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Acute and Subacute Toxicity Studies of A Siddha Formulation - Azhinjiyathi Kashayam

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Abstract: *Azhinjiyathi kashayam is* a poly herbal *Siddha* formulation mainly indicated for diabetic patients has been screened for toxic effects according to OECD Guidelines. For acute toxicity studies, *Azhinjiyathi kashayam* was administered orally in single dose of 5, 50, 300 and 2000 mg/kg body weight to the rats. For sub-acute toxicity study, different doses of *Azhinjiyathi kashayam* (5ml/kg and 10ml/kg body weight) were administered orally to the rats once daily for 28 days. Animals were observed for physiological and behavioural responses, mortality, food and water intake and body weight changes. All the animals were sacrificed on 29th day and changes in organ weights and histology were examined. No mortality was observed up to 2000 mg/kg body weight of *Azhinjiyathi kashayam* in acute toxicity study. Daily administration of as high as 10ml / kg dose of *Azhinjiyathi kashayam* did not result in any mortality or changes in gross behavior, body weight, weight and histology of different organs. According to these results, *Azhinjiathi kashayam* could be concluded as no-observed-adverse-effect level (NOAEL). It showed the safety of the drug which proved its utility in long time administration without any harm to the human being.
Keywords: *Azhinjiyathi kashayam* Polyherbal formulation, Siddha medicine, Diabetes mellitus, acute and sub-acute toxicity.

INTRODUCTION

Plants remain a good source for drug discovery. India is a repository of herbal medicines, and there are confirmations of herbs being used in the management of diseases and for revealing various body systems in all ancient civilizations.

Plant medicines are in great demand both in the developed as well as developing countries for primary health care because of their wide range of biological and medicinal activities, higher safety margin and low cost. Toxicity testing can reveal some of the risks that may be associated with use of herbs, therefore avoiding potential harmful effects when used as medicine. The primary aim of toxicological assessment of any herbal medicine is to identify adverse effects and to determine limits of exposure level at which such effects occur. *Azhinjiyathi kashayam is* a poly herbal *Siddha* formulation containing Dried fruit of *Phyllanthus emblica Linn,* (Nelli mulli), Bark of *Cassia auriculata.Linn,* (Aavaram pattai), Bark *of Salacia reticulata. Wight,* (Kadalazhinjil pattai*),* Outer skin of *Terminalia bellirica Roxb,* (Thandrikkai thol), *Bark* of Smilax *china Linn,* (Parangi pattai) and Seed of *Strychnos potatorum.Linn.f,* (Thaetran vithai mainly indicated for diabetic patients [1]. The drug has been screened for toxic effects according to OECD Guidelines. The siddha formulation Azhinjiyathi kashayam was safe up to the dose level of 2000mg/kg in oral administration, which showed that the safety of the drug which proved its utility in long time administration without any harm to the human being.

ACUTE ORAL TOXICITY

METHODOLOGY

Institutional Animal Ethical Committee Approval

The acute and sub acute toxicity studies were approved by Institutional Animal Ethical Committee of at K.K.College of Pharmacy and the approval no: KKCP/ 2015/ /031. The study was conducted at K.K.College of Pharmacy, Gerugambakkam, Chennai. Acute toxicity studies were carried out according to the OECD (Organization of Economic Co-operation and Development) guidelines 423.

Number of animals and dose levels

Healthy female rats, weighing 150–200 g, were selected and oral administration of the single doses of *Azhinjiyathi kashayam*. 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 2000mg/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.

Administration of doses

Azhinjiyathi kashayam was administered as a single oral dose by gavage using a feeding needle. Animals were fasted prior to dosing. They were deprived of food, but not water 12 h prior to the administration of the test substance. Following the period of fasting, the animals were weighed and then the test substance was administered. An oral (p.o) dose of 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg was administered step by step according to the guidelines. After the substance has been administered, food was withheld for a further 3-4 hours. The principle of laboratory animal care was followed.

OBSERVATIONS

Observations were made and recorded systematically and continuously observed as per the guideline after substance administration. The general behaviours of the rats were continuously monitored individually after dosing at least once during the first 30 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. The visual 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. Finally, the number of survivors was noted after 24 h and these animals were then maintained for further 14 days and observations made daily. The toxicological effect was assessed on the basis of mortality.

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

Parameters observed	5mg/kg	50mg/kg	300mg/kg	2000mg/kg
Alertness	-	-	-	-
Aggressiveness	+	+	+	+
Alopecia	-	-	-	-
Circling	-	-	-	-
Diarrhoea	-	-	-	-
Oedema	-	-	-	-
Touch response	+	+	+	+
Grip strength	+	+	+	+
Grooming	+	+	+	+
Lacrimation	-	-	-	-
Loss of writing reflex	-	-	-	-
Tremors	-	-	-	-
Nasal sniffing	-	-	-	-
Pile erection	-	-	-	-
Hypnosis	-	-	-	-
Righting reflex	-	-	-	-
Seizures	-	-	-	-
Catatonia	-	-	-	-
Mortality	-	-	-	-

SUB ACUTE TOXICITY

METHODOLOGY

Randomization, Numbering and Grouping of Animals

Sub-acute toxicity studies were carried out according to OECD 407. Ten Rats (Five Male and Five Female) in each group randomly divided into three groups for dosing up to 28 days. Animal's 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.

Justification for Dose Selection

The results of acute toxicity studies in rats indicated that Azhinjiyathi kashayam was nontoxic and no behavioural changes were observed up to the dose level of 10ml / kg body weight. The oral route was selected for use because oral route is considered to be a proposed therapeutic route.

Preparation and administration of dose

Test Substance	:	Azhinjiyathikashayam
Animal Source	:	Animal house of King Institute of Preventive Medicine
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 Godrej foods Pvt Ltd, Bangalore
Water	:	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%,
Dark and light cycle	:	Each of 12 hours.

Azhinjiyathikashayam at two dose level 5ml/kg and 10ml/kg respectively were prepared. The test substance was 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.

OBSERVATIONS

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. (Table -2)

Food and water Consumption

The quantity of food consumed by groups consisting of ten animals of for different doses was recorded at weekly interval. Food consumed per animal was calculated for control and the treated dose groups (Table-3 & 4).

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.

Laboratory investigation:

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 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.

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 packed cell volume (PCV). From the estimated values of RBC count (millions/mm3) and PCV (volumes percent), mean corpuscular volume (MCV) was calculated.

Biochemical Investigations:

Serum and Urine was used for the estimation of biochemical parameters. Samples of control and experimental rats were analyzed for protein, bilirubin, urea, uric acid, creatinine, triglyceride, cholesterol and glucose levels by 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.

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. The relative organ weight of each animal was then calculated as follows;

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rats on sacrifice day (g)}} \times 100$$

Histopathology

Histopathological investigation of the vital organs was done. The organ pieces (3-5µm thick) of the highest dose level of 10ml /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 technical 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 heart, kidneys, liver, spleen and pancreas of the animals were preserved they were subjected to histopathological examination.

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 (GraphPad Prism) [2].

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

Table-2: Signs of toxicity in sub acute toxicity (28 days)

Parameters observed	Day-2	Day-4	Day-6	Day-8	Day-10	Day-12	Day-14	Day-16	Day-18	Day-20	Day-22	Day-24	Day-26	Day-28
Alertness	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aggressiveness	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Alopecia	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Circling	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Diarrhoea	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oedema	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Touchresponse	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Grip strength	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Grooming	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lacrimation	-	-	-	-	-	-	-	-	-	-	-	-	-	-
writing reflex	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tremors	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nasal sniffing	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pile erection	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Analgesia	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Righting reflex	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Seizures	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypnosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mortality	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table-3: Body weight (g) of albino rats exposed to Azhinjiyathikashayam for 28 days

Dose (ml/kg/day)		Days			
	1	7	14	21	28
Control	118.20±4.10	116.11±2.44	114.20±2.24	112.16±2.26	112.02±1.24
5ml/kg	120.26±1.04	119.22±1.46	120.40±1.28	120.66±0.42	122.04±0.26
10ml/kg	117.28±0.42	118.24±1.68	119.28±1.28	120.49±1.66	122.10±2.66

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

Table-4: Water (ml/day) intake of albino rats exposed to Azhinjiyathi Kashayam for 28 days.

Dose(ml/kg/day)	Days(ml/rat)				
	1	7	14	21	28
Control	44.02±1.04	44.48±2.01	45.66±1.23	44.68±0.56	45.64±0.86
5ml/kg	48.20±0.24	50.32±1.44	52.32±1.24	52.62±2.48	54.68±1.44
10ml/kg	49.27±1.32	50.66±2.89	50.89±2.42	52.66±1.28	52.87±0.32

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

Table-5: Food (g/day) intake of albino rats exposed to AzhinjiyathiKashayamfor 28 days.

Dose (ml/kg/day)	Days (gms/rats)				
	1	7	14	21	28
Control	40.04±2.22	40.21±1.06	40.46±1.23	40.42±0.32	40.23±0.44
5ml/kg	40.34±0.68	41.42±0.42	41.88±0.64	42.46±1.24	41.44±1.06
10ml/kg	40.21±1.24	40.46±2.62	40.20±1.68	41.69±1.20	42.46±0.46

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

Table-6: Hematological parameters after 28days treatment with Azhinjiyathi Kashayam in rats

Parameters	Red blood cell(mm^3)	HB(%)	Leukocyte ($x10^6$/ml)	Platelets/ul	MCV(gl)
Control	7.42±1.44	14.12±0.22	10146±104.32	1421±20.46	52.46±1.02
5ml/kg	8.01±1.02	15.02±0.24	10180±204.28	1282±22.64	54.23±2.40
10ml/kg	8..04±1.20	15.24±1.04	10226±220.12	1290±30.28	56.24±2.20

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

Table-7: LFT

Parameters	Control	5ml/kg	10ml/kg
Total Bilirubin(mg/dl)	0.210±0.21	0.212±0.11	0.214±0.26
Bilirubin direct(mg/dl)	0.1±0.02	0.1±0.02	0.1±0.04
Bilirubin indirect(mg/dl)	0.1±00	0.1±00	0.1±00
ALP(U/L)	380.01±10,20	362.20±12.21	350.22±12.68
SGOT(U/L)	160.02±1.04	156.43±1.06	154.20±1.04
SGPT(U/L)	40.20±0.12	42.22±0.26	44.20±0.04
Total protein(g/dl)	10.20±0.86	9.48±0.46	9.22±1.20
Albumin(g/dl)	3.09±0.02	3.10±0.06	3.12±0.10
Globulin(g/dl)	6.02±0.26	5.84±0.20	5.28±0.40

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

Table-8: RFT

Parameters	**Control**	**5ml/kg**	**10 ml/kg**
Urea(mg/dl)	54.18±1.46	52.24±1.60	52.32±1.20
Creatinine(mg/dl)	0.72±0.01	0.74±0.02	0.74±0.04
Uric acid(mg/dl)	1.4±0.02	1.4±0.02	1.6±0.04
Na m.mol	130.1±5.26	132.1±5.02	136.10±5.23
K m.mol	18.10±1.20	18.24±1.22	19.10±1.07
Clm.mol	98.06±1.89	99.12±2.02	100.80±1.20

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

Table-9: Urine Analysis

Parameters	**Control**	**5ml/kg**	**10ml/kg**
Transparency	Clear	Slightly turbid	Slightly turbid
Specific gravity	1.010	1.010	1.010
PH	>7.0	>7.4	>7.4
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

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

Table-10: Lipid Profile

Parameters	Control	5ml/kg	10ml/kg
Total cholesterol (mg/kg)	35.30±1.47	36.68±1.40	36.20±1.40
HDL(mg/dl)	12.08±1.42	12.24±1.22	12.98±1.20
LDL(mg/dl)	38.20±1.24	40.44±1.82	40.42±1.28
VLDL (mg/dl)	15.82±1.22	15.64±1.46	15.20±1.20
Triglycerides(mg/kg)	78.14±1.04	80.12±1.68	80.24±2.10
TC/HDL ratio (g/dl)	2.46±0.12	3.42±1.23	3.20±0.12
Blood glucose (mg/dl)	124.22±1.04	124.40±1.22	124.02±1.12

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

Table-11: Effect of oral administration of a AzhinjiyathiKashayam on organ weight

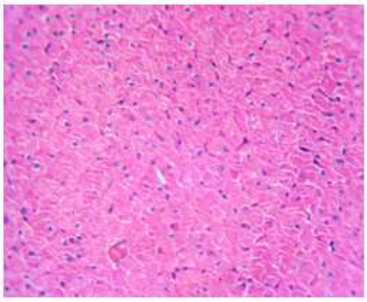
Organs	Control	5ml/kg	10ml/kg
Liver(g)	4.48±0.22	4.52±0.46	4.60±0.28
Heart (g)	0.40±0.01	0.41±0.02	0.42±0.04
Lung(g)	1.30±0.24	1.30±0.68	1.30±0.88
Spleen (g)	0.48±0.16	0.50±0.18	0.52±0.22
Ovary (g)	1.48±0.04	1.50±0.04	1.52±0.04
Testes(g)	1.20±0.13	1.22±0.22	1.22±0.28
Brain(g)	1.36±0.22	1.38±0.24	1.40±0.26
Kidney(g)	0.60±0.04	0.62±0.04	0.64±0.04
Stomach(g)	1.34±0.12	1.36±0.12	1.38±0.14

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

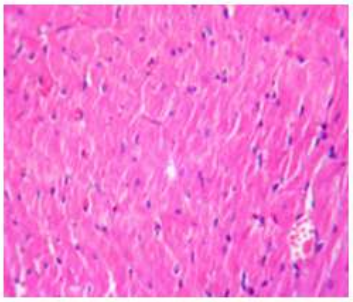
HISTOPATHOLOGY OF VITAL ORGANS

Heart

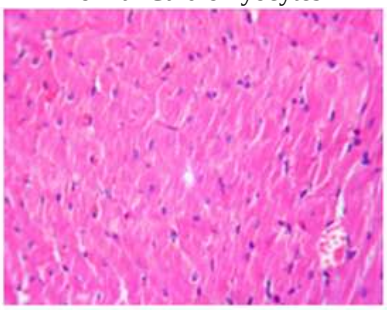
Control Section of ventricle Normal Cardiomyocytes



Low dose Section of ventricle Normal Cardiomyocytes

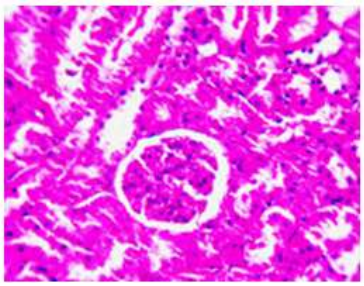


High dose Section of ventricle Normal Cardiomyocytes

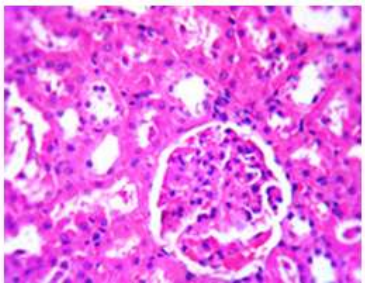


Kidney

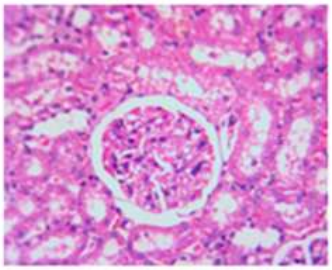
Control (Section of Cortex) Normocellular glomeruli



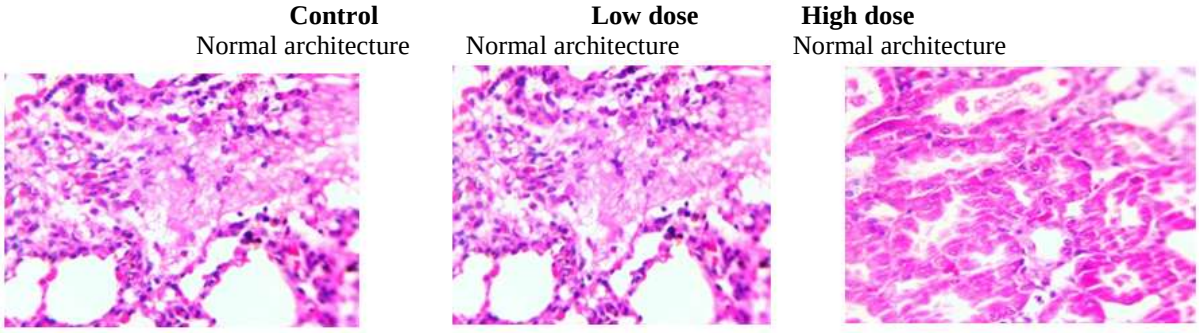
Low dose (Section of Cortex) Normocellular glomeruli



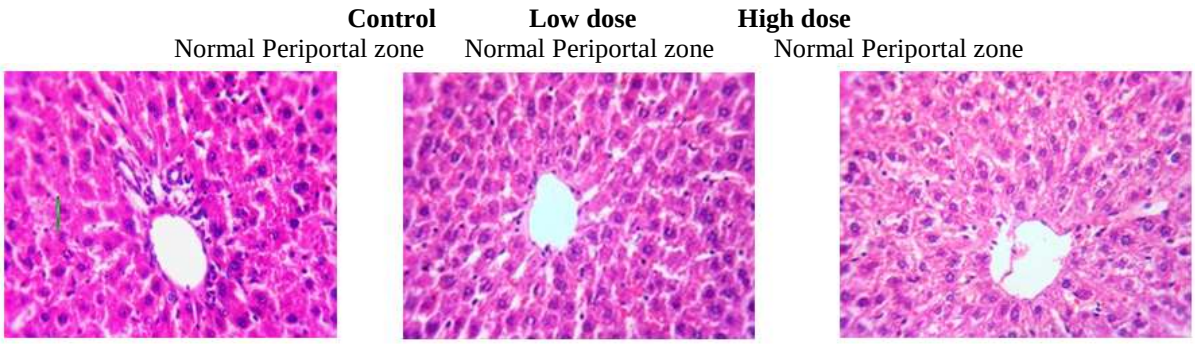
High dose (Section of Cortex) Normocellular glomeruli



Lungs



Liver



RESULTS

All animals from control and all the treated dose groups survived throughout the dosing period of 28 days. 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.

The results of hematological investigations conducted on day 29th day revealed no significant changes in the hematological values when compared with those of respective controls. This gave clear justification that bone marrow and spleen were not influenced by *Azhinjiyathi kashayam* [3].

The clinical biochemistry analysis was done to evaluate the possible alterations in hepatic and renal functions influenced by the test drug [4].

Results of Biochemical investigations conducted on days 29 and recorded in revealed the no significant changes in the values of different parameters studied when compared with those of respective controls; Urea, SGOT,SGPT, Bilirubin were within the limits.

The other cardio vascular risk markers were also within normal ensured that *Azhinjiyathi kashayam* did not influence the Cardio vascular system.

Group Mean Relative Organ Weights are recorded Comparison of organ weights of treated animals with respective control animals on day 29 was found to be comparable with respective control group. Gross pathological examination of animals in control as well as the treated groups did not reveal any abnormalities.

The vital organs such as liver, heart, kidneys, and lungs were removed from the test groups at the end of the study and carefully observed macroscopically to find any observable gross lesions compared with the control group and did not reveal any abnormal macroscopic changes. Gross pathological investigation was carried out and histopathology of vital organ reveled normal histological appearance when compared with the control.

CONCLUSION

The acute toxicity result shows no mortality up to dose level of 2000mg/kg. The normal behavioural changes were observed. In first four hours and no mortality was reported after 14 days observation. Hence the test drug Azhinjiyathi kashayam is a safe up to the dose level of 2000mg/kg in oral administration.According to these results, *Azhinjiathi kashayam* could be concluded as no-observed-adverse-effect level (NOAEL). It showed the safety of the drug which proved its utility in long time administration without any harm to the human being.

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