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Pre Clinical Toxicity Study of Siddha Medicine Pitha Paandu Maathirai on Wistar Albino Rats

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ABSTRACT:-

Background:- Iron deficiency anaemia (IDA) is one of the major disease affecting women from adolescents to elderly persons in our country. PITHA PAANDU MAATHIRAI (PPM) , a siddha medicine used in the treatment of IDA as per literary views. The study was carried out to demonstrate the oral toxicity of PPM in wistar albino rats to prove its safety.

Methods: Acute and 28-day repeated oral toxicity studies were performed following OECD test guidelines 423 and 407. In acute oral toxicity study, PPM was administered at 2000mg/kg b.wt./p.o, animals were observed for toxic signs at 30min, 4hrs, 24 hrs and for next 14 days. Gross pathology was performed at the end of the study. In repeated dose, the 28- days oral toxicity study, PPM was administered at 200 and 400 mg/kg b.wt./p.o/day. Animals were observed for mortality, clinical signs, body weight changes, feed and water intake. Haematological, biochemical, gross pathology, relative organ weight, histopathological examination and urine analysis were performed.

Results: In acute toxicity study, no treatment related death or toxic signs were observed with PPM administration. In the repeated dose study, slight increase in body weight and eosinophil count was observed.

Conclusion: Acute study reveals that the LD50 of PPM is greater than 2000mg/kg, b.wt . 28-days repeated oral toxicity demonstrates that the No Observed Adverse Effect Level of PPM is greater than 400 mg/kg b.wt./day, p.o in rats.

Keywords:- *acute toxicity, sub acute toxicity, pitha paandu.*

I. INTRODUCTION

The name derived from Greek word"anaemia" means "lack of blood". Anaemia refers to reduction in red blood count, haemoglobin content, and packed cell volume. It reflect to the diminished oxygen carrying capacity of blood. (1) It is the most common nutritional deficiency disorder in the world. It is a condition that occurs when the red blood cells do not carry enough oxygen to the tissues of the body.

It occurs because of:

- Decreased production of RBC
- Increased destruction of RBC
- Excess loss of blood from the body.

Globally anaemia affects 1.62 billion people, who corresponds to 24.8% It is prevalent in 12.7% among men, and 47.4% among pre school age children. As per (NFHS) more than half of women in India (55%) have anaemia. (2)

As per WHO, for the year 2011, 43% of children, 38% of pregnant women and 29% of non pregnant women are globally affected by iron deficiency anaemia(3).

In siddha, *anaemia* is defined as *"*paandu*"*. In our oldest epic mahabharatham has the character paandu. He is so pale. Like that paandu noi have the features of pallor of skin, conjunctiva, nails and mucous membrane.

Iron deficiency anaemia is compared with pitha paandu noi. Because its symptoms told in modern aspect may correlated with symptoms referred in *"*yugi vaithiya chinthaamani*"*.(4)

Pitha Paandu Maathirai is a herbo mineral preparation used in the treatement of Pitha Paandu as referred in Sarabendra Vaithya Muraigal (Pandu, Kamali Roga Sigichai) (5) Though PPM is referred as medicine for pitha paandu in literature, no scientific reportwas available on its safety. The pre-clinical toxicity studies are essential to prove its safety for human trials . Present study was undertaken to demonstrate the oral toxicity of PPM, a herbo-mineral siddha formulation in laboratory animals

II. MATERIALS AND METHODS

A) Incredients: PURIFIED INDHUPPU (Rock Salt), MILAGU *(Piper* nigrum*)*, THIPPILI (Piper longum),SEERAGAM*(*Cuminum cyminum),PURIFIED AYAM(Ferrum),KAIYANTHAGARAI JUICE (Eclipta prostata)(6) .

B) Source of Raw Drugs: The required raw drugs are procured from a well reputed indigenous drug shop. The raw drugs will be authenticated by the concerned pharmacognosist at SCRI, Chennai.

C) Purification of Raw Drugs: Raw drugs are purified as mentioned in our siddha text book Gunapadam Thadhu Jeevam.(7)

D) Preparation: The raw drugs are purified and finely powdered .The powdered drugs are rubbed it with karisalai juice till it becomes dry and attains the stage to made into pills. Then it made into 500mg pills.

AIM: Aim of the study is to evaluate the acute and sub-acute toxicity of the siddha drug '*PITHA PAANDU MAATHIRAI*'.

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III. EXPERIMENTAL ANIMALS

Albino rats (wistar rats) of either sex ,weighing (150-200 g) were procured from animal housing facility, K.K college of pharmacy ,Gerugambakkam ,Chennai. All animals were placed in a polypropylene cages in a controlled room temperature 24℃±1℃ and relative humidity of 60-70 % in animal house. The animals were maintained in standard pellet diet and water ad libitum. They were acclimatized to laboratory condition for seven days before commencement of the experiment.

All the protocols and the experiments conducted in strict compliance according to ethical principles and guidelines provided by committee for the purpose of control and Supervision of Experiments on Animals **(kkcp/2013/006)** .Animal experimentation protocols are approved by Institutional Animal Ethical Committee.

IV. ACUTE ORAL TOXICITY – OECD-423 GUIDELINES

A. Drug and preparation of stock solution:

The aqueous suspension of Pitha Paandu Maathirai was prepared in 1% carboxymethycellulose (CMC) solution in distilled water prior to oral administration to animals.It was used within seven days and stored at 8˚C while for futher use ,freshly prepared solution was used. The vehicle alone served as negative control.

B. Number of animals and dose levels:

Three animals are used for each step. The dose level used as the starting dose was selected from one of four fixed levels, 5, 50, 300 and 2000 mg/kg body weight. The starting dose level was most likely to produce mortality in some of the dosed animals. The available information suggests that mortality is likely at the highest starting dose level 2000 mg/kg body weight, so the trial or limit test was conducted. The time interval between treatment groups is determined by the onset, duration, and severity of toxic signs.[8]

C. Limit 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 only above regulatory limit doses. A limit test at one dose level of 2000 mg/kg body weight was carried out with three animals per step. The test substance-related mortality was not produced in animals, so further testing at the next lower level need not be carried out.[9]

D. OBSERVATIONS:

Animals were observed individually after dosing at least once during the first30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed. All observations are systematically recorded with individual records being maintained for each animal. Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somato motor activity and behavior pattern. Attention was directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. The principles and criteria summarized in the Humane Endpoints Guidance Document taken into consideration. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress was humanely killed. When animals are killed for humane reasons or found dead, the time of death should was recorded.

V. REPEATED DOSE 28-DAY SUB-ACUTE ORAL TOXICITY STUDY (OECD – 407 GUIDELINES)

A. *Test Substance: PITHA PAANDU MAATHIRAI*

- Animal Source: Animal house of K.K. COLLEGE OF PHARMACY
- Animals: Male and Female Wistar albino Rats
- Age**:** More than 8 weeks
- Acclimatization**:** Seven days prior to dosing.
- Veterinary examination**:** Prior to and at the end of the acclimatization period.
- Identification of animals**:** By cage number, animal number and individual marking on fur.
- Diet**:** Pelleted feed supplied by Sai meera foods Pvt Ltd, Bangalore
- Water**:** Aqua guard portable water in polypropylene bottles *ad* libitum.
- Housing & Environment: The animals were housed in Polypropylene cages provided with bedding of husk.
- Housing temperature: Between 20 & 24°C,
- Relative humidity: Between 30% and 70%,
- Air changes: 10 to 15 per hour and
- Dark and light cycle: Each of 12 hours.

B. *Justification for Dose Selection:*

The results of acute toxicity studies in wistar rats indicated that pitha paandu maathirai was non toxic and no behavioural changes was observed up to the dose level of 2000 mg/kg body weight. In the literature, therapeutic dosage for pitha paandu maathirai in human is mentioned as 1000 mg. On the basis of body surface area ratio between rat and human, the doses selected for the study were 200 mg/kg (5x) and 400 mg/kg (10x) body weight. The oral route was selected for use because oral route is considered to be a proposed therapeutic route.[10]

C. Preparation and administration of dose:

pitha paandu maathirai at two doses respectively was suspended in 2 ml of 2% CMC in distilled water. It was administered to animals at the dose levels of 200 and 400 mg/kg. The test substance suspensions were freshly

prepared every day for 28 days. The control animals were administered vehicle only. Administration was by oral (gavage), once daily for 28 consecutive days(10).

VI. METHODOLOGY

A. Randomization, Numbering and Grouping of Animals:
Ten rats (Five Male and Five Female) were in each group randomly divided into three groups for dosing up to 28 days. Animals were allowed acclimatization period of 7 days to laboratory conditions prior to the initiation of treatment. Each animal was fur marked with picric acid. The females were nulliporous and non-pregnant.(11)

VII. OBSERVATIONS:

A. Experimental animals were kept under observation throughout the course of study for the following:

- *Body Weight:* Weight of each rat was recorded on day 0, at weekly intervals throughout the course of study and at termination to calculate relative organ weights. From the data, group mean body weights and percent body weight gain were calculated.

- *Food and water Consumption:* The quantity of food consumed by groups consisting of six animals of for different doses was recorded at weekly interval. Food consumed per animal was calculated for control and the treated dose groups.

- *Clinical signs*: All animals were observed daily for clinical signs. The time of onset, intensity and duration of these symptoms, if any, were recorded.

- *Mortality:* All animals were observed twice daily for mortality during entire course of study.

- *Ophthalmoscopy*: The eyes of experimental animals in control as well as treated groups given different dose levels were examined prior to the initiation of the dosing and in 4th and the 6th week of the study. Eye examination was carried out using a hand slit lamp after induction of mydriasis with Atropine sulphate solution.

- *Functional Observations*: At the end of the 4th week exposure, 'sensory reactivity' to graded stimuli of different types (auditory, visual and proprioceptive stimuli), 'motor reactivity' and 'grip strength' were assessed.

B. Terminal Studies:

- *Laboratory Investigations:* Following laboratory investigations were carried out on day 29 in animals'fasted over-night. On 29th day, the animals were fasted for approximately 18 h, then slightly anesthetized with ether and blood samples were collected from the retro-orbital plexus into two tubes: one with EDTA for immediate analysis of haematological parameters, the other without any anticoagulant and was centrifuged at 4000 rpm at 4 °C for 10 minutes to obtain the serum. Serum was stored at 20 °C until analyzed for biochemical parameters. On 28th day of the experiment, 24 h urine samples were collected by placing the animals in the metabolic cage with free access to tap water but no feed was given.(12)

- *Haematological Investigations*: Blood samples of control and experimental rats was analyzed for hemoglobin content, total red blood corpuscles (RBC), white blood corpuscles (WBC) count, Mean corpuscular volume (MCV) and platelets. Then the mean values are calculated.

- *Biochemical Investigations*: Serum and Urine was used for the estimation of biochemical parameters. Samples of control and experimental rats were analyzed for protein, albumin, globulin, bilirubin, urea, uric acid, creatinine, potassium, chlorine and sodium levels was carried using standard methods. Activities of glutamate oxaloacetate transaminase/ Aspartate aminotransferase (GOT/AST), glutamate pyruvate transaminase/ Alanine amino transferase (GPT/ALT) and alkaline phosphatase were estimated as per the colorimetric procedure.

- *Urine analysis*: Urine samples were collected in week 4 and in week 6 and for estimation of normal parameters.

- *Necropsy:* All the animals were sacrificed on day 29. Necropsy of all animals was carried out and the weights of the organs including liver, kidneys, adrenals, spleen, brain, heart, uterus and testes/ovaries were recorded

- *Histopathology:* Histopathological investigation of the vital organs was done. The organ pieces (3-5µm thick) of the highest dose level of 400 mg/kg were preserved and were fixed in 10% formalin for 24 h and washed in running water for 24 h. Samples were dehydrated in an auto technic and then cleared in benzene to remove absolute alcohol. Embedding was done by passing the cleared samples through three cups containing molten paraffin at 50°C and then in a cubical block of paraffin made by the "L" moulds. It was followed by microtome and the slides were stained with Haematoxylin-eosin. The organs included brain, heart, kidneys, liver, lungs, spleen, and uterus of the animals were preserved they were subjected to histopathological examination.(3)

- *Statistical analysis:* Findings such as clinical signs of intoxication, body weight changes, food consumption, hematology and blood chemistry were subjected to One-way Anova followed by

dunnet't' test using a computer software programme -INSTAT-V3 version.

VIII. RESULTS:

The results of acute toxicity study of *pitha paandu maathirai* revealed no mortality, abnormal signs and behavioural changes in rats at the dose of 2000 mg/ kg body weight a administered orally .(table:1)

The median lethal dose for pitha paandu maathirai should be above 2000 mg/kg and it comes under unclassified. All animals from control and all the treated dose groups survived throughout the dosing period of 28 days for sub acute toxicity study. The results for body weight determination of animals from control and different dose groups show comparable body weight gain throughout the dosing period of 28 days. During dosing period, the quantity of food and water consumed by animals from different dose groups was found to be comparable and normal with that by control animals (Table2,3,4).

The results of haematological investigations revealed no significant changes in the values when compared with those of respective controls (Table 5). Among the differential count of WBC, only the Eosinophil's count was slightly increased at the dosage of 200 mg/kg. This might be occurred due to stress. Results of Biochemical investigations conducted on days 29 , revealed the following significant changes in the values of different parameters studied when compared with those of respective controls Table (6,7,8); Urea, SGOT,SGOT, Bilirubin were within the limits. LDL level was elevated in animals of 200 mg/kg dose group ($P<0.05$) and at the dosage of 400mg/kg, total cholesterol level was slightly increased but these were within the normal limits.

The other cardio vascular risk markers were also within normal ensured that pitha paandu maathirai did not influence the Cardio vascular system. Urine analysis data of control group and treated group of animals determined in week 4 did not reveal major abnormalities rather than transparency, pH and deposits(Table 9) . Organ weights of treated animals with respective control animals on day 29 was found to be comparable with respective control group (Table 10).

Gross pathological examination of animals did not reveal any abnormalities. Histopathology examination did not reveal any abnormal macroscopic changes (Panel 1-3).

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	5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	50	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	300	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	2000	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 1:Dose finding experiment and its behavioural Signs of Toxicity

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

Dose (mg/kg/day)		Days			
	1	7	14	21	28
Control	112.02±2.04	114.22±2.04	116.24±2.46	118.32±3.48	122.06±4.22
200	114.46±3.09	116.20±1.02	118.44±1.86	120.66±0.68	122.04±0.21
400	116.28±0.14	117.34±1.04	118.11±1.02	120.12±1.62	122.10±0.66

Table 2:Body weight (g) of albino rats exposed to Pitha Paandu Maathirai for 28 days

Values are mean of a 6 animals ± S.E.M (Dunnet's test) *$p<0.05$;**$p<0.01$.N=6

	Days(ml/rat)				
Dose(mg/kg/day)	1	7	14	21	28
Control	46.02±1.22	48.48±2.01	50.66±1.20	52.11±0.97	53.64±0.61
200	52.44±0.63	52.62±0.62	53.22±0.42	54.62±1.28	55.65±1.02
400	54.20±0.62	55.19±0.44	57.15±1.68	58.02±1.86	59.01±1.29

Table 3.Water (ml/day) intake of albino rats exposed to Pitha Paandu Maathirai for 28 days.

Values are mean of a 6 animals ± S.E.M (Dunnet's test) *$p<0.05$;**$p<0.01$.N=6

Dose (mg/kg/day)	Days (gms/rats)				
	1	7	14	21	28
Control	42.02±1.22	42.88±0.44	44.24±1.23	46.04±1.80	47.82±1.66
200	42.23±10.21	43.68±0.24	44.12±1.88	45.26±1.06	46.10±2.64
400	45.20±1.68	46.01±2.62	46.98±2.02	47.88±1.02	47.90±0.24

Table4.Food (g/day) intake of albino rats exposed to Pitha Paandu Maathirai for 28 days.

Values are mean of a 6 animals ± S.E.M (Dunnet's test)* p<0.05 ;**p<0.01.N=6

Parameters	Control	200mg/kg	400mg/kg
Red blood cell(mm^3)	6.28±0.12	8.68±1.28	9.22±1.42
HB(%)	14.34±0.12	15.68±0.89	16.28±1.62
Leukocyte ($x10^6$/ml)	10162±121.54	10220±208.87	10362±231.34
Platelets/ul	1421±21.08	1108±30.24	1128±32.14
MCV(gl)	55.02±1.46	56.02±2.86	57.11±2.24

Table 5.Hematological parameters after 28days treatment with Pitha paandu maathirai in rats

Values are mean of a 6 animals ± S.E.M (Dunnet's test)* p<0.05 ;**p<0.01.N=6

LFT

Dose(mg/kg)	Control	200mg/kg	400mg/kg
Total Bilirubin(mg/dl)	0.212±0.10	0.216±0.12	0.218±0.22
Bilirubin direct(mg/dl)	0.1±0.02	0.1±0.04	0.1±0.05
Bilirubin indirect(mg/dl)	0.1±00	0.1±00	0.1±00
ALP(U/L)	386.02±12.68	368.46±16.44	342.68±60.24
SGOT(U/L)	160.64±1.06	160.02±1.21	159.23±1.04
SGPT(U/L)	46.46±0.44	46.24±0.26	46.60±0.11
Total protein(g/dl)	10.22±0.86	9.28±0.16	9.02±1.22
Albumin(g/dl)	3.10±0.02	3.12±0.04	3.14±0.06
Globulin(g/dl)	6.08±0.21	5.22±0.22	5.01±0.24

Table 6:Effect of treatment with Pitha paandu maathirai on biochemical parameters:

Values are mean of a 6 animals ± S.E.M (Dunnet's test)* p<0.05 ;**p<0.01.N=6

Dose (mg/kg)	Control	200mg/kg	400mg/kg
Urea(mg/dl)	56.21±1.23	55.80±1.02	53.09±1.21
Creatinine(mg/dl)	0.76±0.02	0.76±0.04	0.76±0.06
Uric acid(mg/dl)	1,6±0.02	1.6±0.03	1.6±0.06
Na m.mol	136.1±5.01	138.1±5.24	140.12±5.28
K m.mol	18.11±1.25	19.24±1.22	19.26±1.42
Cl m.mol	98.21±1.24	99.20±2.10	100.68±1.64

Table 7: RFT

Values are mean of a 6 animals ± S.E.M (Dunnet's test) *p<0.05 ;**p<0.01.N=6.

Parameters	Control	200mg/kg	400mg/kg
Total cholesterol (mg/kg)	37.29±1.02	38.22±1.28	39.28±1.20
HDL(mg/dl)	12.08±1.62	12.22±1.22	12.98±1.10
LDL(mg/dl)	38.30±1.28	40.68±1.90	40.00±1.64
VLDL (mg/dl)	15.90±1.40	15.42±1.28	15.02±1.64
Triglycerides(mg/kg)	78.24±1.03	80.12±1.68	82.46±2.12
TC/HDL ratio (g/dl)	2.89±0.12	3.02±1.46	3.68±0.22
Blood glucose (mg/dl)	125.22±1.02	125.40±1.66	126.02±1.20

Table 8: Lipid Profile

Values are mean of a 6 animals ± S.E.M (Dunnet's test) *p<0.05 ;**p<0.01.N=6

Parameters	Control	200mg/kg	400mg/kg
Transparency	Clear	Slightly turbid	Slightly turbid
Specific gravity	1.010	1.010	1.010
PH	>7.2	>7.8	>7.8
Protein	Nil	2+	2+
Glucose	Nil	Nil	Nil
Bilirubin	-ve	-ve	-ve
Ketones	-ve	-ve	-ve
Blood	Absent	Absent	Absent
Urobilinogen	Normal	Abnormal	Abnormal
Pus cells	0-cells/HPF	0-cells/HPF	1-cells/HPF
RBC	Nil	Nil	1-cells/HPF
Epithelial cells	Nil	1-cells/HPF	Nil
Crystals	Nil	Nil	Nil
Casts	Nil	Nil	Nil
Others	Bacteria seen	Bacteria seen	Bacteria seen
Colour	Yellow	Yellow	Yellow

Table 9:Urine Analysis

Values are mean of a 6 animals ± S.E.M (Dunnet's test)* p<0.05 ;**p<0.01.N=6

Dose (mg/kg)	Control	200mg/kg	400mg/kg
Liver(g)	4.61±0.69	4.72±0.22	5.02±0.21
Heart (g)	0.60±0.02	0.60±0.04	0.62±0.02
Lung(g)	1.46±0.16	1.42±0.11	1.42±0.14
Spleen (g)	0.56±0.04	0.56±0.12	0.58±0.14
Ovary (g)	1.56±0.12	1.58±0.02	1.60±0.02
Testes(g)	1.26±0.22	1.28±0.69	1.32±0.56
Brain(g)	1.48±0.12	1.38±0.14	1.50±0.16
Kidney(g)	0.68±0.02	0.70±0.02	0.72±0.02
Stomach(g)	1.36±0.12	1.38±0.10	1.38±0.12

Table 10:.Effect of oral administration of a pitha paandu Maathirai on organ weight

Values are mean of a 6 animals ± S.E.M (Dunnet's test) *p<0.05 ;**p<0.01.N=6

IX. HISTOPATHOLOGY SLIDE

A . A Light Photomicrography View of Organs From Control Group Animals

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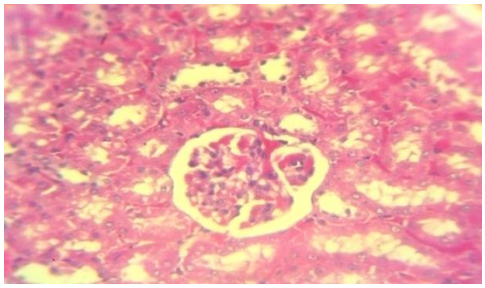
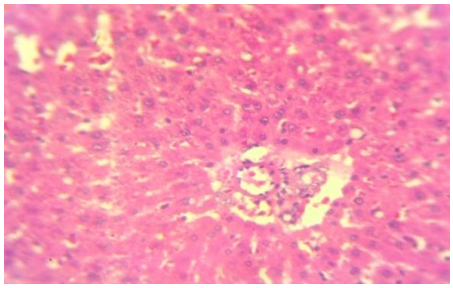


KIDNEY



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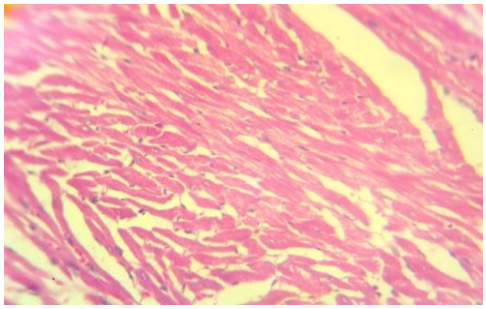

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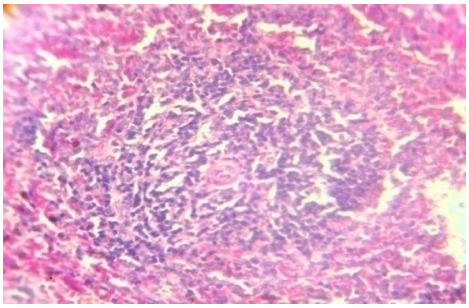

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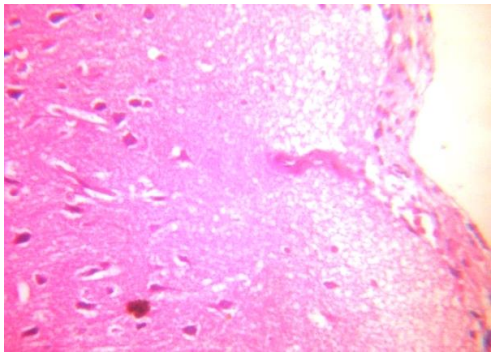

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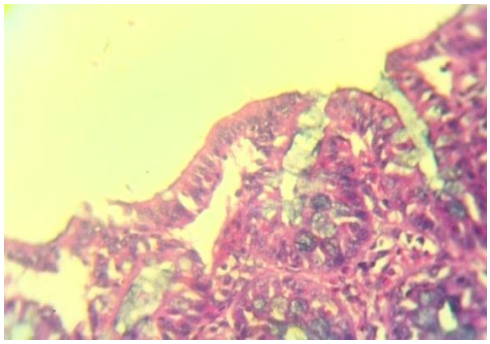
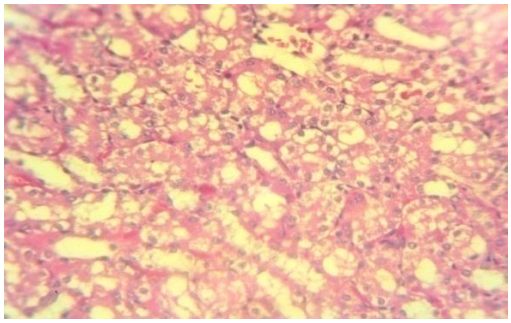
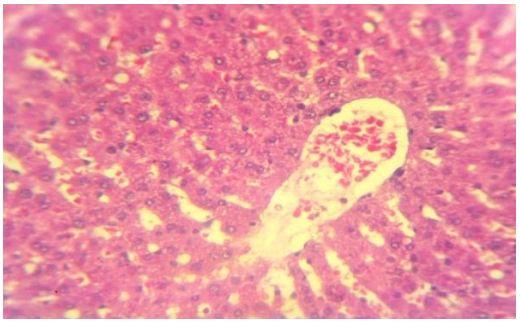


STOMACH

B. A Light Photomicrography View of Organs From Animals of Low Dosage Group.



KIDNEY



LIVER

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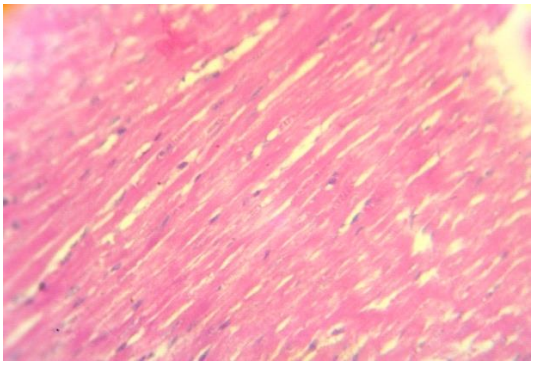
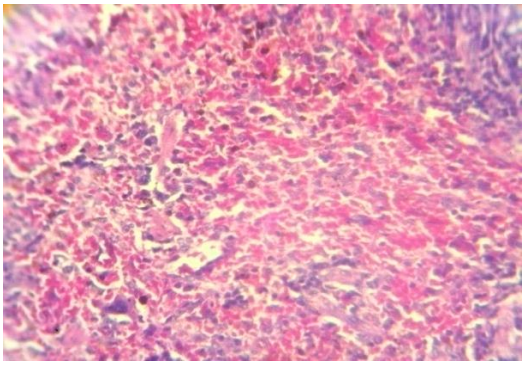
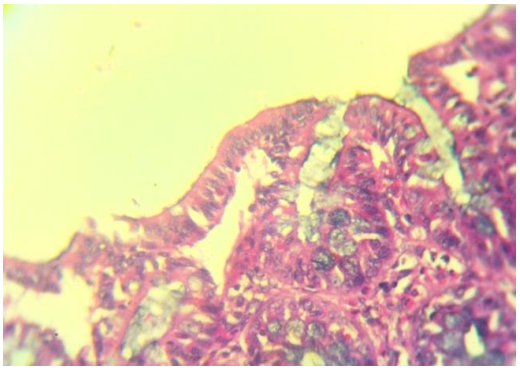


HEART



SPLEEN



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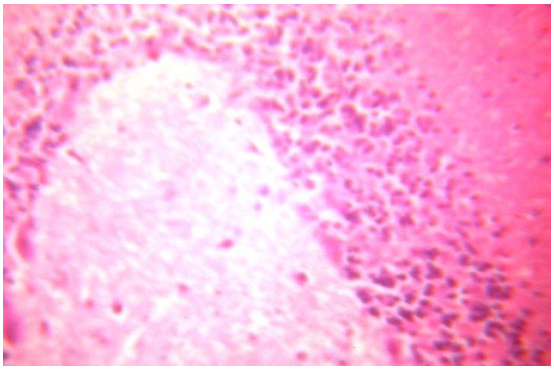
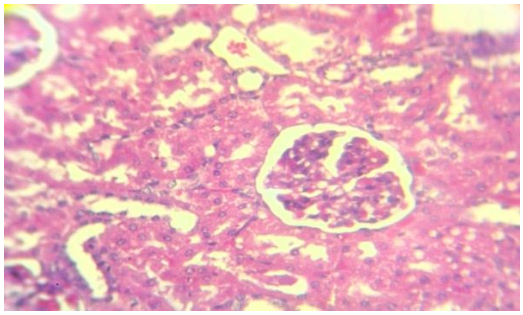
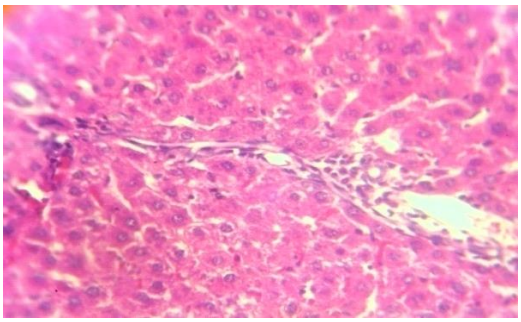


BRAIN

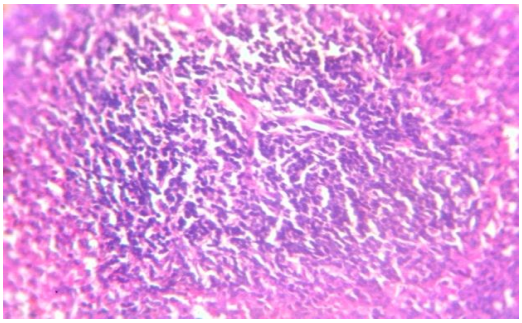
C. A Light Photomicrography View of Organs From Animals of High Dosage Group.



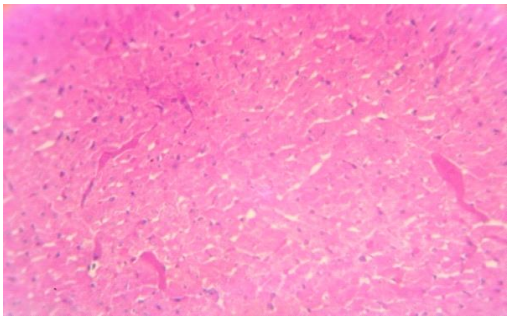
KIDNEY



LIVER



HEART



SPLEEN

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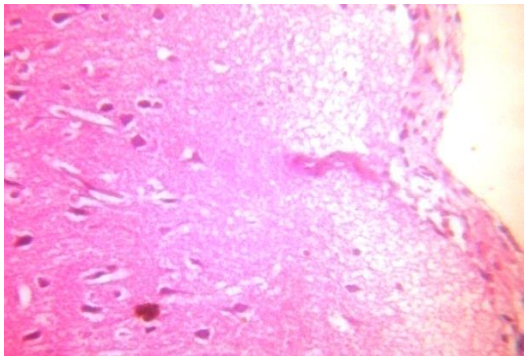

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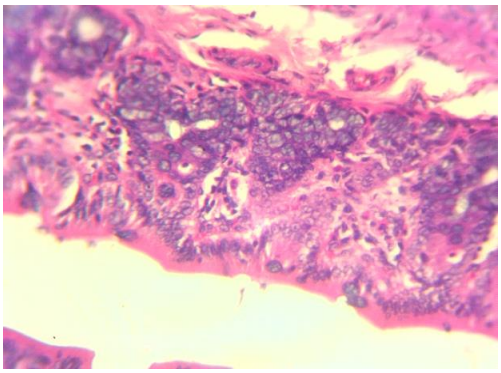

BRAIN

X. CONCLUSION

From the pre clinical Toxicological studies, PITHA PAANDU MAATHIRAI reveals no toxicity upto 2000mg/kg. hence its proved to be safe for the therapeutic use in human at the dosage level 500mg.

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