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TOXICOLOGICAL SAFETY EVALUATION IN ACUTE AND 21 DAYS STUDIES OF SWASANANDAM GUTIKA

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ABSTRACT

Swasanandam gutika is one among the popular herbomineral formulations practiced in Kerala. It contains *Hingula* (Cinnabar), *Vatsanabha (Aconitum ferox)* and *Karpoora (Cinnamomum camphora)* levigated in Triphala kwatha. All the ingredients are having toxic potential. This formulation is indicated especially for respiratory ailments of children. In the light of these data, it will be apt to conduct a reverse pharmacological study to assess the safety evaluation of this yoga. Hence an attempt is made in this study to provide a statistical data regarding the clinical manifestations, biochemical parameters and histopathological changes that may occur during acute toxicity testing and 21 days administration of Swasanandam gutika in therapeutic and higher doses.

Acute toxicity test was conducted according to the Organization for Economic Cooperation and Development (OECD) guidelines- 425. The drug passed the limit test and did not produce any mortality. On in-vivo sub-acute toxicity evaluation, the group administered with the therapeutic dose did not show any statistically significant variation in the liver function tests and renal function tests and in histopathology. At the same time the groups treated with double and four times the therapeutic dose showed statistically significant elevation in the level of liver enzymes. Even though the elevated enzyme levels were noticed, the histopathology results did not reveal changes of toxicological significance in any of the study groups.

Keywords- Swasanandam gutika, acute toxicity, limit test, sub-acute toxicity, histopathology

1.INTRODUCTION

Rasashastra is a branch of Ayurveda that deals with the use of metals and minerals in therapeutics. The drugs are advised to use after specific shodhana[1] methods and sophisticated processing techniques to ensure safety and efficacy. However certain allegations have been raised by the international community against the Ayurvedic system of medicine regarding its safety, due to the presence of mercurials and heavy metals[2] in it. As per the present-day scenario, intake of mercury compounds and their products are claimed to be highly toxic. But according to Ayurvedic science, consumption of any processed drug at a prescribed dose is safe[3]. The doubts and suspicions must be clarified to increase the acceptance of herbomineral drugs globally. In this instance, strategies from pre-clinical safety evaluation criteria of modern drug development should be adopted.

Swasanandam gutika[4] is one among the popular herbomineral formulations practiced in Kerala. It contains Hingula, Vatsanabha and Karpoora levigated in Triphala kwatha. Hingula is Cinnabar, when taken orally is relatively less toxic than that of other mercurial compounds. The drug *Vatsanabha* is one of the deadliest poisonous plants in the world. It is administered therapeutically after subjecting to various processing termed

as *sodhana*. Also, camphor is reported to be having toxicological effects[5]. Swasanandam gutika is generally administered to children in respiratory illness and hence there is a need of toxicity profiling to ensure safety

In the light of these data, it will be apt to conduct a reverse pharmacological study to assess the safety evaluation of this yoga. Hence an attempt is made in this study to provide a statistical data regarding the clinical manifestations, biochemical parameters and histopathological changes that may occur during acute toxicity testing and 21 days administration of Swasanandam gutika in therapeutic and higher doses.

2.MATERIALS AND METHODS

2.1 PREPARATION OF SWASANANDAM GUTIKA

10g each of *sodhita Hingula* (purified Cinnabar), *sodhita Vatsanabha choorna* (Aconitum ferox) and *Karpoora* (Cinnamomum camphora) were taken. 30 ml of *Triphalakwatha* was prepared from 10g each of *Haritaki* (Terminalia chebula), *Vibhitaki* (Terminalia bellerica) and *Amalaki* (Embilica officinalis). Then the powders of *sodhita Hingula* and *sodhita Vatsanabha* were triturated well by adding enough *Triphala kwatha*. On proper grinding, powdered *Karpoora* was added and triturated well. It was subjected to three *bhavana* (levigation) in *Triphala kwatha* and then pills weighing 125mg were rolled out. 140 pills were obtained and it was dried in shade. After proper drying it was kept in air-tight glass bottles.

2.2 ACUTE TOXICITY TESTING

The animal experiment was conducted in the animal house working under Dravyaguna department of Ayurveda College, Trivandrum. The study was conducted with the consent of institutional animal ethical committee (IAEC No. 14/IAEC/AVC/2012). For acute toxicity study, adult wistar albino rats weighing about 200 to 225 g were collected from animal house, working under Agadatantra department of Govt Ayurveda College at Poojappura, Trivandrum, Kerala.

2.2.1**Maintenance of experimental rats**

The rats were kept in properly numbered large polypropylene cages with stainless steel top grills having facilities for pelleted food. The animals were maintained in 12 hours light and dark cycle at 25^0C ± 2^0C in a well-ventilated animal house under natural conditions in large polypropylene cages and they were acclimatized to laboratory conditions for 7 days before the commencement of the experiment. Animals were fed with standard pellets and water ad-libitum. All animal experiments were performed according to the ethical guidelines suggested by the institutional animal ethics committee (IAEC). Saw husk was used as bedding material and changed twice a week.

2.2.2 Methodology

Paragraph 22 of OECD Guideline 425[6] suggests two types of acute oral toxicity tests i.e. limit test and main test. The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity below regulatory limit doses. However, in those situations where there is little or no information about its toxicity, or in which the test material is expected to be toxic, only the main test should be performed.

Limit test

Using the normal procedure, a sighting study starting dose of 2000mg/kg (or exceptionally 5000mg/kg) followed by dosing of a further four animals at this level serves as a limit test for this guideline.

Healthy Wistar albino rats of both sexes weighing between 200-225g maintained under standard laboratory conditions were used for the acute toxicity test according to the Organization for Economic Cooperation and Development (OECD) guidelines 425. A total of five female rats were used and each received a single oral-dose of 2000 mg/ kg body weight of Swasanandam gutika. The stock solution was prepared by dissolving 2000mg of drug in 5ml of milk. 1ml of the solution was administered to a rat of 200g body weight.

Animals were kept overnight fasting prior to drug administration by oral gavage. After administration of drug sample, food was withheld for further 3-4 h. Animals were observed individually at least once during

first 30 min after dosing, periodically during first 24 h (with special attention during the first 4 h) and daily thereafter for a period of 7 days.

Outcome variable

Daily observations on the changes in skin and fur, eyes, and mucus membrane (nasal), respiratory rate, circulatory signs, autonomic effects (salivation, lacrimation, perspiration, piloerection, urinary incontinence, and defecation) and central nervous system (ptosis, drowsiness, gait, tremors, and convulsion) changes were noted. Mortality up to 24 hours, feed and water intake, body weight and the system wise observation and examination were noted and signs were recorded.

Physical parameters

Physical parameters (body weight, food, and water intake) and local injury were studied during treatment of animals. Mortality was also recorded during treatment of all groups. Autopsy was done if animals died during treatment.

Hematological parameters

Blood was collected from the carotid vessels. Blood samples were analyzed for routine hematological parameters. Blood cell count was done with blood smears.

Biochemical parameters

Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), Total protein, serum albumin, blood urea nitrogen (BUN), creatinine, and blood sugar levels were estimated in plasma sample. All parameters were studied by semi auto analyzer by using Erba analytical kits.

2.3 21 DAYS TOXICITY TESTING

Male and female Wistar rats weighing 200-225g were used. They were allowed to acclimatize to the laboratory conditions for seven days. The animals were maintained on standard animal feeds and provided with water *ad libitum.* The two sexes were kept separate from each other throughout the study. The animals were weighed and divided into four groups of six animals each. Each group comprises 3 male and 3 female rats.

Dose fixation

The therapeutic dose of Swasanandam gutika was fixed as 250 mg/day for an adult human. The dose for sub-acute study groups was fixed as equivalent to therapeutic dose, double and four times the therapeutic dose. One group was assigned as control receiving only the vehicle or adjuvant.

Conversion formula as per SOP:

The highest possible human dose of Swasanandam gutika is 250 mg/ human/ day was converted to the dose in rats as mg/kg.

– Human dose is 125 mg, BD, i.e., 250 mg

– Total clinical dose (a) x conversion factor (b) 0.018 = (c) per 200 gm of rat

250mg x 0.018 = 4.5 mg / 200 g of rat

4.5 x 1000/200 = 22.5 mg / kg.

22.5mg/kg, 45mg/kg and 90mg/kg were the doses fixed for the study groups for 21 days administration. The anupana mentioned in the yoga was sita(sugar) and sthanya(milk) and hence vehicle used was cow's milk mixed with sita and the drug was administered as suspension form.

TABLE-1

Group number	Groups	Dose of Swasanandam gulika mg/kg b.w	No of males	No. of females
I	Control	vehicle only	3	3
II	T. D	22.5	3	3
III	2 T. D	45	3	3
IV	4 T. D	90	3	3

T.D – Therapeutic dose

B.W- Body weight

The animals were weighed every seven days, from the start of the treatment, to note any weight variation. The following parameters were assessed during the study

1. Changes in body weight.
2. Changes in the physiological behaviour.
3. Changes in the fur, eyes etc.
4. Changes in food and water intake.

At the end of the experiment, the animals were starved overnight and on the 22nd day, they were made unconscious by ether anaesthesia. The blood was collected by dissecting the carotid artery to assess the haematological and biochemical parameters. The statistical analysis was done using ANOVA.

Tissue Histology

The organs were fixed in 10% buffered formalin for ten days before embedding in paraffin wax. Each organ tissue was sectioned at 5µm and stained with Haematoxylin and Eosin (H and E) stain. The slide specimens were examined under light microscope at high power magnification for changes in organ architecture and photomicrographs were taken.

3. RESULTS AND DISCUSSION

3.1 ACUTE TOXICITY STUDY

Animals were observed continuously during the first 30 min after dosing and observed periodically (with special attention given during the first 4 hours) for the next 24 hours and then daily thereafter, for 14 days. All observations were systematically recorded with individual records being maintained for each animal. Observations included changes in skin and fur, eyes and mucous membranes and behavioural pattern. Attention was given for observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep, coma, and mortality. Individual weights of animals were recorded before the administration of drug on 1st day of the study and thereafter on the 7th and 14th day of the experiment.

All parameters were normal except there was a lethargy observed in the rats as soon as the administration of the drug. But it disappears within 1 hour and became active later. Microscopic examination of vital organs including the liver, kidneys, heart, and spleen did not show any changes. Neither mortality nor clinical alterations occurred, so the LD50 value of Swasanandam gutika was assessed to be more than 2000 mg/kg.

TABLE-2- Observations recorded on first 24 hours

Observations	30min	4 hours	24 hours	72 hours	1 week	2 weeks
Skin and fur	**N**	**N**	**N**	**N**	**N**	**N**
Eyes	**N**	**N**	**N**	**N**	**N**	**N**
Mucous membrane	**N**	**N**	**N**	**N**	**N**	**N**
Salivation	**N**	**N**	**N**	**N**	**N**	**N**
Lethargy	**present**	**A**	**A**	**A**	**A**	**A**
Sleep	**N**	**N**	**N**	**N**	**N**	**N**
Coma	**A**	**A**	**A**	**A**	**A**	**A**
Convulsions	**A**	**A**	**A**	**A**	**A**	**A**
Tremors	**A**	**A**	**A**	**A**	**A**	**A**
Coma	**A**	**A**	**A**	**A**	**A**	**A**
Mortality	**A**	**A**	**A**	**A**	**A**	**A**

N- Normal

A-Absent

3.2 SUB-ACUTE TOXICITY STUDY

Weight variation of the control versus study groups

The body weight of control group and study group was noted once in every week during the study period. The weight variation was significant ($P<0.01$) on comparing the study groups and control groups except with group IV. The study groups showed a statistically significant weight gain when compared with control group. As the group IV administered with 4 times the therapeutic dose did not show any significant variation with the control group, it can be assumed that the higher dose of the drug was not influencing the general health of the animals.

TABLE-3 **Changes observed in the albino rats after administration of drugs for 21 days**

Observation	Before administration	After 21 days of drug administration			
		Group I	**Group II**	**Group III**	**Group IV**
Colour of eyes	Deep red	Deep red	Deep red	Deep red	Deep red
Oedema of eyes	Absent	Absent	Absent	Absent	Absent
Activity	Normal	Normal	Normal	Normal	Normal
Water intake	Normal	Normal	Normal	Normal	Normal
Food intake	Normal	Normal	Normal	Normal	Normal
Paralysis	Not observed	Not observed	Not observed	Not observed	Not observed

Stool colour	Black	Black	Black	Black	Black
Stool nature	Hard	Hard	Hard	Hard	Hard

GRAPH-1 Changes observed in body weight of the albino rats after administration of drugs for 21 days

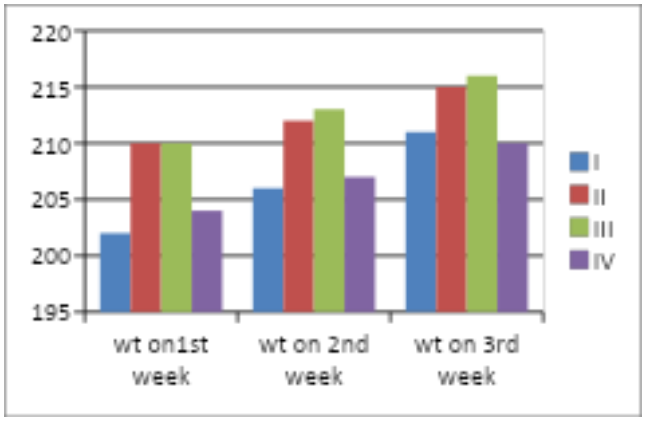


Table-4

Effect of Swasanandam Gulika on heamatological parameters in wistar rats

Parameter	Group I	Group II	Group III	Group IV
Total WBC count/mm^3	4996.67±10.33	4671.50±14.36**	4778.33±50.37**	5093.33±77.89**
% of DC lymphocyte	76.50± 1.64	79.50 ±1.87*	78.50± 1.76^{ns}	76.33± 2.16^{ns}
% of DC neutrophil	23.50± 1.64	20.50 ±1.87*	21.50 ±1.76^{ns}	23.67 ±2.16^{ns}
Hb in g/dl	13.13± 0.12	12.68± 0.12**	13.28 ±0.13^{ns}	13.38± 0.21**
RBS in mg/dl	52.62± 1.14	42.22± 1.85**	71.83± 1.94**	66.10± 2.92**

N=6, Values in mean± standard deviation,

** highly significant p< 0.01,

*Significant p<0.05, ns not significant

GRAPH-2 Comparison of hematological parameters

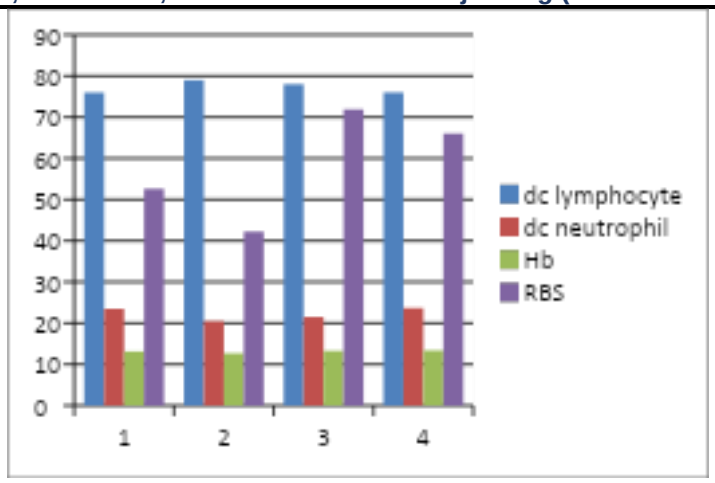


Table -5 Comparison of biochemical parameters

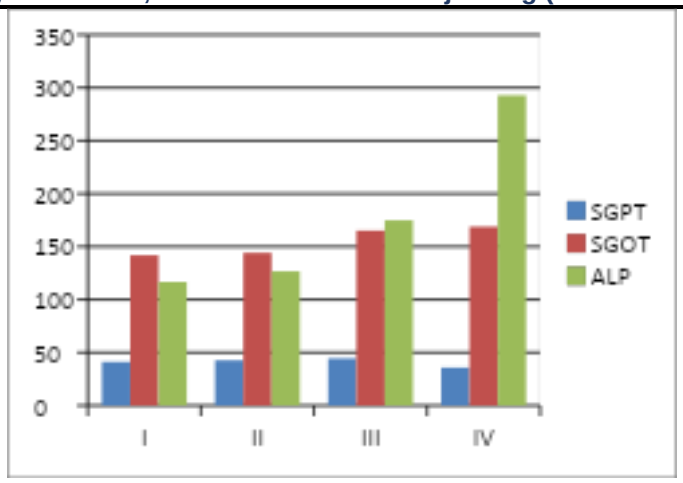
Parameter	Group I	Group II	Group III	Group IV
SGPT/ALT U/L	41.17± 0.75	42.67± 2.34^{ns}	44.50 ±7.42^{ns}	35.83± 3.06*
SGOT/AST U/L	141.83 ±1.17	144.17± 2.64^{ns}	165.17± 3.82**	168.83 ±1.47**
ALP U/L	117.67± 2.58	126.67 ±2.16^{ns}	174.83 ±3.13**	293± 14.27**
Bilirubin total mg/dL	0.49 ±0.01	0.49± 0.01^{ns}	0.55 ±0.03**	0.58 ±0.02**
S. urea mg/dL	20.33± 0.52	20. ±0 1.1^{ns}	20.67± 0.82^{ns}	21.17± 0.75^{ns}
S. creatinine mg/dL	0.72± 0.01	0.70± 0.02*	0.77± 0.01**	0.71± 0.02^{ns}
S. protein g/dL	7.22± 0.02	7.07 ±0.09*	7.14± 0.09^{ns}	6.19± 0.18**
S.albumin g/dL	3.21± 0.01	3.03 ±0.02**	3.00 ±0.03**	2.86± 0.07**

N=6, Values in mean± standard deviation,

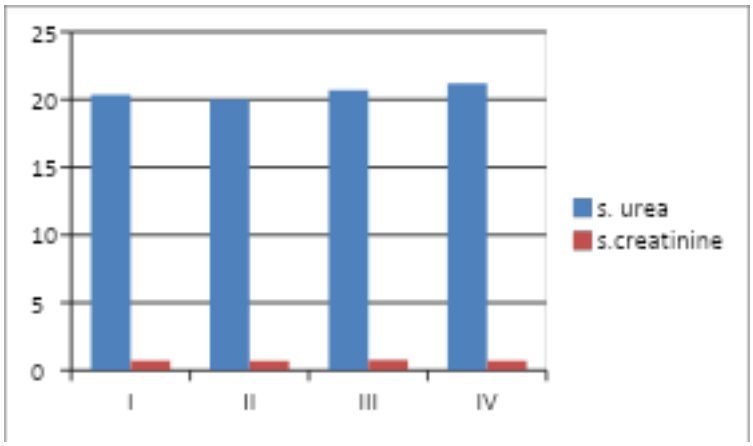
** highly significant p< 0.01,

*Significant p<0.05, ns not significant

GRAPH-3 Comparison of liver enzymes



GRAPH-4 Comparison of renal function parameters



HISTOPATHOLOGY MICROGRAPHS OF LIVER

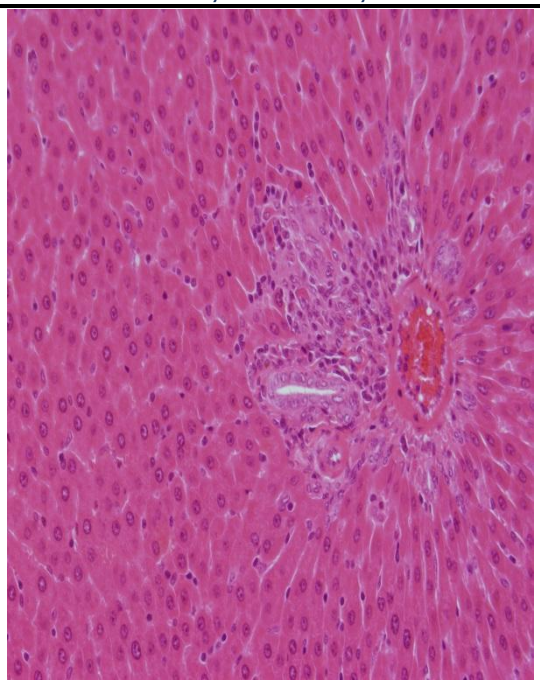


Fig 9.1 Group-I

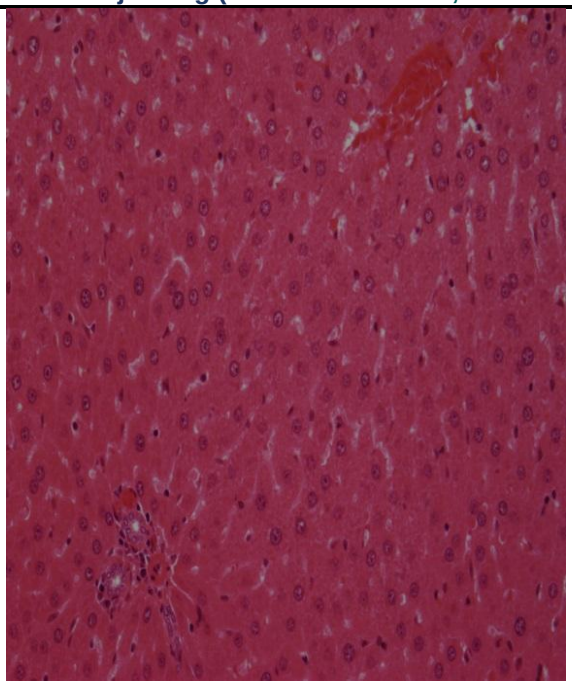


Fig 9.2 Group-II

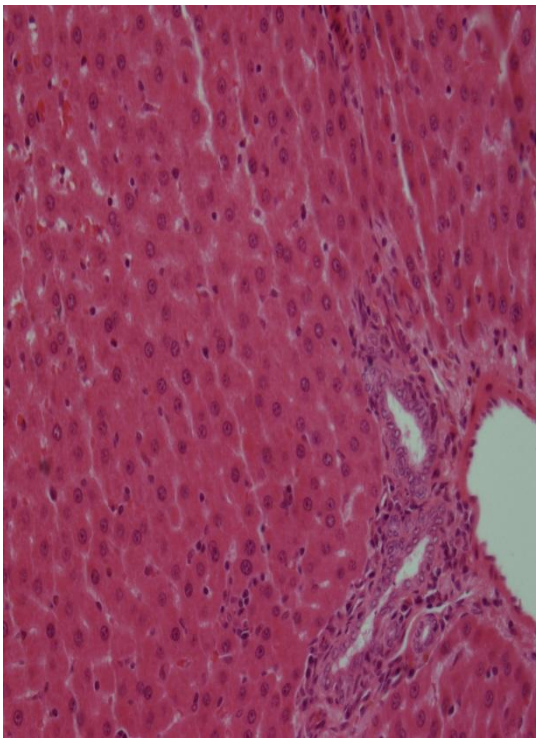


Fig 9.3 Group-III

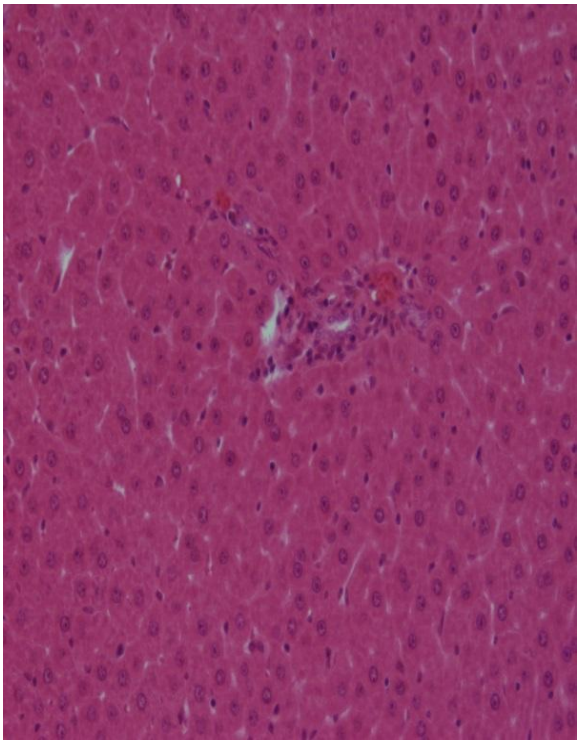


Fig 9.4 Group-IV

HISTOPATHOLOGY MICROGRAPHS OF SPLEEN

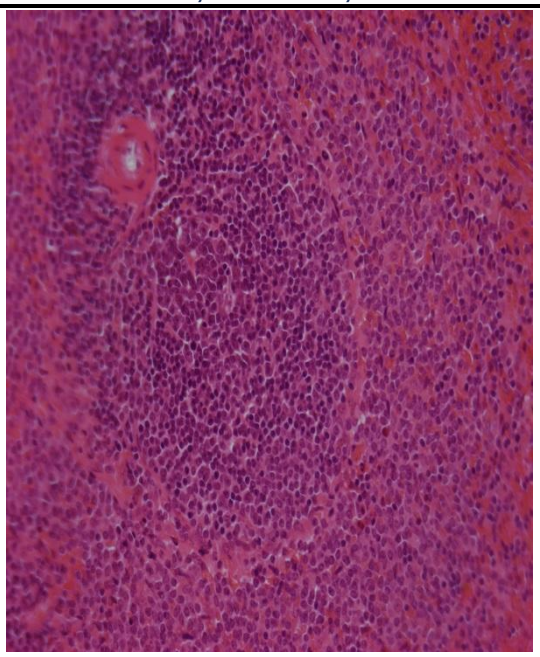


Fig 9.5 Group I

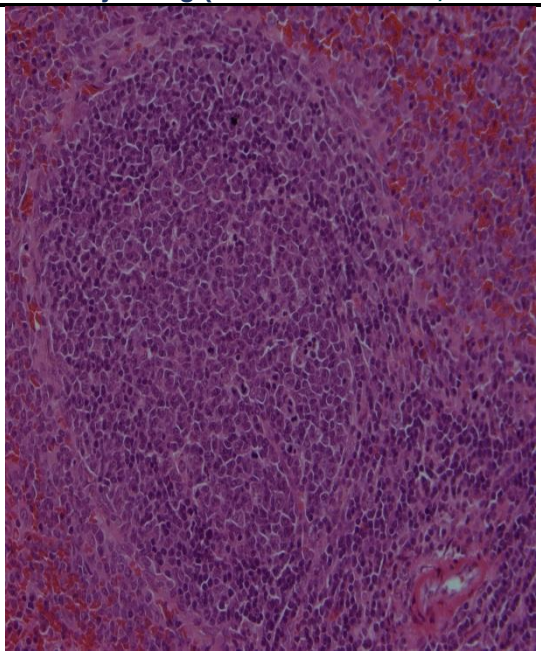


Fig 9.6 Group II

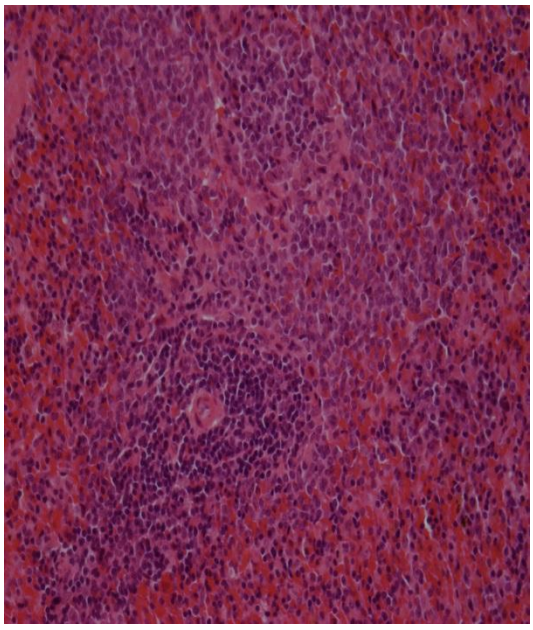


Fig 9.7 Group III

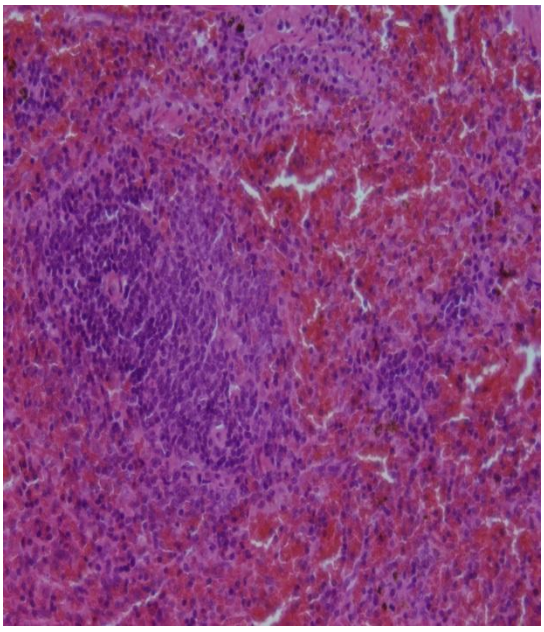


Fig 9.8 Group IV

HISTOPATHOLOGY MICROGRAPHS OF KIDNEY

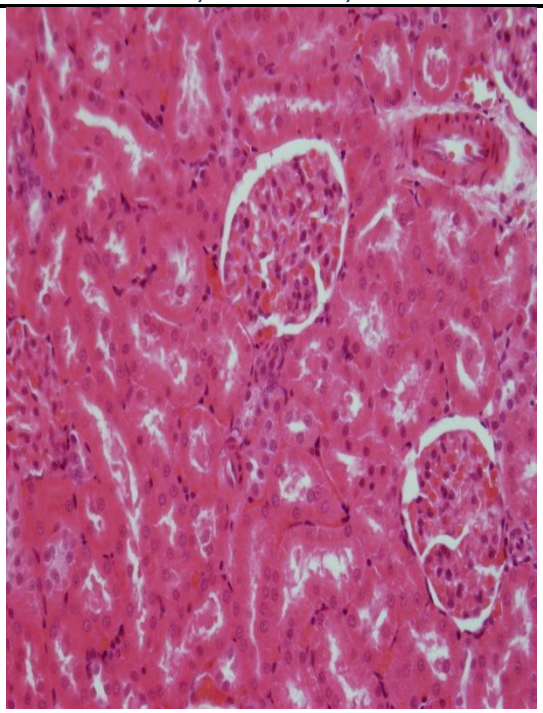


Fig 9.9 Group I

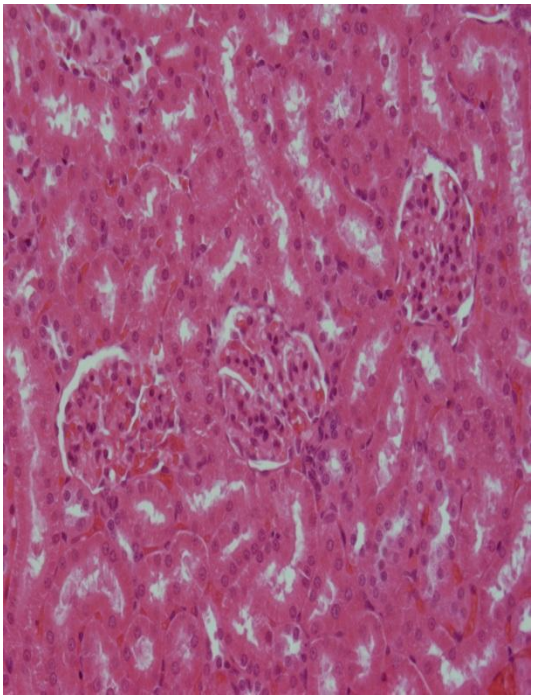


Fig 9.10 Group II

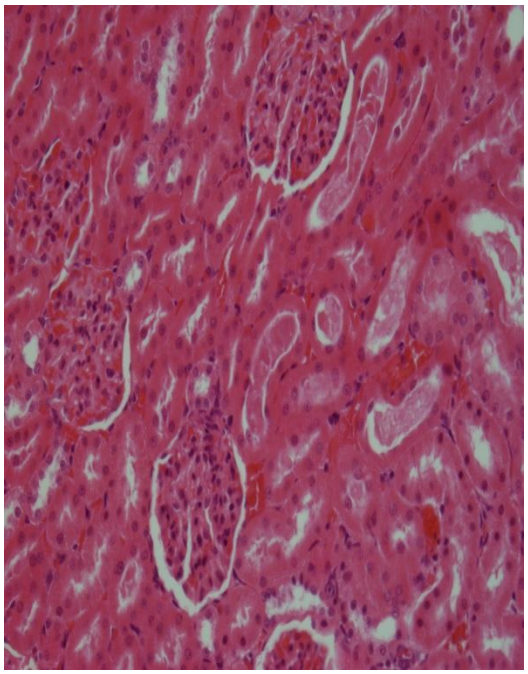


Fig 9.11 Group III

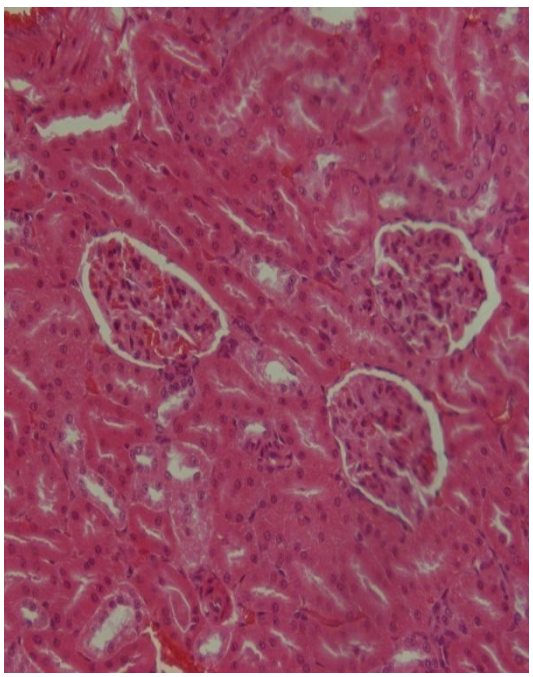


Fig 9.12 Group IV

HISTOPATHOLOGY OF HEART

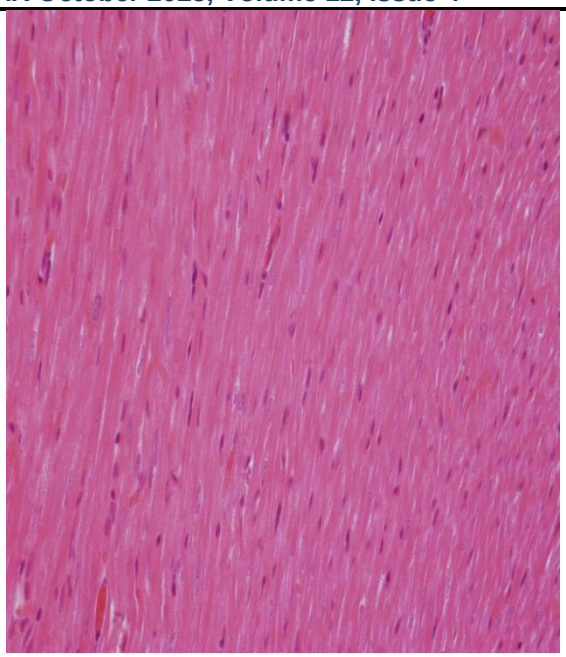


Fig 9.13 Group I

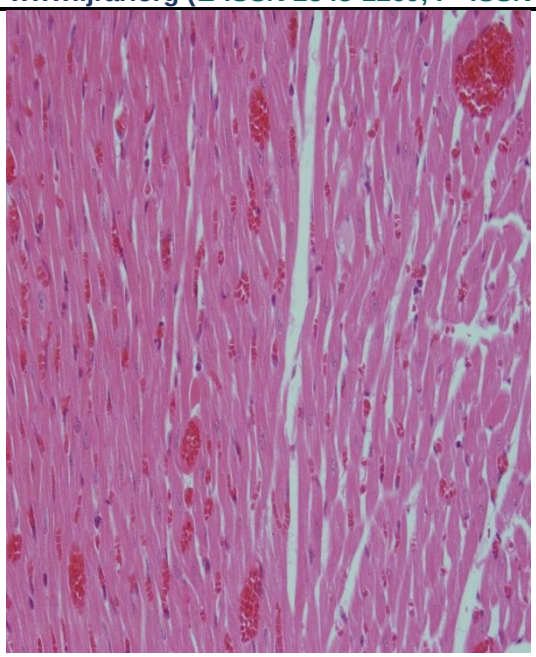


Fig 9.14 Group II

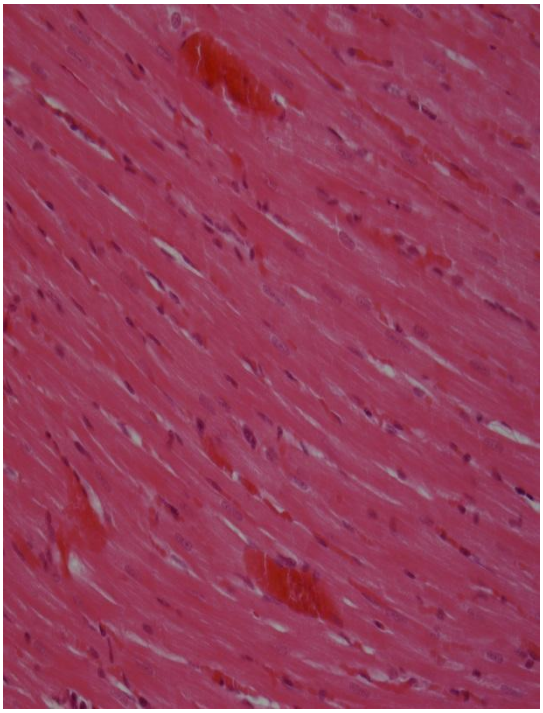


Fig 9.15 Group III

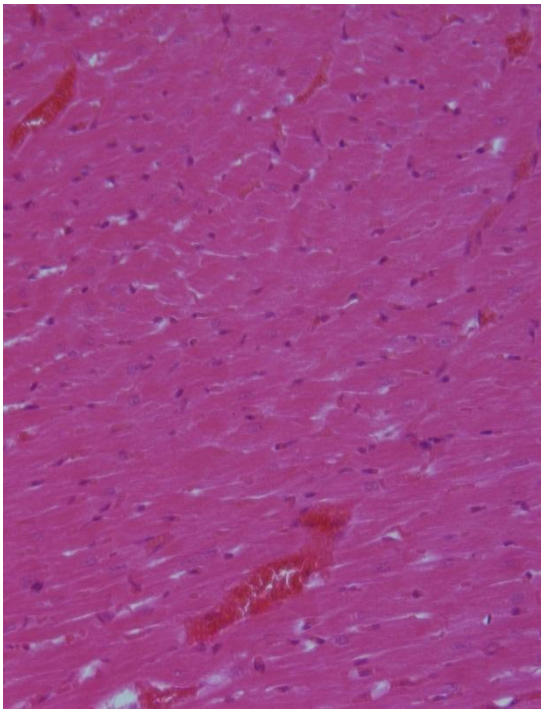


Fig 9.16 Group IV

HISTOPATHOLOGY OF BRAIN

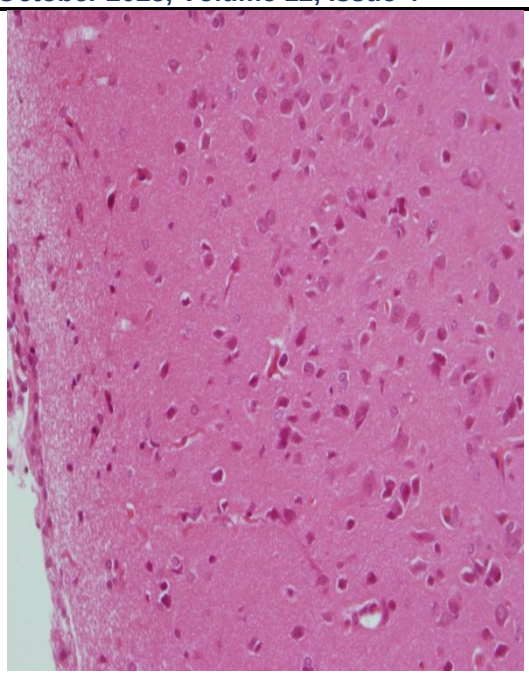


Fig 9.17 Group I

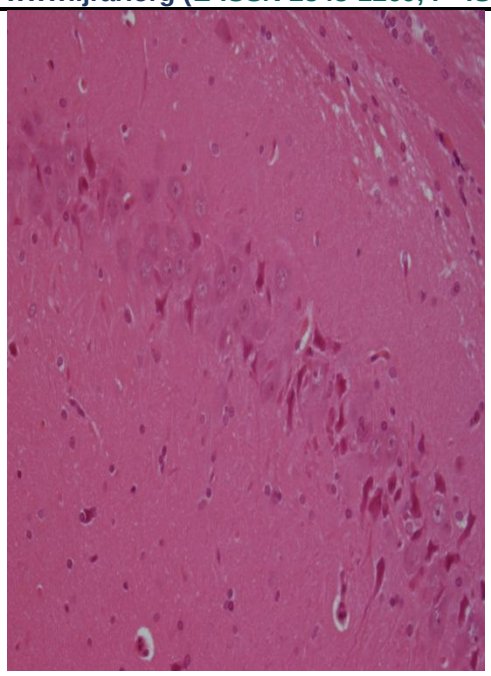


Fig 9.18 Group II

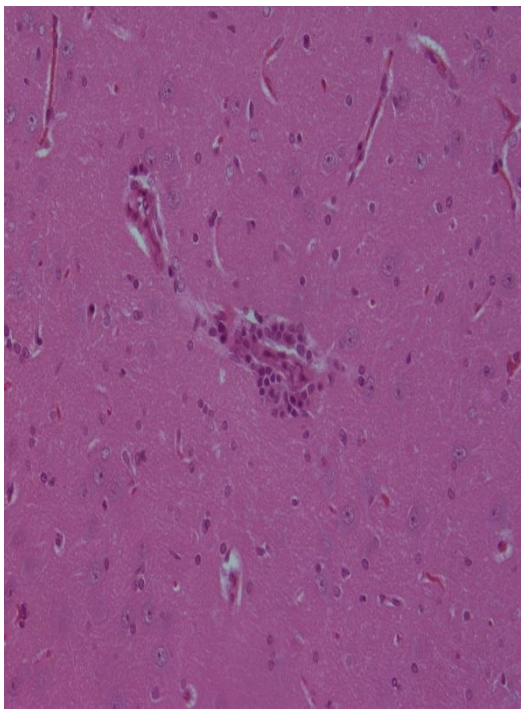


Fig 9.19 Group III

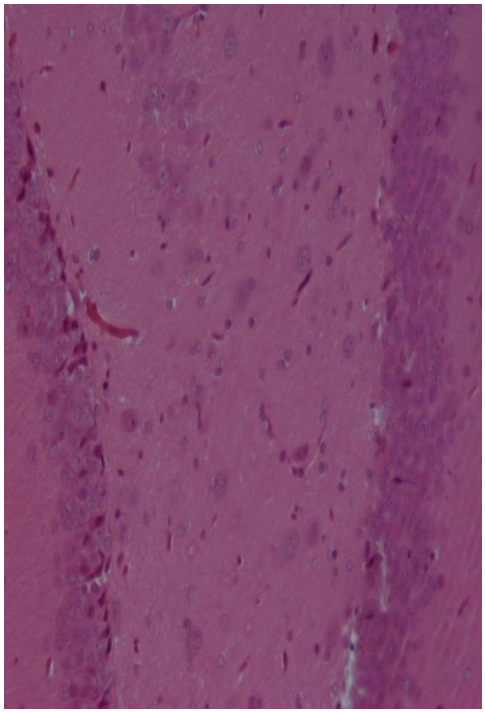


Fig 9.20 Group IV

MICROGRAPHS OF RAT TREATED WITH 2000mg/kg

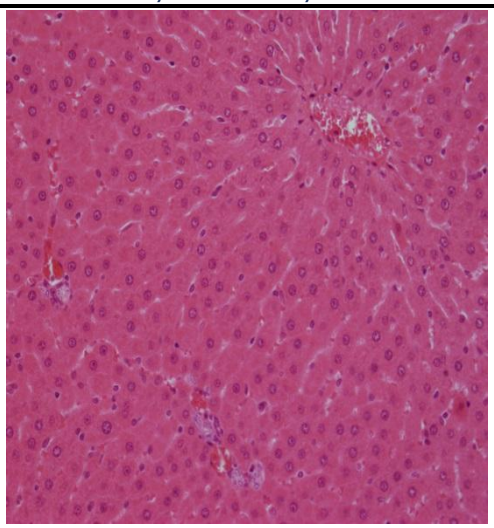


Fig 9.21 Liver

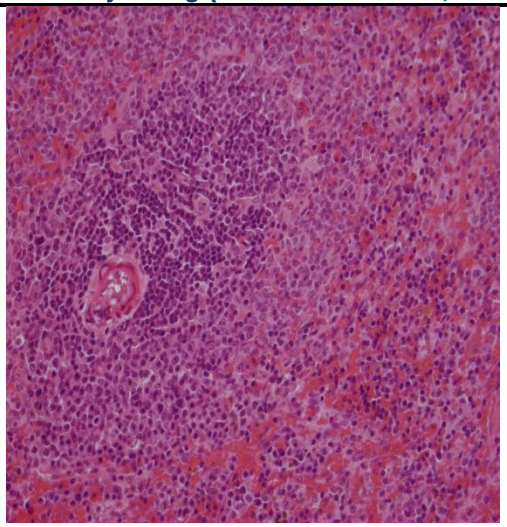


Fig 9.22 Spleen

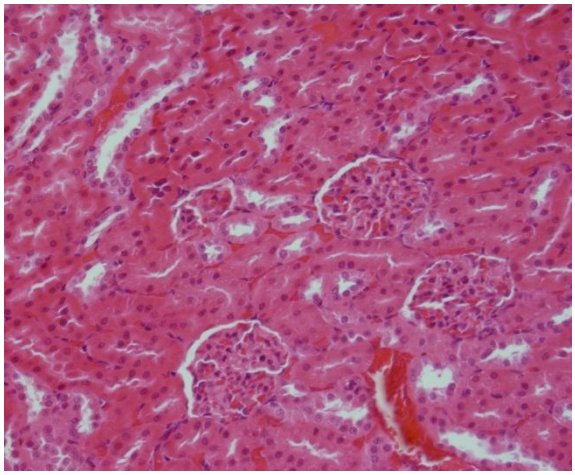


Fig 9.23 Kidney

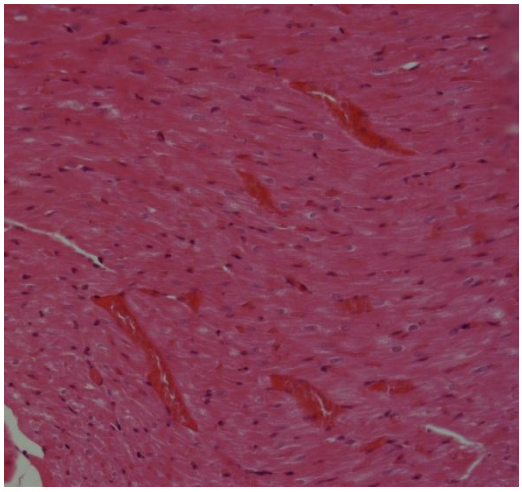


Fig 9.24 Heart

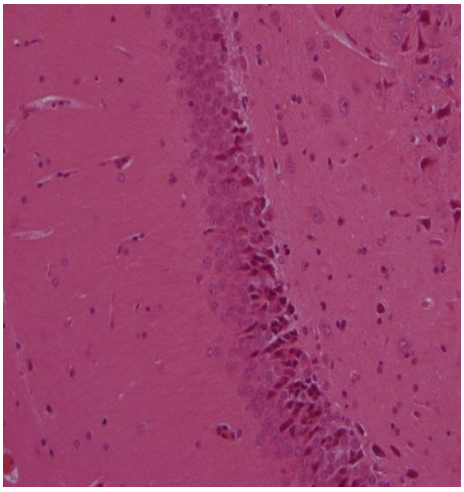


Fig 9.25 Brain

Histopathology results

The organs sampled for histopathology studies include liver, spleen, kidney, heart, and brain. The results are interpreted as follows:

1. Liver-The control and study groups did not show any variation in the tissue architecture. The differences were negligible among study groups dose dependently. Healthy sinusoids and normal portal triad area with canaliculi and Kupfer cells were noticed. No infiltration, vacoulisation or necrosis was seen.

2. Spleen-The spleen of both study group and control group were normal when compared with the control. The lymphoid tissues were healthy with well differentiating red pulp and white pulp.

3. Kidney-The renal cytoarchitecture was normal in control group and study group. The glomerulus is of normal size and cellularity, no increase in mesangium or thickening of glomerular basement membrane seen in control group and in study groups. There was the presence of proteinaceous casts observed in the study groups.

4. Heart-The heart muscles were normal in the control group and study groups.

5. Brain-The cells were of normal cytology in control group and in study groups.

4. DISCUSSION

A drug when administered in to a biological system may exert different types of interactions and a series of dose related responses. Some of those responses will result in therapeutic effect while others may cause harm to the subject. The types of toxicity tests which are routinely performed are acute and sub-acute toxicity evaluation. Acute toxicity test will estimate the LD50 (the dose which has proved to be lethal to 50 percent of the tested group of animals). Determination of acute toxicity is usually a screening step to assess the toxic potential of a drug. Assessment of sub-acute toxicity was done by assessing the haematological, biochemical, and histopathological parameters.

The drug passed the limit test according to the OECD-425 guidelines and a dose of 2000mg/kg did not produce any mortality. Hence the drug was safe for therapeutic administration. The sub-acute toxicity was conducted to evaluate the effect of therapeutic doses and higher doses of Swasanandam gutika on albino rats for a period of 21 days. The animals were randomly selected and grouped in to 4; each group comprised of 6 animals. One group was assigned as control receiving the milk mixed with sugar prescribed as anupana in the yoga. The study groups were treated with the rodent equivalent dose of the therapeutic dose, double and four times the therapeutic doses of Swasanandam gutika. The therapeutic adult dose of the drug was fixed as 250mg in the study.

The hematological and biochemical blood parameters were analyzed using semi-auto analyzers and ERBA analyzing kits. Histopathological evaluation was done and photomicrographs were taken.

On statistical analysis, the renal function tests were insignificant on comparing the study groups with the control group. The liver function tests were statistically insignificant in the group treated with the therapeutic dose. But there was a statistically significant rise in the liver enzymes (AST, ALT, and ALP) in the groups treated with higher doses of Swasanandam gutika. But no changes of toxicological significance were observed in the histopathology reports.

5.CONCLUSION

On in-vivo sub-acute toxicity evaluation, the group administered with the therapeutic dose did not show any statistically significant variation in the liver function tests and renal function tests and in histopathology. At the same time the groups treated with double and four times the therapeutic dose showed statistically significant elevation in the level of liver enzymes. Even though the elevated enzyme levels were noticed, the histopathology results did not reveal changes of toxicological significance in all the study groups. The hepatotoxic nature of camphor may be responsible for the alteration of liver enzyme levels. From this toxicological safety evaluation, it becomes evident that the Swasanandam gutika when used in the prescribed therapeutic dose can be considered safe.

6.ACKNOWLEDGEMENT

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