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Subacute Toxicity Studies of Methanolic Extract of Entada phaseoloides seeds in Rats

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ABSTRACT:
Background and Objective: Entada phaseoloides is an herbal medicinal plant commonly used in Ayurvedic medicine for decades. Researchers explored this medicinal plant with diversified characteristics and enormous data about the bioactive compounds and its pharmacological properties have been reproduced till now. However, references regarding the toxicity study of E. phaseoloides seed is lacking. **Materials and Methods:** To study the subacute toxicity of methanolic extract of E. phaseoloides seeds in Sprague dawley rats, four experimental groups were designed and intended to serve as the control and treated groups to which methanolic extract of E. phaseoloides seeds at doses of 100, 200 and 1000 mg kg-1 bodyweight, were given daily for 28 days. Animals of all groups were sacrificed on 28th day of experimentation. Blood samples were taken to determine haemoglobin, WBC count, RBC count, PCV, ESR, bleeding time, clotting time, blood glucose, serum creatinine, urea, total bilirubin, and direct bilirubin. Gross and histological examination has also been carried out. **Results:** Haematological and clinical blood chemistry revealed no toxicity effects of the extract. Pathologically, the extract was found to be safe as it did not not reveal any gross abnormalities and histopathological changes. **Conclusion:** Methanolic extract of E. phaseoloides was found to be nontoxic when oral subacute toxicities in rats were performed.

KEYWORDS: Entada phaseoloides, Methanolic extract, Subacute toxicity, Biochemical analysis, Haematological analysis, Histopathology.

I. INTRODUCTION:

Entada phaseoloides (E. phaseoloides) a creeper, of genus Entada which consists of 30 species of trees, shrubs and tropical lianas. Out of 30 species, 21 itself are from Africa, six from Asia, two from the American tropics and one with a pan tropical distribution [1]. The plant is distributed throughout the sub-Himalayan tract and in the monsoon forest of Western and Eastern Ghats. It is also abundant in Andaman & Nicobar Islands. Karbi tribes of Assam and Oceanic group of tribes such as Onges and Great Andamanese in India, consumed the boiled seeds of ghila (E. phaseoloides) bean as their native food. The use of E. phaseoloides as folk lore/traditional remedy for various disease conditions and multifarious medicinal properties has been reported from time to time. Almost all of the parts this plant is used in indigenous systems of medicine, the people in tropical and sub-tropical regions of countries made preparations for remedy of a wide variety of illnesses, including haemorrhoids, stomach ache, toothache, spasm, gastritis, and lymphadenitis [2]. The extract was found to possess anti-inflammatory property as it acts on proliferative phase of inflammation [3]. In addition to this, it showed anti-arthritic [4], anti-ulcer [5], anti-complement and antimicrobial [6] properties and molluscidal activities [7]. The seeds of E. phaseoloides are very high in protein,

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carbohydrates, and lipids contents [8]. A tremendous investigation of the seeds of E. phaseoloides has also been done by the author itself and found very effective with its diverse pharmacological properties. Some of such reporting's are the phytoconstituents, nutritive value and antioxidant activity of E. phaseoloides seeds [9] , seeds of E. phaseoloides attenuates scopolamine induced memory impairment, neuro-inflammation and neurodegeneration via BDNF/ TRKB/ NFKB pathway [10], seeds of E. phaseoloides are also effective in chronic restrain stress in mice [11], besides, methanolic extract of E. phaseoloides seeds found to possess analgesic property against different nociceptive animal model [12] and methanolic extract of E. phaseoloides inhibit colorectal carcinoma cell proliferation of HT-29 cell by modulating mitochondria apoptotic pathway [13].

Despite these numerous pharmacological potentials of E. phaseoloides seeds, the sub-acute toxicity effects of methanolic extract of E. phaseoloides seeds (MEEPS) have not been thoroughly investigated. Therefore, it is crucial to investigate the sub-acute toxicity of MEEPS in rats, with the hope that the results would provide information on the safety of this extract prior to the evaluation of its therapeutic efficacy in humans.

II. MATERIALS & METHODS:

Animals:

The Sprague dawley rats weighing 250 to 300 grams of either sex were procured from College of Veterinary Science, Assam Agricultural University, Khanapara, Assam and they were fed with standard pelleted laboratory diet and ordinary tap water. Polycarbonate plastic cages were used to kept the rats individually and allowed to acclimatize to the housing conditions for 7 days. The housing was conditioned with temperature of 22-25°, humidity at 40 %-70 % and equal 12 h light/ dark cycle [14]. All animals used were approved by CPCSEA and IAEC committee of College of Veterinary Sciences, Assam Agricultural University, Khanapara (no.770/ac/CPCSEA/FVSc, AAU/IAEC/20-21/568). and its ethical rules were followed throughout the experimental procedures.

Plant material:

Seeds of E. phaseoloides was collected in June-July, 2022 from local sources and identified by taxonomist Dr. Iswar Chandra Barua, Principal Scientist, Department of Agronomy, Assam Agricultural University, Jorhat, Assam.

Extraction of the plant material:

After collection, the kernel of the seeds was properly removed and dried under shade. The seeds were then finely ground to powder, weighed, and stored in an airtight container away from sunlight.

Then, 250 gm of powdered seed was soaked in 1000 ml of methanol for 72 h was stirred after every 18 h with the help of a sterile glass rod. The mixture was then subjected to filtration (with the help of Whatman filter paper no. 1) and the solvent was removed using a rotary evaporator (BUCHI, R-210, Labortechnik AG, Meierseggstrasse Switzerland) under reduced pressure, leaving behind a dark brown residue (MEEP). The Extract so obtained is stored in an airtight container at 4 °C. The percentage yield of the methanolic extract was 10.11% (w/w).

Precisely, 250 gm of powdered seed was soaked in 1 lit methanol for 72 hr was stirred after every 18 hr with the help of a sterile glass rod. The mixture was then subjected to filtration (with the help of Whatman filter paper no. 1) and the solvent was removed using a rotary evaporator (BUCHI, R-210, Labortechnik AG, Meierseggstrasse Switzerland) under reduced pressure, leaving behind a dark brown residue. The percentage yield of the methanolic extract was 10.11% (w/w).

Experimental design:

According to OECD TG407 (OECD, 2001b) guideline, rats were divided into 4 groups of 12 animals (6 male and 6 female). The MEEPS was administered to the first, second and third group of rats at a dose of 100, 200 and 1000 mg/kg/day for 28 days, whereas an equal volume of vehicle was given to the control group. The toxic manifestation such as signs of toxicity, mortality and the body weight changes were monitored daily. Heparinized blood samples were collected on day 29 by anaesthetizing the rats to determine complete blood count, red blood cell count, platelet count and red cell indices. The serum from non-heparinized blood was carefully collected for blood chemistry and enzyme analysis. The rats were euthanized to collect the important internal organs and tissues which were further weighed to determine relative organs weights and observed for gross lesions.

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Study parameters:

Observational parameters:

The animals were observed every day, for any sign, such as mortality if any, motor activity, tremors, convulsions, posture, spasticity, opisthotonicity, ataxia, righthing reflex, sensation, piloerection, ptosis, lacrimation, exopthalmos, salivation, diarrhoea, writhing, skin colour, respiratory rate, daily food intake. weight of animals was recorded every 7^{th} day.

Biochemical parameters:

Collection of blood was done by capillary tube from the inner canthus of eye in clean test tubes containing Ethylenediamine Tetra acetic Acid (EDTA) anticoagulant for the analysis of haematology and blood chemistry parameters [15], [16]. Plasma samples required for blood biochemical analysis was obtained by the method described by [16]. The plasma was subsequently analysed using fully automated clinical chemistry analyser (BioLis 24i Chemistry Analyzer, Japan) for the concentration of urea, creatinine, total bilirubin, direct bilirubin and glucose.

Haematological parameters:

Automated haematology analyser (ABC Vet®, ABX Diagnostics, France) was used for the complete blood count analyses.

Histopathological examination:

After sacrificing the rats scientifically, the internal organs and tissues were observed for gross lesions if any. Lesions were evaluated at 4x, 10x, 20x and 40x objective lenses of light microscope [15], [16] from each of the processed sample. All tissues were preserved in 10% buffered formaldehyde solution for histological examination.

Statistical analysis:

Data were expressed in Mean ± SEM. Data were analysed by analysis of variance (ANOVA) followed by Tuckey's HSD procedure. The statistical program used was KY Plot for window version 2.0 beta 15.

III. RESULTS AND DISCUSSION:

The rats in subacute toxicity received repeated doses of MEEPS for 28 days. No signs of toxicity and mortality were observed in the extract treated groups. Table 1 shows the effects of MEEPS on average feed and water intake comparing with control group. No significant difference has been observed with the control group.

Table 2 shows the effects of MEEPS on body weight. Body weight of male rats were found to be increased significantly both @ week 3 and 4 and the recorded values were (232.22±5.30* and 255.45±5.59*gm) for MEEPS treatment group (200 mg/kg b. wt) in comparison to control group (218.22±6.25 and 230.26±4.55gm). On the other hand, for MEEPS treatment group (1000 mg/kg b. wt) the observed values were (236.28±3.70* and 265.30±5.19*gm) at week 3 and 4 which were significantly different ($P < 0.05$) from the control group (218.22±6.25 and 230.26±4.55gm). Similarly, in female rats, the values were (223.65±2.50* and 239.10±4.06*gm) which were found to be significantly different ($P < 0.05$) from control group values (198.50±2.25 and 211.90±1.59gm) at week 3 and 4 respectively.

Effects of MEEPS on organ weight is depicted by Table 3. The results showed no significant difference among MEEPS treated groups and control group ($P < 0.05$).

The status of bone marrow activity and intravascular effects were monitored by haematological examination as summarized in Tables 4. During haematological examination, WBC count ($16.84±1.33X10^3/\mu L$) was increased significantly ($P < 0.05$) in male MEEPS treatment group (100 mg/kg b. wt) in comparison to control ($13.71±1.21X10^3/\mu L$) which was not significantly different in females in extracts treated groups. Likewise, there was significant ($P < 0.05$) increase in RBC count ($8.90±0.44X10^6/\mu L$) in the females treated with MEEPS treatment group (100 mg/kg b. wt) in comparison to the control group ($7.22±0.88$ $X10^6/\mu L$) but the value appeared to be lower ($6.22±0.95$ $X10^6/\mu L$) in females treated with MEEPS treated group (200 mg/kg b. wt) when compared with control group. Again, haemoglobin concentration (mean value) is significantly ($P < 0.05$) higher (15.66±0.48 and 16.00±0.30 g/dl) in males MEEPS treatment group (200 and 1000 mg/kg b. wt) while comparing to control group (13.66±1.06 g/dl). Again, the bleeding time value appeared to be more (33.77±1.01 sec) but not significant in female MEEPS treatment group (100 mg/kg b. wt) than the control (32.70±1.29 sec). Nevertheless, the values were found to be lies within the normal range, and therefore it is considered normal for this animal species. Thus, such changes are not indicative of toxicity of the plant extract in subacute toxicity study.

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The levels of the plasma biochemical parameters of the rats treated with varying doses of MEEPS for 28 days are shown on Table 5. In blood chemistry, serum glucose level (99.40±2.10, 97.10±3.20 and 94.23±1.30 mg/dl) was decreased significantly ($P < 0.05$) in male MEEPS treatment groups (100, 200 and 1000 mg/kg b. wt) when compared with control (106.61±1.21 mg/dl). Likewise, there was significant ($P < 0.05$) decrease in serum glucose level (90.20±2.44 and 88.13±1.95 mg/dl) in female MEEPS @ 200 and 1000 mg/kg b. wt group on comparing with control group (95.70±2.30 mg/dl), but the value appeared to be in a normal range. Again, total bilirubin (mean value) appeared to be lower (0.22±0.08, 0.22±0.03 and 0.21±0.05 mg/dl) significantly ($P < 0.05$) in male MEEPS treatment group (100, 200 and 1000 mg/kg b. wt) than the control group (0.25±0.05 mg/dl) whereas total bilirubin (mean value) (0.18±0.03 mg/dl) was decreased significantly ($P < 0.05$) in female MEEPS @ 100 mg/kg b. wt while comparing to control group (0.20±0.03 mg/dl). On the other hand, the direct bilirubin (0.05±0.02, 0.05±0.07 and 0.05±0.03 mg/dl) were observed to be lower significantly ($P < 0.05$) in male MEEPS @ 100, 200 and 1000 mg/kg b. wt when compared to control group (0.07±0.05 mg/dl). Nevertheless, all values lay within the normal range, the results are considered as normal for this animal species. Thus, it can be inferred that the aqueous leaf extract of the plant is devoid of subacute toxicity.

Moreover, figures (1,2,3,4 & 5) predict no pathological features in treated groups as monitored by histopathological analysis of the internal organs. Liver on histological examination revealed normal architecture with normal appearance of radiating hepatocytes and portal triad. No signs of fatty changes were noticed in the liver of extract treated animals. Again, no signs of renal toxicity were observed on histological examination of kidney of extract treated animals, as renal tissue section showed normal appearance of renal parenchyma, tubules and glomeruli with clear bowman's space**.** Likewise, heart of extract treated animals revealed normal myocardial striations with normal myocytes suggest MEEPS is non-toxic. Also, normal splenic architecture with normal white and red pulp and normal cerebral architecture with granular and molecular cell layer revealed that the extract is nontoxic to spleen and brain as well.

From our study it is revealed that MEEPS does not possess hepatic toxicity in rats. In support of the safety profile of MEEPS, the biochemical and haematological parameters revealed no abnormal value when compared with control group. Moreover, no abnormality has been observed on histopathological examination of various internal organs. Therefore, the results obtained suggest that MEEPS is nontoxic.

IV. CONCLUSION:

In summary, it is concluded from the study that MEEPS was found to be nontoxic when oral subacute toxicities in rats were performed. Chronic toxicity may be carried out in future in order to rule out its toxic effect on chronic use for further support the safe use of this plant.

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CONFLICT OF INTEREST: The authors declared no conflict of interest.

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ILLUSTRATION:

Table 1: Effects of MEEPS on average feed and water intake

Treatment	Sex	Average food intake (g/day)	Average water intake (ml/day)
Control	Male	20.03±1.66	25.55±1.06
	Female	15.30±1.43	19.19±1.78
MEEPS 100 mg/kg	Male	20.56±1.90	18.89±1.65
	Female	16.07±2.56	16.29±1.90
MEEPS 200 mg/kg	Male	19.45±1.00	22.60±1.05
	Female	15.90±2.12	16.34±1.86
MEEPS 1000 mg/kg	Male	19.25±1.21	23.40±1.41
	Female	16.00±2.05	16.86±1.90

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Values are expressed as mean ± standard error; n = 10 (5 females or 5 males). No significant difference between MEEPS (Methanolic extract of E. phaseoloides seed) treatment groups and control group (P < 0.05).

Table 2: Effects of MEEPS on body weight

Group	Body weight				
	Week 0 (gm)	Week 1 (gm)	Week 2 (gm)	Week 3 (gm)	Week 4 (gm)
Male					
Control	168.15±6.58	184.26±3.89	200.35±2.10	218.22±6.25	230.26±4.55
MEEPS 100mg/kg	170.22±4.36	190.05±4.46	210.68±1.55	221.22±7.59	246.11±4.33
MEEPS 200 mg/kg	172.30±3.49	190.28±2.22	213.12±2.59	232.22±5.30*	255.45±5.59*
MEEPS 1000 mg/kg	169.20±1.19	188.08±1.23	218.16±1.49	236.28±3.70*	265.30±5.19*
Female					
Control	160.19±4.39	170.25±6.89	179.25±4.65	198.50±2.25	211.90±1.59
MEEPS 100mg/kg	162.56±3.59	175.56±2.15	194.01±2.12	205.26±2.12	221.14±3.10
MEEPS 200 mg/kg	159.68±3.89	175.43±6.22	197.56±5.59	215.86±3.00	234.58±1.66
MEEPS 1000 mg/kg	160.22±1.23	178.14±1.89	208.10±2.45	223.65±2.50*	239.10±4.06*

Values are expressed as mean ± standard error; n = 10 (5 females and 5 males). No significant difference between MEEPS (Methanolic extract of E. phaseoloides seed) treatment groups and control group (P < 0.05).

Table 3: Organ weights of rats in sub-acute toxicity of MEEPS

Control				MEEPS 100mg/kg		MEEPS 200mg/kg		MEEPS 1000mg/kg	
Organ name	Sex	Average organ weight (gram)	Relative organ weight (%)	Average organ weight (gram)	Relative organ weight (%)	Average organ weight (gram)	Relative organ weight (%)	Average organ weight (gram)	Relative organ weight (%)
Heart	M	0.85±0.08	0.37%	0.88±0.03	0.33%	0.89±0.17	0.32%	0.86±0.05	0.31%
	F	0.77±0.03	0.32%	0.70±0.06	0.22%	0.74±0.08	0.28%	0.72±0.07	0.29%
Liver	M	8.20±0.30	3.04%	7.90±0.10	3.51%	8.30±0.40	3.91%	8.90±0.37	3.63%
	F	6.75±0.45	2.32%	6.30±0.20	2.20%	6.30±0.30	2.56%	6.80±0.40	2.78%
Lung	M	1.70±0.22	0.82%	1.27±0.04	0.57%	1.35±0.13	0.37%	1.33±0.02	0.50%
	F	1.30±0.11	0.88%	1.16±0.18	0.48%	1.24±0.19	0.57%	1.26±0.09	0.48%
Kidney	M	2.68±0.05	0.65%	2.70±0.57	0.79%	1.86±0.33	0.68%	1.92±0.36	0.78%
	F	1.60±0.10	0.34%	1.81±0.08	0.39%	1.26±0.25	0.40%	1.29±0.15	0.48%
Spleen	M	0.81±0.05	0.48%	0.73±0.06	0.29%	0.72±0.09	0.21%	0.74±0.02	0.25%
	F	0.40±0.07	0.53%	0.35±0.05	0.14%	0.47±0.06	0.19%	0.50±0.04	0.16%

Values are expressed as mean ± standard error; n = 10 (5 females and 5 males). No significant difference between MEEPS (Methanolic extract of E. phaseoloides seed) treatment groups and control group (P < 0.05).

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Table 4: Haematological values of rats in sub-acute toxicity of MEEPS

Group	Body weight						
	WBC ($X10^3$/µL)	RBC ($X10^6$/µL)	PCV (%)	ESR (mm/hr)	Hb (g/dl)	Bleeding time (sec)	Clotting time (min)
Male							
Control	13.71±1.21	6.69±0.75	36.79±3.11	2.2±0.1	13.66±1.06	32.65±2.09	4.66±0.12
MEEPS 100 mg/kg	16.84±1.33*	6.95±1.48	35.24±3.57	2.1±0.1	14.34±0.68	32.40±2.37	4.24±0.44
MEEPS 200 mg/kg	13.57±3.02	6.89±0.54	35.74±4.20	2.2±0.1	15.66±0.48*	32.08±0.98	4.74±0.18
MEEPS 1000 mg/kg	11.07±2.12	6.74±0.30	36.32±3.27	2.2±0.1	16.00±0.30*	32.35±0.17	4.64±0.26
Female							
Control	14.53±1.53	7.22±0.88	35.69±3.29	2.2±0.1	13.77±0.21	32.70±1.29	4.55±0.26
MEEPS 100 mg/kg	13.59±1.48	8.90±0.44*	34.60±4.05	2.2±0.1	14.20±0.24	33.77±1.01	4.02±0.10
MEEPS 200 mg/kg	14.25±1.98	6.22±0.95	34.84±1.91	2.1±0.1	14.77±0.47	32.11±1.25	4.10±0.30
MEEPS 1000 mg/kg	14.10±2.32	6.15±0.88	34.64±1.51	2.2±0.1	14.90±0.77	32.70±1.80	4.17±0.38

Values are expressed as mean ± standard error; n = 12 (6 females and 6 males). MEEPS (Methanolic extract of E. phaseoloides seed) treatment groups and control group *P=0.05 significance difference.

Table 5: Blood chemistry values of rats in sub-acute toxicity of MEEPS

Group	Glucose (mg/dl)	BUN (mg/dl)	Total Billirubin (mg/dl)	Direct Billirubin (mg/dl)	Creatinine (mg/dl)
Male					
Control	106.61±1.21	31.40±0.55	0.25±0.05	0.07±0.05	0.38±0.04
MEEPS 100mg/kg	99.40±2.10*	30.18±1.21	0.22±0.08*	0.05±0.02*	0.37±0.08
MEEPS 200 mg/kg	97.10±3.20*	31.32±0.24	0.22±0.03*	0.05±0.07*	0.38±0.05
MEEPS 1000 mg/kg	94.23±1.30*	30.44±2.01	0.21±0.05*	0.05±0.03*	0.37±0.08
Female					
Control	95.70±2.30	28.05±0.78	0.20±0.07	0.05±0.06	0.34±0.05
MEEPS 100mg/kg	93.14±3.10	28.10±0.11	0.18±0.03*	0.05±0.03	0.34±0.03
MEEPS 200 mg/kg	90.20±2.44*	27.32±0.67	0.19±0.06	0.04±0.02	0.35±0.09
MEEPS 1000 mg/kg	88.13±1.95*	27.22±0.54	0.19±0.04	0.04±0.03	0.34±0.07

Values are expressed as mean ± standard error; n = 12 (6 females and 6 males). MEEPS (Methanolic extract of E. phaseoloides seed) treatment groups and control group *P=0.05 significance difference.

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Figure 1: Showing normal hepatic architecture of liver with normal appearance of radiating hepatocytes and portal triad (black arrow – portal vein, yellow arrow - bile duct, arrow head- hepatic artery). H & E, × 10X

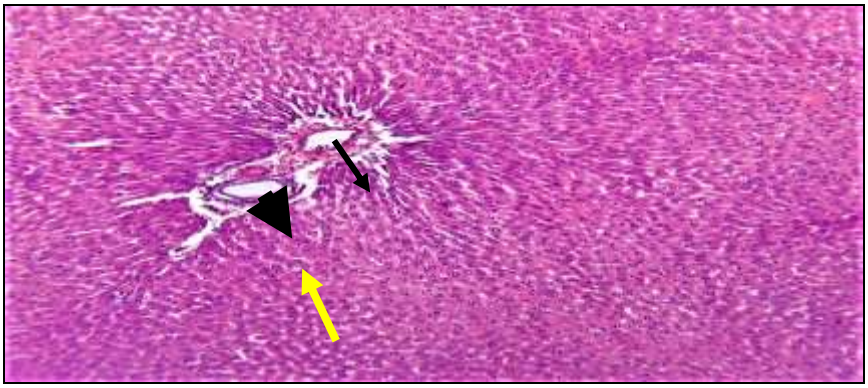

Figure 2: Showing normal appearance of renal parenchyma, tubules and glomeruli with clear bowman's space. H & E, × 10X

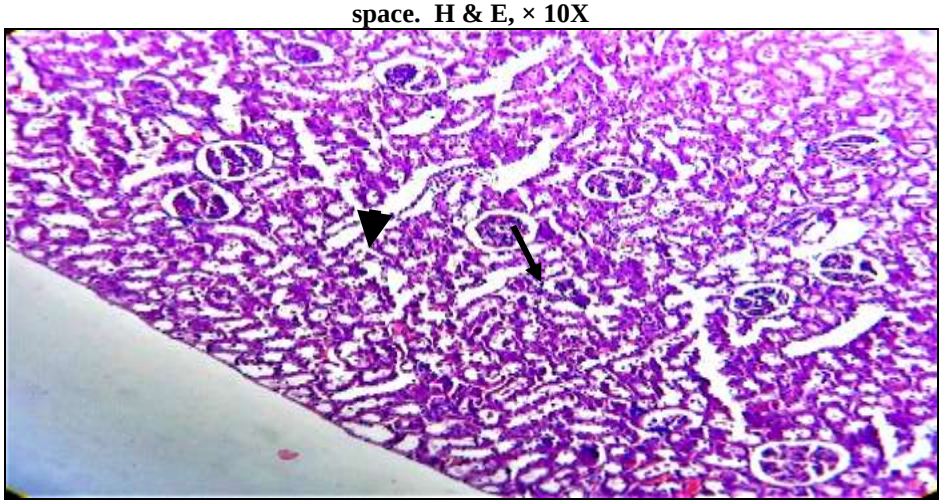

Figure 3: Showing Normal myocardial striations with normal myocytes .H & E, × 10X

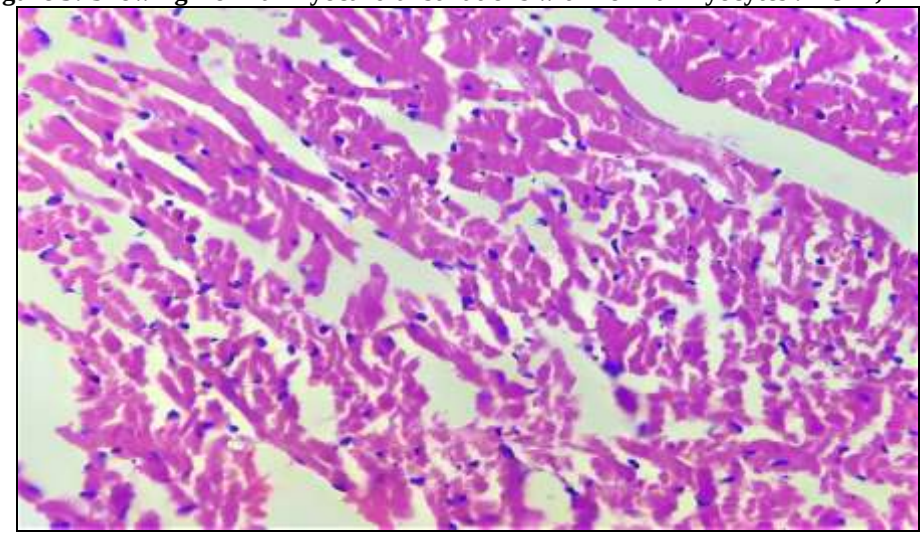

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Figure 4: Showing Normal splenic architecture with normal white and red pulp. H & E, × 10X

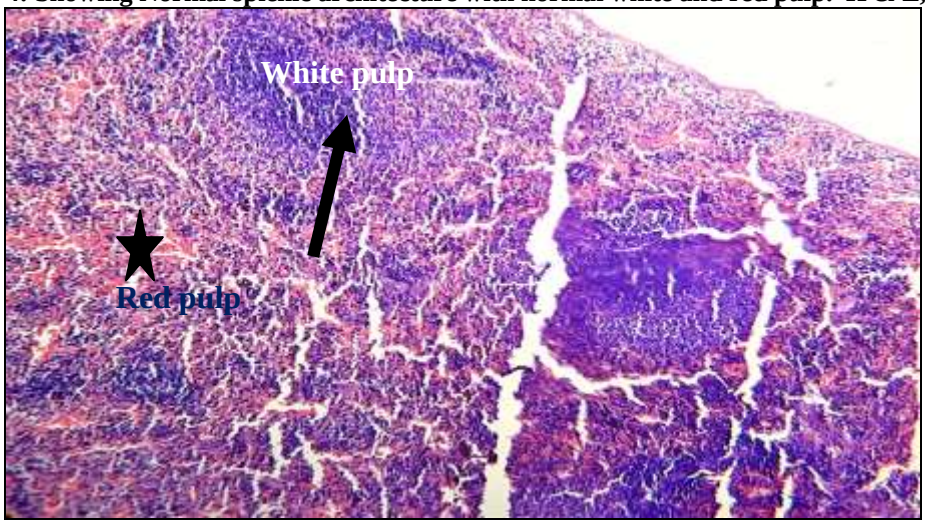

Figure 5: Showing Normal cerebral architecture granular and molecular cell layer (arrow – pyramidal neurons, arrow head – glial cell). H & E, × 40X