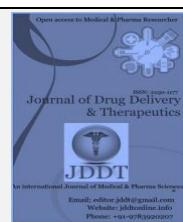


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Research Article

## 28 Days repeated oral toxicity study of *Rosmarinus officinalis* in Wistar Rats

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### Abstract

*Rosmarinus officinalis* has long been used as a traditional oriental medicine. It is traditionally used as antioxidants as well as essential oil. *Rosmarinus officinalis* mainly contains the phenolic compounds which is responsible for the antioxidant property. In the present study, 28-day subacute oral dose toxicity studies of hydroalcoholic extracts of the plant of *Rosmarinus officinalis* were performed in Wistar rats. The repeated oral toxicity study was carried out to detect the no-observed adverse effect level (NOAEL). In this study, a total of 48 rats were classified into the control, low dose (300 mg/kg), medium dose (500 mg/kg) and high dose (1000 mg/kg) treatment groups. The extract was administered daily from day 1 until day 28. At the end of the study, the animals were humanely sacrificed and assessed for the effect extract of *Rosmarinus officinalis* plant on body weight and relative organ weights, biochemical, haematological and histopathological parameters. The biochemical parameters for the assessment of kidney and liver injuries were carried out. Results of haematological and biochemistry results showed no changes in the control and treated groups. In the histopathology, evaluation of kidney tissues in all treated groups showed no significant ( $p > 0.05$ ) lesions. The results conclude that hydro-alcoholic extract of leaves *Rosmarinus officinalis* was found to be safe at highest dose level of 1000mg/kg for 28 days of oral administration.

**Keywords:** Repeated toxicity study, *Rosmarinus officinalis*, biochemical analysis, histopathological study

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### INTRODUCTION:

Medical developments have supported work that explores the creation and innovative use of medicinal plants due to their biological properties and availability at regional and around the world. A variety of natural materials are commonly used as raw materials for treatments, fitness-conscious meals and home remedies <sup>1,2</sup>. Herbal extracts are believed to be more secure than chemical products. Therefore, toxicity studies of natural substances do not usually receive as much attention as studies of chemical products. However, a few natural substances are probably poisonous and can be dangerous to human health. Furthermore, issues concerning the actual safety of natural substances are constantly being discussed<sup>3-4</sup>. Therefore, systematic safety studies are also vital for compounds which can be herbal-based drug treatments or practical health ingredients.

The extract of the rosemary (*Rosmarinus officinalis*) contains phenolic acids (2 to 3% rosmarinic, chlorogenic, and caffeic acids), phenolic diterpenoid bitter substances (up to 4.6% carnosic acid, carnosol, rosmarinidiphenol, and rosmanol),

triterpenoid acids (oleanolic and ursolic acids), flavonoids (apigenin, luteolin, nepetin, and nepitrin), 1.2 to 2.5% volatile oils (15 to 50% 1,8-cineole, 15 to 25% -pinene, 12 to 24% -terpineol, 10 to 25% camphor, 5 to 10% camphene, 1 to 6% borneol, 1 to 5% bornyl acetate), and tannins <sup>5,6,7,8</sup>. Although phenolic diterpenes, carnosic acid, carnosol, rosmanol, and epi- and iso-rosmanol are antioxidant compounds in rosemary leaves. These extracts have been used in the treatment of arthritis, kidney damage due to diabetes, mental tiredness, fibromyalgia, gum disease (gingivitis), hypotension, opioid withdrawal etc. For the therapeutics purpose, the plant extract need to administer daily for several days and there may be safety concern for its repeated use. So in the present investigation our study was undertaken to evaluate safety profile (qualitative and quantitative examinations) of hydro-alcoholic extract of the rosemary in terms of 28 days repeated oral administration in wistar rats.

### MATERIAL AND METHODS:

#### Plant Material:

The plant samples were collected (September 2019) in the city of Pune, state of Maharashtra, India. The plant was

identified and authenticated by M/s. Shamantak Enterprises, Dr. Gautam, Botanist, Pune, India.

#### Extraction:

A weighed quantity (50g) of the air-dried powdered plants of *R. officinalis* was drawn and then it was extracted with 90% ethanol in a Soxhlet extractor. The hydroalcoholic extract was concentrated in a rotary flash evaporator at a temperature not more than 50°C to get a solid sticky residue. Different concentration (300mg/kg, 500mg/kg and 1000mg/kg p.o.) of hydroalcoholic extract of plant of *R. officinalis* was given according to body weight of animals.<sup>3,4</sup>

#### Animals:

The study was undertaken at the AISSMS College of Pharmacy, Pune. The Institutional Animal Ethical Committee approved the protocol for the study. Wistar male and female rats (150-200g) of about 6 months were used. They were maintained at 25±2°C and relative humidity of 45 to 55% and under light dark cycle (12 h light: 12 h dark cycle). The animals had free access to food and water ad libitum throughout study. All experiments were carried out between 9:00 – 16:00 hours.

#### Sub-acute toxicity:

This protocol was performed according to the Organization for Economic Cooperation and Development (OECD) Test Guidelines with slight modifications (OECD, 407). <sup>10,11</sup> Healthy male and female rats were randomly divided into four groups (n = 5/group). Animals received vehicle orally (water; control group) or *Rosmarinus officinalis* in doses of 300, 500 and 1000 mg/kg/day for 28 consecutive days. Body weight was weekly recorded and food consumption and water intake were daily monitored. Animals were observed for signs of abnormalities during the whole treatment. At the end of the treatment, animals fasted overnight, but with water ad libitum. They were then anesthetized with pentobarbital sodium 0.035 g/kg (i.p.) and blood samples were obtained by retro-orbital puncture, using capillary tubes for hematological and biochemical studies, with and without anticoagulant ethylenediaminetetraacetic acid (EDTA), respectively.

#### Haematological and biochemical analysis:

The hematological analysis was performed using an automatic hematological analyser. The parameters included: red blood cell count (RBC), white blood cell count (WBC),

hemoglobin (Hb), hematocrit (Hct), platelet count and mean platelet volume (MPV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW)<sup>11,12</sup>. The differential leukocyte count was performed using staining with optical microscopy and, in each case, 100 cells were counted.

In the biochemical analysis, the blood was centrifuged at 1500 rpm for 10 min to obtain serum, which was stored at -20°C and used for the analysis of glucose, blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglycerides, alkaline phosphatase (ALP), total protein, albumin, total and direct bilirubin; chloride, potassium and sodium.<sup>14</sup>

#### Histopathological Examination:

Liver and kidneys excised from each treatment group were subjected to histopathological examinations. After fixing the tissues in 10 % formalin, they were dehydrated and mounted in paraffin blocks. The sections of 3-5 μ thickness were cut and stained with hematoxylin-eosin stain.

#### Statistical Analysis:

Statistical analysis was carried out using the GraphPad InStat 3. All the data are shown as the mean ± standard error of the mean (S.E.M) and were analyzed using one-way analysis of variance (ANOVA). Significant differences between the control and treatment groups were determined using Tukey - Kramer's all comparison test, P<0.05 was considered significant.

#### RESULT AND DISCUSSION:

The study of subacute toxicity of the *Rosmarinus officinalis* extract was performed as per OECD Guideline 407. The respective oral doses (300, 500 and 1000 mg/kg) of hydroalcoholic extract of leaves of *Rosmarinus officinalis* was given for 28 days. There were no any signs of toxicity observed in any treatment group.

Table 1 depicts the effect of the *Rosmarinus officinalis* on the food and water intake in subacute treatment. The single daily administration of the extract at doses 300, 500 and 1000 mg/kg for 28 days have no significant changes (p>0.05) in food and water intakes per animal when compared with control group.

**Table 1: Effect of *R. officinalis* extract on food and water intake per animal**

Treatment	Sex	Average food intake (g/d)	Average water intake (mL/d)
Control	Female	10.67±2.54	17.74±3.45
	Male	16.38±2.87	22.78±3.21
300 mg/kg <i>R. officinalis</i>	Female	13.69±2.94	15.92±2.61
	Male	17.68±1.86	24.22±3.47
500 mg/kg <i>R. officinalis</i>	Female	12.57±2.21	20.64±3.43
	Male	18.34±2.76	22.68±4.29
1000 mg/kg <i>R. officinalis</i>	Female	11.09±1.98	15.37±1.73
	Male	17.90±2.06	25.51±4.24

Results are expressed as mean ± SEM. (n=5) Data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. \*p<0.05.

Table no 2 shows daily administration of *Rosmarinus officinalis* for 28 days did not cause any significant alteration ( $p<0.05$ ) in organ weights in the treatment groups relative

to control group (Table 2). Results have shown that vital organs such as the liver, kidneys, heart and lungs have not been adversely affected throughout the treatment period.

**Table 2: Effect of *Rosmarinus officinalis* extract on relative organ weight**

Group	Heart	Lungs	Liver	Kidneys
<b>Male</b>				
Control	0.53±0.06	0.33±0.09	3.50±1.73	0.78±0.12
300 mg/kg <i>Rosmarinus officinalis</i>	0.46±0.09	0.29±0.06	2.79±1.23	0.80±0.05
500 mg/kg <i>Rosmarinus officinalis</i>	0.48±0.04	0.31±0.10	2.50±1.50	0.77±0.09
1000 mg/kg <i>Rosmarinus officinalis</i>	0.50±0.09	0.28±0.08	2.87±1.13	0.76±0.12
<b>Female</b>				
Control	0.48±0.09	0.34±0.11	3.18±1.01	0.75±0.17
300 mg/kg <i>Rosmarinus officinalis</i>	0.47±0.09	0.32±0.11	2.57±1.54	0.70±0.12
500 mg/kg <i>Rosmarinus officinalis</i>	0.50±0.12	0.33±0.08	2.74±1.87	0.76±0.14
1000 mg/kg <i>Rosmarinus officinalis</i>	0.47±0.13	0.29±0.05	2.88±1.04	0.70±0.09

Results are expressed as mean ± SEM. (n=5) Data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. \* $p<0.05$ .

Table 3 shows the body weight of rats before, during and after the treatment. The daily oral administration of *Rosmarinus officinalis* at doses of 300, 500 and 1000 mg/kg for 28 days did not show any significant change in body

weights compared to their day 0 treatment. If any change occurs in the body weight then there is herbal extract shows significant change and shows toxicity.

**Table 3: Effect of *Rosmarinus officinalis* extract on the body weights (g)**

Group	Day 0	Day 14	Day 28
<b>Male</b>			
Control	160.08±13.78	162.70±13.77	165.18±14.03
300 mg/kg <i>Rosmarinus officinalis</i>	180.66±11.43	182.16±11.76	190.5±13.25
500 mg/kg <i>Rosmarinus officinalis</i>	203.66±15.15	200.33±9.92	201±9.48
1000 mg/kg <i>Rosmarinus officinalis</i>	200±8.87	205.66±15.00	200.66±11.87*
<b>Female</b>			
Control	160.87±11.60	162.87±10.60	163.5±10.72
300 mg/kg <i>Rosmarinus officinalis</i>	200±10.52	198.66±7.03*	204.83±4.41*
500 mg/kg <i>Rosmarinus officinalis</i>	190.33±10.51	189.16±3.28*	190.66±3.57*
1000 mg/kg <i>Rosmarinus officinalis</i>	200.5±8.01	202±8.06*	200.33±8.46*

Results are expressed as mean ± SEM. (n=5) Data was analysed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. \* $p<0.05$ .

#### Hematological parameters:

The effects of the subacute oral administration of the *Rosmarinus officinalis* extract on hematological parameter are represented in Table 4. The evaluation of hematological parameters is of great importance in determining the health status of an individual. These parameters do not only depict the harmful effects of herbal remedies, but also reveal the blood-relating potential. We observed that there were no noticeable hemolytic changes on WBC, RBC, Hb, PCV, MCH, MCV, MCHC, granulocytes and leukocytes<sup>10,11,12,13</sup>. The

increased release of WBC's is a notable biomarker of stress and also aids in defending the body against some inflammatory conditions, such as bacterial infections, leukemia and hemorrhage. The result obtained from this study revealed that *R. officinalis* extract did not cause any significant changes in the levels of WBC count, or in their subtypes, including neutrophils, lymphocytes, monocytes and eosinophils, at any of the doses, relative to control group (Table 4). This suggests that the *R. officinalis* extract is nontoxic.

**Table 4: Effect of Rosmarinus officinalis extract on the hematological parameters.**

Parameters	Control	200 mg/kg	500 mg/kg	1000 mg/kg
<b>Male</b>				
Hb (gm %)	15.85±2.58	20.025±2.29	17.52±1.39	22.85±07
RBC (x 10 <sup>3</sup> /cm <sup>2</sup> )	8.61±0.23	8.20±0.04	6.84±0.132	6.47±0.19
WBC (x 10 <sup>3</sup> /cm <sup>2</sup> )	13.6±1.34	15.7±0.17	12.95±0.59	14.35±1.15
PLT (x 10 <sup>5</sup> /cm <sup>2</sup> )	40.52±0.28	41.85±0.37	41.77±0.20	41.17±0.43
PCV (%)	57.24±1.67	55.15±1.11	58.65±0.40	57.02±1.18
MCV (fl)	52.71±1.20	33.67±0.80	34.17±0.99	34.52±0.96
MCH (pg)	17.33±0.41	17.05±0.32	17.77±0.16	18.24±0.36
MCHC (gm/dl)	666.75±16.07	640.5±14.25	720.75±4.88	670.5±8.12
Neutrophils (N %)	18.82±0.36	21.97±0.24	19.55±0.68	23.55±0.51
Eosinophils (E %)	8.08±0.17	7.10±0.17	6.87±0.05	6.62±0.11
Lymphocytes (L %)	12.7±0.14	13.7±0.09	13.7±0.18	13.07±0.23
Monocytes (M %)	41.97±0.33	42.27±0.23	41.3±0.40	41.50±1.47
<b>Female</b>				
Hb (gm %)	19.35±1.49	22.17±0.78	20.2±1.12	21.72±0.21
RBC (x 10 <sup>3</sup> /cm <sup>2</sup> )	7.00±0.09	6.96±0.08	6.32±0.11	6.85±0.04
WBC (x 10 <sup>3</sup> /cm <sup>2</sup> )	11.32±1.26	13.14±0.38	13.20±0.35	13.98±0.26
PLT (x 10 <sup>5</sup> /cm <sup>2</sup> )	42.42±0.85	39.87±0.47	37.07±0.53	37.7±0.84
PCV (%)	59.8±0.18	60.5±0.38	57.02±0.28	56.92±0.20
MCV (fl)	49.8±0.52	35.6±0.29	35.12±1.00	35.8±0.24
MCH (pg)	20.55±0.89	21.3±0.28	18.55±0.21	20.09±0.07
MCHC (gm/dl)	675.75±24.28	678.5±21.74	675.75±30.95	678.5±10.89
Neutrophils (N %)	15.9±0.27	15.5±0.55	15.45±1.24	15.5±0.65
Eosinophils (E %)	7.56±0.23	6.47±0.08	6.35±0.18	6.65±0.09
Lymphocytes (L %)	12.52±0.34	12.76±0.30	13.7±0.31	12.67±0.11
Monocytes (M %)	40.35±0.45	37.6±0.73	37.75±0.37	39.52±0.38

Results are expressed as mean ± SEM. (n=5) Data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. \*p<0.05.

The effects of the subacute oral administration of the *Rosmarinus officinalis* on biochemical parameters are represented in Table 5.

The retention of creatinine, electrolytes, urea and uric acid in the body is indicators of kidney damage. Alteration in the levels of some electrolytes such as Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup> and Mg<sup>2+</sup> can also be a sign of renal injury or kidney disease. Our findings revealed that there was no significant difference in the level of creatinine, urea or uric acid in the three dose groups,

when compared with the control group, in both sexes of the rats [14].

Any alteration in the ALT, AST and ALP parameters shows sign of the liver toxicity but in the present study results did not show any alteration in this parameter. While the results of total protein show to significant increased, these results may be because of plant extract contain protein. Hence it concludes that extract *R. officinalis* did not produce any sign or no liver toxicity while its 28 days oral administration.

**Table 5: Effect of Rosmarinus officinalis extract on the biochemical parameters**

Parameters	Control	200 mg/kg	500 mg/kg	1000 mg/kg
<b>Male</b>				
<b>Urea (mmol/L)</b>	6.22±0.20	5.53±0.27	5.881±0.46	6.±0.31
<b>Urea Nitrogen (mmol/L)</b>	7.51±0.23	5.44±0.22*	5.04±0.50*	5.06±0.28*
<b>Creatinine (µmol/L)</b>	46.97±1.47	40.11±2.44	44.70±2.13	49.45±1.59
<b>Total protein (g/L)</b>	78.81±2.01	78.20±2.59	81.52±2.38*	90.345±4.54*
<b>Albumin (g/L)</b>	35.6±1.19	28.66±0.99*	30.95±0.91*	32.94±0.09
<b>ALP (U/L)</b>	283.46±6.37	279.14±0.29	316.56±5.31	308.89±12.98
<b>AST (U/L)</b>	118.22±6.86	120.97±5.02	128.65±4.52	129.58±8.26
<b>ALT (U/L)</b>	49.20±2.22	50.39±0.87	48.32±1.20	53.84±1.73
<b>Bilirubin (Total) (mg/dl)</b>	0.50±0.04	0.49±0.38	0.45±0.03	0.54±0.05
<b>Female</b>				
<b>Urea (mmol/L)</b>	5.19±0.20	5.58±0.17	5.94±0.46	6.20±0.31
<b>Urea Nitrogen (mmol/L)</b>	6.98±0.23	5.79±0.30*	5.87±0.35*	5.43±0.21*
<b>Creatinine (µmol/L)</b>	42.15±1.17	41.44±1.77	40.33±2.40	47.77±1.14
<b>Total protein (g/L)</b>	69.81±1.01	79.40±3.38	85.48±3.96*	88.00±3.23*
<b>Albumin (g/L)</b>	37.3±1.19	35.84±1.31	32.85±1.14	33.55±0.72
<b>ALP (U/L)</b>	276.46±5.37	358.89±13.70*	280.30±20.39	240.82±9.82
<b>AST (U/L)</b>	120.22±6.86	126.15±7.39	120.97±5.19	131.94±5.99
<b>ALT (U/L)</b>	49.20±1.22	50.00±1.43	52.29±0.73	54.60±0.72
<b>Bilirubin (Total) (mg/dl)</b>	0.54±0.04	0.45±0.03	0.46±0.03	0.68±0.04

Results are expressed as mean ± SEM. (n=5) Data was analysed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. \*p>0.05.

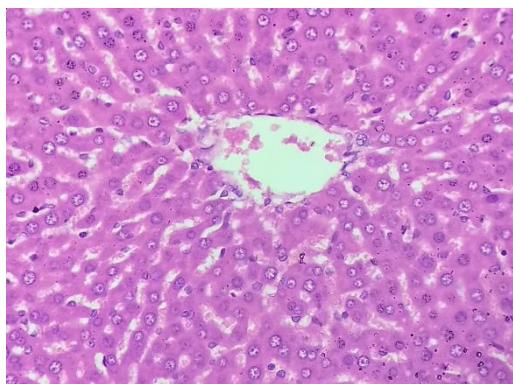
#### Histopathological Examination:

Histological studies are used as benchmarks for determining pathological changes in tissues and organs. Histological analysis of liver and kidney revealed no abnormalities in cellular architecture of these vital organs. The results of histopathology are also supported with the results of biochemical estimation of biomarkers of liver and kidney injury which were normal in all treatment groups.

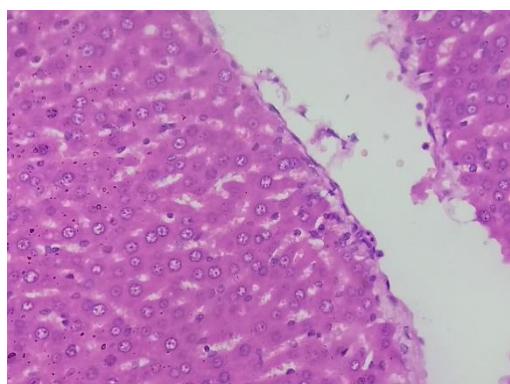
Microscopic examination of Liver from both male and female of all groups did not show any lesion of pathological

significance when compared with respective control group. On the basis of histopathology findings, it can be concluded that test compound did not produce any abnormality of pathological significance.

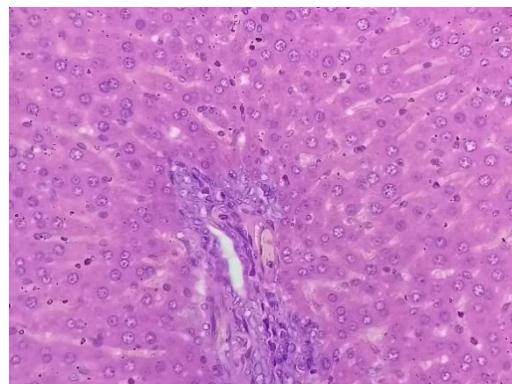
The results of the present study conclude that hydroalcoholic extract of leaves of *Rosmarinus officinalis* was found to be safe at highest dose level of 1000mg/kg for 28 days of oral administration.



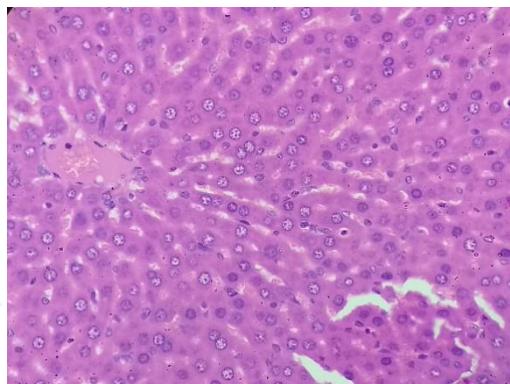
**Control; Male; Liver;** Showing normal periportal hepatocytes. 40 X, H & E



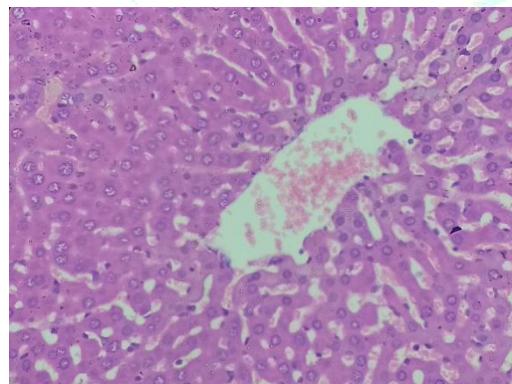
**Control; Female; Liver;** Showing normal Centrallobular hepatocytes. 40 X, H & E



**Low Dose; Male; Control; Liver;** Showing normal hepatocellular parenchyma. 40 X, H & E

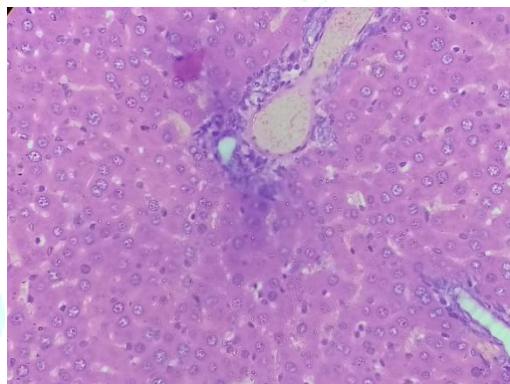


**Low Dose; Female; Control; Liver;** Showing normal hepatocellular parenchyma 40 X, H & E



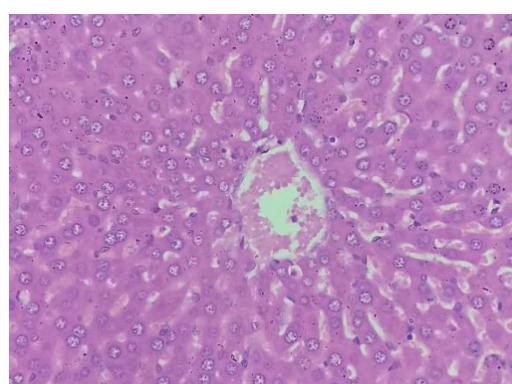
**Medium Dose; Male; Liver;** Showing normal hepatocellular parenchyma

40 X, H & E



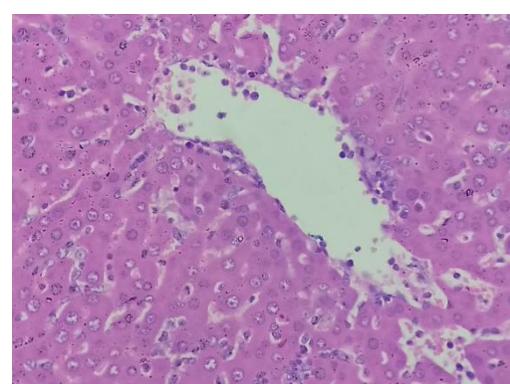
**Medium Dose; Female; Liver;** Showing normal hepatocellular parenchyma

40 X, H & E



**High Dose; Male; Liver;** Showing normal hepatocellular parenchyma

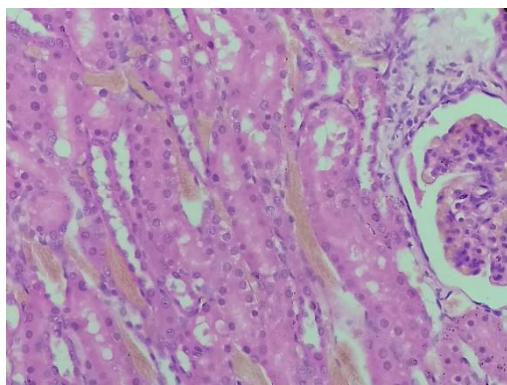
40 X, H & E



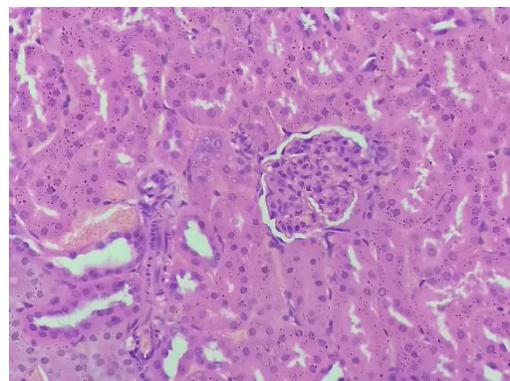
**High Dose; Female; Liver;** Showing normal hepatocellular parenchyma

40 X, H & E

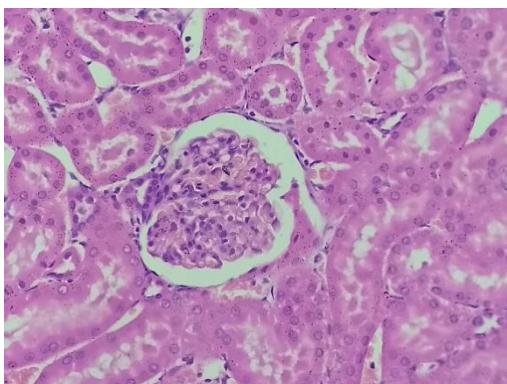
**Figure 1: Histopathological examination of Liver of rats in subacute oral toxicity study.**



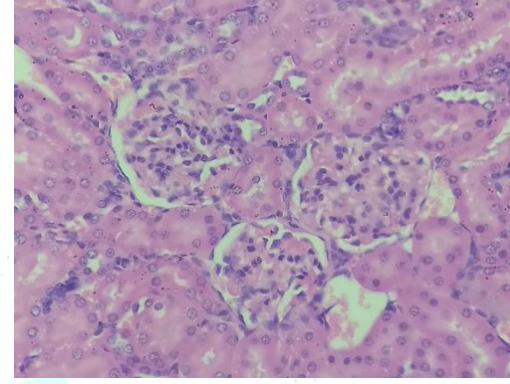
**Control; Male; Kidneys;** Showing normal glomerulus and tubules. 40 X, H & E



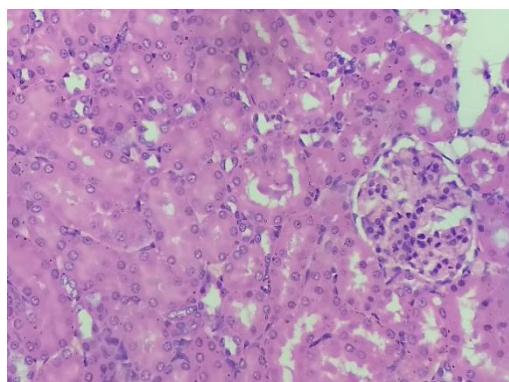
**Control; Female; Kidneys;** Showing normal glomerulus and tubules. 40 X, H & E



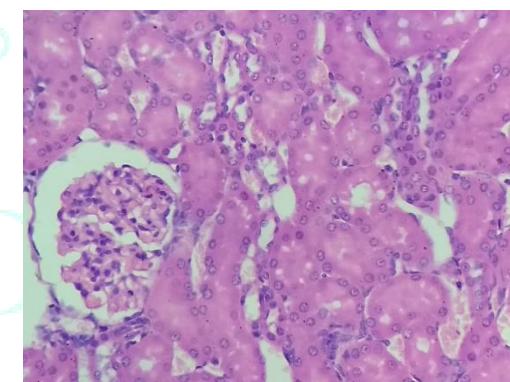
**Low Dose; Male; Kidneys;** Showing normal glomerulus and tubules. 40 X, H & E



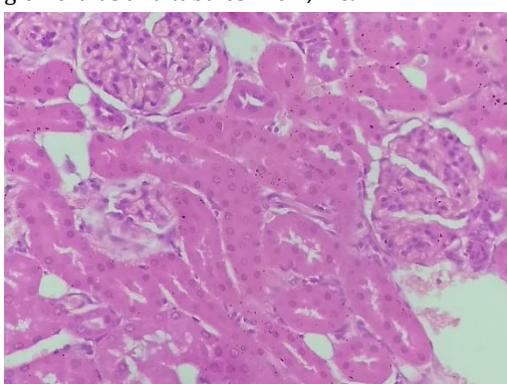
**Low Dose; Female; Control; Kidneys;** Showing normal glomerulus and tubules. 40 X, H & E



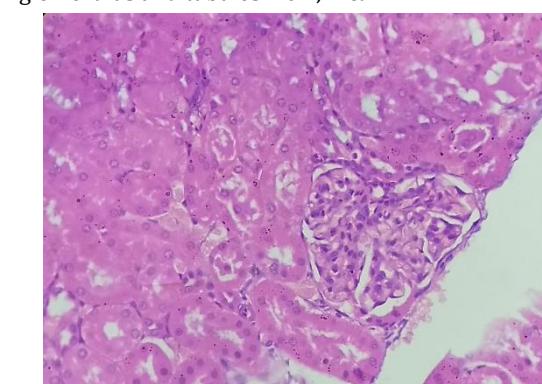
**Medium Dose; Male; Kidneys;** Showing normal glomerulus and tubules. 40 X, H & E



**Medium Dose; Female; Kidneys;** Showing normal glomerulus and tubules. 40 X, H & E



**High Dose; Male; Kidneys;** Showing normal glomerulus and tubules. 40 X, H & E



**High Dose; Female; Kidneys;** Showing normal glomerulus and tubules. 40 X, H & E

**Figure 2: Histopathological examination of Kidney of rats in subacute oral toxicity study.**

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