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Biochemical and Ultrastructural Studies for Toxicity of Kaempferol Derivative Recovered from the Plant *Lysimachia ramosa*

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ABSTRACT

Leaves of *Lysimachia ramosa* are used by various tribes of Meghalaya to cure intestinal helminth infections. Preliminary investigations disclosed impressive anthelmintic effects of the kaempferol derivative, an active component of the plant, however, toxic effects on its consumers, if any, are not known. Therefore, the present study has been undertaken to investigate the acute and sub-acute toxic effects of kaempferol derivative, of the plant, taking Wistar rats as a model. Following OECD 407, 250 mg, 500 mg, and 1000 mg/kg body weight doses of the active component have been selected to treat the animals for 28 days. On the 29th day, the animals have been sacrificed to assess different toxicological effects on animals. The LD_{50} value of the anthelmintic component was found to be more than 5000 mg/kg body weight of rats. Histological, ultrastructural, haematological, biochemical, and organo-somatic (HSI and RSI) studies demonstrate changes in surface characteristics of various cellular organelles of different vital organs such as the liver, kidney, and intestine. Alterations were also recorded in different vital enzymes such as AST, ALT, and ALP in the phytochemical exposed rats at higher doses. The results revealed that treatment with the active component at a higher concentration may lead to toxicological effects if treatment persists for a longer period.

Keywords: Lysimachia ramosa; Kaempferol derivative; Sub-acute toxicity; Light microscopy; Electron microscopy; Haematology; Wistar rats

1. INTRODUCTION

Plants have an inordinate perspective for offering new drugs of great use against many diseases. Plant-derived drugs also known as "green medicine" are cheap, easily available, more reliable, and safer to consume, but many have intolerable after-effects.[1] It is considered that about 25 per cent of promulgated drugs are plant origins and more or less 80 per cent of the human inhabitants in progressing nations depend on conventionally used medicinal flora for curing various kinds of parasitic infections.[2-3] Stipulation for medicinal plants is increasing worldwide market including in India.[4] Fifty percent of the entire flora of India has been found as medicinal plants of extensive potential such as allelopathic[5], anthelmintic potential, etc.[6]

Amidst many parasites, *Raillietina echinobothrida* is one of the cestodes accountable for large-scale mortality in poultry.[7] *Lysimachia ramosa* Wall is a herb, leaves extract of which is consumed by various ethnic people of Meghalaya to control various intestinal worm infections.The leaves of the plant have been found to have different natural products such as alkaloids, tannins, saponins, and phlobatannins. The anthelmintic potential of the leaf extract has also been established by Challam, *et al.*[8] Roy, *et al.* showed that the leaf extract at a concentration of 100 mg/day for 14, days causes deformation of the liver and kidney along with alteration of liver and kidney markers in experimental mice.[9] Dey and Roy discovered that the n-butanol fraction of the crude leaf extract was the most effective anthelmintic fraction of *L. ramosa*as it alters the glycogen content and many other tegumental enzymes of the parasite.[10-11]

Recently, Dey *et al.* showed that kaempferol derivative, an active principle of the n-butanol fraction of the plant is responsible for anthelmintic activity.[12] However, a review of literature divulged that numerous traditional phytoproducts deploy undesirable effects on animals.[13-14] Sarkar and Roy established that at higher dosages of kaempferol derivative isolated from *Lysimachia ramosa*, the component affects the reproductive system in male Wistar rats in a negative way.[15] Furthermore, it is also imperative to establish a safety profile of the isolated natural component as a chaperon for the execution of its applications. The present study, therefore, was planned to evaluate the *in-vivo* toxic effects, if any, of the active anthelmintic component kaempferol derivative isolated from the n-butanol fraction of leaf of *L.ramosa*. This novel finding will help the researchers in further work

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on the possible uses of the component in human welfare.

2. METHODOLOGY

2.1 Processing of Plant Materials and Isolation of the Active Component

Leaves of the plant *Lysimachia ramosa* were collected from different areas of East Khasi hills (25.45º/92.19º), Meghalaya, and air-dried. Dry leaves were then ground into a fine powder and soaked in methanol. Later the crude methanolic extract was obtained from the filtered solvent by a rotary evaporator. The N-butanol fraction was obtained from the crude methanolic extract using the fractional distillation method.[16] From the active n-butanol fraction, the active anthelmintic component "5,7-dihydroxy-3-(((2R,3R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yl)oxy)-2-(4-(((2R,4S,5S,6R)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy) phenyl)-4H-chromen-4-one", a kaempferol derivative was isolated with the help of column chromatography technique using silica gel as stationary phase, petroleum ether, ethyl acetate and methanol in different ratios as a mobile phase by running the n-butanol fraction through the column and identified using NMR, DEPT, and HMBC as described earlier.[12] (Fig.1)

2.2 Experimental Animals

The investigation was carried out on Wistar rats ensuing animal ethics committee's guidelines. The animals were put up in a grilled enclosure at a steady temperature of 22°C-25°C. Proper food and water as per requirement were provided to the animals. All the animals were conformed to the environment 14 days earlier than the beginning of the trial. The animals were given oral doses gently with the help of an oral gavage. Special care was taken while handling and dosing the animals.

2.3 Acute Oral Toxicity Study

An acute toxicity investigation was conducted following OECD 423 for testing of chemicals.[17] Institutional Ethical Committee's permission was obtained to perform the experiments on the animals. To study the acute toxicity of the isolated component, its single oral dose was given to animals to know the LD_{50} value of the kaempferol derivative component. The animals were kept under observation for about 24 hours to check for any kind of behavioural fluctuation.

2.4 Sub-acute Oral Toxicity Study

Sub-acute toxicity was conducted following OECD 407 for checking the long-term effects of the chemical.[18] All the animals were clustered into four groups. They were the control group and the other three groups were treated with 250 mg, 500 mg, and 1000 mg/kg bw of the isolated component, respectively. After giving treatment continuously for 28 days, on the 29th day, the animals were sacrificed and different body parts and blood were obtained for haematological, biochemical, histological, and ultrastructural studies.

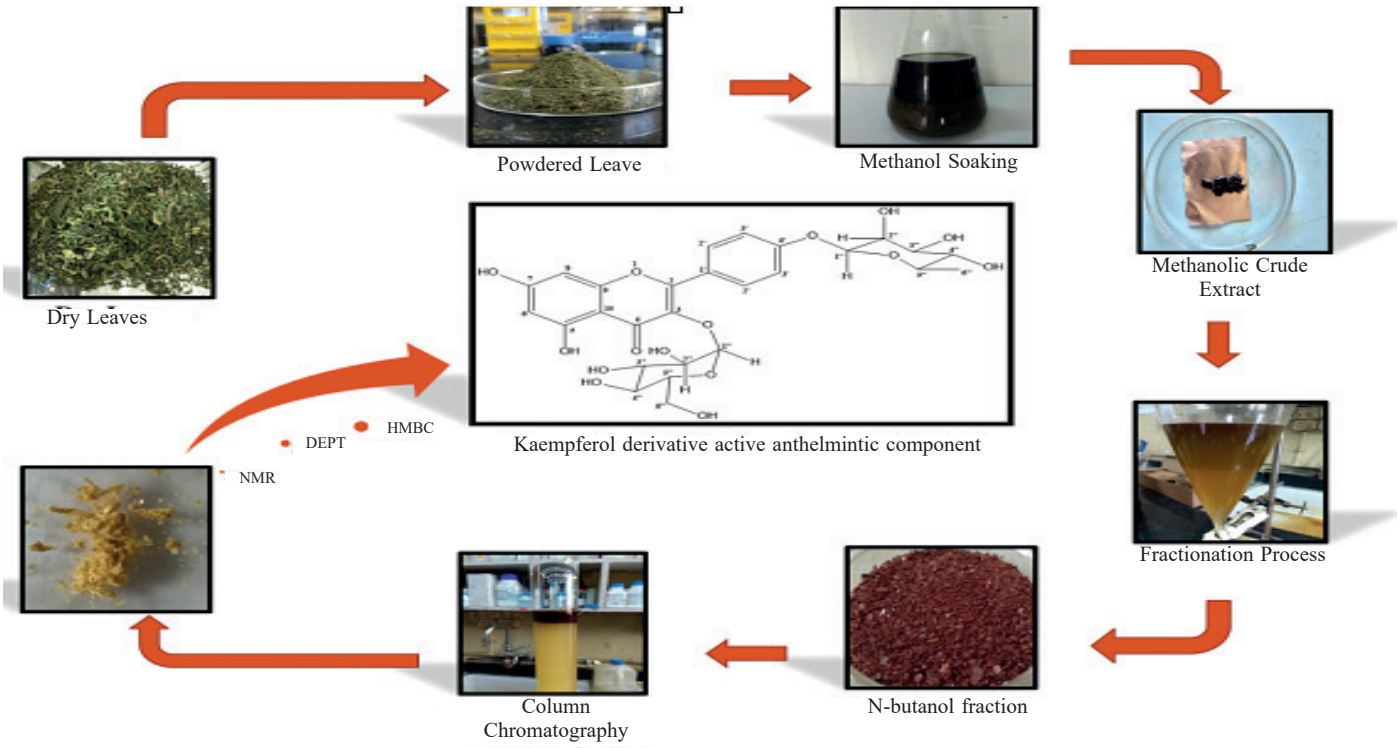

Figure 1. Flow-chart showing the process of isolation of the kaempferol derivative active anthelmintic component from leaves of *L. ramosa*.

2.4.1 Study of the Hepato-somatic Index (HSI) and Reno-somatic Index (RSI)

To assess the HSI and RSI, liver and kidney were solicited from the animals and weighed. The index was calculated using the following formula[19] :

$$\text{Organo-somatic index} = \frac{\text{Weight of an organ}}{\text{Weight of the body}} \times 100\%$$

2.4.2 Haematological and Biochemical Study

With the help of a syringe, blood was reaped from the hind limb vein of the experimental rats into an ampoule encompassing anticoagulant to tally RBCs, WBCs, and haemoglobin concentration.[20] Biochemical indices were achieved using a semi-automated biochemical analyser.

2.4.3 Histological Study

For the histological study, tissues (liver, kidney, and intestine) were poised and perpetuated in a 10 per cent formalin solution. Next through microtome, tissue sections were received on clean slides which were then rolled through different alcoholic grades of different strength, stained with haematoxylin-eosin, and finally studied under a compound microscope.

2.4.4 Ultrastructural Study

For investigation of alterations on the surface structures of different tissues, samples were fixed in 2 per cent glutaraldehyde followed by air-drying in TMS.[21] After coating with gold, tissues were observed in JEOL JSM 6360 at 25 kV. To know about the cellular level basic inconsistencies, little areas of tissues were settled in Karnovsky's fixative post-obsession were worn out with 1 per cent osmium tetraoxide, got dried out in acetone, and inserted in Araldite. Ultrathin areas were coloured with uranyl acetic acid derivation taken after lead citrate. After going through the process of ultra microtomy and staining, the samples were viewed under JEOL JEM 2100 as described earlier.[22]

2.5 Statistical Analysis

The comes about were communicated as mean±SEM. Factual investigations were performed utilising student's t-tests. Contrasts were considered factually noteworthy at the esteem $p \leq 0.05$.

3. RESULTS

3.1 Acute Toxicity Study

LD_{50} of the kaempferol derivative was found to be above 5000 mg/kg bw of animals. Maximum animals were observed having loss of appetite, wriggling movement apart from irritation, and anorexia before death.

3.2 Sub-acute Toxicity Study

3.2.1 Changes in Body Weight

It had been perceived that with an increase in concentrations of the kaempferol derivative component, the weights of the animal bodies had declined at the end of the dosing period as compared to the control group of animals.

Table 1. Effect of kaempferol derivative isolated from *L. ramosa* on the growth of rats

Group	Body weight (gram) Day 0	Day 29
Control	160.7±2.19	240.4±3.23
250 mg/kg	159.5±1.32	237.8±2.30
500 mg/kg	160.4±1.65	230.1±3.46*
1000 mg/kg	161.2±2.47	210.4±2.63*

Values are communicated as mean±SEM, *p values are critical at ≤0.05

3.2.2 Study of the Hepato-somatic Index and Reno-somatic Index

Results showed that there was a significant upsurge in the hepato-somatic index (HSI) tracing the larger liver in animals when treated with a higher dose of the isolated kaempferol derivative whereas the reno-somatic index (RSI) established the fact that kidneys got damaged when treated with the higher doses.

Table 2. Effect of kaempferol derivative on hepato-somatic index and reno-somatic index in rats

Group	HSI	RSI
Control	4.30±0.09	0.85±0.01
250 mg/kg	4.09±0.04	0.78±0.03
500 mg/kg	4.82±0.06*	0.65±0.01*
1000 mg/kg	5.67±0.01*	0.55±0.05*

Values are communicated as mean±SEM, *p values are critical at ≤0.05

3.2.3 Haematological Responses

The kaempferol derivative had been found pragmatic in raising the number of RBCs and thereby valuable in significant haemoglobin gram percentage increment when treated with 250 mg/kg bw of the component. RBC count decreased significantly when treated with 1000 mg/kg bw of the component. The WBC (Total Leucocyte Count) also cut back significantly when treated with 500 and 1000 mg/kg bw of the isolated component.

3.2.4 Biochemical Responses

All the three enzymes AST, ALT, and ALP had been recorded to be boosted significantly when the animals were exposed to 500 and 1000 mg kaempferol derivative/kg bw of the rat as set aside by the side of the control group.

3.2.5 Histological Study

Histological observations on the control intestine

Table 3. Haematological changes in rats exposed to kaempferol derivative isolated from *L. ramosa*

Group	RBC (mil/cmm)	WBC (mil/cmm)	Haemoglobin (gm per cent)
Control	5.50±0.11	6.54±0.05	15.07±0.06
250 mg/kg	5.58±0.17	6.51±0.06	15.80±0.57*
500 mg/kg	5.36±0.06	6.29±0.01*	15.10±0.08
1000 mg/kg	4.55±0.11*	5.48±0.06*	15.00±0.03*

Values are communicated as mean±SEM, *p values are critical ≤0.05

Table 4. Biochemical effect of the kaempferol derivative isolated from *L. ramosa*

Group	AST (U/L)	ALT (U/L)	ALP (U/L)
Control	39.00±0.28	30.35±0.13	256.20±0.15
250 mg/kg	40.20±0.07	31.83±0.44	258.80±0.10
500 mg/kg	50.87±0.05*	53.00±0.11*	308.00±0.33*
1000 mg/kg	63.53±0.10*	57.96±0.50*	322.00±0.57*

Values are communicated as mean±SEM, *p values are critical at ≤0.05, AST= Aspartate aminotransferase, ALT= Alanine aminotransferase, ALP= Alkaline phosphatase, U/L= Units per liter

showed normal intestinal villi along with regular lamina propria typical intestinal glands, and ordinary muscularis (Fig 2A). Rats when treated with 250 mg isolated component/kg bw, demonstrated median villi with colossal intestinal glands (Fig 2B). However, deteriorating villi apart from damaged lamina propria had been observed upon treatment with 500 mg component/kg bw rat (Fig 2C). Rats treated with 1000 mg component/kg bw showed pulverised villi and intestinal gland (Fig 2D). The control liver was revealed to have intact hepatocytes (H), natural central vein (CV), and average sinusoids (S) (Fig. 2E). But on treatment with 250 mg isolated component/kg bw rat, it showed an inflated central vein (Fig 2F).

When rats were exposed to 500 mg component/kg bw, they exhibited widened central vein and stretched sinusoid (Fig 2G). After treatment with 1000 mg component/kg bw rat, irregular order of hepatocytes dilated central vein and development of vacuole (V) were observed (Fig 2H). The kidney of the control group had normal conditions of capsular cavities (CC), intact glomerulus (G), and

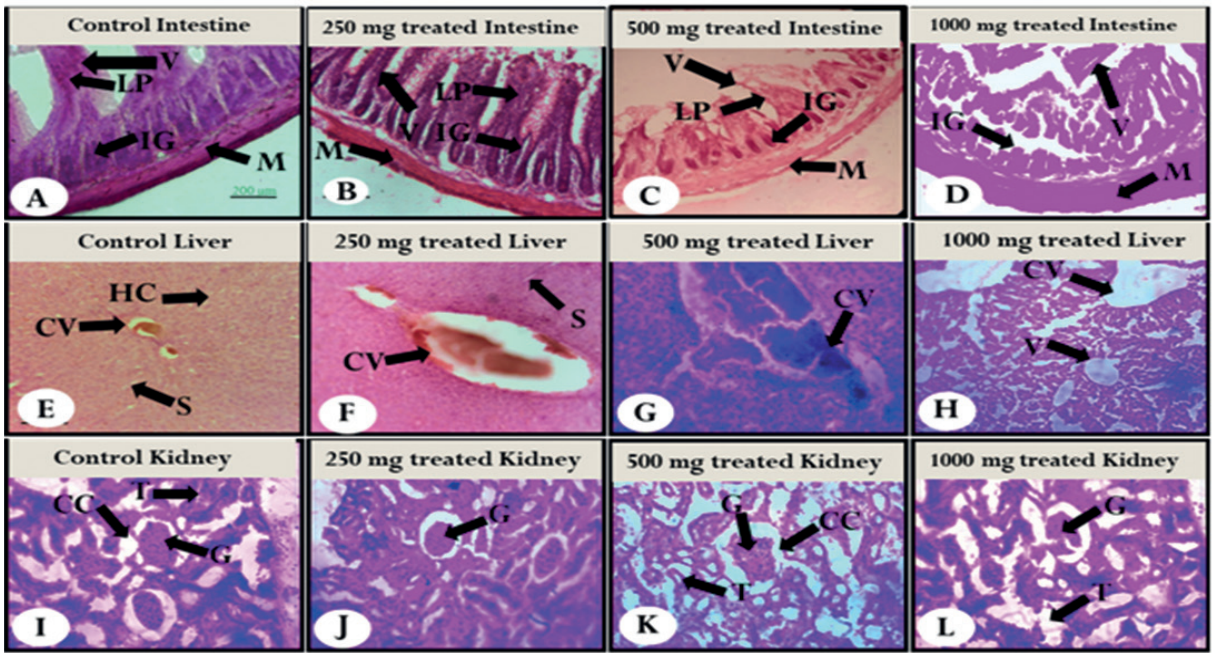

Figure 2. Light microscopic views of the small intestine (A-D), liver (E-H), and kidney (I-L). A, E, I show control small intestine, liver, and kidney, respectively. B, C, D sections show gradually increasing damages in villi (V), lamina propria (LP), intestinal gland (IG), muscularis (M) of the small intestine on treatment with 250 mg, 500 mg, and 1000 mg isolated component/kg bw of rat, respectively. F, G, H sections show deformed central vein (CV), and sinusoid (S) along vacuole (V) formation after treatment with 250 mg, 500 mg, and 1000 mg component/kg bw of rat, respectively. J, K, L sections showingnormal glomerulus (G) on treatment with 250 mg component/kg bw of rat which gets deteriorated with a dilated tubular wall (T) and capsular cavity (CC) after treatment with 500 mg and 1000 mg component/kg bw of rats, respectively. (Scale bar: 200µm).

ordinary shaped tubules (T) (Fig. 2I). On treating rats with 250 mg isolated component/kg bw, they showed routine glomerulus (Fig 2J). However, the disturbed structure of the glomerulus along with dilated capsular cavity and glomerular gaps with dilated tubules were noticed when rats were treated with 500 mg and 1000 mg component/kg bw, respectively (Fig 2K-2L).

3.2.6 Scanning Electron Microscopic Study

The existence of normal villi all over the internal surface in their legitimate provision had been unveiled in the surface topography of the control small intestine. Natural folding through which absorption of nutrients occurs could be observed on the villi surface (Fig. 3A). Rats on treating with 250 mg isolated component/kg bw had small intestine owing to little breaching over the villi surface in some range of small intestine (Fig 3B). After treatment with 500 mg component/kg, bw rat caused the depressed structure of normal columnar-shaped villi (Fig 3C).

However, rats exposed to 1000 mg component/kg bw revealed dematerialised villi (Fig 3D). The surface of the control liver demonstrated the presence of intact parenchymal cells (PC) along with central vein (CV) and a good histological relationship between liver parenchymal cells and sinusoidal vascular bed (SVB) (Fig. 3E). A bit of flexure in the structure of the central vein had been observed on treating with 250 mg component/kg bw rat (Fig 3F). After treatment with 500 mg component/kg bw of rat, the liver had disjunction of central vein and discontinuity of the liver parenchymal cell layer (Fig 3G).

However, exhaustive debasement of parenchymal cells along with central vein was noticed when treated with 1000 mg isolated component/kg bw rat (Fig 3H). Control kidney reveals the occupancy of the intact glomerulus (G) with the perfect bowman's capsule cavity and salubrious urinary tubules (T) (Fig. 3I).The conventional structure of glomerulus in the kidney with no glitch was found on treatment with 250 mg component/kg bw rat (Fig 3I). After treatment with 500 mg component/kg, the bw rat had a deteriorating glomerular barricade (Fig 3K). Anyhow, massive rupture of glomerular blood vessels and dilation of the capsular cavity (CC) were detected when rats were treated with 1000 mg component/kg bw (Fig 3L).

3.2.7 Transmission Electron Microscopic Study

In transmission electron pictograph of the control rat's villi, microvilli (MV) were distinctly noticed on the surface of enterocytes. Mitochondria (M) were in typical condition. Goblet cells (GC) were found with secretory vesicles (SV) in a regular form which secreted mucus over the intestinal surface (Fig 4A). Normal pictures of enterocytes except for non-uniform distribution of microvilli were noted when rats were treated with 250 mg isolated component/kg bw (Fig 4B).

In 500 mg component/kg bw of the treated rat group, plentiful vacuolisation had been recognised in the enterocytic cytoplasm along with severe microvilli damage (Fig 4C). However, after treating with 1000 mg component/kg bw rat, large-scale anomalies were noticed in the regular architecture of cells in the form of disintegrated goblet cells, shattering of the normal nucleus, and exhaustive eradication of microvilli from enterocytes (Fig 4D).

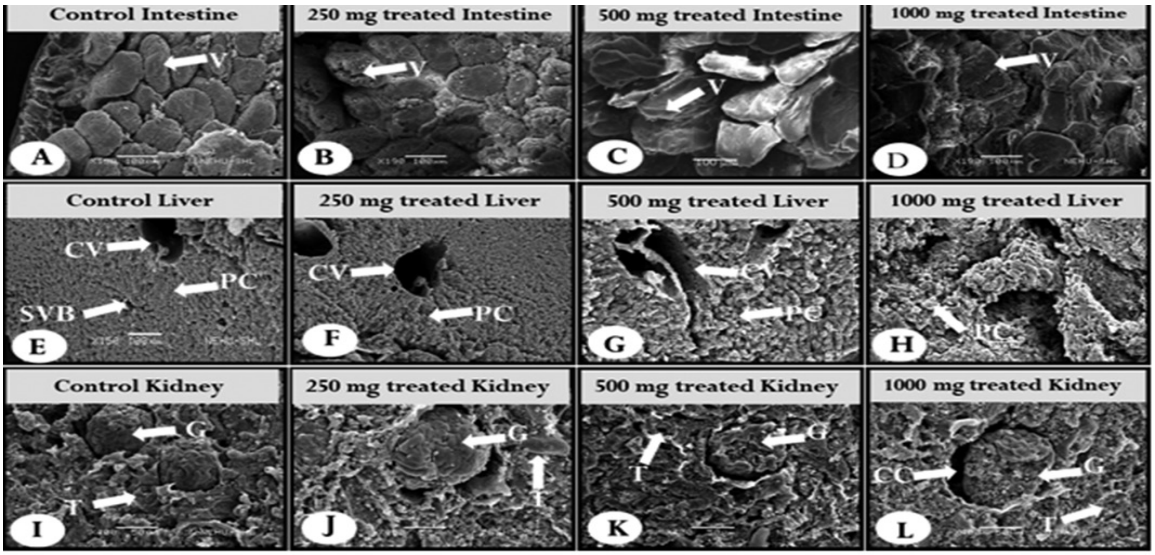

Figure 3. Scanning electron micrographs of the small intestine (A-D) (Scale bar: 100µm), liver (E-H) (Scale bar: 100µm), and kidney (I-L) (Scale bar: 50µm). A, E, I show control small intestine, liver, and kidney, respectively. B, C, D sections show gradually increasing bleachings as well as erosions of villi (V) in the small intestine on treatment with 250 mg, 500 mg, and 1000 mg isolated component/ kg bw of rat, respectively. F, G, H sections show broadened and deformed central vein (CV) after treatment with 250 mg, 500 mg, and 1000 mg component/kg bw of rat, respectively. J, K, L sections showing normal glomerulus (G) and tubule (T) on treating with 250 mg component/kg bw of rat which gets deteriorated with dilated tubules and capsular cavity (CC) after treatment with 500 mg and 1000 mg component/kg bw of rats, respectively.

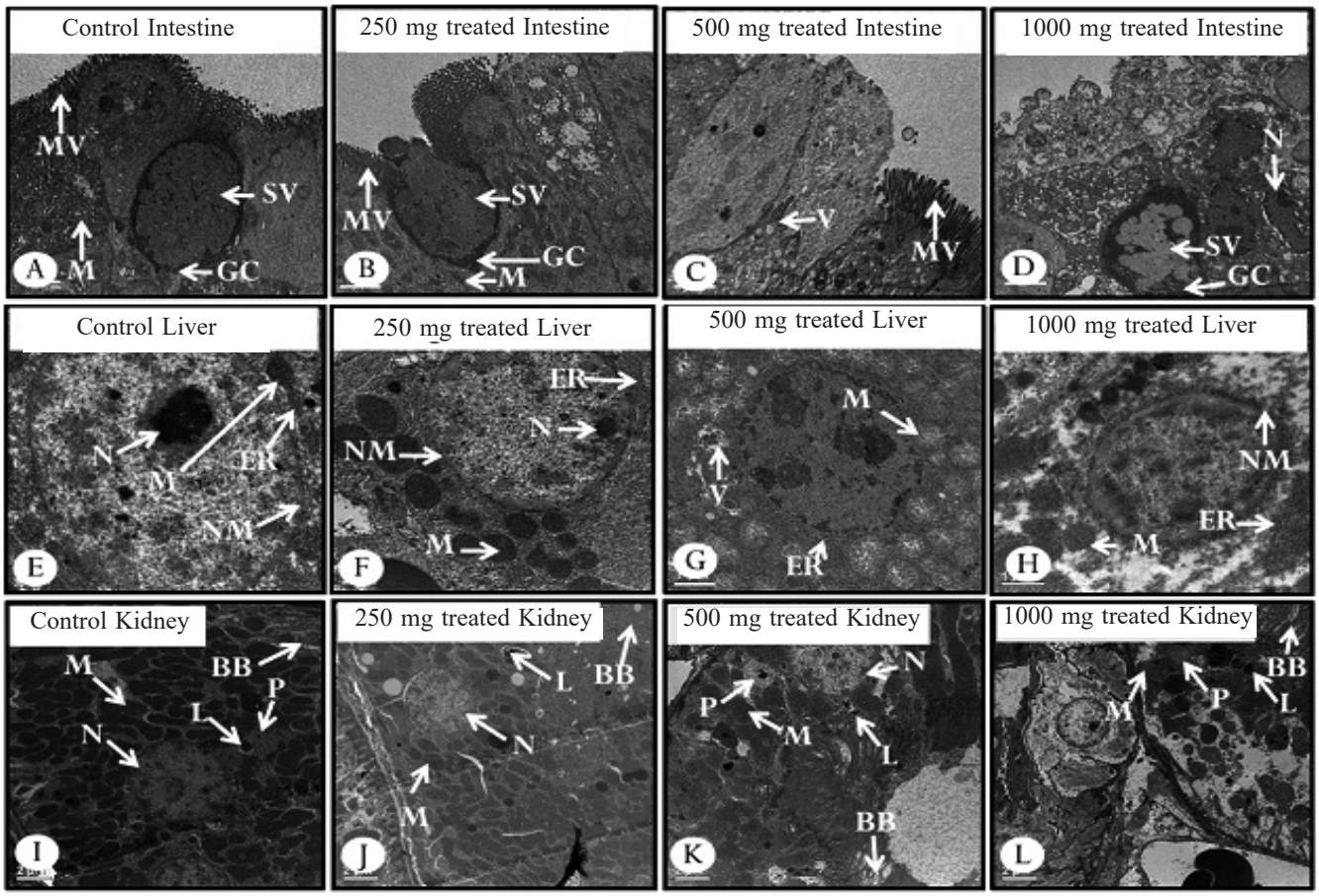

Figure 4. Transmission electron micrographs of the small intestine (A-D) (Scale bar: 1µm), liver (E-H) (Scale bar: 1µm), and kidney (I-L) (Scale bar: 2µm). A, E, I show control small intestine, liver, and kidney, respectively. B, C, D sections show gradual erosion of microvilli (MV), increasing damages in goblet cell (GC) as well as secretory vesicles (SV) in enterocytic cells of the small intestine on treatment with 250 mg, 500 mg, and 1000 mg isolated component/kg bw of rat, respectively. F, G, H sections show deteriorations in mitochondria (M), nuclear membrane (NM), endoplasmic reticulum (ER) which are associated with vacuolisation (V) after treatment with 250 mg, 500 mg, and 1000 mg component/kg bw of rat, respectively. J, K, L sections showing a normal structure of mitochondria (M), nucleus (N), lysosome (L), brush border (BB) on treating with 250 mg compound/kg bw of rat which gets degenerated adversely after treatment with 500 mg and 1000 mg component/kg bw of rat, respectively.

In transmission electron pictograph of the control liver traditional structure of the liver cell (hepatocyte) including routine double-layered nuclear membrane (NM), centrally located nucleolus (N), solid structure of parallel endoplasmic reticulum (ER), and mitochondria (M) were perceived (Fig 4E). As the rats were treated with 250 mg component/kg bw rat, no exclusive variation was noticed in the cellular structure of hepatocytes aside from a few vacuole formations (Fig 4F). When the rats were treated with 500 mg component/kg bw rat, nucleolar fragmentation was observed. Cristae of mitochondria were noted to be crippled (Fig 4G). A comprehensive evaluation of cytoplasmic material, the disordered structure of nucleus with the dissociated nucleolus, and partially expounded endoplasmic reticulum were recorded when treated with 1000 mg component/kg bw rat (Fig 4H). In the control structure of proximal convoluted tubule (PCT) region from kidney nephron of rats, it was noticed to be associated with orderly nucleus (N), properly defined mitochondria (M), peroxisomes (P) known for providing compartmental oxidation reaction, cellular recycle bin-lysosomes (L) and typical brush border (BB) (Fig 4I). While treating rats with 250 mg component/kg bw rat, no basic structural alterations were observed (Fig 4J). Treatment with the higher dose of 500 mg component/kg bw rat, led to shrinkage in the brush border area of PCT along forwarded with vacuolisation near the nuclear region (Fig 4K). However, the devastation of the basic layout of the columnar structure of PCT cells having large scale empty spaces deteriorating the cell organelles interconnection was observed when treated with 1000 mg isolated component/kg bw rat (Fig 4L).

4. DISCUSSION

In the present study, no mortality, lethargy, or other behavioural changes were detected after the single-dose oral administration of the component up to 5000 mg/kg bw, implying the LD_{50} value to be more than 5000 mg/kg bw. But it cannot be surely attributed that the isolated component is not toxic at this much of high

dose. The unfavourable reaction of the component at this single high dose cannot come about, maybe due to fast absorption in the intestine or first-pass metabolism in the liver through which toxic substances would have been transformed into harmless forms.[23] Although flavonoids are very good for health excess intake of flavonoids such as kaempferol and saponins may cause toxic effects in various aspects.[24] Therefore the sub-acute toxicity study was carried out involving 250 mg, 500 mg, and 1000 mg phytochemical/kg bw of rats for 28 days which revealed a decrease in body weights of the animals exposed to higher dosages as a contrast to the control group, suggesting adverse effects on the consumers.[25-26]

Treatment with higher dosages has been noticed to cause extensive damage to the villi of the small intestine which are the main absorber of nutrients in the body resulting in body weight decrease in the animals. The detrimental villi are found to have fragmented microvilli, which are plasma-membrane protuberances of enterocytes when observed under the transmission electron microscope. Robins and Brooker found comparable influences on sheep when they served the animals with higher doses of *Acacia aneura* leaf extract.[27] The liver and kidney are among the prime body parts involved in the detoxification of the toxic substances present in our bodies. Any alteration in them points towards the toxic nature of the chemical under investigation.[28-29] Therefore special attention is given to these organs while estimating the extent of toxicity of a substance under study. In our study, it has also been observed through light microscopy and SEM that treating animals with 500 mg and 1000 mg component/kg bw causes exhaustive debasement of parenchymal cells, central vein, and development of vacuole in the liver and deteriorating (ruptured) blood vessels in glomerulus respectively. Elevated values of hepato-somatic index propose a larger proportion of the liver, which takes place due to the stretching and dilation of central vein and hepatocytes, which in turn increases the enzyme secretion. Such kind of effects on liver, kidney, and hepato-somatic index was also observed when Wistar rats were continuously treated with higher doses of an anthelmintic oxyclozanide.[30]

Moreover in liver histology, widening of the central vein has been observed especially when treated with higher doses which may indicate congestive hepatopathy.[31] Similar to our observations, the nuclei of the liver got impaired with other organelles when mice were dosed with active ingredients present in acaricide Frontline.[32] Reno-somatic index reinforces the fact that its low value as recorded in our present investigation may be due to structural and cellular damages undergone in the experimental animal. Cellular level investigation of the kidney validated the ravage in the nuclear region, cristae of mitochondria, endoplasmic reticulum of cells, and in tubule's columnar cells of the kidney, justifying the lower value of the reno-somatic index.

Similar observations were also made when mice were given platinum particles of size lesser than 1 nm intravenously to check induction of nephrotoxicity.[33] Blood parameter analysis in toxicity analysis is crucial to figure out jeopardize to the hematopoietic system when it is related to those findings in humans.[34-35] In this case, all three enzymes AST, ALT, and ALP levels increased significantly when treated with the higher dosages. AST is ubiquitous involving the heart, brain, and blood cells.[36] An increase in the synthesis of ALP indicates bile duct obstructions in the liver.[37] An increase in these enzymes in our study supports the altered hepatic function in animals of higher dosed groups. Similar kinds of observations were made in the higher dose treatment with silver nanoparticles synthesised by *Azadirachta indica* in rats.[38] On treating with 1000 mg/kg body weight of the component, the RBC count decreased significantly. Naturally, haemoglobin concentration also decreased with the highest dose but it increased significantly when treated with the lowest dose suggesting the isolated component to be a blood tonic at a lower level only.[39] Total WBC count decreased significantly when treated with higher dosages. These results suggest that consumption of higher doses of kaempferol derivative may cause anaemia and weaken the immune system making the consumers more vulnerable to infection.[40-41]

5. CONCLUSION

The present study revealed that at 250 mg/kg body weight, the kaempferol derivative isolated from the plant *L. ramosa* is not harmful. However, 500 mg or more/kg, if consumed for the long term may cause an increase indeformity of the surface of the liver, intestinal villi, and kidney, also their cellular infrastructure along with alterations in blood constituents. Therefore, for further experiments involving the kaempferol derivative for chemotherapeutic purposes, the doses of the phytochemical should be considered low, preferably below 500 mg/kg body weight.

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In the present study, he framed and conceptualised the work and guided in the final draft preparation. He also provided guidance in carrying out the research work and preparation of the manuscript.