International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG(USA) Volume 4, Issue 11 - 2017

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2017.04.11.004

Acute and subacute oral toxicity study of Rasa chenduram

R.Kalaimani*[1], M.Mohammed Mustafa [2]

[*1] P.G.Scholar, Post Graduate Department of Sirappu Maruthuvam, Government Siddha Medical College, Arumbakkam, Chennai 600 106, Tamil Nadu, India.

[2] Reader and Head of the Department of Sirappu Maruthuvam, Government Siddha Medical College, Arumbakkam, Chennai 600 106, Tamil Nadu, India.

Corresponding author: *kalaisiddha@gmail.com*

Abstract

Back ground: Rasa chenduram is one of the siddha shastricherbomineral formulation containing Rasam [hydragyram], Gandhagam [sulphur], and aridharam [arsenic trisulphide] were indicated for many chronic skin diseases like parangisogai, kiranthi, kuttarogam, thaadhunattam, lingaputtru, vippuruthi from the classical text pulippanivaidhiyam undergone preclinical study and clinical study with the approval from iec committee from our institution.

Materials and methods: Acute oral toxicity 423 and sub acute oral toxicity studies 407 were carried out in wister albino rats of body weight 150-200 gms of 6-8 weeks with the proper approval from IAEC committee.

Conclusion: this study reveals the oral administration of rasa chenduram in wister albino rats with the dose of 9mg/kg body weight showed no significant changes in haematological, pathological, behavioural movements.

Keywords: Siddha Medicine, Rasa chenduram, Acute oral toxicity, wister albino rats.

Introduction

The acute toxic class method is a stepwise procedure with the use of 3 animals of a single sex.Depending on the mortality and/or the moribund status of the animals, on average 2-4 steps may be necessary to allow judgement on the acute toxicity of the test substance. This procedure is reproducible, uses very few animals and is able to rank substances in a similar manner to the other acute toxicity testing methods. The acute toxic class method is based on biometric evaluations with fixed doses, adequately separated to enable a substance to be ranked for classification purposes and hazard assessment.

In principle, the method is not intended to allow the calculation of a precise LD50, but does allow for the determination of defined exposure ranges where lethality is expected since death of a proportion of the animals is still the major endpoint of this test.The method allows for the determination of an LD50 value only when at least two doses result in mortality higher than 0% and lower than 100%. The use of a selection of pre-defined doses, regardless of test substance, with classification explicitly tied to number of animals observed in different states improves the opportunity for laboratory to laboratory reporting consistency and repeatability.

Principle of the Test:

It is the principle of the test that based on a stepwise procedure with the use of a minimum number of animals per step, sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered orally to

a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex. Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.no further testing is needed, dosing of three additional animals, with the same dose, dosing of three additional animals at the next higher or the next lower dose level. The method will enable a judgment with respect to classifying the test substance to one of a series of toxicity classes.

Methodology

Selection of Animal Species

The preferred rodent species is the wistar albino rat, although other rodent species may be used. Healthy young adult animals are commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Each animal, at the commencement of its dosing, should be between 6 to 8 weeks old and the weight (150-200gm) should fall in an interval within±20 % of the mean weight of any previously dosed animals.

Housing and Feeding Conditions

The temperature in the experimental animal room should be 22ºC ± 3ºC. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal.

Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions

Test Animals and Test Conditions

Sexually mature Female Wistar albino rats (150-200gm) were obtained from TANUVAS, Madhavaram, Chennai. All the animals were kept under standard environmental condition (22±3°C). The animals had free access to water and standard pellet diet (Sai meera foods, Bangalore).

Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions

Preparation for Acute Toxicity Studies

Rats were deprived of food overnight (but not water 16-18 h) prior to administration of the, *Rasa chendhuram.*

The principles of laboratory animal care were followed and the Institutional Animal Ethical Committee approved the use of the animals and the study design

IAEC No: IAEC/XL VIII/30/CLBMCP/2016

Test Substance: Rasa chendhuram
Animal Source: TANUVAS Madhavaram, Chennai.
Animals : Wister Albino Rats (Female-3+3)
Age **:** 6-8 weeks
Body Weight on Day 0:150-200gm.
Acclimatization **:** Seven days prior to dosing.
Veterinary examination: Prior and at the end of the acclimatization period.
Identification of animals: By cage number, animal number and individual marking by using Picric acid.
Number of animals : 3 Female/group,
Route of administration: Oral
Diet **:** Pellet feed supplied by Sai meera foods Pvt Ltd, Bangalore
Water: Aqua guard portable water in polypropylene bottles.
Housing & Environment: The animals were housed in Polypropylene cages provided with bedding of husk.
Housing temperature: between 22ºC ±3ºC.
Relative humidity: between 30% and 70%,
Air changes **:** 10 to 15 per hour and
Dark and light cycle **:** 12**:**12 hours.
Duration of the study: 14 Days

Administration of Doses:

Rasa chendhuram (Pulipani) was suspended in water and administered to the groups of wistar albino rats in a single oral dose by gavage using a feeding needle. The control group received an equal volume of the vehicle. Animals were fasted 12 hours prior to dosing. Following the period of fasting, the animals were weighed and then the test substance was administered.

Three Female animals are used for each group. The dose level of 9mg/kg body weight was administered. After the substance has been administered, food was withheld for a further 3-4 hours. The principle of laboratory animal care was followed. Observations were made and recorded systematically and continuously as per the guideline after substance administration. The visual observations included skin changes, mobility, aggressively, sensitivity to sound and pain, as well as respiratory movements. Finally, the number of survivors was noted after 24 hrs and these animals were then monitored for a further 14 days and observations made daily. The toxicological effect was assessed on the basis of mortality.

Observations

Animals are observed 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, 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 somatomotor activity and behavior pattern. Attention was directed to observations of tremors, convulsions, salivation, diarrhoea, 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 humanly killed. When animals are killed for human reasons or found dead, the time of death was recorded.

Acute oral toxicity study of Rasa chendhuram

Table 1: Dose finding experiment and its behavioral Signs of acute oral Toxicity

Observation done:

Sl	Group Control	Observation	Sl	Group Test group	Observation
1	Body weight	Normal	1	Body weight	**Normally increased**
2	Assessments of posture	Normal	2	Assessments of posture	**Normal**
3	Signs of Convulsion Limb paralysis	Normal	3	Signs of Convulsion Limb paralysis	**Absence of sign (-)**
4	Body tone	Normal	4	Body tone	**Normal**
5	Lacrimation	Normal	5	Lacrimation	**Absence**
6	Salivation	Normal	6	Salivation	**Absence**
7	Change in skin color	No significant color change	7	Change in skin color	**No significant color change**
8	Piloerection	Normal	8	Piloerection	**Normal**
9	Defecation	Normal	9	Defecation	**Normal**
10	Sensitivity response	Normal	10	Sensitivity response	**Normal**
11	Locomotion	Normal	11	Locomotion	**Normal**
12	Muscle gripness	Normal	12	Muscle gripness	**Normal**
13	Rearing	Mild	13	Rearing	**Mild**
14	**Urination**	**Normal**	**14**	**Urination**	**Normal**

Behaviour

The animals will be observed closely for behaviour in the first four hours which includes abnormal gait, aggressiveness, exophthalmos, ptosis, akinesia, catalepsy, convolusion, excitation, head twitches, lacrimation, loss of corneal reflex, loss of traction, piloerection reactivity of touch, salivation, scratching, sedation, chewing, head movements, sniffing, straub, tremor and writhes, diarrhea, leathery, sleep and coma.

Body Weight

Individual weight of animals was determined before the test substance was administered and weights will be recorded at day 1, 7, and 14 of the study. Weight changes were calculated and recorded. At the end of the test, surviving animals were weighed and humanly killed.

Food and water Consumption

Food and water consumed per animal was calculated for control and the treated dose groups.

Mortality

Animals were observed for mortality throughout the entire period.

Repeated dose 28-day oral toxicity (407) study of *Rasa chendhuram*

Test Substance : **Rasa chendhuram**
Animal Source : TANUVAS, Madhavaram, Chennai.
Animals : Wister Albino Rats (Male -24, and Female-24)
Age **:** 6-8 weeks
Body Weight **:** 150-200gm.
Acclimatization **:** Seven days prior to dose.
Veterinary examination **:** Prior and at the end of the acclimatization period.
Identification of animals **:**Bycage number, animal number and individual marking by using Picric acid
Diet **:** Pellet feed supplied by Sai meera foods Pvt Ltd, Bangalore
Water **:** Aqua guard portable water in polypropylene bottles.
Housing & Environment **:** The animals were housed in Polypropylene cages provided with bedding of husk.
Housing temperature **:** between 22ºC ±3ºC.
Relative humidity **:** between 30% and 70%,
Air changes **:** 10 to 15 per hour
Dark and light cycle **:** 12**:**12 hours.
Duration of the study **: 28 Days.**

Table 1

Groups	No of Rats
Group I Vehicle control (Water)	**12(6 male,6 female)**
Group II RCMp- low dose X (9mg)	**12 (6 male,6 female)**
Group III RCMp- Mid dose 5X (45mg)	**12 (6 male,6female)**
Group IV RCMp- High dose 10X(90mg)	**12 (6 male,6female)**

RCM -Rasa chendhuram

Methodology

Randomization, Numbering and Grouping of Animals:

48 Wistar Albino Rats (24M + 24F) were selected and divided into 4 groups. Each group consists of 12 animals (Male -6, and Female-6). First group treated as a control and other three groups were treated with test drug (low, mid, high) for 28 days. Animals were allowed acclimatization period of 7 days to laboratory conditions prior to the initiation of treatment. Each animal was marked with picric acid. The females were nulliparous and non-pregnant.

Justification for Dose Selection

As per OECD guideline three dose levels were selected for the study. They are low dose (X), mid dose dose (5X), high dose (10X). X is calculated by multiplying the therapeutic dose (488mg) and the body surface area of the rat (0.018).i.e X dose is (9mg), 5X dose is 45mg/animal, 10X dose is 90mg/animal.

Preparation and Administration of Dose

Rasa chendhuram suspended in prescribed solvent, it was administered to animals at the dose levels of X, 5X, 10X. The test substance suspensions were freshly prepared every two days once for 28 days. The control animals were administered vehicle only. The drug was administered orally by using 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.

Food and water Consumption:

Food and water 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.

Necropsy:

All the animals were sacrificed by excessive anaesthesia on day 29. Necropsy of all animals was carried out.

Laboratory Investigations:

Following laboratory investigations were carried out on day 29 in animals fasted over-night. Blood samples were collected from orbital sinus using sodium heparin (200IU/ml) for Bio chemistry and potassium EDTA (1.5 mg/ml) for Hematology as anticoagulant. Blood samples were centrifuged at 3000 rpm for 10 minutes.

Haematological Investigations:

Haematological parameters were determined using Haematologyanalyzer.

Biochemical Investigations:

Biochemical parameters were determined using auto-analyzer.

Histopathology:

Control and highest dose group animals will be initially subjected to histopathological investigations. If any abnormality found in the highest dose group than the low, then the mid dose grothiup will also be examined. Organs will be collected from all animals and preserved in 10% buffered neutral formalin for 24 h and washed in running water for 24 h. The organ sliced 5 or 6µm sections and were dehydrated in an auto technicon 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 red.

Statistical analysis:

Findings such as body weight changes, water and food consumption, hematology and blood chemistry were subjected to One-way ANOVA followed by dunnet test using a computer software programme – Graph pad version 7.

Results of oral toxicity study of *Rasa chendhuram*

All data were summarized in tabular form showing for each test group the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test ,description of toxic symptoms, weight changes, food and water intake.

No of animals in each group:3

Table 2 Observational study Results

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.	**Control**	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.	9mg	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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.Diarrhea 18.Writhing 19. Respiration 20.Mortality.
(+ Present, - Absent)

Table 3 (Body weight Observation)

Dose	**Days**		
	1	7	14
Control	280.2±42.30	281.4 ± 64.12	282.6 ±26.18
High dose	280.4± 21.24	281 ± 3.64	281.4 ± 2
P value (p)*	NS	NS	NS

Table 4 (Water intake (ml/day) of Wistar albino rats group exposed to Rasa chendhuram

Dose	**Days**		
	1	6	14
Control	61 ± 1.12	62±2.22	63.9±1.14
High dose	62.2±1.1	63±1.14	64.20±24
P value (p)*	NS	NS	NS

N.S- Not Significant, **(p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

Table 5: Food intake (gm/day) of Wistar albino rats group exposed to Rasa chendhuram

Dose	**Days**		
	1	7	14
Control	56.24±2.22	56.2±7.42	58.4±3.46
High dose	60.6±1.63	60.6±2.62	64.1±5.38

Table 6: Body weight of wistar albino rats group exposed to Rasa chendhuram

Dose	**Days**				
	1	7	14	21	28
Control	230.2±15.45	231.5 ± 25.15	231.5 ± 15.50	232.5± 15.16	232.4 ± 15.15
Low dose	235.2 ± 15.15	235.7 ± 32.22	236.6± 46.14	236 ± 62.18	236.41± 15.24
Mid dose	200.4± 06.64	200.3 ± 16.24	201.2 ± 18.12	201.2 ± 11.26	202.4 ± 24.10
High dose	210.6± 24.24	210.6 ± 10.42	211.4 ± 12.24	211 ± 14.38	212 ± 54.61
P value (p)*	NS	NS	NS	NS	NS

NS- Not Significant, **(p > 0.01),*(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

Table 7: Water intake (ml/day) of Wistar albino rats group exposed to Rasa chendhuram

Dose	Days				
	1	6	14	21	28
Control	50.1 ± 4.32	50±4.12	50.2±1.10	50±1.12	50.4±1.12
Low dose	52.1±1.11	52.8±2.22	52.6±1.42	53.2±2.26	52.4±1.21
Mid dose	53.1±1.12	53.3±1.11	53.1±2.21	53.4±1.12	53.4±1.42
High dose	54.1±1.41	54.2±1.42	54.4±1.44	54.6±1.52	55.8±2.82
P value (p)*	NS	NS	NS	NS	NS

N.S- Not Significant, **(p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

Table 8: Food intake (gm/day) of Wistar albino rats group exposed to Rasa chendhuram

Dose	Days				
	2	7	23	22	28
Control	30±5.14	31.2±2.12	31.3±2.18	31.2±1.14	32±2.12
Low dose	30.2±1.14	31.3±1.31	31.1±1.21	31.5±1.32	31.5±1.62
Mid dose	32.1±2.22	32.2±3.40	32.2±2.24	32.2±2.16	33.2±1.24
High dose	33.1±1.12	33.1±1.14	33.6±2.26	34.2±1.10	34.6±3.42
P value (p)*	Ns	Ns	Ns	Ns	Ns

N.S- Not Significant, **(p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test

Table 9: Haematological parameters of Wistar albino rats group exposed to Rasa chendhuram

Category	Control	Low dose	Mid dose	High dose	P value (p)*
Haemoglobin(g/dl)	16.7±0.71	16.60±0.24	16.5±0.23	16.82±0.16	N.S
Total WBC ($\times 10^3$ l)	10.81±0.32	10.64±0.21	10.54±0.42	9.60±1.12	N.S
Neutrophils (%)	31.12±0.01	31.02±0.12	32.11±1.22	33.02±6.21	N.S
lymphocyte (%)	72.12±1.24	72.12±1.32	73.10±2.34	73.20±2.44	N.S
Monocyte (%)	0.9±0.02	0.9±0.01	0.9±0.04	0.9±0.03	N.S
Eosinophil (%)	0.5±0.03	0.5±0.04	0.5±0.05	0.5±0.08	N.S
Platelets cells10^3/µl	680.17±3.13	682.41±4.12	682.13±2.02	684.10±2.34	N.S
Total RBC 10^6/µl	8.42±0.12	8.46±0.53	8.49±0.44	8.74±0.46	N.S
PCV%	42.12±0.2	42.62±1.02	43±1.20	44.40±2.10	N.S
MCHC g/dL	34.5±1.20	34.2±1.10	34.8±1.70	34.33±1.30	N.S
MCV fL(µm^3)	58.2±4.02	59.2±1.10	58.9±1.40	58.8±1.20	N.S

N.S- Not Significant, **(p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

Table 10 : Biochemical Parameters of Wistar albino rats group exposed to Rasa chendhuram

Biochemical Parameters	Control	Low dose	Mid dose	High dose	P value (p)*
Glucose (r) (mg/dl)	125.11±3.2	125.12±2.10	126.10±13.08	128.12±4.2	N.s
T.cholesterol(mg/dl)	120.16±1.20	120.25±1.30	122.60±1.18	123.24±1.30	N.s
Trigly(mg/dl)	54.16±1.52	54.12±1.42	56.15±1.23	56.16±1.23	N.s
Ldl	72.4±2.14	72.12±2.54	73.10±1.32	3.24±10.20	Ns
Vldl	11.2±1.30	11.20±2.21	11.22±1.24	11.14±12.14	Ns
Hdl	27.14±6.12	27.42±2.30	28.16±2.60	28.17±2.14	Ns
Ratio 1(t.cho/hdl)	3.41±1.16	3.42±1.40	3.74±1.04	3.64±2.03	Ns
Ratio 2(ldl/hdl)	1.92±1.14	1.91±1.12	1.71±2.20	1.96±08.02	Ns
Albumin (g/dl)	5.43±0.16	5.50±0.52	5.04±9.30	5.42±9.48	Ns

NS- Not Significant, **(p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test

Table 11: Renal function test Wistar albino rats group exposed to Rasa chendhuram

Parameters	Control	Low dose	Mid dose	High dose	P value (p)*
Urea (mg/dl)	26.70±0.19	26.50±0.26	27.16±1.28	27.68±1.24	N.s
Creatinine(mg/dl)	0.22±0.02	0.21±0.04	0.22±0.05	0.24±0.07	N.s
Bun(mg/dl)	17.1±0.01	17.10±0.64	17.6±0.52	17.86±1.02	Ns
Uric acid(mg/dl)	6.04±0.34	6.06±0.51	6.6±0.15	6.42±0.20	N.s

NS- Not Significant, **(p > 0.01), * (p >0.05) , n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

Table 12: Liver Function Test of Wistar albino rats group exposed to Rasa Chendhuram

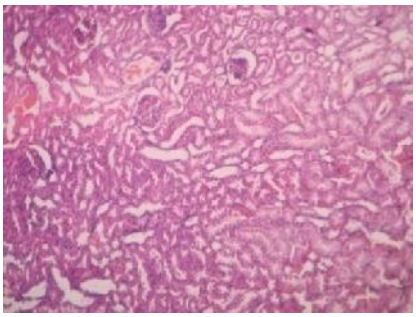
Parameters	**Control**	**Low dose**	**Mid dose**	**High dose**	**P value (p)***
T bilirubin (mg/dl).	0.07±0.01	0.07±0.02	0.07±0.01	0.07±0.03	N.s
Sgot/ast(u/l)	81.14±1.63	81.31±0.02	82.01±1.24	82.64±1.63	N.s
Sgpt/alt(u/l)	78.12±1.08	78.21±1.24	78.14±1.26	77.68±0.01	N.s
Alp(u/l)	119.21±3.16	119±32.10	119±12.14	120.03±8.32	N.s
T.protein(g/dl)	6.2.10±0.04	6.2±0.11	6.2±0.10	6.4±0.46	N.s

NS- Not Significant, **(p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test

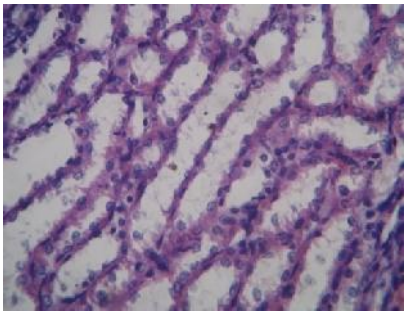
Histopathology

Control group

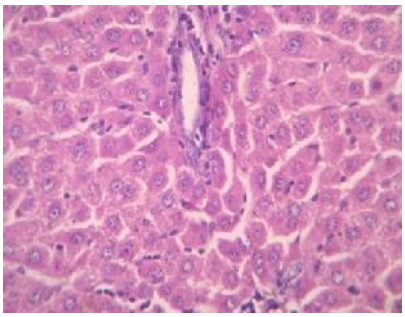
Kidney



Liver

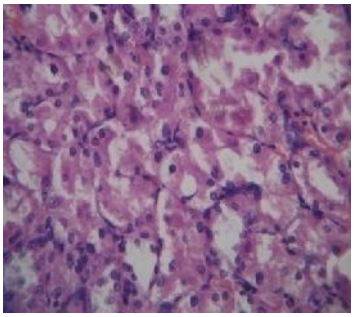


Spleen

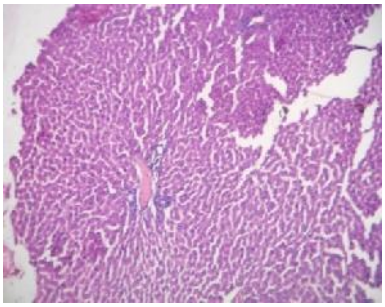


High dose

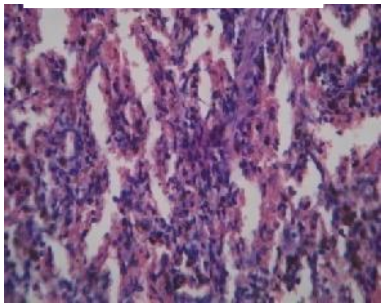
Kidney



Liver



Spleen



Discussion

The acute and repeated 28 days oral toxicity is studies of rasa chenduram showed no toxicity signs in wister albino rats. No mortality and morbidity is seen in experiment. Three varied doses of drug includes 9mg 45 mg and 90 mghelped to find the lethal dose of the drug rasa chenduram. Haematological analysis reveals that no other variable changes seen. Histopathological studies of liver, kidney and spleen doesnot reveal any vascular changes. No other toxicological signs were noted during the study.

Conclusion

Based on the observed result no toxicological signs were seen upto the heavy dose of 90 mg of rasa chenduram through orally for a period of 28 days. Hence the study concludes that the use of rasachenduram is safe in human and ensureits effectiveness for various chronic skin diseases.

Acknowledgements

The author is greatfulto Dr. P. Muralidharan, CL baidmetha college of Pharmacy, Principal and Faculties of Sirappu Maruthuvam, Govt. Siddha medical college Chennai and Dr. C. S. Rakhavi, M.D (S)

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DOI:10.22192/ijarbs.2017.04.11.004	

How to cite this article:
R.Kalaimani, M.Mohammed Mustafa. (2017). Acute and Subacute oral toxicity study of Rasa chenduram. Int. J. Adv. Res. Biol. Sci. 4(11): 22-31.
DOI: http://dx.doi.org/10.22192/ijarbs.2017.04.11.004