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ACUTE AND SUBACUTE TOXICITY STUDY ON SPERMATOGENIC SIDDHA DRUG 'ISAPPUKOL CHOORANAM' (IC)

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ABSTRACT: Herbal medicines have been broadly used in developed countries hence they are natural and comparatively safe. They contain plant materials as their pharmacologically active components. Infertility is one of the most extremely tragic all over the world. Despite recent advances in the treatment of male infertility, the problem has not been satisfactorily tackled. The male infertility is mainly due to an inadequate number of spermatozoa in the semen, the failure of the spermatozoa to move with sufficient vigor towards their goal. Aim of the study is to evaluate the acute and sub-acute toxicity of the spermatogenic siddha drug Isappukol Chooranam (IC) (siddha drug).For acute studies, different doses of IC were administered orally to rats once daily for one week. Forsub-acute studies, different doses of IC were administered orally to rats once daily for 28 days in various doses at 50,100,200 mg/kg of body weight. Detailed hematological, biochemical, necropsy and Histopathological evaluation of organs was performed for all animals. Histopathological analysis revealed that Spleen, Testes, Pancreas, Lung, Liver, Brain, Heart, Stomach, Intestine, Bone, Ovary, and Kidney tissues of treated groups did not show any signs of toxicity. No impairment in hepatic, renal, haemopoietic functions were observed throughout the study. No mortality was observed up to 200 mg/kg of body weight in acute and sub-acute toxicity studies.

INTRODUCTION: Worldwide use of complementary and alternative medicines, including herbal products for various health benefits, has recently increased [1, 2]. Infertility is the failure of a couple to conceive a pregnancy after trying to do so for at least one full year. Approximately 20% of couples struggle with infertility at any given time.

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Infertility has increased as a problem over the last 30 years [3]. Male reproductive health has social and psychological impact on normal life [4]. Male infertility is commonly due to deficiencies in the semen and semen quality is used as a surrogate measure of male fecundity [5].

Male reproductive medicine and surgery remains one of the most actively evolving areas in urology, with a variety of therapeutic modalities under investigation [6]. Conventional treatment for infertility usually involves invasive and, expensive procedures. There are many alternative treatments available that can increase the chance of conception [7]. Herbs have been in use since long time to treat various diseases [8].

However, many issues related to a lack of scientific evidence about the efficacy and safety of herbal remedies remainsunresolved [9, 10].

Pre-clinical toxicity studies are essential to determining a safe dose for human trial [11]. Prior to the initiation of human clinical trials of novel drugs, the safety of their application is to be proved. Generally this is accomplished by the implementation of general preclinical toxicity experiments to uncover potential poisonous effects of any drug in animals [12].

The present study was conducted to evaluate the acute and sub-acute toxicity of the siddha drug 'Isappukol Chooranam'. The interventional drug Isappukol Chooranam has been quoted by Koshayi Anuboga Vaithiya Brahma Ragasiyam for Aan maladu. The drug is chosen for the treatment of Aanmaladu (Male Infertility) quoted by Yugi Muni [13]. This report aims to provide vital information about the efficacy and safety of the siddha drug "Isappukol Chooranam". Adjutants of natural sources like *Psyllium* husk are preferred over synthetics materials due to their non-toxicity, low cost, ease of availability, high affinity of water (swelling index is about 20 times in volume), chemically inert & purely mechanical action in the Alimentary canal and the body do not assimilate this [14].

Hygrophila auriculata extract showed no signs of acute toxicity as evidenced by the absence of mortality or visible adverse effects in the animals during the study period. Nomacroscopic alterations were noted in theviscera of the treated rats [15]. Butprolonged use of *Myristica fragrans* at high doses (400-500 mg/kg) could be very toxic to the studied organs [16].

MATERIALS & METHODS:

Preparation of the Isappukol Chooranam: 2 parts of seed of Isappukol (*Plantago ovata*), 1 part of Jadhikkai (Myristicafragrans), 2 parts of seed of Neermulli (*Hygrophila auriculata*), and 6 parts of karkandu (Rock candy) were dried and powdered& mixed well.

Aim: Aim of the study is to evaluate the acute and sub-acute toxicity of the siddha drug 'Isappukol Chooranam'.

Chemicals: The necessary chemicals and reagents were obtained from Sigma chemicals. All other solvents and Analytical Kits were of analytical grade and obtained from qualigen fine chemicals and Artek laboratories.

Animals: Mice of either sex weighing 25-30g and rats weighing 210-240g were obtained from the animal house of Vel's University. All experimental procedures described were reviewed and approved by the Institutional Animal Ethical Committee of Vel's College of Pharmacy, Chennai-117 on 11.08.2012 bearing no. (XIII/VELS/PCOL/27/ 2000/CPCSEA/IAEC/08.08.2012). They were fed with a balanced standard pellet diet and maintained under standard laboratory conditions, providing 24-28^{o}C temperature, standard light cycle (12 h light, 12 h dark) and water ad libitum.

Animals were kept in cages with raised floors of wide mesh to prevent coprophagy. Animal welfare guidelines were observed during the maintenance period and experimentation. The rats were randomly assigned to control and different treatment groups, six animals per group. The animals were acclimatized for one week under laboratory conditions.

Acute toxicity study-OECD 425 guidelines [17]: Acute oral toxicity test for the Isappukol Chooranam was carried out as per OECD Guidelines 425. As with other sequential test designs, care was taken to ensure that animals are available in the appropriate size and age range for the entire study. The test substance is administered in a single dose by gavage using a stomach tube or a suitable intubation cannula. The fasted body weight of each animal is determined and the dose is calculated according to the body weight. After the substance has been administered, food was withheld for a further 2 hours in mice.

The animals were observed continuously for the first 4 h and then each hour for the next 24 h and at 6 hourly intervals for the following 48 h after administering of the test drug, to observe any death or changes in general behavior and other physiological activities. Single animals are dosed in sequence usually at 48 h intervals. However, the time interval between dosing is determined by the onset, duration, and severity of toxic signs.

Treatment of an animal at the next dose was delayed until one is confident of survival of the previously dosed animal.

Observation of toxicity signs: General behavior, respiratory pattern, cardiovascular signs, motor activities, reflexes, change in skin and fur, mortality and the body weight changes were monitored daily. The time of onset, intensity, and duration of these signs, if any, was recorded [18, 19].

Sub-Acute Toxicity: In 28-days sub-acute toxicity study, twenty four either sex rats were divided into four groups of 6 rats each. Group I that served as normal control was administered with distilled water (p.o.) while groups II, III and IV were administered daily with the Isappukol Chooranam (p.o.) for 28 days at a dose of 50, 100 and 200mg/kg respectively. The animals were then observed daily for gross behavioral changes and any other signs of sub-acutetoxicity [**Table 1**]. The weight of each rat was recorded on day 0 and weekly throughout the course of the study [**Table 2**], food and water consumption per rat was calculated [**Table 3 & 4**]. At the end of the 28 days they were fasted overnight, each animal was anaesthetized with diethyl ether, following which they were then dissected and blood samples were obtained by cardiac puncture into heparinized tubes. The blood sample collected from each rat was centrifuged with 3000 X g at 40^{o}C for 10 min to separate the serum and used for the biochemical assays.

TABLE 1: DOSE FINDING EXPERIMENT AND ITS BEHAVIORAL SIGNS OF TOXICITY

S. No	Dose (mg/kg)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	**500**	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	**1000**	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	+	-
3	**2000**	+	-	-	+	-	+	+	-	+	-	-	-	+	-	-	-	+	+	+	-

1. Alertness 2. Aggressiveness 3. Pile erection 4. Grooming 5. Gripping 6. Touch Response 7. Decreased Motor Activity 8. Tremors 9. Convulsions 10. Muscle Spasm 11. Catatonia 12. Muscle relaxant 13. Hypnosis 14. Analgesia 15.Lacrimation 16. Exophthalmos 17. Diarrhoea 18. Writhing 19. Respiration 20. Mortality

TABLE 2: BODY WT (G) OF ALBINO RATS EXPOSED TO ISAPPUKOL CHOORANAM FOR 28DAYS.

Dose (mg/kg/day)	Days				
	1	**7**	**14**	**21**	**28**
Control	210.50±6.52	212.16±6.40	215.48±5.42	221.52±8.00	223.11±5.88
50	215.71±6.43	218.20±5.18	220.51±5.70	226.64±7.02	226.50±6.00
100	213.42±5.45	216.14±6.18	220.00±6.61	224.10±7.10	227.72±7.12
200	212.10±6.25	217.28±5.42	219.23±6.49	222.22±6.43	225.25±7.23

Values are mean of 6 animals ± S.E.M. (Dunnet's test). $^{ns}P>0.05$.

TABLE 3: FOOD (G/DAY) INTAKE OF ALBINO RATS EXPOSED TO ISAPPUKOL CHOORANAM FOR 28DAYS.

Dose (mg/kg/day)	Days (gms/rats)				
	1	**7**	**14**	**21**	**28**
Control	47.06±2.55	48.48±2.51	46.24±2.50	45.82±2.54	47.20±3.42
50	46.24±2.17	46.43±2.40	46.40±2.46	49.14±2.90	48.52±3.05
100	44.34±2.28	45.00±2.92	44.25±2.45	45.18±3.21	46.04±3.00
200	45.60±2.55	45.20±2.18	46.51±2.82	45.31±2.40	45.14±3.14

Values are mean of 6 animals ± S.E.M. (Dunnet's test). $^{ns}P>0.05$.

TABLE 4: WATER (ML/DAY) INTAKE OF RATS EXPOSED TO ISAPPUKOL CHOORANAM FOR 28DAYS.

Dose (mg/kg/day)	Days (ml/rat)				
	1	**7**	**14**	**21**	**28**
Control	52.00±2.85	52.20±3.32	54.20±3.12	52.20±3.14	51.22±3.22
50	53.10±2.63	50.12±3.04	45.21±4.02	46.50±3.00	44.52±2.48
100	49.17±2.18	42.25±3.72	45.48±3.34	42.10±2.92	41.12±3.28
200	54.30±3.52	54.72±3.00	52.42±3.82	48.20±3.12	47.32±3.62

Values are mean of 6 animals ± S.E.M. (Dunnet's test). $^{ns}P>0.05$.

Hematological and blood biochemical analyses: At the end of the study, all animals were kept fasted for 16-18 h and then anesthetized with anesthetic ether on the 28th day. Blood samples for hematological and blood chemical analyses were taken from retro orbital vein. Heparinized blood samples were taken for determining complete blood count [**Table 5**] (white blood cell count, differential white blood cell count, platelet count, red blood cell count, hematocrit, and hemoglobin) by semi-automated hematology analyzer. The serum from non-heparinized blood was carefully collected for blood chemistry and enzyme analysis glucose, Creatinine, total protein, albumin, total and direct bilirubins, serum glutamate-oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALP) were automatically determined using auto analyzer [20] [**Table 6, 7, 8 & 9**].

TABLE 5/: HEMATOLOGICAL PARAMETERS AFTER 28DAYS TREATMENT WITH IC IN RATS

Parameter	Control	50mg/kg	100mg/kg	200 mg/kg
Red blood cell (mm^3)	7.02±0.71	7.92±0.62	7.18±0.75	8.00±0.60
HB (%)	15.21±0.43	15.10±0.41	15.56±0.42	15.00±0.44
Leukocyte ($x10^6$/mL)	10.00±1.6	10.35±2.2	10.02±2.1	10.36±1.32
Platelets (lakhs/cu.mm)	1.25±0.49	1.62±0.54	1.72±0.62	1.25±0.56
MCV (gm/dl)	54.42±5.20	53.12±4.22	54.00±5.24	55.10±4.80
Neutrophils ($x10^6$/mL)	5.45±1.23	5.42±1.21	5.18±0.96	5.14±3.72
Lymphocyte	92.13±2.82	91.70±3.18	93.20±3.22	92.30±3.12
Monocyte	2.2±0.30	2.0±0.23	2.21±0.24	2.30±0.28
Eosinophil	1.00±0.00	1.0±0.22	1.0±0.11	1.00±0.11
Basophil	0	0	0	0
ESR (mm)	1±00	1±00	1±00	1±00
PCV	45.30±2.42	45.26±2.12	45.04±3.02	45.42±3.42

Values are mean of 6 animals ± S.E.M. (Dunnet's test). $^{ns}P>0.05$.

TABLE 6: EFFECT OF TREATMENT WITH ISAPPUKOL CHOORANAM BIOCHEMICAL PARAMETERS

Dose (mg/kg)	Control	50 mg/kg	100 mg/kg	200 mg/kg
Total Bilirubin (mg/dL)	0.210±0.05	0.210±0.06	0.212±0.05	0.214±0.04
Bilirubin direct (mg/dL)	0.1±0.04	0.1±0.05	0.1±0.04	0.1±0.05
Bilirubin indirect(mg/dL)	0.1±00	0.1±00	0.1±00	0.1±00
ALP (U/L)	376.42±11.10	370.12±10.30	374.12±10.00	372.1±12.32
SGOT (U/L)	164.20±6.20	162.16±6.82	158.80±5.40	159.21±6.78
SGPT(U/L)	45.8±3.42	45.2±3.24	45.00±2.84	46.22±3.14
Total Protein(g/dl)	10.00±1.00	9.88±0.90	9.54±0.87	9.28±0.96
Albumin(g/dl)	3.70±0.28	3.92±0.44	3.40±0.30	3.42±0.32
Globulin(g/dl)	6.00±0.15	5.85±0.16	5.58±0.20	5.80±0.26

Values are mean of 6 animals ± S.E.M. (Dunnet's test). $^{ns}P>0.05$ Vs. control

TABLE 7: RFT

Dose (mg/kg)	Control	50 mg/kg	100 mg/kg	200 mg/kg
Urea (mg/dL)	56.20±1.37	55.74±3.65	55.42±2.45	54.82±2.30
Creatinine (mg/dL)	0.77±0.05	0.76±0.05	0.77±0.06	0.78±0.05
Uric acid (mg/dL)	1.5±0.14	1.6±0.15	1.6±0.14	1.6±0.18
Nam.mol	140.50±5.52	140.42±4.00	140.21±5.22	141.18±5.02
Km.mol	20.15±2.81	19.58±1.82	20.10±1.68	20.52±2.24
Clm.mol	101.05±4.42	101.00±4.18	99.48±4.42	100.00±5.10

TABLE 8: LIPID PROFILE

Dose (mg/kg)	Control	50 mg/kg	100 mg/kg	200 mg/kg
Total cholesterol (mg/dL)	41.60±2.52	41.28±2.40	40.11±3.12	42.00±3.02
HDL (mg/dL)	13.40±1.54	13.22±1.47	13.40±1.80	13.40±2.13
LDL (mg/dL)	42.56±2.48	44.52±3.16	45.43±3.02	44.22±3.28
VLDL (mg/dl)	16.30±2.28	15.72±2.53	16.00±1.42	15.00±1.20
Triglycerides (mg/dl)	86.42±3.02	85.62±2.42	85.20±3.32	86.42±2.42
TC/HDL ratio (g/dl)	3.52±0.25	3.60±0.28	3.55±0.30	3.45±0.28
Blood glucose (mg/dl)	127.30±6.00	126.02±5.20	126.10±5.00	124.88±4.56

Values are mean of 6 animals ± S.E.M. (Dunnet's test). ^{ns}P>0.05.Vs. control

TABLE 9: URINE ANALYSIS

Parameters	Control	50 mg/kg	100 mg/kg	200 mg/kg
Colour	Yellow	Yellow	Yellow	Yellow
Transparency	Clear	Slightly turbid	Slightly cloudy	Slightly turbid
Specific gravity	1.010	1.010	1.010	1.010
PH	>7.2	>8.0	>8.0	>9.0
Protein	Nil	3+	3+	3+
Glucose	Nil	Nil	Nil	Nil
Bilirubin	-ve	-ve	-ve	-ve
Ketones	-ve	+ve	+ve	+ve
Blood	Absent	Absent	Absent	Absent
Urobilinogen	Normal	Abnormal	Abnormal	Abnormal
Pus cells	0-cells/HPF	1-cell/HPF	2-cells/HPF	1-cell/HPF
RBCs	Nil	Nil	0-1cells/HPF	Nil
Epithelial cells	Nil	1-cell/HPF	Nil	1-cell/HPF
Crystals	Nil	Nil	Nil	Nil
Casts	Nil	Nil	Nil	Nil
Others	Bacteria seen	Bacteria seen	Bacteria seen	Bacteria seen

Necropsy: All rats were sacrificed after the blood collection. The positions, shapes, sizes and colors of internal organs were evaluated. The Spleen, Testes, Pancreas, Lung, Liver, Brain, Heart, Stomach, Intestine, Bone, Ovary, and Kidney tissues were excised from all rats to visually detect gross lesions, and weighed to determine relative organs' weights and preserved in 10% neutral formalin for histopathological assessment [**Table 10**]. The tissues were embedded in paraffin, and then sectioned, stained with haematoxylin and eosin and were examined microscopically [**Figure-1, 2, 3 & 4**].

TABLE 10: EFFECT OF ORAL ADMINISTRATION OF ISAPPUKOL CHOORANAM ON ORGAN WEIGHT

Dose (mg/kg)	Control	50 mg/kg	100 mg/kg	200 mg/kg
Liver (g)	5.21±0.14	5.28±0.12	4.98±0.10	5.12±0.14
Heart (g)	0.60±0.04	0.60±0.05	0.57±0.04	0.58±0.04
Lung (g)	1.45±0.15	1.46±0.12	1.48±0.14	1.50±0.14
Spleen (g)	0.65±0.05	0.65±0.04	0.64±0.04	0.65±0.05
Ovary (g)	1.70±0.14	1.72±0.15	1.78±0.18	1.74±0.15
Testes (g)	1.47±0.10	1.49±0.12	1.56±0.15	1.54±0.15
Brain (g)	1.57±0.15	1.56±0.13	1.56±0.14	1.55±0.14
Kidney (g)	0.72±0.04	0.71±0.04	0.70±0.04	0.71±0.05
Stomach (g)	1.35±0.13	1.36±0.10	1.35±0.11	1.35±0.10

Values are mean of 6 animals ± S.E.M. (Dunnet's test). ^{ns}P>0.05.Vs control.

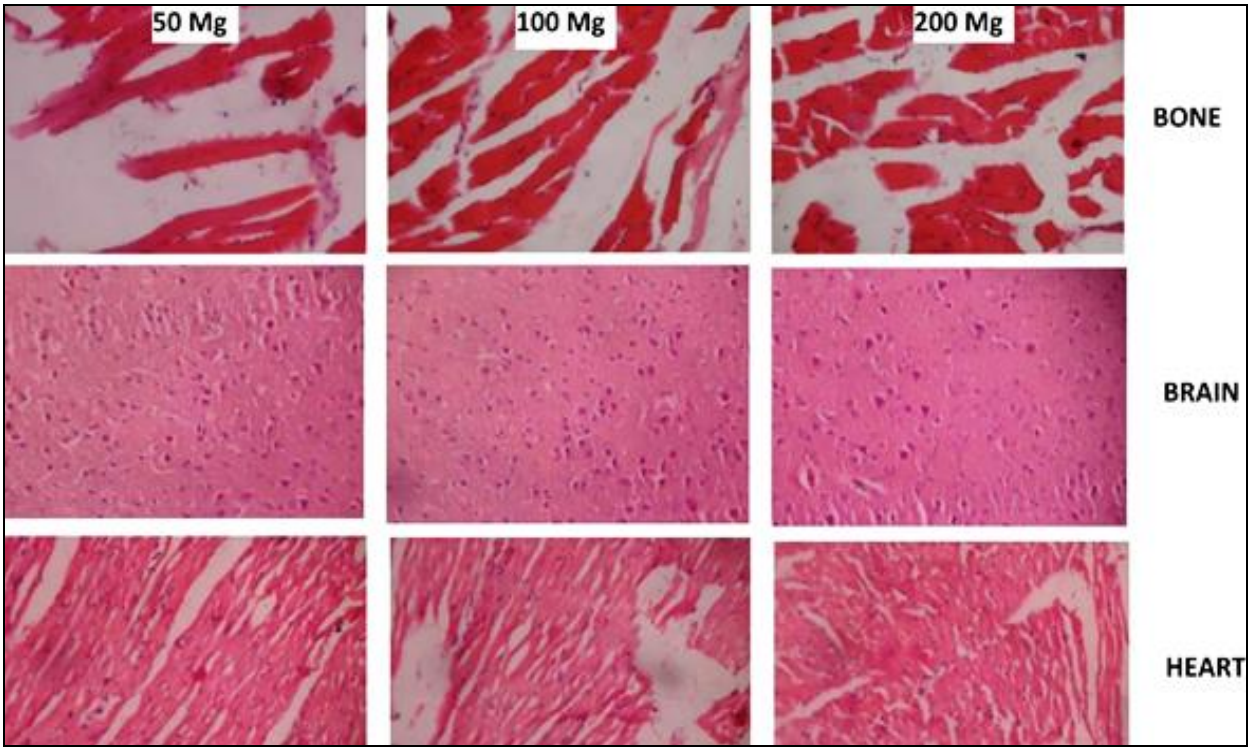

FIGURE 1

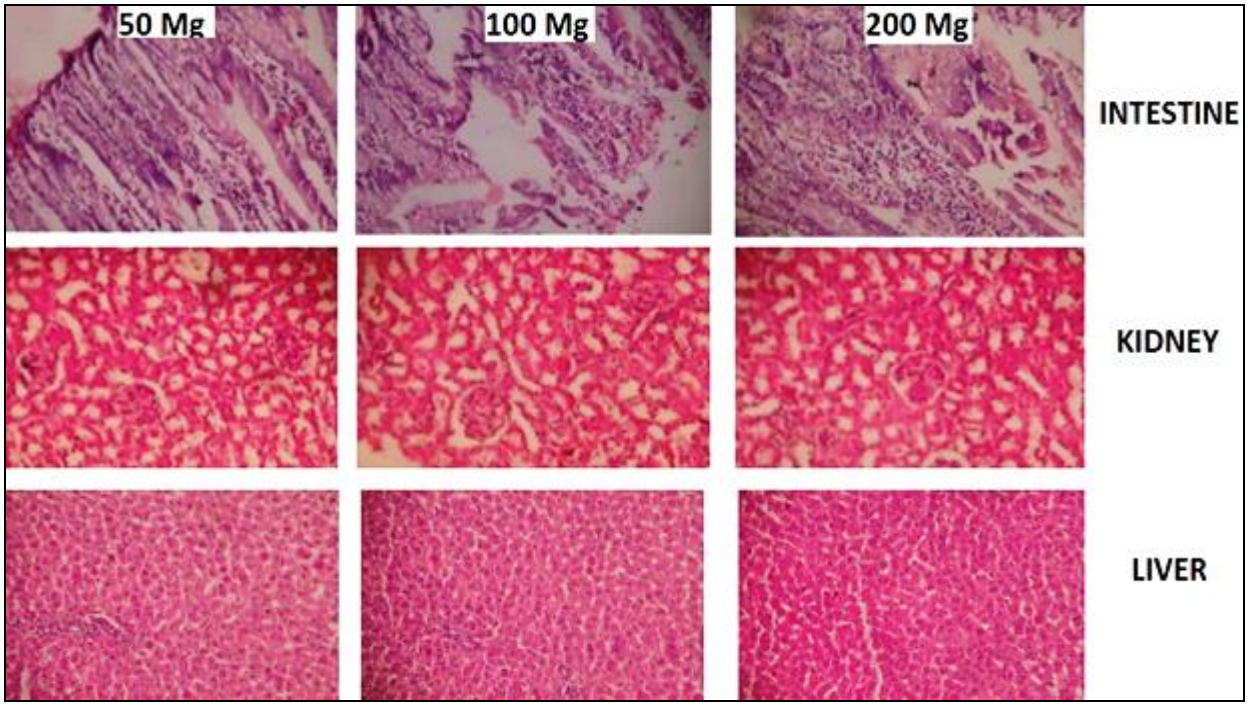

FIGURE 2

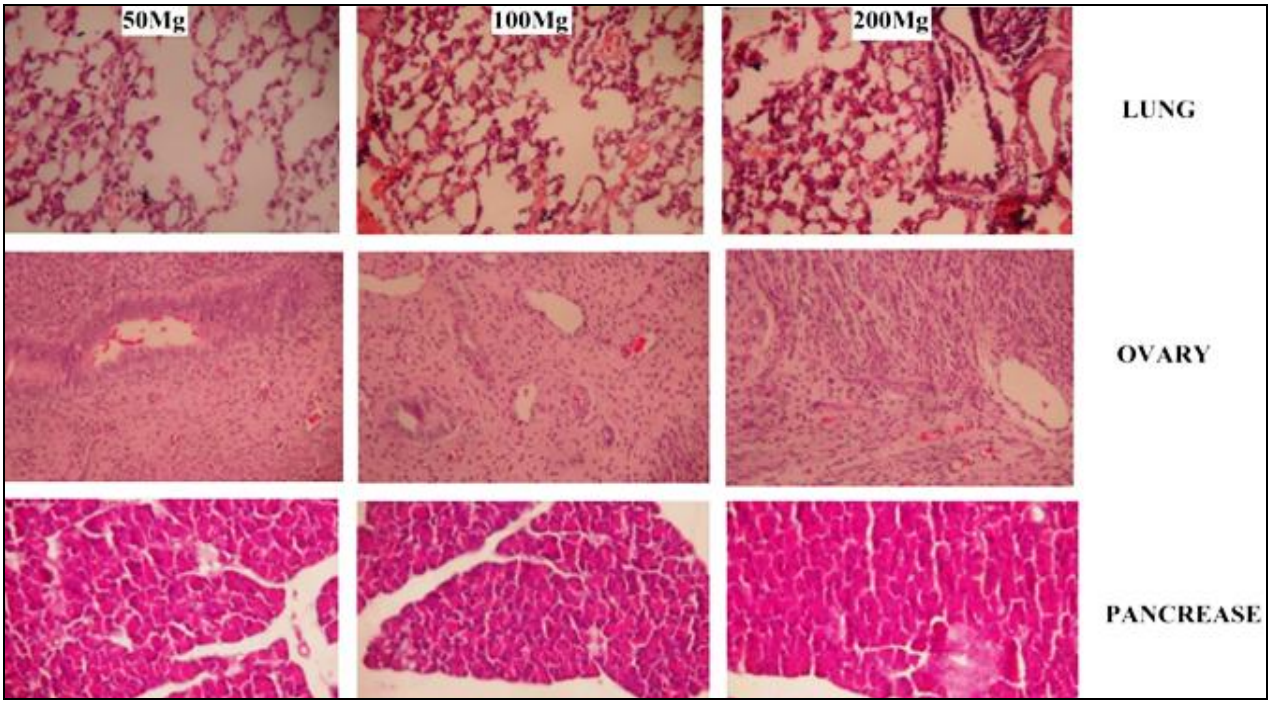

FIGURE 3

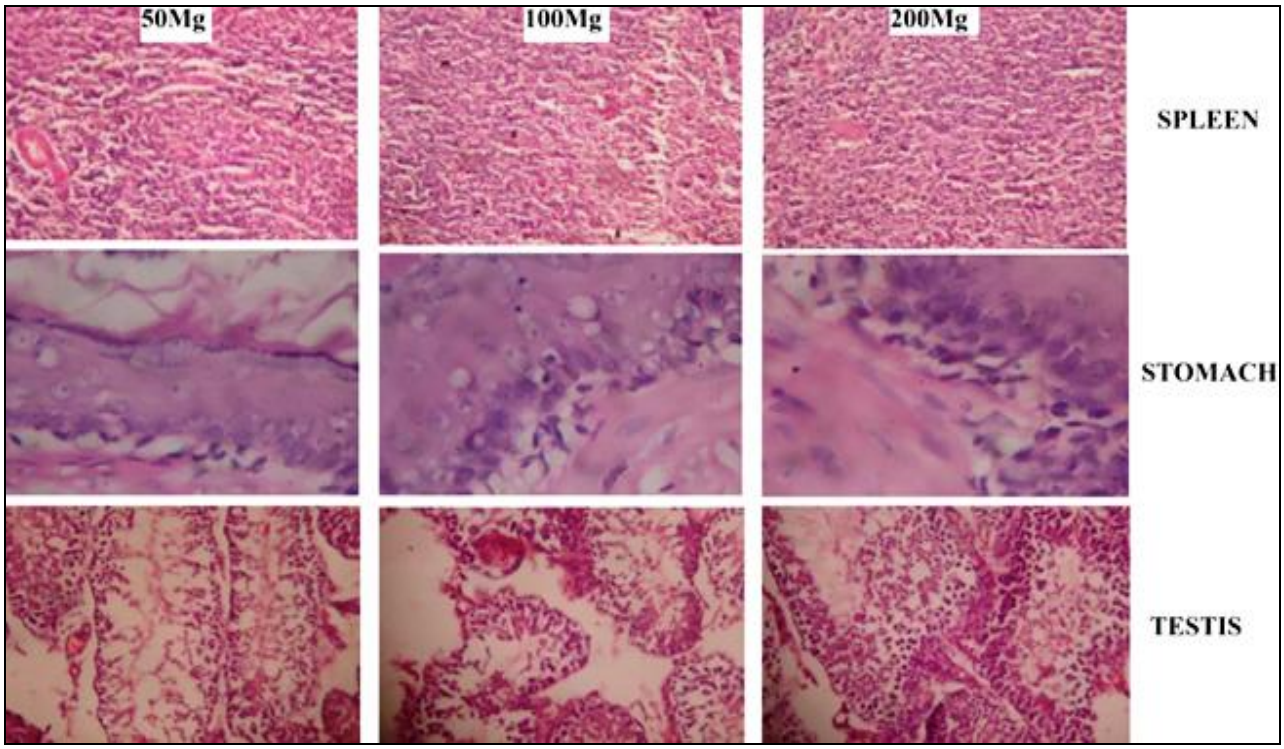

FIGURE 4

Statistical Analysis: Values were represented as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) and group means were compared using the Tukey-Kramer Multiple Comparison Test using Instat-V3 software. P values < 0.05 were considered significant.

RESULTS AND DISCUSSION:

1) All the animals in both control and the dose treated groups up to 200 mg/kg survived throughout the dosing period of 28 days.

2) No signs of major or significant intoxication were observed in animals from lower to higher dose groups during the dosing period of 28 days.

3) Animals from all the treated dose groups exhibited comparable body weight gain with that of controls throughout the dosing period of 28 days.

4) Food consumption of control and treated animals was found to be comparable throughout the dosing period of 28days.

5) Ophthalmoscopic examination, conducted prior to and at the end of dosing period on animals from control and all the treated dose groups did not reveal any abnormality.

6) Hematological analysis conducted at the end of the dosing period on day 28, revealed no significant abnormalities attributable to the treatment.

7) Biochemical analysis conducted at the end of the dosing period on day 28, revealed no remarkable abnormalities attributable to the treatment.

8) Functional observation tests conducted at termination revealed no abnormalities.

9) Urine analysis, conducted at the end of the dosing period in week 4 and at the end of recovery period in week 6, revealed no abnormality attributable to the treatment.

10) Organ weight data of animals sacrificed at the end of the dosing period was found to be comparable with that of respective controls.

11) Gross pathological examination did not reveal any abnormality.

12) Histopathological examination did not reveal any abnormality.

CONCLUSION: Based on these findings, no toxic effects were observed up to 200mg/kg of Isappukol Chooranam treated via oral route over a period of 28 days. So, it can be concluded that the Isappukol Chooranam can be prescribed for therapeutic use in human with the dosage recommendations of up to 200mg/kg of body weight p.o.

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