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Ninety days repeated dose oral toxicity study of *Maruthampattai kudineer* in Wistar rats

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Abstract

Context: *Maruthampattai kudineer* (MP) is polyherbal siddha formulation recommended for diabetes mellitus in the classical text Agathiyar 2000. The present study was carried out to evaluate the repeated dose oral toxicity of MP to generate evidence for safety and global acceptance of the drug. **Aim:** The objective was to evaluate toxicological profile and to find no observed adverse effect level (NOAEL) in rats after oral administration for ninety consecutive days. **Materials and Methods:** *Maruthampattai kudineer* was administered to male and female Wistar rats for ninety consecutive days at 900, 1800, 3600mg/kg body weight. All relevant biochemical and hematological changes were observed. At termination, all the rats were sacrificed and necropsy was performed. Histopathological evaluation was also performed. **Statistical Analysis Used:** Values are expressed as mean ± SD Statistical significance (p) calculated by one way ANOVA followed by dunnett"s. *P*< 0.05 considered as significant by comparing treated group with control group using Graph Pad Prism 3.0. **Results:** The repeated dose study did not show evidence of any treatment related changes in all observations up to the high dose level, when compared with the control. Histopathological examination revealed no abnormalities in high dose group. This study provides scientific validation for the safety of MP.

Keywords: *Maruthampattai kudineer*, diabetes mellitus, oral toxicity, Histopathological evaluation.

Introduction

The currently available oral anti-hyperglycaemic agents for clinical use have characteristic profile of side effects, drug dependency and drug resistance in other systems. The management of Diabetes with agents devoid of any side effects is still a challenge to the health care sector. Hence people are turning towards herbal medicine in recent days. Herbal medicines have attained the widespread acceptability as natural therapeutic agents for various diseases like diabetes, arthritis, renal and liver diseases, obesity and cardiovascular disorders. *Maruthampatti kudineer* is polyherbal formulation with potent anti-diabetic and antioxidant properties. Therefore toxicological studies on such a drug should be done to ensure its safety.

Repeated dose 90-days oral toxicity study was conducted as per OECD-408 Guideline. The 90 days study provides information on the possible health Hazard likely to raise from repeated exposure over a prolonged period of time covering post- weaning maturation and growth well into adulthood.

Materials and Methods

Preparation of the trial drug:

The required raw drugs for the preparation of *Maruthampattai kudineer* were procured from the raw drug shop, Parrys, Chennai. The ingredients were identified and were authenticated by, Medicinal Botanist at NIS, Tambaram sanatorium, Chennai.

Ingredients:

1. Maruthampattai (*Terminalia arjuna*) -350 gms
2. Navalpattai (*Syzygium cumini*) - 350 gms
3. Karuvellampattai (*Acacia nilotica*) - 350 gms
4. Athipattai (*Ficus racemosa*) - 350 gms
5. Avaraithol (*Cassia auriculata*) - 350 gms
6. Kadalalinjilpattai (*Salacia reticulata*)- 700 gms
7. Thetrankottai (*Strychnos potatorum*) - 35 gms
8. Kalipakku (*Areca catechu*) -35 gms
9. Kadukkai thol (*Terminalia chebula*) -35 gms
10. Nellivatral (*Phyllanthus emblica*) - 35 gms
11. Thandrikai thol (*Terminalia bellirica*)- 35 gms

The above ingredients were ground into coarse powder

Approval of the study:

The protocol of the study was approved by the Institutional Animal Ethical Committee .Approval no. NIS/IAEC-III /11/29092016.

Test System Detail

Young adult wistar rats of 8-12 weeks old weighing 140-160 gms of both the sex was used for study. The body weight range should be within ± 20% of the mean body weight at the time of Randomization and grouping. Animals were housed in four groups (5/cage/sex) in polypropylene cages in a well-ventilated room under a temperature of 22 ± 3°C and 30 - 70% relative humidity, with a 12-hr light/dark artificial light cycle. The rats were purchased from TANUVAS, Madhavaram, Chennai and housed in standard laboratory condition in Polypropylene cages, provided with food water adlibitum

Acclimatization:

The animals were selected after veterinary examination by the veterinarian. All the selected animals were kept under acclimatization for a week.

Randomization & grouping:

One day before the initiation of treatment (last day of acclimatization), the selected animals were randomly grouped into four different groups containing 10 male animals and 10 female animals per group.

Numbering and Identification:

Animals were housed with appropriate identification by colouring the fur with picric acid solution prepared in water and with cage cards. The group no., cage no., sex of the animal and animal no. were identified as indicated below using cage label and body marking on the animals

Numbering and Identification of animals in repeated dose 90-days oral toxicity study

Cage no	Group No	Animal	Sex
1	I Control	1-10 10-20	Male Female
2	II Low dose	21-30 31-40	Male Female
3	III Mid dose	41-50 51-60	Male Female
4	IV High dose	61-70 71-80	Male Female

Dose level repeated dose 90-days oral toxicity study:

Test Group	Dose To Animals (mg/ kg.b.wt)	No.of Animals
Group I	Control distilled water(10ml/kg.b.wt)	20 (10male and 10female)
Group II	900mg /kg.bwt	20 (10male and 10female)
Group III	1800 mg /kg.bwt	20 (10male and 10female)
Group IV	3600 mg /kg.bwt	20 (10male and 10female)

Dose Preparation:

Maruthampattai kudineer was prepared at the calculated dose of a required concentration.

Administration:

The test drug was administered orally to each rat as single dose using a needle fitted onto a disposable syringe of appropriate size at the following different doses. The concentration was adjusted according to its body weight; the volume not exceeding 2 ml/kg body weight. Variability in test volume was minimized by adjusting the concentration to make sure a constant volume at all dose levels.

Observations:

The observations included but were not restricted to changes in skin and the eyes and mucous membranes and in the respiratory, circulatory, central and autonomous nervous systems and behaviour.

Clinical signs of toxicity:

All the rats were observed at least two times daily with the purpose of recording any symptoms of ill- health or behavioural changes and clinical signs of toxicity daily for 90 days.

Food and water intake:

A measured amount of feed was kept in the cages and then after 24 hrs. The left out amount of feed was measured to calculate the amount of food consumed by the rats. Water intake was observed by visual observation during the Study. In addition, the water consumption in each cage was observed daily for a period of 90 days

Body weight:

The body weight of rats were recorded one week before the start of treatment, and during the course of the treatment on day one, 7^{th}, 14^{th}, 21^{st} , 28^{th}, 35^{th} , 42^{nd} , 49^{th} , 56^{th} , 63^{rd}, 70^{th} , 77^{th} ,84^{th} , 90^{th} day (day of sacrifice)

Pre-terminal deaths:

All rats were observed twice daily for any pre terminal deaths.

Blood Collection:

Blood was collected through retro-orbital sinus from all the animals of four groups on 90th day. The blood was collected in tubes containing as an anticoagulant (Heparin/EDTA). Animals were fasted overnight prior to the blood collection.

Laboratory studies:

During the last day of treatment, blood were withdrawn from the orbital sinus of animals from each group, under thiopental sodium anaesthesia. The collected blood samples also centrifuged 10000 rpm in 10 minutes to separate the serum. The separated serum used to evaluate biochemical parameters like SGOT, SGPT, ALP and BILIRUBIN etc. The following hematological parameters were analysed (Autoanalyser) Haemoglobin (g %) Packed Cell Volume, White Blood Corpuscles (x103/cmm), Red Blood Corpuscles (x106/cmm) Blood Platelet count (x103/cmm) Differential WBC count.

The following clinical Bio parameters were analysed using Auto analyser. Total serum protein (g/dl), Alanine amino transferase (U/L), Aspartate amino transferase (U/L) Alkaline serum phosphatase (U/L), Cholesterol (mg/dL) Triglyceride

Sacrifice and macroscopic examination:

At the end of study period, the overnight fasted animals were anaesthetized with thiopental sodium and blood samples were collected from retro-orbital sinus. After blood collection, the animals in group 1 to 4 were sacrificed on 90th day.

Histopathology:

The target organs from control and drug treated animals were preserved in 10 % buffered neutral formalin for histopathological examination. Control and highest dose animals were initially subjected to histopathological investigation. If any abnormality was found in the highest dose group, then the low and mid group will also be examined. All deviations from normal histology were recorded and compared with corresponding controls.

Statistical analysis:

Values are expressed as mean ± SD Statistical significance (p) calculated by one way ANOVA followed by dunnett‟ s. $P< 0.05$ considered as significant by comparing treated group with control group using Graph Pad Prism 3.0

Results

In repeated oral toxicity study all the animals were well oriented and active during the trial period and survived until 90-days treatment period. No signs of clinical toxicity attributable to MP were observed throughout the study. No changes were observed in food intake and water consumption in the treated groups. No significant ($P > 0.05$) change was observed in the weight of rats after 90 days although there was a general substantial increase in the weight of rats of both the vehicle control and drug treated groups. (Figure 1). The absolute weights of both vehicle control and test group rats were found to have no significant differences (Table 1). There was no statistically significant difference found in hematological parameters and biochemical parameters. (Table 2) between vehicle control and test groups. All organs such as brain, heart, stomach, lung, liver, kidney, thymus, spleen, testis, and ovary were revealed to have no abnormalities

Fig 1:Effect of PCC on body weight of control and MP treated rats in repeated oral toxicity study.

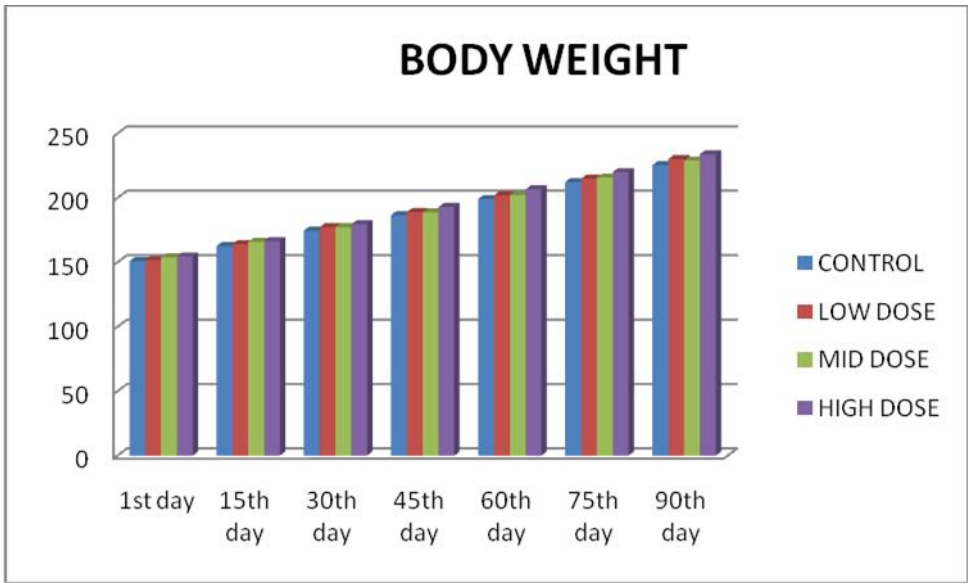

Fig 2 Histopathology of vehicle control heart tissue (b) Histopathology of high dose (3600 mg/kg b.w.) heart tissue.

a) Control b) High dose

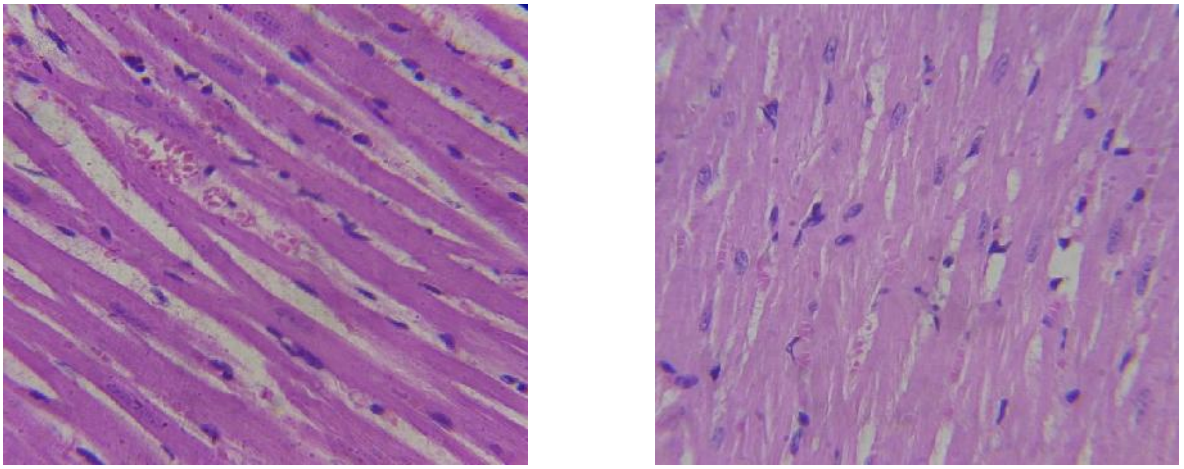

Fig 3 Histopathology of vehicle control lung tissue (b) Histopathology of high dose (3600 mg/kg b.w.) lung tissue.

a) Control b) High dose

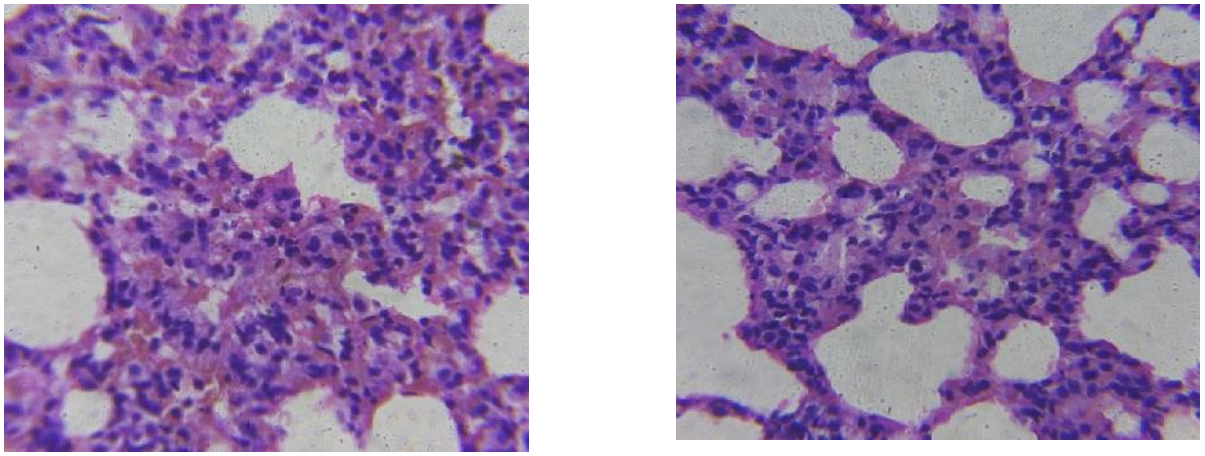

Fig 4 Histopathology of vehicle control liver tissue (b) Histopathology of high dose (3600 mg/kg b.w.) liver tissue.

a) Control b) High dose

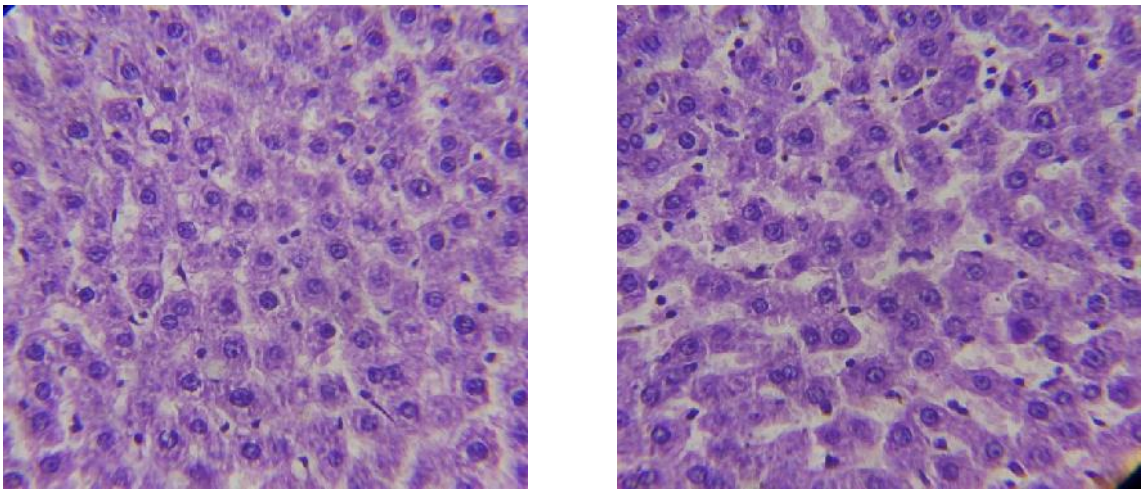

Fig 5 Histopathology of vehicle control kidney tissue (b) Histopathology of high dose (3600 mg/kg b.w.) kidney tissue.

a) Control b) High dose

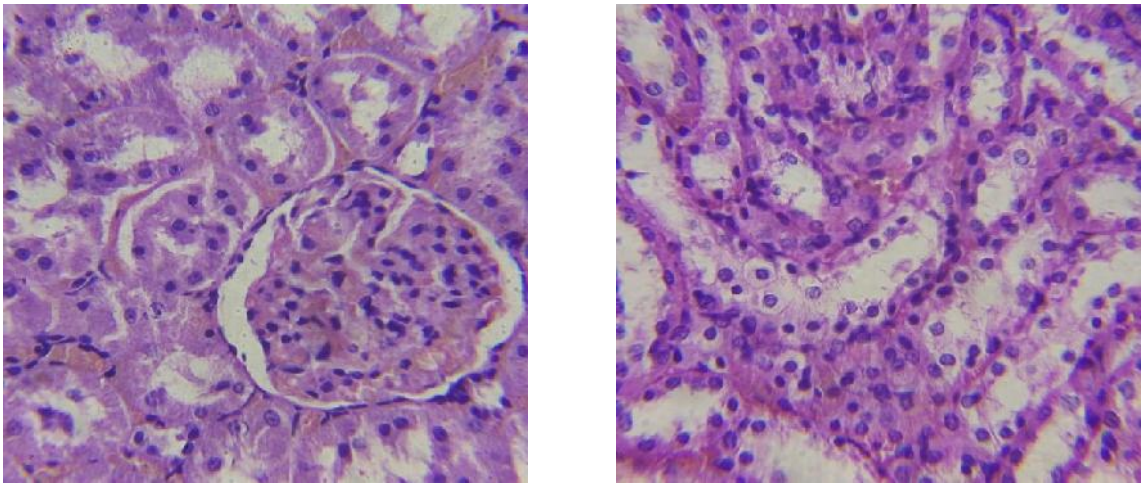

Fig 6 Histopathology of vehicle control spleen tissue (b) Histopathology of high dose (3600 mg/kg b.w.) spleen tissue.

a) Control b) High dose

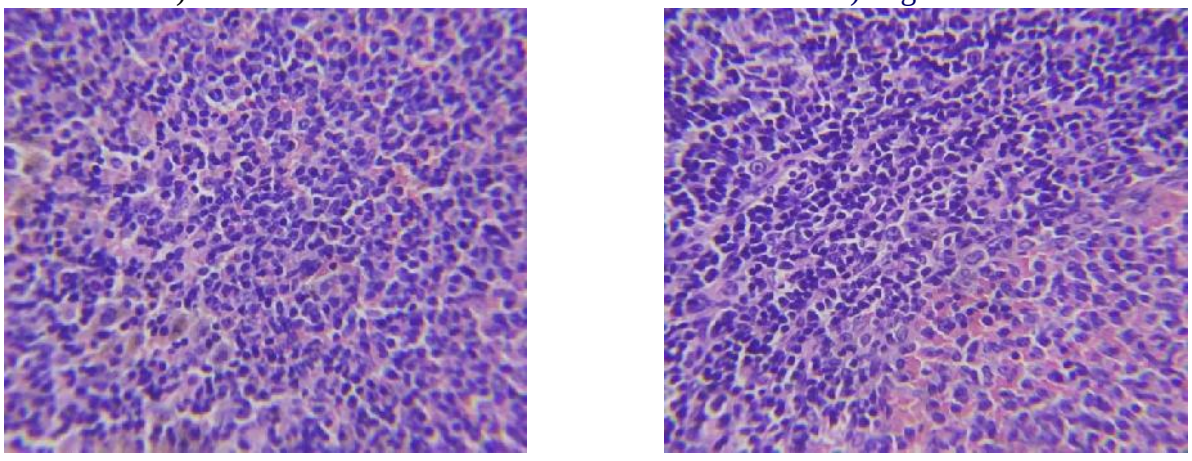

Fig 7. Histopathology of vehicle control testis tissue (b) Histopathology of high dose (3600 mg/kg b.w.) testis tissue

a) Control b) High dose

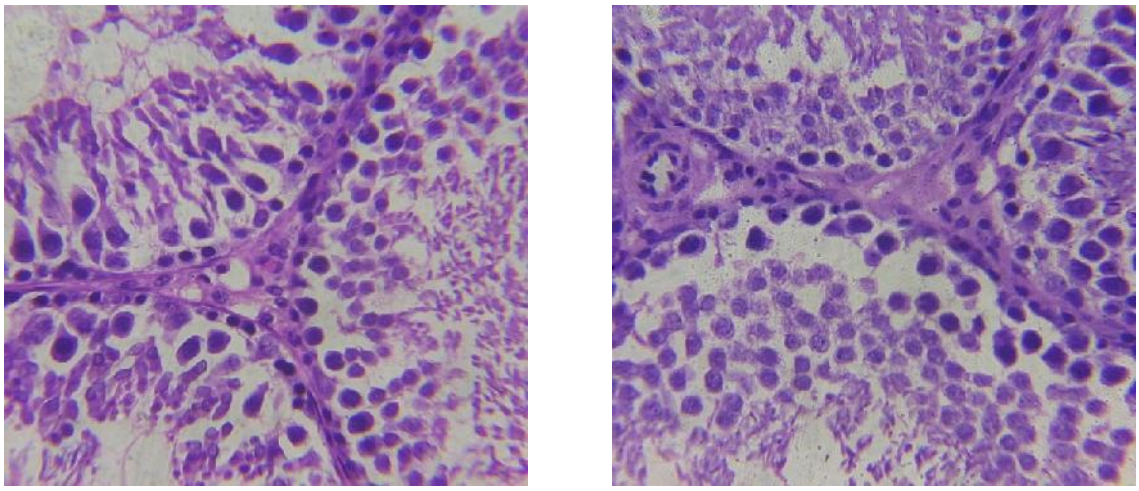

Fig 8. Histopathology of vehicle control ovary tissue (b) Histopathology of high dose (3600 mg/kg b.w.) ovary tissue

a) Control b) High dose

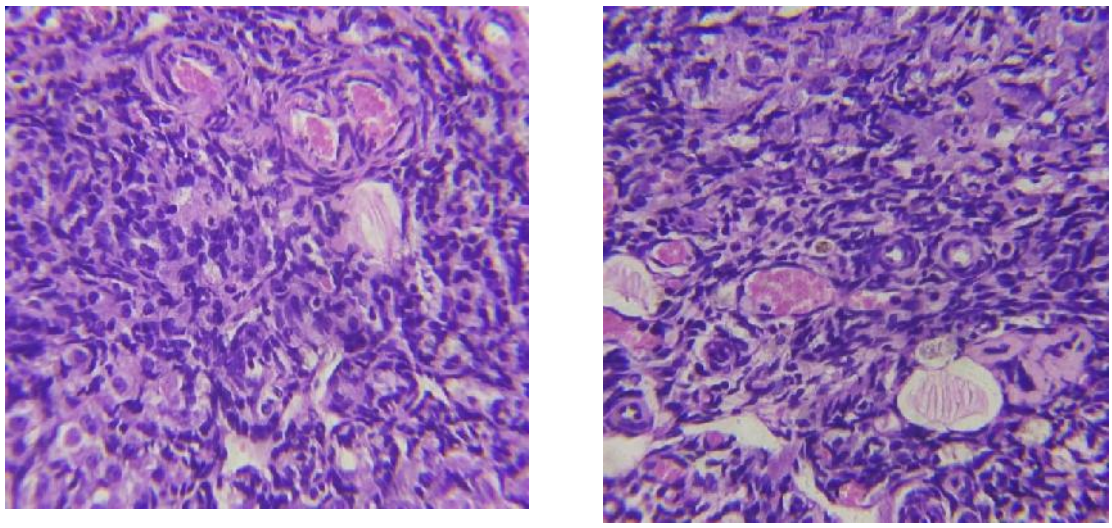

Table 1 :Effect of *Maruthampattai kudineer* on Body weight of experimental Wistar rats in 90 days repeated oral toxicity study

Sl. No	Day	Control	Low dose	Mid dose	High dose
1.	Initial day	150.9± 6.57	151.5 ± 5.19	153.8 ±5.88	154.5 ± 4.83
2.	15thday	162.6± 6.63	164.1 ± 6.31	166.9 ± 8.10	168.4 ± 6
3.	30thday	174.5 ± 6.91	177.2± 6.35	178.2 ± 9.95	180.5 ± 6.81
4.	45thday	186.5± 7.30	188.9± 6.80	188.7 ± 11.70	192.9 ± 7.60
5.	60thday	199.1± 8.71	201.9 ± 7.59	204.2 ± 13.02	206.6 ±8.01
6.	75thday	212.3± 10.14	214.9± 8.39	216.8 ± 14.06	220 ± 9.11
7.	90thday	225.4± 11.39	230.1 ± 9.26	229 ± 15.43	235.9 ±10.53

Table 2: Effect of *Maruthampattai kudineer* of haematological and biochemical parameters of experimental Wistar rats in 90 days repeated oral toxicity study.

Blood parameters	Control	Low dose	Mid dose	High dose
RBC	5.66±0.34	6.85±0.69	6.24±0.55	6.61±1.11
WBC	9.07±2.17	9.26±2.18	9.38±2.11	8.30±1.5
Platlet	769.75±80.65	726.6±126.17	626.6±91.55	757.55±110.6
HB	12.12±1.7	12.81±1.63	12.56±1.59	12.85±1.38
T.Cholesterol	108.92±13.4	113.55±18.43	132.54±9.81	129.74±12.86
TGL	49.35±5.1	40.25±7.9	40.65±8.06	38.5±10.88
Creatinine	0.58±0.19	0.72±0.16	0.69±0.21	0.68±0.21
SGOT	88.2±7.14	90.35±20.5	92.15±23.30	95.45±16.28
SGPT	28.85±7.43	30.85±8.02	37±9.13	32.35±8.96
T.Bilirubin	0.35±0.16	0.51±0.36	0.44±0.25	0.93±1.08

Discussion

MP is a potent polyherbal anti-diabetic drug. To evaluate the safety profile of MP, 90 days repeated oral toxicity studies were performed. Acute toxicity data are of limited clinical application since cumulative toxic effects do occur even at very low doses. Hence, multiple dose studies are usually helpful in evaluating the safety profile of phytomedicines. 90-days repeated oral toxicity study was therefore carried out.

Body weight changes are an indicator of adverse side effects, as the animals that survive cannot lose more than 10% of the initial body weight. There were no significant changes in body weight between vehicle control and test groups in both acute and repeated oral drug treatment (table 1). Repeated dose toxicity studies were conducted to evaluate the adverse effects of test drug MP and were carried out to provide information about the possible health hazards likely to arise from repeated exposure over a relatively limited period of time, the possibilities of cumulative effects, and an estimate of the dose at which there is no observed adverse effect. Determination of food consumption was important in the study of safety of a product with therapeutic purpose as proper intake of nutrients is essential to the physiological status of the animals and to the accomplishment of the proper response to the drug tested instead of a false response due to improper nutritional conditions. In water and food consumption, no significant changes were observed in MP treated groups and this reveals that it did not adversely affect the basic metabolic processes of the experimental animals.

Clinical biochemistry and haematological data hold significant role in determining the toxicity induced by drugs. Blood parameters analysis is relevant to risk evaluation as the haematological system has a higher predictive value for toxicity in humans (91%) when assays involve rodents and non-rodents. Blood forms the main medium of transport for many drugs and xenobiotics in the body and for that matter components of the blood such as red blood cells, white blood cells, haemoglobin, and platelets are at least initially

exposed to significant concentrations of toxic compounds. Damage to and destruction of the blood cells are inimical to normal functioning of the body There is no significant alteration in haematological parameters which indicate that MP did not affect blood cells production. There is no significant dose dependent increase in the white blood cell count, red blood cell count, and haemoglobin concentration (Table 2). There is no haematological variation noted between vehicle control and test drug treated groups.

Bilirubin is formed by the breakdown of haemoglobin in the liver, spleen, and bone marrow. An increase in tissue or serum bilirubin concentrations occurs as a result of increased breakdown of RBC (haemolysis) or liver damage, for example, hepatitis or bile duct obstruction. The normal levels of serum bilirubin concentrations at all doses of the MP used in this study are indicative of no adverse effects of the test drug on haemoglobin metabolism pathways. The results of the haematological parameters were within the normal expected range.

The plasma creatinine concentrations in normal individuals are usually affected by a number of factors such as the muscle mass, high protein diet, and catabolic state; thus serum urea concentration is often considered the more reliable renal function predictor. In the present study there were no significant changes in the levels of creatinine, urea with different doses and therefore it is considered nonnephrotoxic.

There were no changes in the biomarkers of liver function, SGPT and SGOT levels which reveal that MP did not affect liver function.

The evaluation of histopathological changes in organs remains a cornerstone in safety assessment of medicines. No abnormality was recorded with respect to gross or histopathological examinations of all other organs examined.

Conclusion

Since there were no signs of toxicity with respect to hematology, clinical biochemistry, gross weight and histopathological examinations it can be inferred that the test drug *Maruthampattai kudineer* (MP) is a safe drug for human consumption

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