	230.6 ± 8.18	160.0 ± 3.71	130.1 ± 6.55	111.6 ± 5.39	114.5 ± 7.81
	12th	351.5 ± 5.77	305.0 ± 9.94	237.6 ± 7.01	232.9 ± 8.21	158.5 ± 5.45	132.4 ± 4.68	112.6 ± 14.49	119.0 ± 7.73
	13th	353.2 ± 5.78	309.6 ± 9.40	240.3 ± 6.89	233.0 ± 8.21	118.9 ± 4.39	111.3 ± 2.79	85.40 ± 5.24	84.00 ± 5.37

Values are expressed as mean ± SEM (n = 5 in subacute dermal toxicity study and n = 10 in subchronic dermal toxicity study). No significant differences were observed among the groups.

tingling of tongue, mouth, and throat followed by numbness, while neurological manifestation includes vertigo, restlessness, headache, and giddiness. The causes of death in vatsanabha toxicity are ventricular arrhythmia, asystole, paralysis of the heart, and respiratory center [25]. The toxic manifestations of *Datura* leaves and seeds are called "Anticholinergic Toxicidrome" due to toxic effects of anticholinergic compounds such as atropine, scopolamine alkaloids which include mydriasis, dry mouth, tachycardia and fever, and erythema. These effects are dose-dependent and become more profound as the dose increases. Abusers of *Datura* for psychoactive effects experience the troublesome peripheral effects before the development of the psychoactive CNS effects (e.g. hallucinations). Both atropine and scopolamine produce CNS effects that include delirium, hallucinations, agitation, and excitation [4].

Hence, it becomes very essential to evaluate the toxicity of LVT, if these constituents are not purified and prepared as per the procedures mentioned in the Ayurvedic

classics. In the present study, initially, LVT was undertaken for toxicity assessment and to substantiate the safe use of LVT in clinical practices. The present investigation showed that LVT did not cause any deleterious effect on parameters observed during acute, and 28 and 90 days repeated dose dermal toxicity study.

The results of all the physicochemical and analytical parameters are presented in Tables S2–S7 (Supplementary material). The results of all the parameters were found within pharmacopoeial limits. Based on the results, the test item (LVT) is found quality complaint product.

In the acute dermal toxicity study, the test drug LVT did not cause signs of alterations in any of the parameters observed during 14 consecutive days of cage-side observation compared with vehicle control rats. No mortality or moribund stage was observed throughout the study period. There was no significant ( $P > 0.05$ ) difference observed in the weekly body weight of both male and female rats of the test and vehicle control group (Table 1).

**Table 3.** Effect of 28 and 90 days repeated dose application of LVT on hematological parameters

Parameters	Subacute (28 days repeated dose) dermal toxicity study				Subchronic (90 days repeated dose) dermal toxicity study			
	Male		Female		Male		Female	
	Vehicle Control	LVT	Vehicle Control	LVT	Vehicle Control	LVT	Vehicle Control	LVT
WBC ( $10^3 \mu\text{L}$ )	26.92 ± 4.81	24.95 ± 2.75	20.01 ± 1.88	18.83 ± 1.49	23.25 ± 1.85	17.34 ± 1.54	15.16 ± 1.62	14.81 ± 1.40
RBC ( $10^6 \mu\text{L}$ )	8.93 ± 0.14	8.95 ± 0.23	8.79 ± 0.09	8.44 ± 0.41	8.61 ± 0.21	8.47 ± 0.21	7.54 ± 0.23	7.59 ± 0.13
HGB (g/dL)	14.56 ± 0.51	15.04 ± 0.23	15.56 ± 0.07	14.72 ± 0.39	13.90 ± 0.34	13.96 ± 0.29	13.12 ± 0.38	13.31 ± 0.23
HCT (%)	41.24 ± 1.48	42.78 ± 0.73	43.78 ± 0.37	41.24 ± 0.87	40.60 ± 1.01	40.77 ± 0.87	38.86 ± 1.08	39.68 ± 0.64
MCV (fL)	46.14 ± 1.16	47.90 ± 1.33	49.82 ± 0.73	49.18 ± 1.65	47.19 ± 0.56	48.24 ± 0.58	51.59 ± 0.44	52.35 ± 0.63
MCH (pg)	16.28 ± 0.38	16.82 ± 0.35	17.70 ± 0.17	17.54 ± 0.49	16.11 ± 0.14	16.52 ± 0.19	17.41 ± 0.16	17.56 ± 0.20
MCHC (g/dL)	35.34 ± 0.24	35.18 ± 0.39	35.54 ± 0.24	35.68 ± 0.29	34.24 ± 0.19	34.22 ± 0.12	33.75 ± 0.12	33.54 ± 0.16
PLT ( $10^3 \mu\text{L}$ )	1183.4 ± 98.97	1277.6 ± 66.16	1020.2 ± 42.28	1249.8 ± 75.57	1371.8 ± 47.87	1307.1 ± 59.23	1238.7 ± 70.36	1147.1 ± 69.5
RDW-SD (fL)	31.06 ± 0.65	30.28 ± 1.68	25.48 ± 0.44	25.78 ± 0.78	28.55 ± 0.81	28.45 ± 0.70	27.40 ± 0.48	28.23 ± 0.75
RDW-CV (%)	21.52 ± 0.58	20.66 ± 0.52	17.14 ± 0.57	17.14 ± 0.80	19.99 ± 0.65	19.66 ± 0.50	17.26 ± 0.48	18.11 ± 0.56
PDW (fL)	9.24 ± 0.18	8.78 ± 0.29	9.48 ± 0.23	9.36 ± 0.08	7.99 ± 0.11	8.26 ± 0.17	7.98 ± 0.08	8.22 ± 0.14
MPV (fL)	7.76 ± 0.08	7.76 ± 0.16	8.30 ± 0.17	8.12 ± 0.08	7.18 ± 0.08	7.39 ± 0.104	7.28 ± 0.06	7.54 ± 0.10
P-LCR (%)	10.32 ± 0.60	10.24 ± 1.00	13.72 ± 1.26	12.56 ± 0.42	7.24 ± 0.39	8.25 ± 0.61	7.52 ± 0.32	9.05 ± 0.61
PCT (%)	0.92 ± 0.07	0.99 ± 0.05	0.85 ± 0.04	1.018 ± 0.07	0.99 ± 0.032	0.97 ± 0.039	0.90 ± 0.05	0.86 ± 0.05
NEUT ( $10^3 \mu\text{L}$ )	3.96 ± 0.69	3.13 ± 0.17	3.28 ± 0.56	3.854 ± 0.48	4.93 ± 0.90	3.47 ± 0.36	2.58 ± 0.36	2.99 ± 0.36
NEUT (%)	14.84 ± 1.52	12.92 ± 1.04	17.00 ± 3.33	20.42 ± 1.64	20.45 ± 2.41	20.42 ± 2.10	17.65 ± 2.11	20.39 ± 1.75
LYMPH( $10^3 \mu\text{L}$ )	11.08 ± 2.55	19.74 ± 2.53	13.05 ± 1.72	13.30 ± 1.18	16.85 ± 1.20	12.89 ± 1.28	11.63 ± 1.42	11.002 ± 1.14
LYMPH (%)	78.08 ± 2.24	78.64 ± 1.60	74.92 ± 3.22	70.54 ± 1.80	73.27 ± 2.66	73.82 ± 2.02	75.83 ± 2.43	73.85 ± 1.73
MONO ( $10^3 \mu\text{L}$ )	0.60 ± 0.012	0.69 ± 0.05	0.56 ± 0.10	0.43 ± 0.04	0.47 ± 0.14	0.23 ± 0.03	0.28 ± 0.05	0.24 ± 0.04
MONO (%)	2.18 ± 0.01	2.82 ± 0.15	2.72 ± 0.25	2.28 ± 0.08	1.86 ± 0.41	1.34 ± 0.103	1.79 ± 0.16	1.69 ± 0.26
EO ( $10^3 \mu\text{L}$ )	1.24 ± 0.28	1.36 ± 0.21	1.09 ± 0.23	1.03 ± 0.26	0.97 ± 0.12	0.76 ± 0.09	0.66 ± 0.07	0.56 ± 0.07
EO (%)	4.78 ± 0.85	5.50 ± 0.70	5.22 ± 0.64	6.68 ± 1.16	4.31 ± 0.55	4.30 ± 0.49	4.65 ± 0.48	3.91 ± 0.48
BASO ( $10^3 \mu\text{L}$ )	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.002	0.02 ± 0.004	0.02 ± 0.003	0.013 ± 0.004	0.03 ± 0.01
BASO (%)	0.12 ± 0.02	0.12 ± 0.02	0.14 ± 0.02	0.08 ± 0.02	0.11 ± 0.01	0.12 ± 0.01	0.08 ± 0.02	0.16 ± 0.05

Values are expressed in mean ± SEM (n = 5 in subacute dermal toxicity study and n = 10 in subchronic dermal toxicity study). No significant differences were observed among the groups.

During necropsy, no gross morphological changes were observed in skin and internal organs from test groups compared with vehicle control rats. This indicates that the approximate LD<sub>50</sub> of LVT is more than 2000 mg/kg.

In subacute and subchronic dermal toxicity studies, the cage side observation showed that LVT treatment did not cause any signs of alterations in the parameters observed during 28 and 90 consecutive days, respectively, compared with the vehicle control group. No mortality or moribund stage was observed throughout the study period. In both subacute and subchronic dermal toxicity studies, the LVT treatment did not cause any significant ( $P > 0.05$ ) change in body weight of male and female rats compared with vehicle control rats (Table 2). The increase or decrease in the body weight of animals can be used as an indicator of the adverse effects of drugs and chemicals [26]. The LVT-treated rats gained weight with age, and there was no significant change ( $P > 0.05$ ) in their body weight compared with vehicle control rats. It was observed that 28 and 90 days repeated dose topical application of LVT showed a nonsignificant ( $P > 0.05$ ) change in feed consumption in both male and female rats compared with vehicle control rats (Table 2). The feed intake was insignificantly ( $P > 0.05$ ) affected compared with vehicle control rats. It indicates that the LVT

treatment did not cause any alteration in cellular biosynthesis and metabolism which was also evident from the insignificant changes in glucose, cholesterol, and triglycerides levels [27].

The hematological parameters such as hematocrit, hemoglobin, erythrocyte count, total and differential leucocyte count and percent, platelet count, and other related corpuscular parameters remained nonsignificantly ( $P > 0.05$ ) altered by LVT treatment compared with vehicle control rats (Table 3). The changes in the hematological parameters have a higher predictive value for toxicity [28]. The subacute and subchronic treatment of LVT did not cause any significant alterations in the numbers of RBC, total and differential WBC and platelets, as well as packed cell volumes, and hemoglobin concentrations. It indicates LVT treatment did not cause any hematological abnormalities. The effect of LVT was studied on the parameters of liver and kidney functions. Repeated dose of LVT treatment did not cause any significant ( $P > 0.05$ ) change in biochemical parameters such as AST, ALT, ALP, GGT, urea, creatinine, albumin, total bilirubin, total protein, fasting glucose, triglycerides, cholesterol, calcium, phosphorus, chloride, sodium, and potassium compared with vehicle control rats (Table 4). The alterations in the levels of ALT, AST, ALP, GGT,

**Table 4.** Effect of 28 and 90 days repeated dose application of LVT on biochemical parameters

Parameters	Suacute (28 days repeated dose) dermal toxicity study				Subchronic (90 days repeated dose) dermal toxicity study			
	Male		Female		Male		Female	
	Vehicle Control	LVT	Vehicle Control	LVT	Vehicle Control	LVT	Vehicle Control	LVT
AST (IU/L)	113.34 ± 11.71	111.80 ± 11.42	108.10 ± 7.02	112.83 ± 10.62	106.07 ± 3.28	122.95 ± 8.41	109.79 ± 3.22	127.92 ± 6.13
ALT (IU/L)	47.44 ± 3.57	43.48 ± 1.70	39.77 ± 1.52	49.43 ± 4.83	44.94 ± 2.10	43.88 ± 2.74	38.45 ± 2.49	38.48 ± 2.62
ALP (IU/L)	161.20 ± 27.79	168.00 ± 24.80	89.60 ± 9.60	105.80 ± 12.33	165.3 ± 19.65	167.0 ± 21.11	136.1 ± 19.6	161.7 ± 20.77
GGT (IU/L)	1.24 ± 0.35	1.19 ± 0.27	1.11 ± 0.29	1.55 ± 0.38	1.42 ± 0.24	0.66 ± 0.25	0.93 ± 0.28	0.97 ± 0.15
T. Bilirubin (mg/dL)	0.55 ± 0.14	0.62 ± 0.22	0.50 ± 0.11	0.40 ± 0.16	0.50 ± 0.09	0.75 ± 0.06	0.50 ± 0.06	0.65 ± 0.08
Albumin (g/dL)	2.35 ± 0.51	2.49 ± 0.60	2.70 ± 0.64	2.84 ± 0.78	3.38 ± 0.09	3.29 ± 0.12	3.67 ± 0.18	3.59 ± 0.11
T. proteins (g/dL)	6.60 ± 0.15	6.72 ± 0.29	7.15 ± 0.24	7.25 ± 0.29	6.02 ± 0.23	5.83 ± 0.21	6.46 ± 0.24	5.85 ± 0.27
Urea (mg/dL)	46.10 ± 4.63	43.88 ± 2.93	47.11 ± 2.99	41.13 ± 3.97	41.71 ± 2.204	44.49 ± 2.87	45.35 ± 3.66	46.65 ± 4.33
Creatinine (mg/dL)	0.42 ± 0.017	0.43 ± 0.02	0.46 ± 0.02	0.44 ± 0.03	0.59 ± 0.01	0.61 ± 0.01	0.60 ± 0.02	0.62 ± 0.02
Glucose (mg/dL)	124.93 ± 5.59	109.36 ± 8.39	120.61 ± 9.62	117.92 ± 12.72	121.11 ± 3.94	102.32 ± 4.41	129.16 ± 4.16	118.82 ± 5.35
Triglyceride (mg/dL)	33.53 ± 2.17	37.95 ± 7.21	39.23 ± 7.30	27.85 ± 4.60	84.95 ± 12.39	76.36 ± 13.63	85.98 ± 10.05	64.91 ± 4.83
Cholesterol (mg/dL)	32.00 ± 2.17	30.40 ± 4.27	44.00 ± 4.37	34.00 ± 5.57	47.80 ± 2.56	52.00 ± 3.72	49.50 ± 3.99	46.20 ± 3.67
Sodium (mEq/L)	130.28 ± 2.27	136.65 ± 4.68	134.49 ± 2.94	133.94 ± 2.58	158.35 ± 1.45	157.07 ± 1.67	160.51 ± 1.94	158.77 ± 1.36
Potassium (mEq/L)	3.73 ± 0.14	4.08 ± 0.29	3.37 ± 0.20	3.49 ± 0.16	4.86 ± 0.17	4.93 ± 0.19	4.70 ± 0.18	4.69 ± 0.28
Calcium(mg/dL)	10.44 ± 0.35	10.94 ± 0.68	11.40 ± 0.85	11.35 ± 1.16	10.33 ± 0.79	10.40 ± 0.29	10.29 ± 0.48	9.81 ± 0.47
Chloride (mEq/L)	112.27 ± 6.90	96.87 ± 6.64	112.83 ± 8.48	105.00 ± 2.91	106.67 ± 2.53	105.04 ± 2.03	101.43 ± 3.05	109.65 ± 7.61
Phosphorus (mg/dL)	6.97 ± 0.52	7.95 ± 0.50	6.49 ± 0.43	5.70 ± 0.46	6.24 ± 0.48	6.23 ± 0.34	6.25 ± 0.67	5.81 ± 0.34

Values are expressed in mean ± SEM (n = 5 in subacute dermal toxicity study and n = 10 in subchronic dermal toxicity study). No significant differences were observed among the groups.

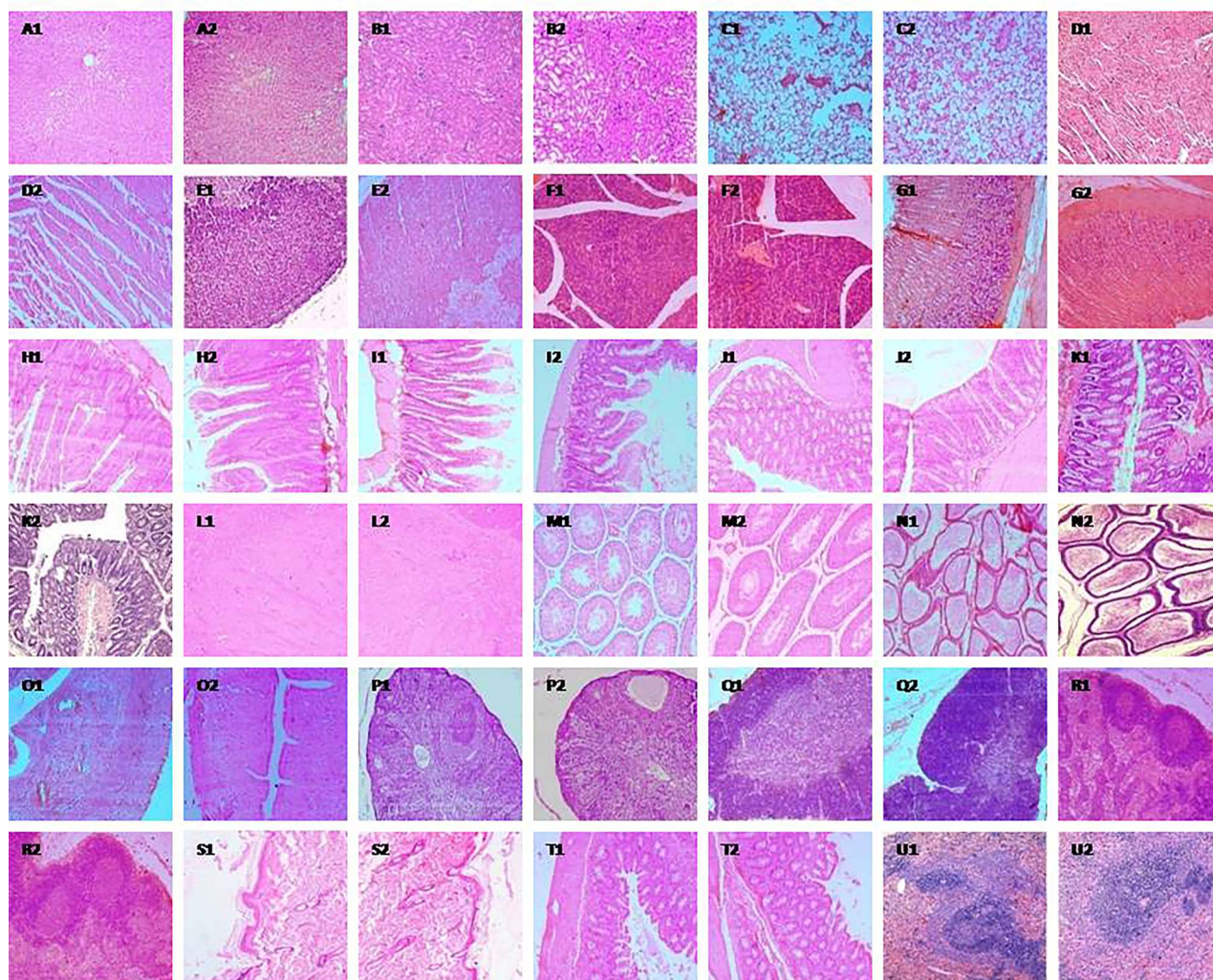
**Table 5.** Effect of 28 and 90 days repeated dose application of LVT on organ weight (g) of rats

Parameters	Subacute (28 days repeated dose) dermal toxicity study				Subchronic (90 days repeated dose) dermal toxicity study			
	Male		Female		Male		Female	
	Vehicle Control	LVT	Vehicle Control	LVT	Vehicle Control	LVT	Vehicle Control	LVT
Liver	8.62 ± 0.54	8.20 ± 0.19	6.99 ± 0.32	5.81 ± 0.30	9.89 ± 0.39	8.20 ± 0.48	6.40 ± 0.25	5.93 ± 0.24
Kidney	1.98 ± 0.03	1.91 ± 0.09	1.56 ± 0.10	1.31 ± 0.04	2.07 ± 0.05	1.76 ± 0.08	1.41 ± 0.06	1.34 ± 0.06
Adrenal	0.05 ± 0.004	0.05 ± 0.004	0.05 ± 0.005	0.04 ± 0.006	0.08 ± 0.03	0.04 ± 0.002	0.05 ± 0.003	0.05 ± 0.002
Spleen	1.05 ± 0.11	0.77 ± 0.07	0.72 ± 0.04	0.68 ± 0.07	1.02 ± 0.07	0.97 ± 0.08	0.77 ± 0.05	0.70 ± 0.02
Heart	0.91 ± 0.03	0.97 ± 0.05	0.71 ± 0.04	0.72 ± 0.03	1.04 ± 0.04	0.87 ± 0.03	0.79 ± 0.03	0.77 ± 0.04
Thymus	0.30 ± 0.03	0.24 ± 0.03	0.20 ± 0.007	0.21 ± 0.01	0.24 ± 0.01	0.24 ± 0.02	0.20 ± 0.02	0.18 ± 0.02
Brain	1.89 ± 0.03	1.90 ± 0.02	1.88 ± 0.02	1.85 ± 0.03	1.88 ± 0.03	1.78 ± 0.05	1.83 ± 0.03	1.85 ± 0.02
Testis	2.67 ± 0.05	2.78 ± 0.05	-	-	2.89 ± 0.05	3.00 ± 0.09	-	-
Ovary with uterus	-	-	3.79 ± 0.33	3.75 ± 0.67	-	-	7.18 ± 0.65	5.62 ± 0.42
Epididymus	4.20 ± 0.17	4.57 ± 0.23	-	-	6.41 ± 0.19	5.97 ± 0.27	-	-

Values are expressed in mean ± SEM (n = 5 in subacute dermal toxicity study and n = 10 in subchronic dermal toxicity study). No significant differences were observed among the groups.

and total protein reflect the liver dysfunction [29], while any change in the level of creatinine and urea may cause deleterious effects on renal function [30]. Repeated dose of LVT treatment did not produce any hepatotoxicity as there was a nonsignificant change in ALT, AST, ALP, GGT, and total protein compared with vehicle control rats. Furthermore, no renal toxicity

was seen due to LVT treatment as evident from the nonsignificant change in the levels of creatinine and urea. Sodium, calcium, potassium, chlorine, phosphate, and magnesium are intracellular ions that play an important role in maintaining homeostasis within the body [31]. They help to regulate muscle contraction, heart and neurological function, fluid balance, oxygen



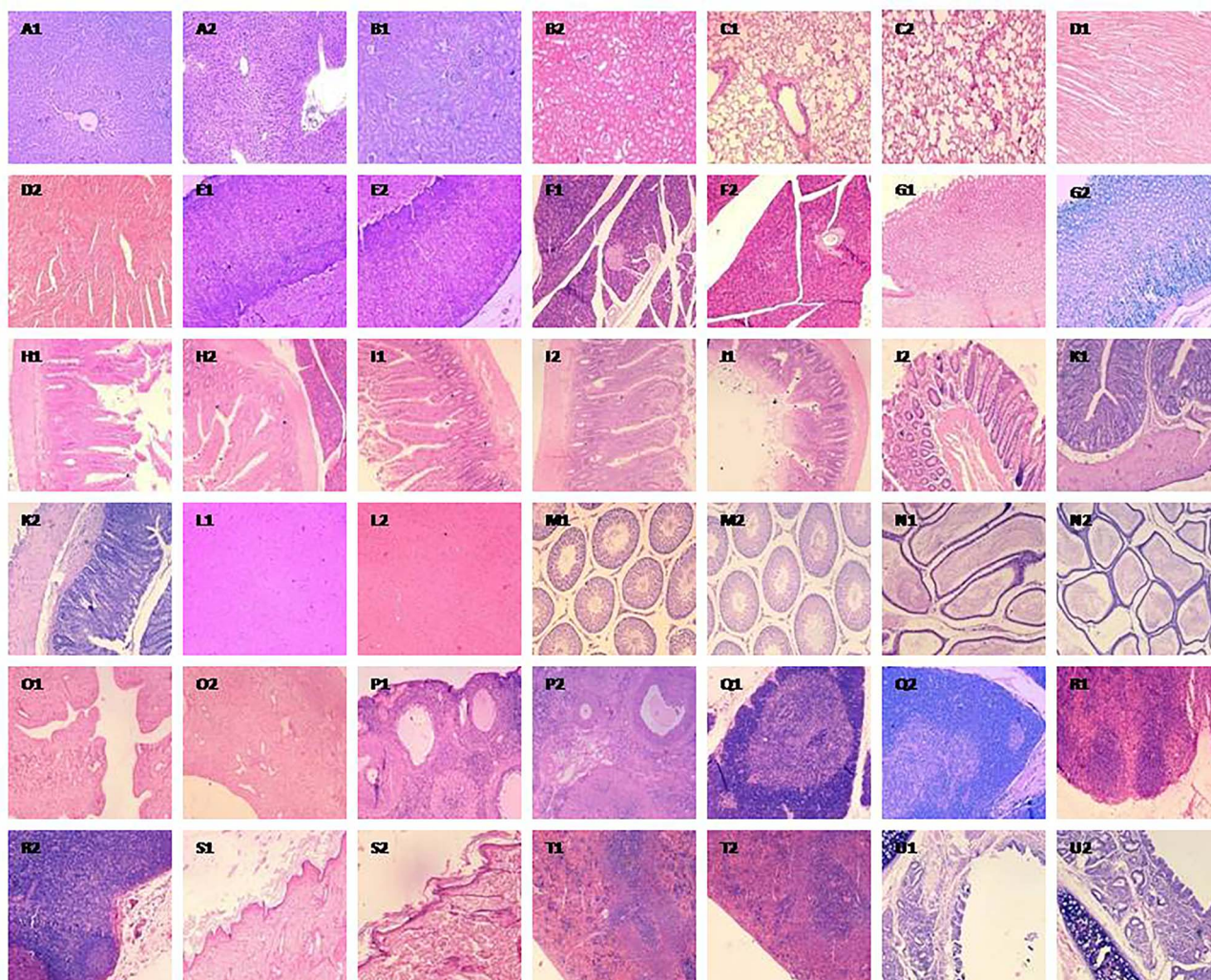
**Figure 1.** Photomicrograph of histopathology of organs/tissues of vehicle control (A1–U1) and LVT-treated (A2–U2) rats in subacute (28 days repeated dose) dermal toxicity study; no treatment-related pathological changes were found in Liver (A1 & A2), Kidney (B1 & B2), Lungs (C1 & C2), Heart (D1 & D2), Adrenals (E1 & E2), Pancreas (F1 & F2), Stomach (G1 & G2), Duodenum (H1 & H2), Jejunum (I1 & I2), Colon (J1 & J2), Rectum (K1 & K2), Brain (L1 & L2), Testis (M1 & M2), Epididymis (N1 & N2), Uterus (O1 & O2), Ovary (P1 & P2), Thymus (Q1 & Q2), Lymph node (R1 & R2), Skin (S1 & S2), Cecum (T1 & T2), and Spleen (U1 & U2); the letters A–U represent the specific organ as listed above and the letter (A–U) with numeric 1 represents the organ of vehicle control group, while 2 represents the organ of LVT-treated group; the histopathology of organs of LVT-treated group were compared with vehicle-treated control group; magnification:  $\times 400$ .

delivery, acid–base balance, and much more [32, 33]. The treatment with LVT did not cause any significant differences in the plasma level of sodium, calcium, potassium, chloride, and phosphate compared with vehicle control rats. It indicates that topical application of LVT did not lead to electrolyte disturbances and also did not produce any deleterious effect on muscle contraction.

During necropsy, no gross morphological changes were observed in the organs collected from LVT-treated groups compared with organs from vehicle control rats. The repeated dose (28 and 90 days) dermal application of LVT did not cause any treatment-related effect on organ weights of male and female rats compared with the vehicle control group (Table 5). Histopathology evaluation of various organs/tissues viz. brain, heart, lungs with trachea, kidneys, adrenals, liver, pancreas, spleen, testes/ovaries, epididymis, thymus, and GIT

of the vehicle control group and LVT-treated group did not reveal any treatment-related effect during 28 days repeated dose (Fig. 1) and 90 days repeated dose (Fig. 2) dermal toxicity study. The skin of control, as well as LVT, the treated group revealed mild-to-moderate hyperkeratosis, which can be attributed to frequent rubbing due to the application of oil for dermal application during the study. But there was no test drug treatment-related increase in hyperkeratosis or any other associated skin lesion observed. Thus, there were no treatment-related histopathological changes seen in any of the organs and indicates that LVT did not produce any adverse effect on the function of the cardiovascular system (heart), central nervous system (brain), respiratory system (lungs and trachea), endocrine system (adrenal and thymus), immune system (thymus), digestive system (liver and GIT), reproductive system (testes/ovaries and epididymis), and skin.





**Figure 2.** Photomicrograph of histopathology of organs/tissues of vehicle control (A1–U1) and LVT-treated (A2–U2) rats in subchronic (90 days repeated dose) dermal toxicity study; no treatment-related pathological changes were found in Liver (A1 & A2), Kidney (B1 & B2), Lungs (C1 & C2), Heart (D1 & D2), Adrenals (E1 & E2), Pancreas (F1 & F2), Stomach (G1 & G2), Duodenum (H1 & H2), Jejunum (I1 & I2), Colon (J1 & J2), Rectum (K1 & K2), Brain (L1 & L2), Testis (M1 & M2), Epididymis (N1 & N2), Uterus (O1 & O2), Ovary (P1 & P2), Thymus (Q1 & Q2), Lymph node (R1 & R2), Skin (S1 & S2), Spleen (T1 & T2) and Trachea (U1 & U2); the letters A–U represent the specific organ as listed above and the letter (A–U) with numeric 1 represents the organ of vehicle control group, while 2 represents the organ of LVT-treated group; the histopathology of organs of LVT-treated group were compared with vehicle-treated control group; magnification:  $\times 400$ .

Thus results of 28 and 90 days study revealed that no treatment-related adverse findings were seen in the clinical signs, body weights, hematology, and biochemical parameters, organ weights, and organ histopathology of animals topically treated with LVT at the limit test dose level of 1000 mg/kg in both male and female Wistar rats. No mortality or moribund stage was recorded, and no significant changes were observed in the general behavior of the LVT-treated groups.

The study further revealed that despite the presence of poisonous plant constituents such as *D. metel* L. leaves and seed, and *A. ferox* Wall. ex Ser. roots, LVT did not exhibit dermal toxicity in rats. This suggests that the test drug used was containing properly processed and purified constituents and also highlights the importance of the “Shodhana” process (purification) while preparing Ayurvedic medicines.

## Conclusion

Topical application of a single and repeated dose of LVT in rats did not exhibit any toxicity or adverse effects, which could compromise its medicinal use in the treatment of painful musculoskeletal disorders. This suggests that approximate  $LD_{50}$  of LVT is more than 2000 mg/kg in the acute dose and NOAEL is more than 1000 mg/kg/day for repeated dose topical application.

## Supplementary data

Supplementary data is available at *TOXRES Journal* online.

## Author’s contribution statement

M.M.W. and B.S. generated the concept and designed the study. M.M.W., M.Y. and Y.D. executed the study, analyzed the data and prepared the manuscript. D.S. assisted in the pharmacology work. S.B.J. performed and interpreted

the histopathology of the samples. S.G., A.D.J. and M.G. corrected the manuscript. All authors checked the final manuscript.

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## Conflict of interest statement

The authors declare no conflict of interest.

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