Evaluation of Acute and Sub-Acute Dermal Toxicity Studies of Ethanolic Leaf Extract of Lawsonia Inermis in Rats

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ABSTRACT

Lawsonia inermis is one of the most significant plants used in traditional medicine. However, many details of the dermal toxicity of L. inermis remain unknown. The objective of this study is to evaluate the in vivo acute and sub-acute dermal toxicity of ethanolic extract of L. inermis leaves. In acute experiment, a total of 20 rats were divided into four groups of five rats. A total of 30 rats were divided into five groups of six rats for the sub-acute experiment. The extract at a single dose of 2000 and 5000 mg/kg of body weight did not produce treatment-related signs of toxicity or mortality in all rats tested during the 14-day observation period. However, in a repeated dose 28-day study, the application of 500, 1000 and 2000 mg/kg of body weight/day of leaves extract revealed no significant change ($p > 0.05$) in bodyweight, haematological and biochemical parameters compared with the control group. Similarly, gross pathology and histopathology examinations of liver, kidneys, and skin did not reveal any morphological alteration. Overall, the results show that the close application of L. inermis leaves extract did not have any critically dangerous impact on rats. Subsequently, the concentrate may be employed for pharmaceutical plans.

Keywords

Lawsonia inermis, leaves extract, acute dermal toxicity, and sub-acute dermal toxicity.

1. INTRODUCTION

Henna (Lawsonia inermis Linn) has attracted the attention of researchers worldwide due to its pharmacological activities ranging from anti-inflammatory to anticancer activities [1]. A native of North Africa and South-West Asia, the plant is now widely cultivated throughout the tropics as an ornamental and dye plant. Henna has been used cosmetically and medicinally for over 9,000 years. Henna leaves, flowers, seeds, stem, bark, and roots are used in traditional medicine to treat a variety of ailments as rheumatoid arthritis, headache, ulcers, diarrhoea, leprosy, fever, leucorrhoea, jaundice, skin diseases, venereal diseases and smallpox, diabetes, and cardiac disease [2, 3]. Exposure to hazardous chemicals to leads to adverse health effects. Widespread usage of medicinal plants in alternative medicine may contribute to the toxicological effects of the toxic compounds present in the plants [4]. Toxicity refers to the state of being poisonous resulted from the interaction between cells and toxicants. In addition, before binding of the toxicants into vital organs, acute toxic effects or signs may be observed. A toxic property evaluation of toxic substances, particularly those obtained from plants, is considered to be essential to the protection of public health. Thus, toxicological assessment becomes imperative for the purpose of achieving potencies and safe formulations for clinically efficient remedies.

In accordance with OECD guidelines, toxicological studies are considered to be very vital in animals such as rats, mice, rabbits, monkeys, guinea pigs, dogs, and so on under different drug conditions to establish the safety and efficiency of a new drug. The decision whether a new drug can be used or not is dependent on toxicological studies. The clinical usage of drug is not allowed without toxicity studies and clinical trial. Practically, the evaluation typically includes acute, sub-acute, sub-chronic, chronic, carcinogenic, and reproductive effects [5]. In this present study, the evaluations on the use of the herbal extract for topical application were tested in both acute and sub-acute dermal toxicity studies. Acute toxicity study determines the immediate toxic effects or lethal dose 50 (LD50), where a single dose of each extract is given in large quantity, while sub-acute toxicity study determines the no observed adverse effect level (NOAEL) of each herb that is given in sub-lethal quantity for 15 to 28 days [6]. In the present study, toxic effects of ethanolic leaf extract of *L. inermis* in Spargue Dawley rats were conducted at dosages of 2000 and 5000 mg/kg body weight for a period of 14 days for the acute toxicity study (followed OECD 402 guideline), and at dosages of 2000, 1000, and 500 mg/kg body weight for a period of 28 days for the sub-acute toxicity study (followed OECD 410 guideline).

2. MATERIALS AND METHODS

2.1 Experimental animals

Eight-week old male and female Sprague Dawley rats with a body weight ranging from between 200 and 250 g were purchased from a local supplier. Upon arrival, the rats were weighed and assigned randomly in polypropylene plastic cages, where one rat was placed in each cage with wood chips for bedding and housed in an animal room with controlled conditions involving these parameters; temperature (22±2°C), humidity (55±10%) and lighting (12 hours light/dark) in the animal house at the Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor.

2.2 Preparation of *Lawsonia inermis* (Henna) leaf extracts

Fresh whole plant samples were obtained from Jerantut, Pahang, Malaysia. The leaves were separated from the plant and dried at a temperature between 25°C to 30°C for three to five days. The dried leaves were ground into powder form using a grinder and kept in a refrigerator at 4°C. The powdered leaves were then macerated with 97% ethanol in a flask. The mixture was left in a water bath at temperature between 55°C to 60°C for one day to allow the chemicals in the leaves to dissolve in the ethanol solution. The ethanol phase was isolated from the mixture by filtration followed by evaporation using a rotor paper evaporator then dried using freeze dryer for one day. The diluted solutions were kept in the refrigerator for later use. The leaf extract was mixed with white soft paraffin (10%).

2.3 Skin preparation for dermal toxicity studies

Skin at the dorsal thoracic area of the rats was clipped under general ketamine (50 mg/kg) and xylazine (5 mg/kg) anaesthesia using an electric clipper, followed by manual shaving using razor blade. Based on OECD guidelines 402 and 410, not less than 10% of the body surface area was cleared for application of the test substance. The herbal extract was applied to the dorsum area.

2.4 Experimental design

In acute and sub-acute dermal toxicity studies, the toxic effects of the ethanolic leaf extract of *L. inermis* in Sprague Dawley rats were examined at dosages of 2000 and 5000 mg/kg body weight for a period of 14 days for acute dermal toxicity study, and at dosages of 2000, 1000 and 500 mg/kg body weight for a period of 28 days for sub-acute dermal toxicity study based on the OECD guidelines 402 and 410, respectively. Different doses were used in dermal toxicity study in order to have a variety of extracts dosage and also to ensure that this extract is not toxic and safe in different concentrations. Base on (OECD) guidelines, female rats are used in acute toxicity studies as they are more sensitive to toxic substance than male. The application of extract in acute toxicity study occurred once. On the other hand, in sub-acute dermal toxicity test, application occurred daily. Thus, female rats were used in the acute dermal toxicity tests and males were used in the sub-acute dermal toxicity tests. In acute dermal toxicity, a total of 20 eight-week old female Sprague Dawley rats were divided into four groups (n=5), namely Groups 1, 2, 3, and 4.

The duration of this study was 14 days. Each group underwent application of the extract once at day 1 and sacrificed at day 14 of the experimental period. For sub-acute dermal toxicity, a total number of 30 eight-week old male Sprague Dawley rats were divided into 5 groups (n=6) namely Groups 1, 2, 3, 4 and 5. Duration of this study was 28 days. Each group was applied with the extract once daily for 28 days, and sacrificed at day 28 of the experimental period.

2.5 Necropsy

All rats were humanely scarified by complete exsanguinations under general anaesthesia with a mixture of 75 mg/kg Ketamine and 10 mg/kg Xylazine.

2.6 Gross pathology

Complete gross examination was conducted to detect any gross changes, especially skin necrosis. The liver and kidneys were blotted dry and weighed immediately after necropsy.

2.7 Histopathology

Skin, liver and kidneys samples were collected and fixed in 10% formalin for 48 hours. After fixation, the samples were sliced to 0.5 cm thickness and placed in plastic cassettes for dehydration using an automated processor (Leica ASP300, Germany), before embedded in paraffin (Leica EG1160, Germany) using the routine paraffin embedding method. The tissue samples were then trimmed and sectioned at 4 µm thicknesses (Leica RM2155, Germany). Then the tissue sections were mounted on glass slides using a hot plate (Leica HI1220, Germany). Subsequently, the tissue sections were deparaffinised by two changes of xylene for 2 minutes each and rehydrated by three changes of different ethanol dilutions (100%, 90% and 70%) for 2 minutes each, respectively. The tissue sections were then further rinsed in tap water and stained with Harris's haematoxylin and eosin (H&E) stain. The slides were observed using a light microscope at 40x, 100x, 200x, 400x and 1000x magnifications.

2.8 Blood biochemistry

Blood samples were withdrawn from posterior vena cava from each rat and collected in serum vacuumed blood collection tubes. The blood samples were centrifuged (Hettichzent- EBA20, Germany) at 5000 rpm for 5 minutes and then the serum were collected in micro centrifuge tubes and stored at -20°C. Serum samples were analysed using an automatic biochemistry analyser (TRX 7070, Biorex, Germany) to determine the levels of alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), albumin, total protein, creatinine, uric acid, urea and bilirubin. Globulin levels were calculated via the following formula: total protein - albumin.

2.9 Haematology

Blood samples were taken from the posterior vena cava of each rat and collected in ethylene diamine tetra acetic acid (EDTA) vacuumed blood collection tubes and were gently mixed immediately to mix the blood with EDTA-anticoagulant material inside the tubes for automatic and manual haematology analyses.

2.9.1 Automatic haematology

Blood samples were analysed using an automatic haematology analyser (Cell Dyn, 3700, Abbot, USA) for the total number of white blood cells (WBC), red blood cells (RBC) and haemoglobin concentration.

2.9.2 Manual haematology

A capillary microhematocrit tube was filled to about three-fourths of its length with EDTA blood by capillary action. The dry end of microhematocrit tubes was sealed by melting with heat then placed in a micro-centrifuge machine (Hettich Haematokrit 210, Germany) and centrifuged at 10,000 rpm for 5 minutes to separate the RBCs from plasma. The plasma was at the top and the RBCs were at the bottom of the microhematocrit tube. The centrifuged microhematocrit tubes were used for determination of packed cell volume (PCV), icteric index and plasma protein concentration. For the PCV, the microhematocrit tube was placed in holder slots of the microhematocrit tube reader (Hawksley). The base of red blood cell was intersected with base line of reader and the top of plasma was intersected with the top line of the reader by moving the holder left or right, before the middle line of the reader was adjusted to intersect with the top of the RBCs and the measuring ruler. The PCV results were obtained from the middle line and the measuring ruler point (e.g.: 24% is equal to 0.24 L). The icteric index results were obtained by comparing the plasma colour in the microhaematocrit tube with the icteric index standard board colour degree. Blood plasma protein concentration was obtained by dropping the plasma on the refractometer glass (Atago T2-NE, Japan) to obtain the result from the measuring ruler and the plasma unit was read in terms of protein in grams divided by plasma in litres (e.g.: 6.2 % is equal to 62 g/L).

2.10 Peripheral blood smear

A peripheral blood smear was conducted by dropping a small blood drop on a glass slide, after which the drop was drawn by a cover slip on the slide surface and left to dry for 20 minutes at room temperature. The blood smear was stained using standard Wright's stain method. The blood smear slide was examined using a light microscope at 100x, 200x, 400x and 1000x magnifications. The manual WBC differential count was conducted by counting one hundred WBCs on the blood smears. The values of each WBC type in these one hundred cells were converted to percentage and multiplied by the automated total WBC count to obtain the absolute WBC differential count (× 109/L) for each cell type.

3. STATISTICAL ANALYSIS

The data obtained was statistically analysed by using Statistical Package for Social Science (SPSS) software version 20. The values were expressed as mean ± standard deviation (SD) for different parameters. Repeated measurements of analysis of variance (ANOVA) tests were done to compare the differences of data between and within the groups. Post hoc analysis using a Duncan test was used to determine level of statistical significance, which was set at $P < 0.05$.

4. RESULTS

4.1 Acute dermal toxicity

4.1.1 General sign and behaviour of the rats

The toxic effects of ethanolic extracts of *L. inermis* on the appearance and the general behavioural pattern of the rats are shown in Table 1 and Table 2, respectively. No toxic signs or mortality were observed in any animals, which survived up to 14 days after applying of the extracts once on the first day at single dose level of 5000 and 2000 mg/kg body weight. The behavioural patterns of animals were observed first 6 hours and 14 hours after applying the extracts. The animals in both vehicle treated and extract-treated groups were normal and did not display significant changes in behaviour, skin effects, breathing, impairment in food intake and water consumption, postural abnormalities and hair loss. In the treated groups, in the first 6 hours after applying the extract rapid heartbeat was observed, but then normalized. This may be due to the stress of handling.

4.1.2 Organ and body weight

There were no gross lesions observed in the liver, spleen and kidneys of all rats. In addition, body weight of the rats are shown in Figure 1 and Table 3, respectively. There were no significant changes in the body weight. All rats showed a normal increment in the body weight which was not significantly ($p > 0.05$) different between both control and treated groups. Similar to the body weights, there were no significant ($p > 0.05$) differences in the changes of relative organ weights between groups.

4.1.3 Haematology evaluation

All erythron parameters were normal in all rat, as shown in Table 4. Normal results were also observed for the leukon parameters, as shown in Table 5.

4.1.4 Blood biochemistry

Tables below show the serum biochemical parameters of liver and muscle enzymes (Table 6), kidney parameters (Table 7), and protein concentration (Table 8) of female rats of control and *L. inermis* extract groups. There were no significant ($p > 0.05$) changes observed in all biochemical parameters.

4.1.5 Histopathology examination

Microscopic structures of the liver, kidneys, and skin are depicted in Figure 2 which shows unnoticeable differences between the control and treatment groups. The microscopic examination revealed that, all the organs from the extract treated rats did not show any alterations in cell structures or any unfavourable effects when viewed under the light microscope using multiple magnification powers.

4.2 Sub-acute dermal toxicity

4.2.1 General sign and behavioural analysis

The toxic effects of ethanolic extracts of *L. inermis* on the appearance and the general behavioural pattern of rats are shown in Table 9 and Table 10, respectively. No toxic signs or mortality were observed in any animals that survived up to 28 days after application of each ethanol extract daily at three different doses; 2000, 1000 and 500 mg/kg body weight. The behavioural patterns of animals were observed 6 hours and 14 hours after applying the extracts. The animals in both vehicle-treated and extract-treated groups were normal and did not display any significant changes of behaviour, skin effects, breathing, impairment in food intake and water consumption, postural abnormalities and hair loss. In the treated groups, for the first 6 hours after each extract was applied on the skin, rapid heartbeat was observed, which might be related to handling stress.

4.2.2 Organ and body weight

There were no gross abnormalities observed in the liver, spleen, or kidneys of any rats. However, body weight of the rats are shown in Figure 3 and Table 11, respectively. There were no significant changes in the body weight. All rats had shown a normal increment in the body weight which was not significantly ($p > 0.05$) different between both control and treated groups. Similar to body weights, there were no significant ($p > 0.05$) differences in the changes of relative organ weights between groups.

4.2.3 Haematology evaluation

All the erythron parameters were normal in all rats, as shown in Table 12. Normal results were also observed for the leukon parameters, as shown in Table 13.

4.2.4 Blood biochemistry

The tables below show the serum biochemical parameters of liver and muscle enzymes (Table 14), kidney parameters (Table 15), and protein concentration (Table 16) of male rats of control and *L. inermis* extract groups. There were no significant ($p > 0.05$) changes observed in all the biochemical parameters.

4.2.5 Histopathology examination

Microscopic structures of liver, kidneys and skin depicted in Figure 4 which shows unnoticeable differences between control and treatment groups. All organs from the extract-treated rats did not show any alteration in cell structure or any unfavourable effects viewed under the light microscope using multiple magnification power.

5. DISCUSSION

Phytotherapeutic products from medicinal plants have become universally popular in primary healthcare, particularly in developing countries and some such products have been mistakenly regarded safe as just because they are a natural source. The bioactive products from medicinal plants are supposed to be safe without any compromising health effect and thus widely used as self-medication [7]. The general conception of herbal drugs is that they are very safe and free from side effects; however, this may not be true as some of the plant compounds are very toxic, such as the most cytotoxic anti-cancer plant-derived drugs, digitalis, the pyrrolizidine alkaloids, ephedrine, phorbol esters, and so on [7]. The adverse effects of most herbal drugs are moderately less frequent when the drugs are used appropriately compared with synthetic drugs [8]. A toxic substance might elicit interesting pharmacological effects at a lower non-toxic dose. Toxicity results from animals will be crucial in definitively judging the safety of medicinal plants if they are found to have sufficient potential for development into pharmacological products [9]. As the use of medicinal plant increases and there is a lack of proven scientific study on the toxicity and adverse effect of most of the products, *in vivo* experimental screenings of the toxicity of the plants are vitally needed, not only to identify the range of doses that could be used subsequently but also to reveal the possible toxicity signs elicited by the substances [10]. Acute and sub-acute toxicity testing in laboratory animals are used to evaluate natural remedies for different pharmacological activities [11]. It is one of the necessary studies need to be performed for the toxicological analyses of medicinal plants.

The toxicity effects of the plants are evaluated through qualitative and quantitative analyses of blood and histopathology samples of the laboratory animal [11]. The present study was designed to investigate the toxicity of ethanolic extracts of *L. inermis,* for topical application via acute and sub-acute dermal toxicity analyses. Results in this study indicated no signs of toxicity and deaths observed throughout the experimental period in both acute and sub-acute toxicity studies. All rats applied topically with the ethanolic extracts of *L. inermis* did not show any organ or systemic toxicity at the dose levels of 2000 mg/kg and 5000 mg/kg in the acute dermal toxicity study and dose levels of 500 mg/kg, 1000mg/kg and 2000 mg/kg in sub-acute dermal toxicity study. The rats were monitored daily until day 14 in the acute study and day 28 in the sub-acute study for any toxic signs and mortality. During the 14 and 28 days of period acute and sub-acute toxicity evaluation, the rats showed no overt signs of distress and there were no observable symptoms of either toxicity or deaths; clinical abnormality is one of the major important observations to indicate the toxicity effects on organs in the treated groups [12]. All of the rats displayed no significant changes in behaviour. In addition, physical appearance such as skin, fur, and eyes

were normal and as the body weight of the rats was increased, this indicates that applying of the crude extracts on the skin had a negligible level of toxicity on the growth of the rats. Moreover, determination of feed intake and water consumption is essential in the study of safety of a product with therapeutic purpose, as proper intake of nutrients is significant to the physiological status of the animal and to the accomplishment of the proper response to the drugs tested [13, 14]. In this study, the feed intake and water consumption were normal. The extract did not induce a decrease in appetite, leading to no disturbance in carbohydrate, protein or fat metabolism of the animals [15].

Generally, the alterations of body weight gain and internal organ weights of rats would reflect the toxicity after exposure to the toxic substances [16]. In 2002, a study done by Raza and his team indicated that the body weight changes are indicators of adverse effects of drugs and chemicals and it will be significant if the body weight loss is more than 10% of the initial weight [17]. Organ weight also is an important index of physiological and pathological status in animals. The relative organ weight is fundamental to diagnose whether the organ was exposed to the injury or not [18]. The liver, kidneys, and spleen are the primary organs affected by metabolic reaction caused by toxicants [19]. In this study, gross appearance of the selected organs of both control and treated groups were normal. The relative and absolute weight of the organs in both control and treated groups showed no significant differences.

Body weight gain was similar in both control and treated groups without statistically significant differences. Local application of ethanolic extracts of *L. inermis* did not show any adverse effects on organs weight of the important organs. Hence, it is suggested that *L. inermis* extracts are virtually nontoxic. On the other hand, the haematopoietic system is very sensitive to toxic compounds and serves as an important index of the physiological and pathological status in both animals and humans [20]. After 14 and 28 days of treatment with ethanolic extracts of *L. inermis*, there were no changes in the haematological parameters in both control and treatment groups. There were also no significant changes in levels of all serum biochemistry parameters which supported the nontoxic nature of ethanolic extract of *L. inermis.* This finding is in the agreement with the safe use of the herbs by the traditional healers. In 1999, a study by Ajagbonna and his team on certain medical herbs indicated that the normal range of haematological parameter can be altered by the intake of toxic plants. Apart from blood analysis, histopathology is a compulsory method need to be done for further confirmation of the alteration in cell structure of the organs as results of toxicity [21]. The histopathological examination is the "golden standard" method for evaluating treatment-related pathological changes in tissues and organs [22].

In this study, histopathological evaluation of acute and sub-acute dermal toxicity studies indicated that ethanolic extracts of *L. inermis* did not induce any structural damage on the morphology of liver, kidneys and skin. The histopathological findings were in agreement with the results of haematological and biochemical analyses. The histopathology results were also collaborated with the results of body weight and relative organ weights. The liver is the main target organ of acute and sub-acute toxicity, as it is exposed to the foreign substances that were absorbed from the intestines and metabolised into other compounds, which may or may not be hepatotoxic [23]. In this study, the liver histology revealed normal hepatocytes. The portal and central vein, bile duct, as well as hepatic artery did not show any alterations in the structure in both controls and treated rats. In contrast to the present study, the histological examination of a study conducted by Harizal and his team using *Mitragyna speciosa* extract revealed morphological changes in liver of mice treated with the extract at dose levels of 100 mg/kg and 500 mg/kg [24]. Meanwhile, another study by Salawu and his team using *Crossopteryx febrifuga* observed inflammatory changes histologically in the liver by infiltration of lymphocytes at portal and central veins of rats treated at dose levels of 500 mg/kg and 1000 mg/kg. Those studies have shown that the extracts exerted deleterious effects on the liver. Liver is capable of regenerating damaged tissues, hence it functions may not be impaired early on following an insult from a toxicant [25].

In addition, toxicity study conducted on *Cassia fistula* pod extract done by Akanmu and his team in 2004 reported that the histological examinations of liver, kidneys and testes showed no potential toxicity or damage to the cell structure of liver, kidneys and testes at a dose of 1000 mg/kg of *C. fistula* pod extract [26]. There was no necrosis, inflammatory reaction, fibrosis, or local fatty degeneration observed in liver and the disarrangement of the hepatocytes. In this study the morphology of liver cells in both control and treated groups were normal and no structural alteration were microscopically observed. Similar to the liver, histology of kidney microphotographs displayed no adverse effects observed in both groups. The glomeruli and Bowman's capsules were normal. In contrast to this study, a study conducted by Alade and his team revealed focal proximal tubular epithelial necrosis in the kidneys [27]. Apart from that study, other study conducted by Akanmu on *C. fistula* pod extract revealed significant changes in the histology of kidneys in rats treated with the extract at a dose of 1000 mg/kg [26]. In this present study, the microscopic examination of the skin of rats treated with the extracts did not indicate any changes in the layers of the skin at the epidermis, dermis and hypodermis as compared to the control rats. Skin lesions were observed due to colloidal nanosilver toxicity at doses of 100, 1000 and 10000 µg/mL in guinea pigs demonstrated decreased thickness of epidermis and dermis, increased numbers of Langerhans and inflammatory cells, decreased papillary layer with regular collagens fibres, acidophilic cytoplasm in muscle fibres, degenerative fibres, and increased levels of macrophage in the endomysium [28].

6. CONCLUSION

The results of this study suggest that ethanolic extracts of *L. inermis* do not cause any apparent *in vivo* toxicity. No death or signs of toxicity were observed in rats treated with the extracts at doses of 5000 and 2000 mg/kg (acute toxicity study), and also at doses of 2000 mg/kg, 1000 mg/kg and 500 mg/kg (sub-acute toxicity study), thus establishing its safety in use. The histology examinations revealed no changes in the architectures of the selected organs of the rats in both control and treated groups. Hence, it is concluded that *L. inermis* can be used as topical medicinal agents at those dosages, especially in rural communities in which conventional drugs are unaffordable because of their high cost.

7. REFERENCES

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TABLES

Table 1: Mortality rate of rats after applied with topical ethanolic extracts of *L. inermis* at 5000 mg/kg and 2000 mg/kg, once, for the acute toxicity study

Group	Mortality rate*(%)
G1	0
G2	0
G3	0
G4	0

* Mortality rate is number of dead rats divided by total number of rats per group.

G1: No treatment, G2: Paraffin, G3: *L. inermis* 5000 mg/kg, G4: *L. inermis* 2000 mg/kg

Table 2: Behavioural patterns and general appearance of rats in all groups

Abnormal sign	Control group: G1 (6 h)	Control group: G1 (14 h)	Treatment groups: G2-G4 (6 h)	Treatment groups: G2-G4 (14 h)
Skin and fur	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal
Mucous membrane	Normal	Normal	Normal	Normal
Behavioural patterns	Normal	Normal	Tachycardia	Normal
Salivation	Normal	Normal	Normal	Normal
Lethargy	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal
Diarrhea	Normal	Normal	Normal	Normal
Coma	NO	NO	NO	NO
Tremors	NO	NO	NO	NO

G1: No treatment, G2: Paraffin, G3: *L. inermis* 5000 mg/kg, G4: *L. inermis* 2000 mg/kg.

NO: Not observed

Table 3: Organ relative weights of rats in all groups

Group	Liver (g)	Kidneys (g)
G1	0.024 ± 0.005	0.005 ± 0.0005
G2	0.020 ± 0.007	0.006 ± 0.0004
G3	0.020 ± 0.007	0.006 ± 0.0007
G4	0.024 ± 0.008	0.006 ± 0.0007

Values are expressed as mean ± SD (n = 5 for each group). Relative organ weight was calculated by organ weight/body weight × 100%. G1: No treatment, G2: Paraffin, G3: *L. inermis* 5000 mg/kg, G4: *L. inermis* 2000 mg/kg

Table 4: Erythron parameters and plasma protein concentration of rats in all groups

Parameter	Unit	G1(Mean ±SD)	G2(Mean ±SD)	G3(Mean ±SD)	G4(Mean ±SD)
RBC	x1012/L	8.25±0.28	8.56±0.61	8.75±0.55	8.12±0.73
Hb	g/L	171±7.91	166±7.79	157±14.00	176±13.75
PCV	L/L	0.45±0.00	0.45±0.01	0.45±0.02	0.45±0.01
MCV	fl	58.2±0.83	57.8±1.30	56.6±1.51	56.8±1.30
PP	g/L	75.4±2.07	77.8±1.92	77.0±0.70	74.8±2.16

Values are expressed as mean ± SD (n = 5 for each group). G1: No treatment; G2: Paraffin; G3: *L. inermis* 5000 mg/kg; G4: *L. inermis* 2000 mg/kg

Table 5: Leukon and thrombon parameters of rats in all groups

Parameter	Unit	G1(Mean ±SD)	G2(Mean ±SD)	G3(Mean ±SD)	G4(Mean ±SD)
WBC	109/L	7.82±0.87	7.68±0.93	7.14±0.92	7.86±1.46
Neutrophils	109/L	1.45±1.14	1.47±0.83	1.48±2.28	1.50±1.48
Lymphocytes	109/L	5.66±1.14	5.66±1.64	5.21±2.73	5.89±2.23
Monocytes	109/L	0.31±1.00	0.32±1.30	0.19±0.83	0.28±1.14
Esoinophils	109/L	0.21±0.83	0.12±0.89	0.15±0.83	0.14±0.83
Basophils	109/L	0.07±0.70	0.09±0.83	0.08±1.09	0.03±0.54
Platelets	109/L	869±40.70	913±28.10	933±32.40	950±15.80

Values are expressed as mean ± SD (n = 5 for each group). G1: No treatment; G2: Paraffin; G3: *L. inermis* 5000 mg/kg; G4: *L. inermis* 2000 mg/kg

Table 6: Serum biochemical parameters of liver and muscle enzymes of rats in all groups

Parameter	Unit	G1(Mean ±SD)	G2(Mean ±SD)	G3(Mean ±SD)	G4(Mean ±SD)
ALT	(U/L)	41.2 ±1.30	43.4 ±1.14	39.8 ±0.83	45.0 ±1.58
ALP	(U/L)	74.0 ±2.73	73.2 ±2.58	72.8 ±2.58	74.6 ±2.07
AST	(U/L)	134.6 ±2.96	139.0 ±2.23	136.4 ±1.51	137.4 ±2.70
CK	(U/L)	172.8 ±2.68	174.6 ±1.81	173.0 ±2.54	174.0 ±2.73

Values are expressed as mean ± SD (n = 5 for each group). G1: No treatment; G2: Paraffin; G3: *L. inermis* 5000 mg/kg; G4: *L. inermis* 2000 mg/kg

Table 7: Serum biochemical parameters of kidney of rats in all groups

Parameter	Unit	G1(Mean ±SD)	G2(Mean ±SD)	G3(Mean ±SD)	G4(Mean ±SD)
Urea	(mmol/L)	6.44 ±0.25	6.30 ±0.30	5.28 ±2.57	6.42 ±0.19
Creatinine	(μmol/L)	36.5 ±1.35	37.5 ±1.46	35.9 ±2.43	35.9 ±2.07

Values are expressed as mean ± SD (n = 5 for each group). G1: No treatment; G2: Paraffin; G3: *L. inermis* 5000 mg/kg; G4: *L. inermis* 2000 mg/kg

Table 8: Serum biochemical parameters of protein concentration of rats in all groups

Parameter	Unit	G1(Mean ±SD)	G2(Mean ±SD)	G3(Mean ±SD)	G4(Mean ±SD)
TP	(g/L)	72.8 ±1.92	73.8 ±1.92	72.2 ±1.78	72.4 ±2.07
Albumin	(g/L)	37.7 ±0.97	38.1 ±0.79	37.8 ±0.60	38.8 ±0.92
Globulin	(g/L)	32.7 ±0.72	33.1 ±0.75	32.3 ±1.13	33.2 ±1.11
A/G ratio	(g/L)	1.14 ±0.03	1.14 ±0.03	1.16 ±0.04	1.16 ±0.05

Values are expressed as mean ± SD (n = 5 for each group). G1: No treatment; G2: Paraffin; G3: *L. inermis* 5000 mg/kg; G4: *L. inermis* 2000 mg/kg

Table 9: Mortality rate of rats after applied with topical ethanolic extracts of *L. inermis* at 2000 mg/kg, 1000 mg/kg and 500 mg/kg, daily, for 28 days

Group	Mortality rate*(%)
G1	0
G2	0
G3	0
G4	0
G5	0

* Mortality rate is number of dead rats divided by total number of rats per group.

G1: No treatment; G2: Paraffin; G3: *L. inermis* 2000 mg/kg; G4: *L. inermis* 1000 mg/kg; G5: *L. inermis* 500 mg/kg

Table 10: Behavioural patterns and general appearance of rats in all groups

Abnormal sign	Control group: G1 (6 h)	Control group: G1 (14 h)	Treatment groups: G2-G5 (6 h)	Treatment groups: G2-G5 (14 h)
Skin and fur	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal
Mucous membrane	Normal	Normal	Normal	Normal
Behavioural patterns	Normal	Normal	Tachycardia	Normal
Salivation	Normal	Normal	Normal	Normal
Lethargy	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal
Diarrhea	Normal	Normal	Normal	Normal
Coma	NO	NO	NO	NO
Tremors	NO	NO	NO	NO

G1: No treatment, G2: Paraffin, G3: *L. inermis* 2000 mg/kg, G4: *L. inermis* 1000 mg/kg, G5: *L. inermis* 500 mg/kg, NO: Not observed

Table 11: Organ relative weight of rats in all groups

Group	Liver (g)	Kidneys (g)
G1	0.026 ± 0.002	0.0062 ± 0.0002
G2	0.031 ± 0.002	0.0062 ± 0.0002
G3	0.028 ± 0.003	0.0064 ± 0.0002
G4	0.028 ± 0.004	0.0063 ± 0.0003
G5	0.026 ± 0.002	0.0063 ± 0.0002

Values are expressed as mean ± SD (n = 6 for each group). Relative organ weight was calculated by organ weight/body weight × 100%. G1: No treatment group; G2: Paraffin group; G3: *L. inermis* (2000 mg/kg); G4: *L. inermis* (1000 mg/kg); G5: *L. inermis* (500 mg/kg)

Table 12: Erythron parameters and plasma protein concentration of rats in all groups

Parameter	Unit	G1 (Mean ±SD)	G2 (Mean ±SD)	G3 (Mean ±SD)	G4 (Mean ±SD)	G5 (Mean ±SD)
RBC	x1012/L	8.60 ±0.44	8.93 ±0.56	9.02 ±0.32	8.33 ±0.55	8.52 ±0.56
Hb	g/L	170 ±8.57	172 ±3.83	171 ±7.12	166 ±9.45	163 ±19.4
PCV	L/L	0.45 ±0.00	0.46 ±0.01	0.46 ±0.00	0.46 ±0.01	0.42 ±0.01
MCV	fl	57.6 ±1.75	57.8 ±0.75	56.0 ±2.75	57.6 ±1.03	57.1 ±1.60
MCHC	g/L	342 ±7.31	345 ±7.74	340 ±9.71	337 ±8.73	346 ±9.24
PP	g/L	77.6 ±1.86	75.3 ±1.03	75.5 ±2.07	74.6 ±2.33	77.5 ±1.87

Values are expressed as mean ± SD (n = 6 for each group). G1: No treatment; G2: Paraffin; G3: *L. inermis* 2000 mg/kg; G4: *L. inermis* 1000 mg/kg; G5: *L. inermis* 500 mg/kg.

None of the values were significantly different at P > 0.05

Table 13: Leukon and thrombon parameters of rats in all groups

Parameter	Unit	G1 (Mean ±SD)	G2 (Mean ±SD)	G3 (Mean ±SD)	G4 (Mean ±SD)	G5 (Mean ±SD)
WBC	109/L	7.45 ±1.31	8.01 ±0.74	6.40 ±1.23	7.63 ±1.07	7.46 ±0.98
Neutrophils	109/L	1.49 ±0.98	1.61 ±0.75	1.28 ±1.32	1.48 ±1.04	1.43 ±1.21
Lymphocytes	109/L	5.43 ±1.54	5.81 ±1.36	4.71 ±1.63	5.76 ±1.72	5.49 ±1.36
Monocytes	109/L	0.29 ±1.09	0.29 ±1.21	0.22 ±0.54	0.24 ±0.75	0.32 ±1.21
Esoinophils	109/L	0.17 ±0.81	0.17 ±0.75	0.13 ±0.75	0.12 ±0.51	0.13 ±0.98
Basophils	109/L	0.04 ±0.81	0.10 ±0.81	0.03 ±0.54	0.06 ±0.75	0.06 ±0.75
Platelets	109/L	864 ±44.9	837 ±27.3	811 ±45.3	741 ±44.8	732 ±92.2

Values are expressed as mean ± SD (n = 6 for each group). G1: No treatment; G2: Paraffin; G3: *L. inermis* 2000 mg/kg; G4: *L. inermis* 1000 mg/kg; G5: *L. inermis* 500 mg/kg. None of the values were significantly different at P > 0.05.

Table 14: Serum biochemical parameters of liver and muscle enzymes of rats in all groups

Parameter	Unit	G1 (Mean ±SD)	G2 (Mean ±SD)	G3 (Mean ±SD)	G4 (Mean ±SD)	G5 (Mean ±SD)
ALT	(U/L)	44.1 ±8.35	42.0 ±6.41	45.6 ±5.95	44.1 ±6.85	45.5 ±3.83
ALP	(U/L)	135 ±12.3	138 ±10.1	134 ±7.00	124 ±13.2	131 ±7.38
AST	(U/L)	149 ±12.4	150 ±16.5	141 ±17.7	137 ±28.4	149 ±20.4
CK	(U/L)	201 ±22.1	221 ±26.3	229 ±21.1	201 ±18.3	238 ±20.1

Values are expressed as mean ± SD (n = 6 for each group). G1: No treatment group; G2: Paraffin group; G3: *L. inermis* (2000 mg/kg); G4: *L. inermis* (1000 mg/kg); G5: *L. inermis* (500 mg/kg. None of the values were significantly at P > 0.05

Table 15: Serum biochemical parameters of kidney of rats in all groups

Parameter	Unit	G1 (Mean ±SD)	G2 (Mean ±SD)	G3 (Mean ±SD)	G4 (Mean ±SD)	G5 (Mean ±SD)
Urea	(mmol/L)	7.71±0.91	6.91±0.57	7.41±0.64	8.00±0.88	7.20±0.67
Creatinine	(µmol/L)	55.5±2.25	55.5±3.08	52.0±3.94	55.1±2.56	52.5±2.50

Values are expressed as mean ± SD (n = 6 for each group). G1: No treatment group; G2: Paraffin group; G3: *L. inermis* (2000 mg/kg); G4: *L. inermis* (1000 mg/kg); G5: *L. inermis* (500 mg/kg. None of the values were significantly different at P > 0.05

Table 16: Serum biochemical parameters of protein concentration of rats in all groups

Parameter	Unit	G1 (Mean ±SD)	G2 (Mean ±SD)	G3 (Mean ±SD)	G4 (Mean ±SD)	G5 (Mean ±SD)
TP	(g/L)	72.2 ±4.09	75.5 ±4.37	74.1 ±4.03	71.3 ±4.40	68.0 ±3.09
Albumin	(g/L)	40.8 ±1.15	43.8 ±2.62	40.4 ±3.13	39.6 ±3.94	38.1 ±1.81
Globulin	(g/L)	31.4 ±3.23	31.6 ±5.26	33.7 ±5.46	32.2 ±6.92	29.8 ±3.00
A/G ratio	(g/L)	1.25 ±0.13	1.36 ±0.26	1.18 ±0.24	1.20 ±0.31	1.23 ±0.16

Values are expressed as mean ± SD (n = 6 for each group). G1: No treatment group; G2: Paraffin group; G3: *L. inermis* (2000 mg/kg); G4: *L. inermis* (1000 mg/kg); G5: *L. inermis* (500 mg/kg. None of the values were significantly different at P > 0.05

FIGURES

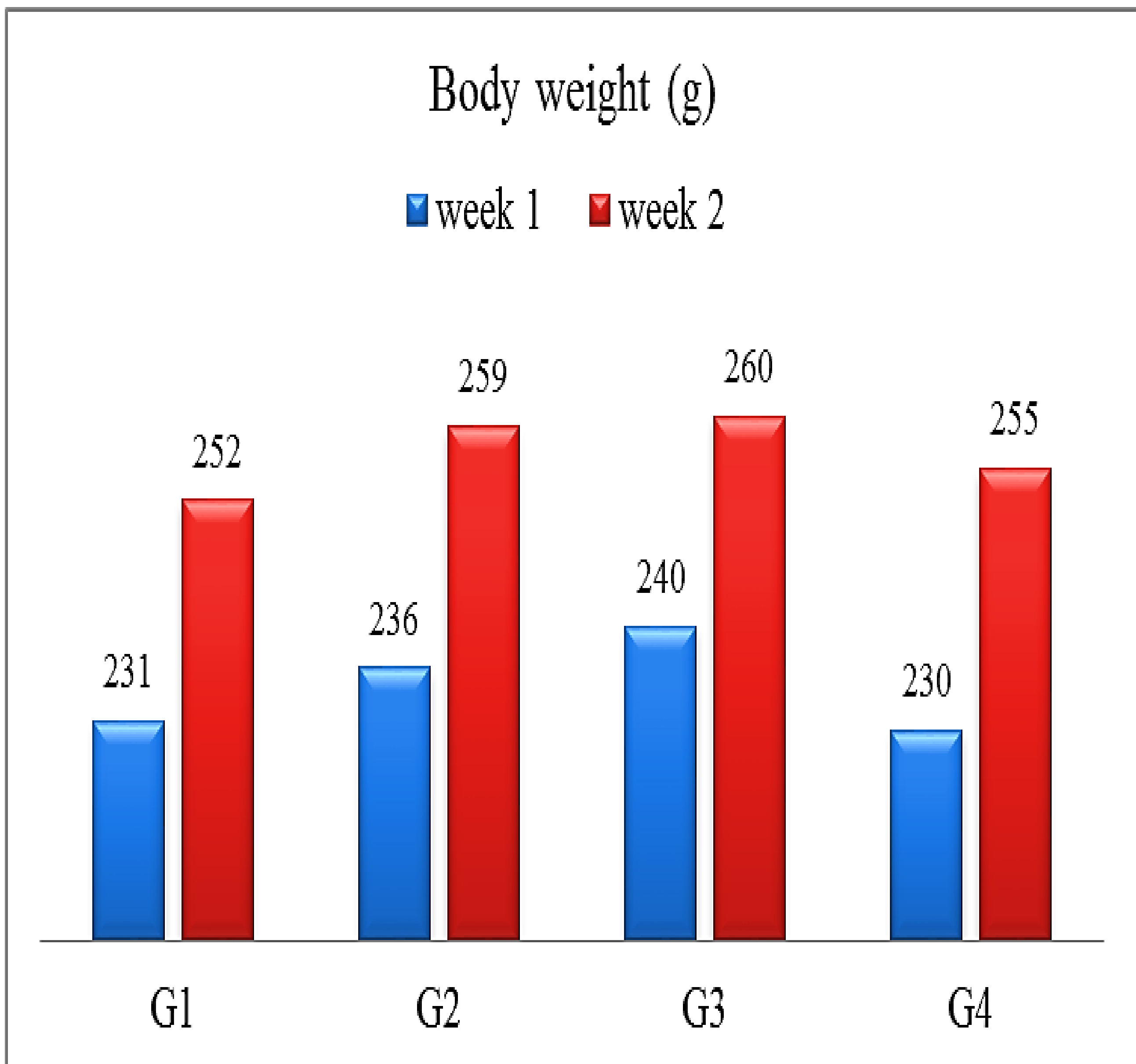

Fig. 1: Mean body weight (g) of rats in all groups. Data collected were recorded and presented as mean ± standard error of mean. G1: No treatment; G2: Paraffin; G3: *Lawsonia inermis* 2000 mg/kg; G4: *Lawsonia inermis* 5000 mg/kg. None of the numbers were seriously variant at p>0.05

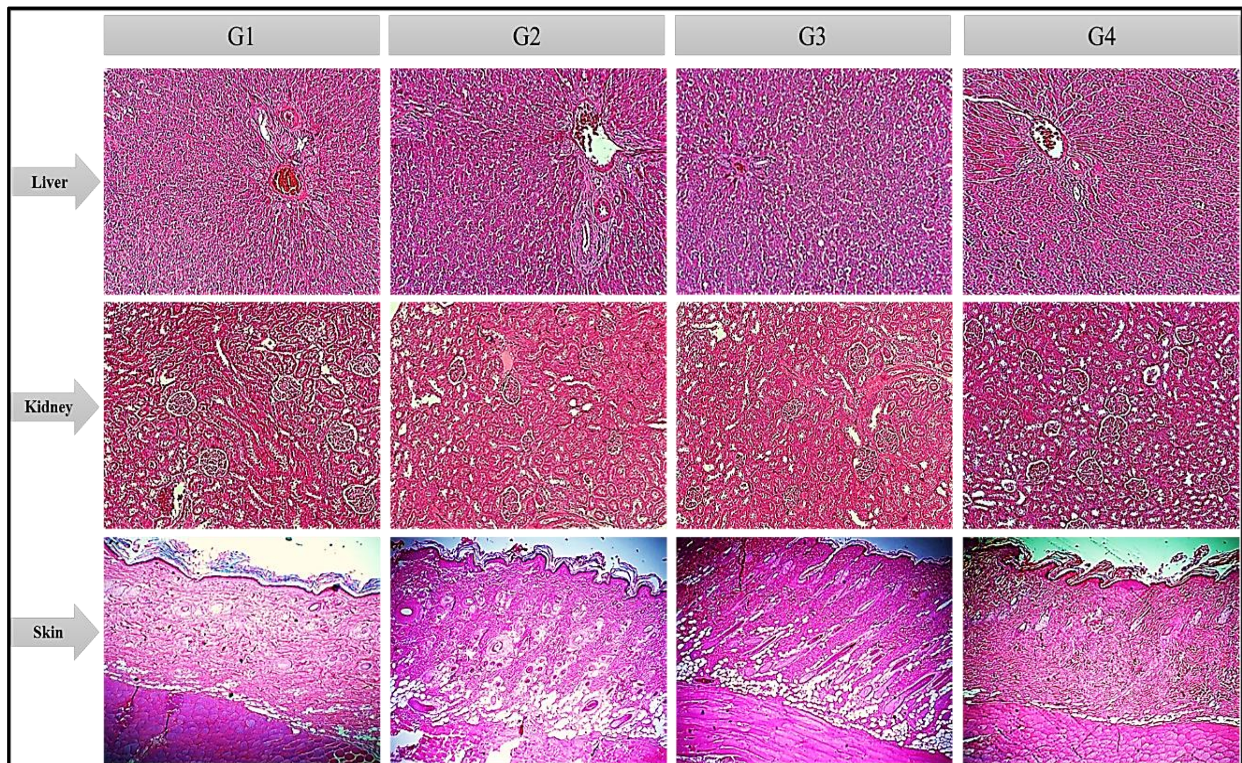

Fig. 2: Histological sections of liver, kidney and skin of rats in all groups. G1: No treatment; G2: Paraffin; G3: *Lawsonia inermis* 2000 mg/kg; G4: *Lawsonia inermis* 5000 mg/kg. Selected photomicrographs ×10 and ×20. Hematoxylin-eosin staining (scale bar: 200 µm)

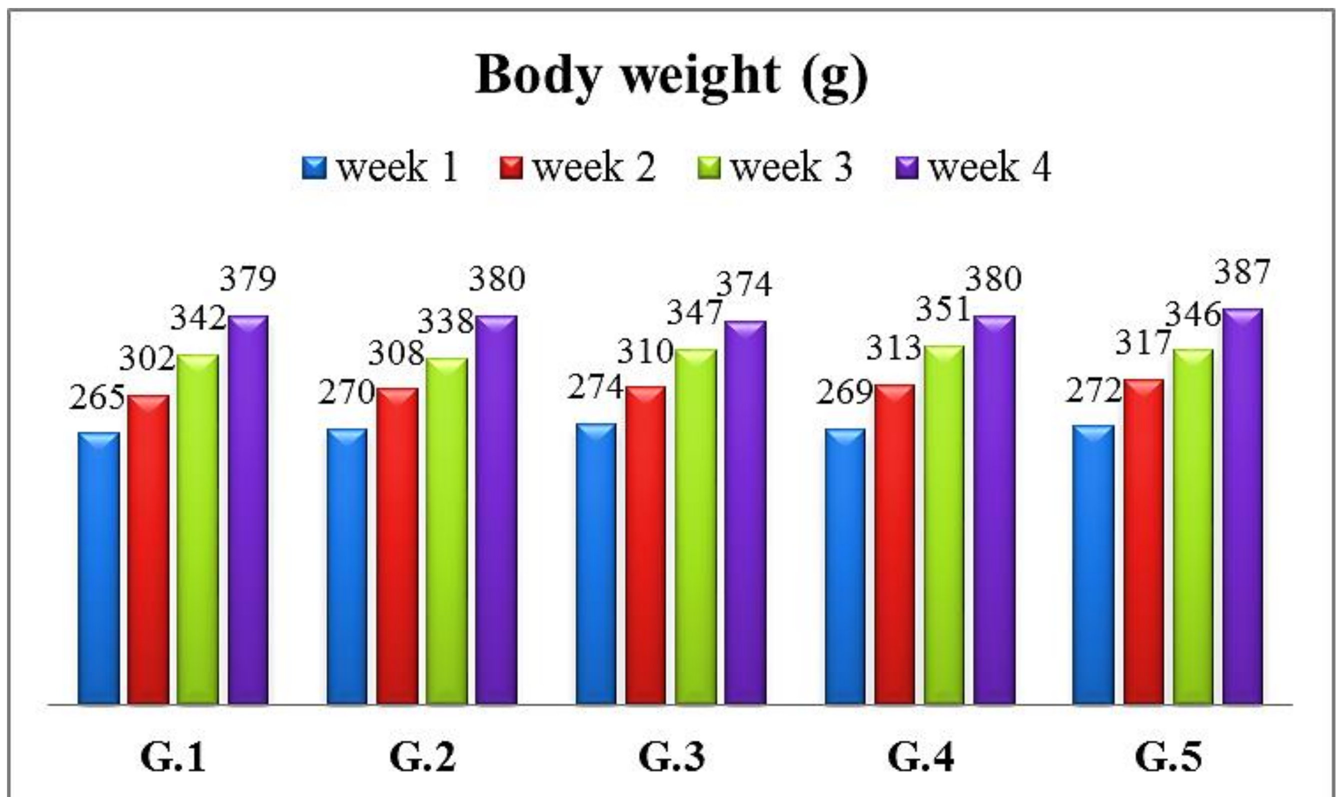

Fig. 3: Mean body weight (g) of rats in all groups. Data collected were recorded and presented as (mean ± SEM). G1: No treatment group; G2: Paraffin group; G3: *L. inermis* (2000 mg/kg); G4: *L. inermis* (1000 mg/kg); G5: *L. inermis* (500 mg/kg)

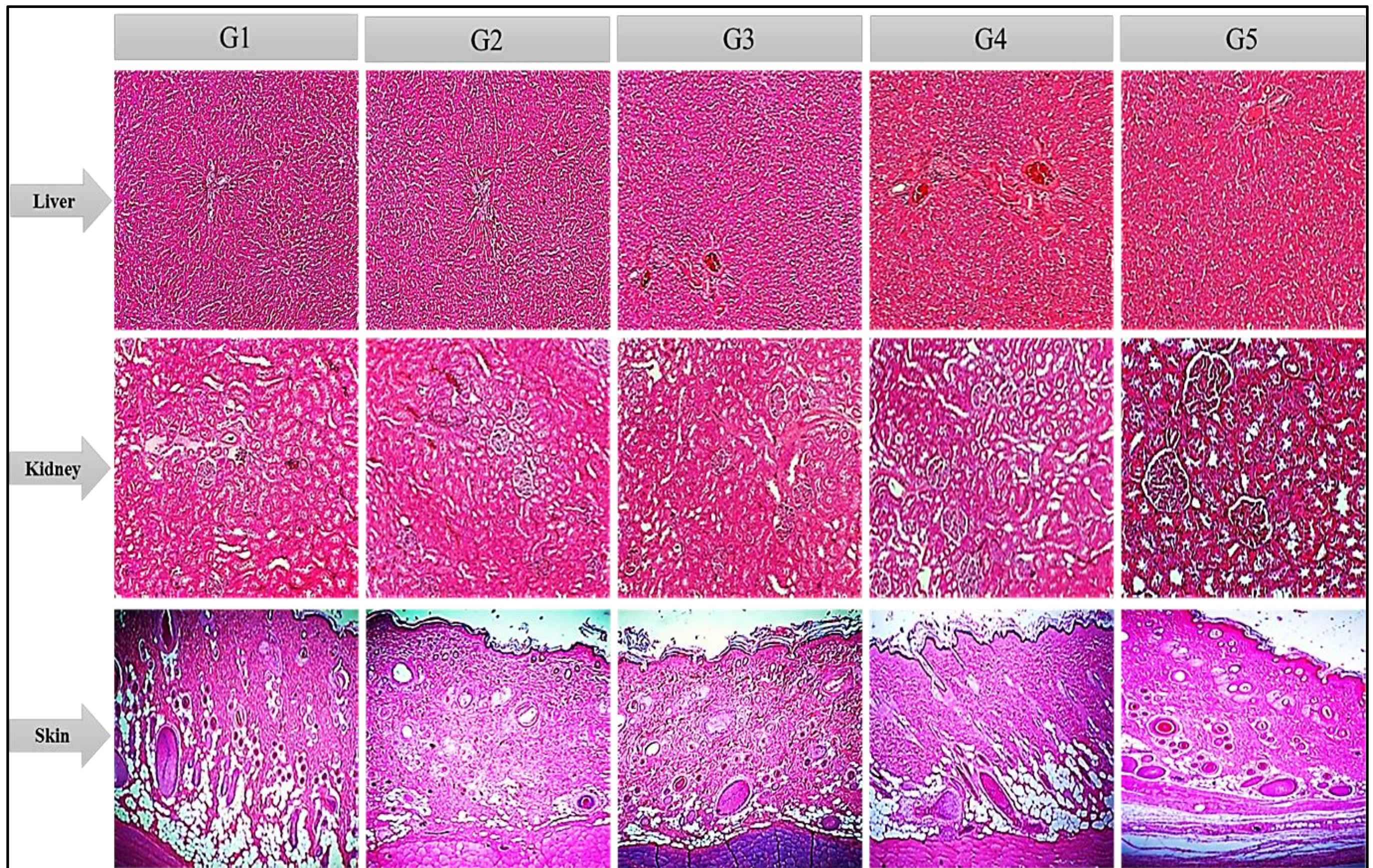

Fig. 4: Histological sections of liver, kidney and skin of rats in all groups G1: No treatment; G2: Paraffin; G3: *Lawsonia inermis* 500 mg/kg; G4: *Lawsonia inermis* 1000 mg/kg; G5: *Lawsonia inermis* 2000 mg/kg. Selected photomicrographs ×10 and ×20. Hematoxylin-eosin staining (scale bar: 200 μm)