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Acute and sub-acute toxicity study of hydro-alcoholic leaves extract of *Reinwardtia indica* in rats

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ABSTRACT

The present study was to assess the toxicity of hydro alcoholic leaves extract of *Reinwardtia indica* in Charles foster rats through an acute and sub-acute study. In the acute study, rats were treated orally with single dose and for sub-acute study different doses were given orally for 28 consecutive days. At the dose of 2000 mg/kg satellite group was also used for 6 weeks as per OECD guidelines-407. General behavioral parameters were assessed in acute toxicity and found no mortality or exterior signs of toxicity. While in the sub-acute study; biochemical, hematological and histopathology along with the body weight, food, and water consumptions parameters were screened in the animals after 14 & 28 days. The study reveals the insignificant ($P < 0.05$) change in treated group in comparison to the control. The hydroalcoholic leaves extract of *Reinwardtia indica* was found non-toxic up to 5000 mg/kg in acute study whereas up to 2000 mg/kg dose level in the sub-acute study.

1. Introduction

Reinwardtia indica (R. indica) Dumort belongs to family Linaceae and commonly known as Pyoli, Basanti, phunili, shivali. It is geographically distributed in a Himalayan region of India, Nepal, China, Western Ghats, and north-east area. Only three species of Reinwardtia is known which are native to Southern Asia namely, *Reinwardtia indica* [1], *Reinwardtia trigyna* [2] and *Reinwardtia sinensis* [3]. It is widely used as folk medicine in India by local people for the treatment of boils, pimples, skin infections and carbuncle. Traditionally, it is mentioned that bright yellow petals of *(R. indica)* were used as tongue cleaner and the crushed leaves were used to treat paralysis as the chemical components would act upon the sodium pump. The paste of the whole plant is used to get relief from backaches [4,5]. It is used in traditional Chinese medicine formulation for the treatment of acute or chronic gastritis [6,7]. *Reinwardtia trigyna* is used as natural herbicide and weedicide as it showed a good phytotoxic activity [2,8,9].

The alcoholic and hydro-alcoholic leaf extracts and hydro-alcoholic stem extract of *(R. indica)* are used for anti-oxidant, anti-microbial, anti-inflammatory activity (unpublished). The investigation was carried out first time to develop standardization parameters of this plant in the performed plant microscopy, powder microscopic characterization, physicochemical analysis, heavy metal analysis, extractive values in different polarity based solvent, preliminary phytochemical screening etc. [10]. Antioxidant, antimicrobial and cytotoxic potential of silver nanoparticles synthesized using flavonoid-rich alcoholic leaves extract of *Reinwardtia indica* has also been reported [11].

Several reports have demonstrated that secondary plant metabolites of this plant exert diverse medicinal biological effects [12]. Main aim of this study was to screen its safety in Charles foster rats at different dose level with hematological and biochemical estimation from blood and histopathology of vital organs at interval of 14th & 28th days.

2. Material and methods

2.1. Plant material and preparation of extracts

R. indica was collected from Uttrakhand (2000–2200 m altitude) the surroundings of the region of the Himalayan in the month of September to February as it flowers in the month of winter [13,14]. The specimen of plant part leaves and stems were identified with the help of Herbarium sheet (*Voucher specimen number Lina.2015/1*) available in Department of Botany, Institute of Science by Professor N. K. Dubey. Extraction of the leaves in the hydro alcoholic solvent (70:30) was done through the soxhlet apparatus with hot percolation method and then filtered followed by evaporation to dryness at a 45 °C with a rotary

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Table 1
Behavioral responses and general appearance of rat treated with single dose of HALERI in acute toxicity study.

Observation	Control group	50 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg	5000 mg/kg
Temperature	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Change In skin	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Eye color change	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Food intake	Normal	Normal	Normal	Normal	Normal	Normal	Normal
General physique	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Diarrhea	Not present	Not present	Not present	Not present	Not present	Not present	Not present
Coma	Not present	Not present	Not present	Not present	Not present	Not present	Not present
Drowsiness	Not present	Not present	Not present	Not present	Not Present	Not Present	Not Present
Breathing difficulty	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
Sedation	No effect	No effect	No effect	No effect	No effect	Observed	Observed
Tremor	Not present	Not present	Not present	Not present	Not present	Not present	Not present
Death	Alive	Alive	Alive	Alive	Alive	Alive	Alive

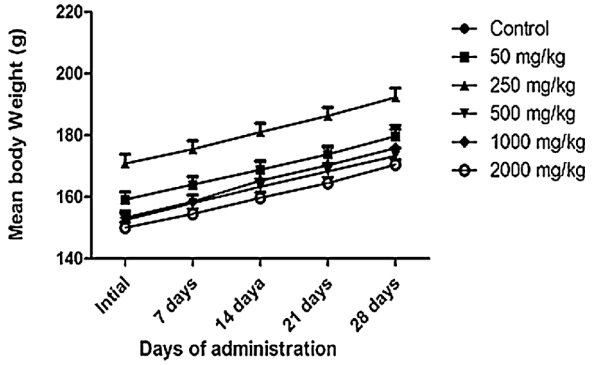

Fig. 1. Body weight assessment of treated rats in sub-acute toxicity study.

evaporator (*Buchi R-210 Advanced, Switzerland*). The hydroalcoholic extract of the leaf of *Reinwardtia indica* (HALERI) was dissolved in water and given to the rats for toxicity study as per WHO guidelines.

3. Experimental design

3.1. Animals

The protocol of this study was approved by the Institutional Animal Ethical Committee of the Institute of Medical Sciences BHU Varanasi, India (Permission number: Dean/2016/CAEC/50). The inbreed albino female rats of Charles foster strain (120–150 g) was purchased from the Central Animal Facility of our Institute and acclimatized in our laboratory conditions for 7 days with free access to normal standard chow diet and tap water. The animals were kept under standard conditions of temperature (23 ± 2 °C) and a relative humidity of 50%.

3.2. Acute toxicity

The oral acute toxicity study was carried out according to the guideline No. 423 provided by the Organization of Economic Cooperation and Development (OECD) for the acute toxicity class method (ATC) procedure with slight modifications. Female rats were allocated in treatment groups (6 animals/per group). Prior to the treatment, animals were weighed, marked and not allowed to take food overnight without suppression of water intake. Animals of the control group received distilled water whereas the treated groups received a single dose of 50 mg/kg, 250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 and 5000 mg/kg body weight of the freshly prepared hydroalcoholic leaves extract of *Reinwardtia indica* (HALERI). After dosing, food was withheld for a further 3–4 h while animals were observed individually during the first 30 min, and then at 2, 4, 6 h post-dosing, and thereafter once daily over 7 days for clinical signs of toxicity such as mortality, respiratory pattern, changes in general behavior, skin, eyes, fur, and somatomotor activity. General characteristics of animals (eye, touch, activeness of animal, and movement etc.) before treatment and after treatment were observed without any specific scoring method or instrument involvement.

Table 2
Effect of HALERI on food intake and water consumption by rat during 28 days treatment and recovery period (satellite group).

Treatment	Average food intake (g/day/rat)	Average water intake (ml/day/rat)
Control	4.29 ± 1.90	4.81 ± 1.20
50 mg/kg	4.12 ± 1.01	3.74 ± 1.74
250 mg/kg	4.05 ± 1.11	4.06 ± 1.35
500 mg/kg	3.20 ± 1.22	4.86 ± 1.80
1000 mg/kg	4.48 ± 1.28	4.74 ± 1.47
2000 mg/kg	4.95 ± 1.90	4.39 ± 1.13
Satellite control	4.23 ± 1.56	4.12 ± 1.81
Satellite (2000 mg/kg)	4.5 ± 1.10	4.19 ± 1.17

Values are expressed in Mean ± SEM, n = 6 animals/group, $p < 0.05$ (ANOVA/ Dunnett's test).

3.3. Sub-acute toxicity study

According to the OECD guidelines no. 407 with slight modification, rats were divided into 6 groups of 6 animals and treated by gavage. Control group received distilled water while, the HALERI treated groups received the extract once daily (10:00–1:00 pm) for 28 consecutive days at the doses of 50, 250, 500, 1000 and 2000 mg/kg. Half of the animals from each group were sacrificed after the 14th day and a half animals were sacrificed after 28 days. Vital organs like heart, liver, kidney, and brain were stored for histological analysis. Satellite group at the dose of 2000 mg/kg was also used for 6 weeks to observe the effect of extract an additional 6 animals.

3.4. Body weight, food, and water consumption

Body weight of the rats in all the groups was recorded before administration of doses, further body weight was taken weekly during the treatment and finally on the day of sacrifice. The amount of food and water intake was recorded daily. The consumed amount of food and water were measured before they provided to each group, their remnants were calculated next day to get the differences, which were recorded as daily food (g./rat/day) and water consumption (ml/rat/day).

3.5. Blood analysis

Blood (1.5 ml) was collected from the retro-orbital region of the rats for measurement of hematological (EDTA-coated tubes) and biochemical (dry tubes) parameters after 14 days and 28 days.

Table 3
Hematological parameters of rat treated with different dose level of HALERI in sub-acute toxicity after 14th day.

Parameters	Normal ranges	Control	50 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
Hemoglobin (%)	**10.2-16.6**	15.73 ± 1.48	13.01 ± 1.48	13.41 ± 1.06	12.15 ± 0.89	13.65 ± 1.97	14.59 ± 1.11
Total RBC (10^6/μL)	**5-10**	6.34 ± 0.12	7.02 ± 0.39	6.54 ± 0.10	8.13 ± 0.18	9.13 ± 0.97	9.52 ± 0.37
WBC (10^3/μL)	**6-15**	11.41 ± 2.42	8.78 ± 1.46	10.05 ± 0.11	12.68 ± 1.22	7.78 ± 2.08	10.19 ± 1.25
Platelets(10^3/L)	**782-985**	909.30 ± 22.04	902.06 ± 52.15	805.77 ± 80.25	888.10 ± 70.25	917.77 ± 34.07	896.18 ± 33.12
PCV (%)	**39-49**	48.05 ± 1.23	39.16 ± 2.24	42.18 ± 1.04	44.10 ± 1.77	43.73 ± 2.20	46.12 ± 1.55
LC (%)	**55-95**	71.09 ± 1.52	66.18 ± 3.37	79.14 ± 1.15	82.11 ± 1.15	79.18 ± 1.25	87.27 ± 1.77
NP (%)	**10-40**	13.23 ± 1.64	24.18 ± 1.64	34.14 ± 1.14	19.01 ± 1.78	26.15 ± 1.46	33.18 ± 2.19
MC (%)	**1-4**	1.05 ± 0.01	1.88 ± 0.15	2.78 ± 1.70	3.09 ± 0.18	2.11 ± 0.16	2.19 ± 0.88
EP (%)	**0-4**	1.86 ± 0.84	2.56 ± 0.18	3.40 ± 0.10	2.82 ± 0.75	2.16 ± 0.58	2.39 ± 0.98

All values are expressed in Mean ± SEM, n = 6 animals/group, $p < 0.05$ (ANOVA/ Dunnett's test).

Table 4
Hematological parameters of rat treated with different dose level of HALERI in sub-acute toxicity after 28th day.

Parameters	Normal ranges	Control	50 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg	**Satellite**
Hemoglobin (%)	**10.2-16.6**	16.02 ± 1.91	14.11 ± 1.88	14.52 ± 1.25	13.85 ± 0.65	14.97 ± 1.51	15.11 ± 1.66	15.95 ± 2.08
Total RBC(10^6/μL)	**5-10**	10.90 ± 0.07	8.12 ± 0.28	9.14 ± 0.14	9.33 ± 0.41	9.89 ± 0.87	8.72 ± 0.48	9.10 ± 0.17
WBC (10^3/μL)	**6-15**	12.94 ± 1.74	10.78 ± 1.05	12.25 ± 0.71	13.37 ± 1.55	9.18 ± 2.88	10.78 ± 1.77	14.16 ± 1.18
Platelets(10^3/L)	**782-985**	922.70 ± 34.02	957.06 ± 32.17	897.17 ± 43.05	908.12 ± 14.05	957.17 ± 29.17	939.16 ± 70.05	980.06 ± 17.60
PCV (%)	**39-49**	49.9 ± 2.16	44.25 ± 2.07	45.07 ± 1.40	46.16 ± 1.08	47.37 ± 2.17	48.16 ± 1.19	49.09 ± 1.11
LC (%)	**55-95**	76.10 ± 3.68	66.18 ± 3.37	79.14 ± 1.15	82.11 ± 1.15	79.18 ± 1.25	66.18 ± 3.37	93.03 ± 1.23
NP (%)	**10-40**	25.32 ± 1.88	34.88 ± 2.06	36.40 ± 1.40	29.11 ± 1.70	36.50 ± 1.48	34.89 ± 1.97	39.10 ± 2.10
MC (%)	**1-4**	1.50 ± 0.15	2.08 ± 0.37	3.80 ± 1.72	3.99 ± 0.82	3.19 ± 0.96	2.87 ± 0.67	3.57 ± 0.38
EP (%)	**0-4**	1.93 ± 0.11	3.50 ± 0.37	3.90 ± 0.10	3.08 ± 0.41	3.56 ± 0.45	3.76 ± 0.32	3.80 ± 0.70

All values are expressed in Mean ± SEM, n = 6 animals/group, $p < 0.05$ (ANOVA/ Dunnett's test).

Table 5
Biochemical estimation from blood serum of rats after 14th day's treatment at different dose level in sub- acute toxicity study.

Parameters	Normal ranges	Control	50 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
SGOT (U/L)	**54-298**	153.6 ± 16.70	179.16 ± 30.46	169.83 ± 25.69	157.5 ± 36.22	192.33 ± 32.22	151.33 ± 27.75
SGPT (U/L)	**17-77**	43.5 ± 7.77	37.33 ± 8.38	37.83 ± 5.57	31.5 ± 6.73	39.66 ± 8	35.5 ± 7.15
ALP (U/L)	**64-128**	95.33 ± 6.71	82.33 ± 4.94	82.66 ± 6.02	96.83 ± 6.61	88.16 ± 8.36	105 ± 6.61
Creatinine (mg/dL)	**0.2-0.9**	0.34 ± 0.035	0.36 ± 0.02	0.33 ± 0.02	0.34 ± 0.04	0.3 ± 0.018	0.37 ± 0.022
Urea (U/L)	**35-96**	52.5 ± 5.07	56 ± 8.44	75.5 ± 8.46	59.5 ± 8.34	62.5 ± 8.62	65.33 ± 7.73
BUN (mg/dl)	**8-33**	24.51 ± 2.36	26.15 ± 3.94	27.31 ± 3.7	27.78 ± 3.89	29.18 ± 4.02	30.51 ± 3.61

All values are expressed in Mean ± SEM, n = 6 animals/group, $p < 0.05$ (ANOVA/ Dunnett's test).

Table 6
Biochemical estimation from blood serum of rats after 28th day's treatment at different dose level in sub- acute toxicity study.

Parameters	Normal ranges	Control	50 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
SGOT (U/L)	**54-298**	163.5 ± 32.5	167.16 ± 32.74	183.33 ± 24.8	178.66 ± 19.2	192.5 ± 35.47	208.66 ± 15.99
SGPT (U/L)	**17-77**	45.5 ± 8.65	44.5 ± 8.09	47 ± 8.23	36.66 ± 10.22	58.16 ± 6.53	45.16 ± 9.24
ALP (U/L)	**64-128**	111.1 ± 9.3	83.83 ± 7.4	103.5 ± 8.09	104.8 ± 8.54	101.6 ± 7.36	107.1 ± 8.17
Creatinine (mg/dL)	**0.2-0.9**	0.39 ± 0.05	0.38 ± 0.02	0.33 ± 0.034	0.4 ± 0.045	0.47 ± 0.055	0.48 ± 0.039
Urea (U/L)	**35-96**	65.16 ± 5.66	58.5 ± 8.07	76.16 ± 10.07	72.83 ± 7.02	73.83 ± 6.33	71.3 ± 8.6
BUN (mg/dl)	**8-33**	30.43 ± 2.64	35.25 ± 3.95	35.56 ± 4.7	34.01 ± 4.27	34.48 ± 2.95	33.3 ± 4.02

All values are expressed in Mean ± SEM, n = 6 animals/group, $p < 0.05$ (ANOVA/ Dunnett's test).

3.6. Hematological analysis

The blood samples collected in heparinized tubes were used for the hematological analyses. The following parameters: red blood cell count (RBC), white blood cell count (WBC), neutrophils (NP), lymphocytes (LC), monocytes (MC), eosinophils (EP), hemoglobin (Hb), platelets (PL) and packed cell volume (PCV) were evaluated by automated analyzer (KX-21-Hematology-analyzer, Sysmex Corporation, USA).

3.7. Biochemical analysis

Dry tubes containing collected blood were centrifuged at 3000 rpm at 25 °C for 15 min to obtain the serum, which was stored at −20 °C until the measurement of biochemical parameters (Erba chem 5 semi-auto analyzer) Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT) (Coral clinical system), Alkaline phosphatase (ALP) (Arkray health care Pvt ltd.), Urea, Creatinine (Coral clinical system) and BUN analysis was performed.

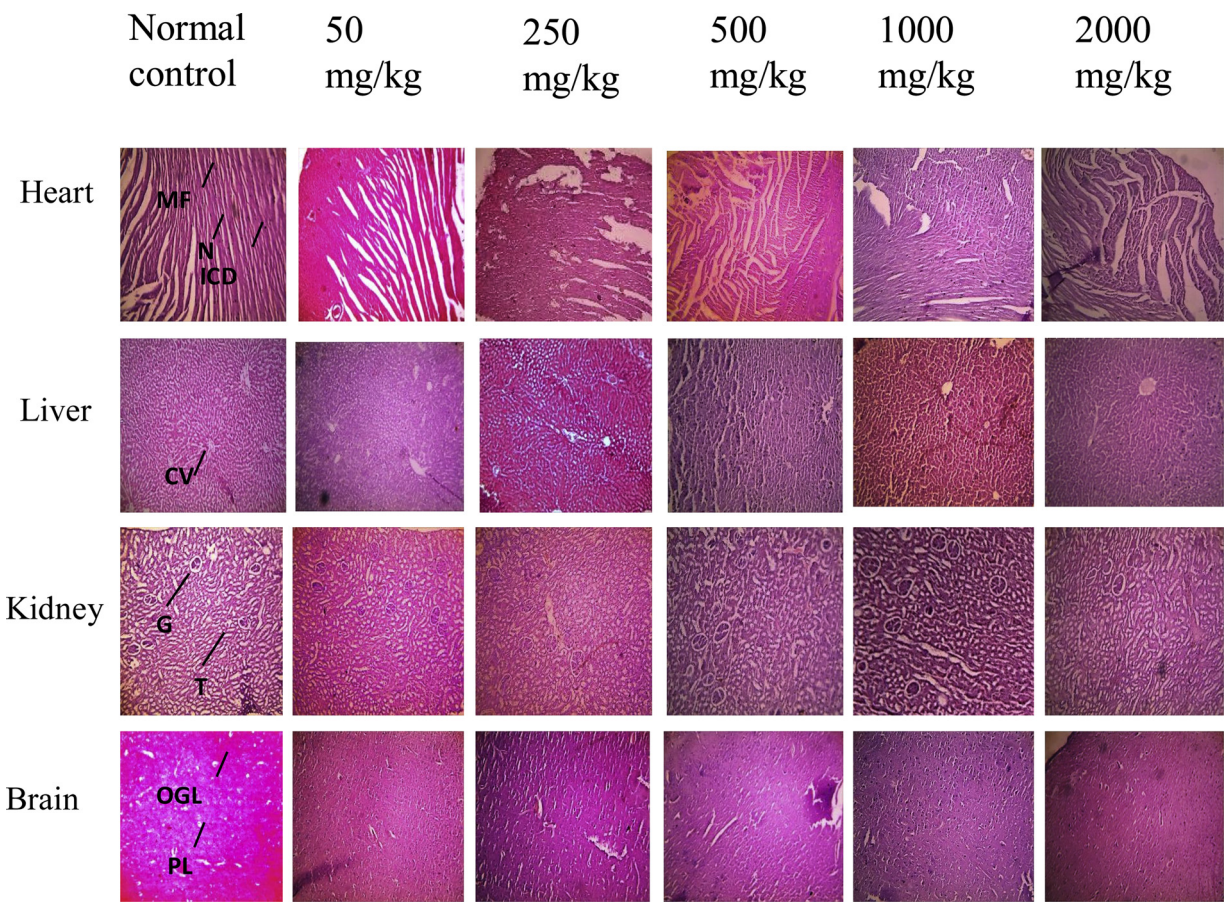

Fig. 2. Histopathology (H&E stain10x) of rat tissues of treated groups rats after 14th days. ICD- Intercalated disc, N- Nuclei, MF- Muscle fiber, CV- central vein, G-glomerulus, T- tubules, OGL- outer granular layer, PL- Polymorphic layer.

3.8. Histological analysis

Organs such as heart, liver, kidney, and brain were excised and fixed in 10% formaldehyde for histological analysis. Paraffin-embedded organs were cut to 5 mm sections and stained with hematoxylin and eosin. Stained sections were visualized and all measurements were performed using a Nikon eclipse e200 microscope equipped with cat cam 300-3.0 M P micro cope camera connected to a computer where the images were transferred and analyzed with the Scope Tek scope photo ×86, 3.1.475 microscopic instrument.

3.9. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Comparison and analysis was performed using the one way ANOVA one way variance followed by Dunnett's multiple comparisons. The p values < 0.05 were considered significant.

4. Results

4.1. Acute toxicological evaluation

To perform acute toxicity, rats were treated orally with single dose of various concentrations (50, 250,500, 1000, 2000 and 5000 mg/kg) of HALERI and obsreved for 24 h and no mortality observed. Clinical signs (temperature, change in skin, eye color change, general physique, diarrhea and sedation) were recorded (Table 1).

4.2. Sub-acute toxicity

For sub-acute toxicity, rats were divided into six groups and treated with control, 50, 250, 500, 1000, 2000 mg/kg/day for 28 days and satellite dose 2000 mg/kg/day for 6 weeks. Body weight, intake of food & water, hematology, serum chemistry, and microscopic findings were recorded after 14 days and 28 days. In satellite treated group, the intake of food, water consumption, hematology and biochemical was assessed after 6 weeks.

4.3. Effect of HALERI on body weight, food intake and water consumption in rat

No significant change was observed in the animal's body weight during the study (Fig. 1). After 28 days of oral administration of HALERI, the food intake and water consumption were also not affected. It indicated that the extract did not show any significant change in appetite and deleterious effect on the growth of the animal. No significant changes were observed in rat's physiological as well as metabolic activity in comparison to control. The negative effect of the extract showed at the particular dose toxicity in the rat. But during dosing (28-day) and the recovery periods, there was no significant change in food and water intake in rats at different dose treated groups as compared to their respective control (Table 2). In satellite group, rats treated with the highest dose (2000 mg/kg body wt.) also did not show any significant change in food and water consumption.

4.4. Effect of HALERI on hematological parameters

Data of hematological analysis of 14^{th} and 28^{th} days were given in

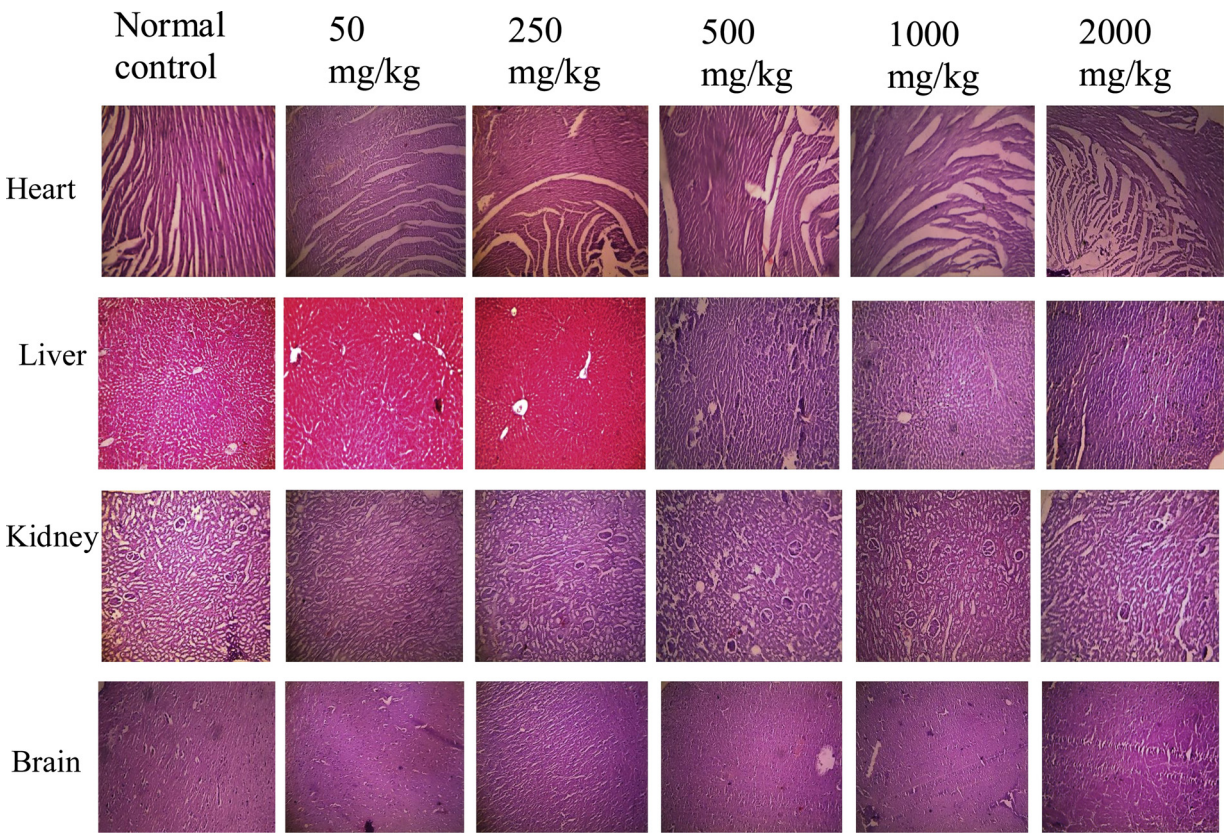

Fig. 3. Histopathology (H&E stain10x) of rat tissues of treated groups rats after 28th days.

Tables 3 & 4 . Results revealed that no significant changes have been shown in the hematological analysis as compared with control. The same result also found in the satellite treated group.

4.5. Effect of HALERI on biochemical parameters

To assess the toxicity of any new compound it is very essential to know the status of renal function test and liver function test, which can be checked through biochemical estimation without sacrificing the rat. For liver function assessment, the SGOT, SGPT and ALP parameters were mainly used. For assessments of kidney function serum urea and creatinine mainly used. After the intake of any compound or molecules if any changes are seen in above-mentioned biomarkers from the normal range, indicates the toxicity of the compounds or molecules in animals [15].

The results suggested from our study that the levels or activities of biochemical parameters in animals after 14^{th} & 28^{th} days have no significant variations occurred in SGOT, SGPT, ALP, urea and creatinine levels at all tested dose in comparison to control. The satellite groups after 6 weeks also showed insignificant changes in the above-mentioned parameters compare to control (Tables 5 & 6)

5. Histopathology

In the histopathological study, it was observed that in all treated groups after 14 days (Fig. 2) and 28 days (Fig. 3) the organs showed no changes at the cellular level in comparison to the control. The histopathological slides also confirmed that the HALERI treated group up to the dose of 2000 mg/kg showed no toxicity in 4 weeks.

6. Discussion

Before pharmacological study and development of phytopharmaceutical product of any medicinal plant, the acute and sub-acute toxicity is mandatory as per the standard guidelines [16]. It is also a necessary process of dose determination at the preclinical level in drug discovery and development [17]. *Reinwardtia indica* is the novel medicinal plant used as a folk medicine in the treatment and management of several diseases on the basis of traditional uses but no scientific report available till date.

Reinwardtia indica is widely used as folk medicine in India. It is also used to increase the lactation period. Due to yellow color of its petal; many people used it to dye clothes. Pharmacologically, this plant belongs to the flax families which are used as antioxidant, wound healing, anti-microbial, anti-inflammatory, and anti-nociceptive agent. Medicinal properties of the plant would be due to the presence of alkaloids, flavonoids, phenolic, tannins, and other phytoconstituents. Several reports have demonstrated that secondary plant metabolites exert diverse medicinal biological effects [12]. In the study, Charles foster rats are used to screen the safety at various dose level of the plant extract with hematological and biochemical estimation from blood and histopathology of vital organs at the interval of 14^{th} & 28^{th} days.

In the acute toxicological study, single dose treatment at various concentrations of HALERI for 24 h, showed no mortality, clinical signs, temperature, change in skin, eye color change, general physique, diarrhea, and sedation were recorded (Table 1).

Sub-acute toxicity study assessed for 28 days with satellite dose 2000 mg/kg/day for 6 weeks and no toxicity or any mortality in any treated groups was found. After 28 days of oral administration of HALERI, the food intake and water consumption were not affected. It indicates that the extract did not affect the appetite and deleterious effect on the growth of animals. No significant changes were observed in rat's physiological as well as metabolic activity in comparison to control. If the changes occur in hematological parameters, it indicates the toxic effects of extract in rat blood either at physiological or pathological level. Most of the nutrients and foreign bodies in the body is

transported through blood [18]. If the extract shows the toxicity in the body, it directly affects its components such as red blood cells, white blood cells, platelets, and hemoglobin. The ranges of these components either decrease or increase significantly from normal. It indicates that the toxicity caused by the extract can affect the body immune as well as the function of organs [19]. Data of hematological analysis at 14^{th} and 28^{th} days are given in Tables 3 & 4 . Results revealed that no significant changes have been found in the hematological analysis when compared with control. The same result also observed in the satellite treated group.

The body has two essential and vital organs for proper functions are liver and kidney. The function of liver and kidney are different as one is used for metabolism of intake and another one is used for excretion of the waste product [20]. To assess the toxicity of any new compound it is very essential to know the status of these two vital organs, which can be checked through biochemical estimation without sacrifice of rats. The satellite group after 6 weeks also showed no significant changes in the above mentioned biochemical with compare to control.

In the histopathological study, we found that in all treated groups after 14 days (Fig. 2) and 28 days (Fig. 3) the organs showed no significant changes at the cellular level in comparison to control. The histopathological images also confirmed that the HALERI treated groups up to the dose of 2000 mg/kg show no toxicity in 28 days.

7. Conclusions

The oral dose upto 5000 mg/kg of hydroalcoholic extract of the leaves of *Reinwardtia indica* show no treatment-related sign of toxicity or mortality in the animals. The repeated dose of HALERI up to 2000 mg/kg for 28-days at the different concentration showed no significant changes in intake of food & water. Hematology, biochemical serum analysis and histopathology at the cellular level also did not show any marked effect. The toxic effect on fertility, mutagenicity, teratogenicity, carcinogenicity potential may be performed in future. This study clearly indicates that the HALERI can be used for further evaluation of pharmacological activities on the above-mentioned doses of extract.

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