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ACUTE AND SUB ACUTE TOXICITY STUDIES OF A FEMALE INFERTILITY SIDDHA DRUG - KARPA CHOORANAM ON MICE

RESEARCH ARTICLE

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ABSTRACT

F*emale Infertility is a pan global public health issue leading to physical, emotional and social stigmatic conditions. Ovulatory factors play a major role in female infertility. The Siddha herbal formulation Karpa Chooranam (KC) has been employed for the treatment of Female Infertility (FI). The safety profile of KC was evaluated in mice using OECD guidelines. In Acute oral toxicity study, a single dose of KC was administered and observed for 14 days. The results of acute toxicity study of Karpa Chooranam revealed no mortality, abnormal signs and behavioral changes in mice at the dose of 2000mg/ kg body weight. Sub-acute toxicity studies were carried in four different groups in which KC was administrated orally to mice once daily for 28 days in various doses ranging from 104, 520 and 1040 mg/kg body weight respectively. Detailed hematological, biochemical, necropsy and histopathological evaluation of organs were performed for all animals. KC was well tolerated and no toxic manifestations were seen in any of the animals. Histopathological analysis revealed that Spleen, Testes, Pancreas, Lung, Intestine, Stomach, Liver, Brain, Heart, Ovary, Uterus and Kidney tissues of treated groups did not show any signs of toxicity. No toxic effect was observed in both acute and sub-acute toxicity studies of Karpa Chooranam.*

Keywords: *Karpa Chooranam (KC), Female Infertility (FI), Acute toxicity, Sub-acute toxicity*

INTRODUCTION

Infertility is defined by an unsuccessful waiting time to pregnancy of 12 months, despite frequent unprotected intercourse[1]. Infertility is a complex disorder with significant medical, psychosocial, and economic problems[2]. Data from population - based studies suggest that 10-15 % of couples in the world experience infertility[3].

The Siddha drug *Karpa Chooranam* (KC) mentioned in the classical literature Yakobu Vaithiya Chinthamani 700 has been used for the treatment of Penn Maladu (FI)[4]. Pre-clinical toxicity studies were essential for determining a safe dose for human trials. Consequently an effort was made to evaluate acute and sub-acute toxicity of the herbal Siddha formulation *Karpa Chooranam* in laboratory animals.

MATERIALS AND METHODS

Preparation of *Karpa chooranam*:

INGREDIENTS:

- Vilvam leaves (*Aegle marmelos* leaves) – 1 part
- Vilvam root (*Aegle marmelos* root) – 1 part
- Athimathuram (*Glycyrrhiza glabra*) – 1 part
- Vendhayam (*Trigonella foenum-graecum* – 1 part
- Seeragam (*Cuminum cyminum*) – 1 part
- Lavangam (*Syzygium aromaticum*) – 1 part
- Elam (*Elettaria cardamomum*) – 1 part

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PREPARATION

The above mentioned seven herbal drugs are taken in equal quantity. The raw drugs are purified by removing foreign matters if any present. Then they are pulverized and made into fine powder. The powdered medicine *Karpa Chooranam* is stored in an air tight container devoid of moisture.

Aim: To evaluate the acute and sub-acute toxicity of the herbal Siddha drug '*Karpa Chooranam*'.

Animals: BALB/C Mice of either sex weighing more than 20gms were obtained from the animal house of King Institute of Preventive Medicine, Guindy, Chennai and maintained in the animal laboratory of Sairam Advanced Centre for Research. The animals were used with the approval of the Institute animal ethics committee (IAEC) of Sairam Advanced Centre for Research, Chennai. Approval No. (1545/PO/a11/CPCSEA/1-9/2013). All the animals were kept under standard environmental condition (23±2°C), standard light cycle (12 h light, 12 h dark). The animals had free access to water and standard pellet diet. The animals were randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to dosing to allow for acclimatization to the laboratory conditions.

Acute Toxicity Study-OECD423 guidelines[5-6]

Karpa chooranam suspended in 2% CMC with uniform mixing and was administered to the groups of Wistar mice in a single oral dose by gavage using a feeding needle. Animals were fasted prior to dosing. Following the period of fasting, the animals were weighed and then the test substance 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 observed 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. They were deprived of food, but not water 16–18 hr prior to the administration of the test suspension. Finally, the number of survivors was noted after 24 hr and these animals were then maintained for a further 14 days and observations made daily. The toxicological effect was assessed on the basis of mortality.

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.

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.

OBSERVATIONS

Animals were 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 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.

ACUTE ORAL TOXICITY IN MICE

Table-1: Dose finding experiment and its behavioral Signs of Toxicity of Karpa Chooranam

Dose mg/kg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2000	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-

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

Result of Acute toxicity of KC

The results of acute toxicity study of *Karpa Chooranam* revealed no mortality, abnormal signs and behavioral changes in mice at the dose of 2000 mg kg/body weight administered orally (Table 1). The median lethal dose for *Karpa Chooranam* should be above 2000 mg/kg and it comes under unclassified. [5-6]

Sub-acute oral toxicity study of *Karpa Chooranam* on mice (OECD– 407 Guidelines)

The results of acute toxicity studies in mice indicated that *Karpa chooranam* was non toxic and no behavioral changes was observed up to the dose level of 2000 mg/kg body weight. In the literature, therapeutic dosage for *Karpa Choranam* in human is mentioned as 3000 mg. On the basis of body surface area ratio between rat and human, the doses selected for the study were 104mg/kg (x), 520 mg/kg (5x) and 1040 mg/kg (10x) 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:

Karpa Chooranam at three doses respectively was suspended in 2 ml of 2% CMC in distilled water. It was administered to animals at the dose levels of 104, 520 and 1040 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.

METHODOLOGY

Randomization, Numbering and Grouping of Animals:

Ten mice (Five Male and Five Female) were in each group randomly divided into four 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 nulliparous and non-pregnant.

OBSERVATIONS

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

- **(i)** ***Body Weight:*** Weight of each mouse 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.
- **(ii)** ***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.
- **(iii)** ***Clinical signs***: All animals were observed daily for clinical signs. The time of onset, intensity and duration of these symptoms, if any, were recorded.
- **(iv)** ***Mortality:*** All animals were observed twice daily for mortality during entire study.
- **(v)** ***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 4^{th} and the 6^{th} week of the study. Eye examination was carried out using a hand slit lamp after induction of mydriasis with Atropine sulphate solution.
- **(vi)** ***Functional Observations***: At the end of the 4^{th} 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. Blood samples were collected from orbital sinus using sodium heparin (200IU/ml) for Blood chemistry and potassium EDTA (1.5 mg/ml) for Haematology as anticoagulant. Blood samples were centrifuged at 3000 r.p.m. for 10 minutes. 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.

The urine was free from fecal contamination. Toluene is used as a preservative while collecting the sample. The sediments present in the urine were removed by centrifugation and the collected urine was used for biochemical estimations. 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.

***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 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. The estimations were performed using appropriate methodology.

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;

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

Histopathology: Histopathological investigation of the vital organs was done. The organ pieces (3-5µm thick) of the highest dose level of 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 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.

The organs included brain, heart, kidneys, liver, lungs, spleen, and uterus 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 -INSTAT-V3 version.

SUB-ACUTE ORAL TOXICITY 28-DAY REPEATED DOSE STUDY IN MICE

Table-2: Body weight (g) changes of mice exposed to Karpa Choornam

Dose (mg/kg/day)	*Days*				
	1	**7**	**14**	**21**	**28**
Control	22.37±3.21	24.14±4.09	28.21±2.17	27.21±5.11	33.32±1.89
104	25.28±3.21	25.21 ±3.25	26.17 ±2.71	28.12 ±3.41	32.22 ±3.54
520	27.22 ±2.45	29.45 ±3.65	30.25 ±3.42	32.25 ±2.14	33.25 ±2.34
1040	27.12 ±3.45	28.45 ±3.75	31.48 ±3.25	31.45 ±2.34	33.45 ±3.25

Values are expressed as mean ± S.E.M. N=10

Table-3: Effect of Karpa Choornam on Haematological parameters in mice

Parameter	Control	104 mg/kg	520 mg/kg	1040 mg/kg
RBC (x 10^6/mm^3)	5.2±0.43	5.6±0.86	6.7±1.2	6.2±0.92
PCV (%)	48.2±1.8	52.4±1.26	56.3±4.6	52.8±6.4
Hb (g/dl)	15±0.3	15±1.06	16.2±1.8	15.46±1.8
WBC (mm^3)	8422±183	9567±110**	9724±263**	10568±106**
Neutrophils (%)	18±2	22±1.2	19±0.9	24±1.6*
Mononuclear cells (%)	78±3	77±2.3	78±1.6	73±0.9

Eosinophils (%)	3±0.3	2±0.08*	1.6±0.12**	2.2±0.4
Platelets (x 10^3/mm^3)	645±6.2	752±15.6**	796.3±17.2**	810±25.4**

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 *vs* control; N=10

Table-4: Effect of Karpa Choornam on biochemical parameters in mice

Parameters	Control	104 mg/kg	520 mg/kg	1040 mg/kg
Protein (g/dl)	6.33±0.3	5.72±1.4	6.4±2.4	5.4±0.8
Albumin (g/dl)	2.8±0.2	2.2±0.18	3.2±0.5	2.7±0.24
BUN (mg/dl)	21.33±4.6	20.4±1.6	19.3±1.2	26.4±2.4
Urea (mg/dl)	63±4.3	52±2.2	54±5.2	66.4±3.8
Creatinine (mg/dl)	0.53±0.03	0.52±0.04	0.65±0.007	0.83±0.04
Total Cholesterol(mg/dl)	111.53±13.17	106±18.2	98.6±7.8	121.4±22.8
Triglycerides (mg/dl)	97.56±14.5	84.3±11.2	96.2±15.4	96.6±9.8
Glucose (mg/dl)	113.4±12.2	87.2±9.6	93.4±10.2	126.5±13.4
Total Bilirubin (mg/dl)	0.9±0.08	0.72±0.04	0.62±0.08	0.93±0.12
SGOT (U/L)	86.5±5.0	95.4±8.2	97.2±4.6	110.3±13.3
SGPT(U/L)	46.5±6.2	36.4±7.3	42.4±5.8	57.4±16.2
Alkaline phosphatase (U/L)	48.6±7.2	42.6±13.4	52.8±1.2	46.3±3.6
Sodium (mEq/L)	147.3±5.8	132.4±8.2	148.4±10.4	128.32±7.2
Potassium (mEq/L)	5.3±0.4	4.6±0.2	5.1±0.63	4.8±0.59

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 *vs* control; N=1

Table-5: Effect of Karpa Choornam on Organ weight in mice

Organ	Control	104 mg/kg	520 mg/kg	1040 mg/kg
Liver (mg)	2115.9±210.5	2232.7±113.6	2462.4±231.5	2672.8±214.4
Heart (mg)	139.8±15.4	146.2±13.2	152.6±21.2	154.8±13.4
Lung (mg)	175.2±16.6	178±22.4	186.3±14.6	192.4±23.8
Spleen (mg)	145.5±19.3	152.3±13.4	164.6±22.9	168.3±13.5
Brain (mg)	455.6±14.2	472.4±23.6	502±19.4	556.4±28.4
Kidney (mg)	632.7±84.2	720.68±73.2	696.4±36.2	782.9±46.4

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 *vs* control; N=10

Table-6: Effect of Karpa Choornam on Urine parameters in mice

Parameters	Control	X	5x	10x
Colour	Yellow	Yellow	Yellow	Light Yellow
Transparency	Clear	Clear	Clear	Light Yellow
Specific gravity	1.010	1.02	1.01	0.99
pH	6.4	7.2	7.0	6.2
Protein	Nil	Nil	Nil	Nil
Glucose	Nil	Nil	Nil	Nil
Bilirubin	-ve	- ve	- ve	- ve
Ketones	-ve	- ve	- ve	- ve
Blood	Absent	Absent	Absent	Absent
RBCs	Nil	Nil	Nil	-
Epithelial cells	Nil	Nil	Nil	Occasional
Casts	Nil	Nil	Nil	Nil

RESULTS

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 no significant changes throughout the dosing period of 28 days and this I shown in the Table 2.

The results of hematological investigations such as Erythrocytes, Total Leucocytes and Platelets count (Table 3) conducted on day 29, revealed significant increase in leucocyte count (P<0.01) in the values when compared with those of respective controls. This gave clear justification that bone marrow and spleen were not influenced by *Karpa Chooranam.*

Among the differential count of WBC, only the Eosinophil's count was slightly decreased (P<0.05) at the *Karpa Chooranam* dosage of 104 mg/kg and at the dose of 520 mg/kg. Eosinophil count was significantly decreased. (P<0.01) Platelets count also significanty increased (P<0.01). The other parameters were within the normal limits.

Results of Biochemical investigations conducted on days 29 and recorded in Table 4 revealed no significant changes in the values of different parameters and these are within normal limits when compared with those of respective controls.

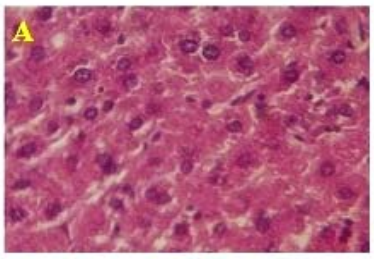
Group Mean Relative Organ Weights (% of body weight) are recorded in Table 5. Comparison of organ weights of treated animals with respective control animals on day 29 was found to be comparable with respective control group.

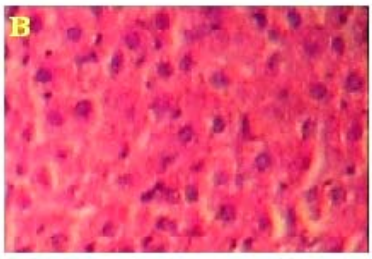
Urine analysis data (Table 6) of control group and treated group of animals determined in week 4 did not reveal major abnormalities rather than colour and transparency.

Gross pathological examination of animals in control as well as the treated groups did not reveal any abnormalities.

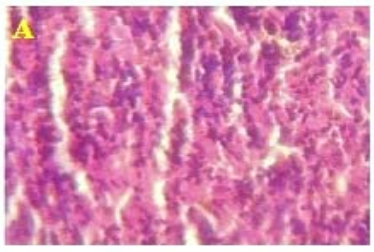
Histopathology: The vital organs such as liver, heart, Spleen and kidneys were removed from the test groups at the end of the study and carefully observed macroscopically to find any observable gross lesions compared with the control group and did not reveal any abnormal macroscopic changes. Microscopically, these organs of the test groups revealed normal histological appearance when compared with the control group (Panel 1-4).

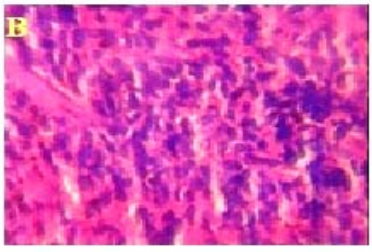
Histopathology of Karpa Chooranam



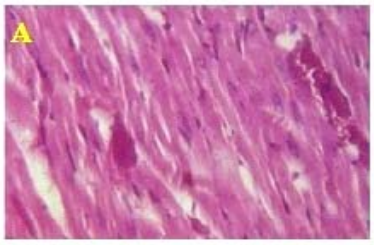


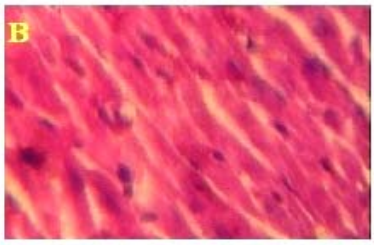
Panel 1: Light photomicrography of liver of control mice
Figure A – Control
Figure B – Treated on high dose, no abnormality is seen in hepatocytes, sinusoids.



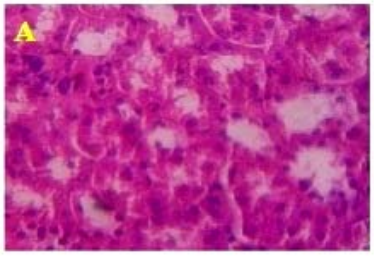


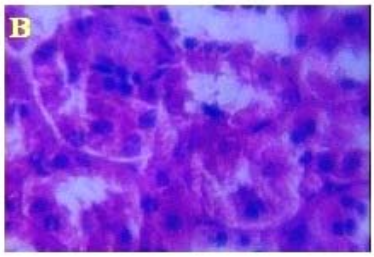
Panel 2: Light photomicrography of Spleen of control mice
Figure A – Control
Figure B – Treated on high dose, no abnormality is seen in trabeculae, capsule.





Panel 3: Light photomicrography of Heart of control mice
Figure A – Control
Figure B – Treated on high dose, no abnormality is seen in nuclei of Myocytes, myocardium





Panel 4: Light photomicrography of Kidney of control mice
Figure A – Control
Figure B–Treated on high dose, no abnormality is seen in glomeruli, Bowman's capsule, capillaries.

CONCLUSION

From these studies the authors conclude that the acute and sub-acute toxicity studies of *Karpa Chooranam* revealed no toxicity by oral route over a period of 28 days. So, the female infertility Siddha medicine *Karpa Chooranam* can be prescribed therapeutically up to a human dose of 3gm, in infertile women as per the dosage recommended in literature.

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