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Acute Toxicity and 28 days repeated Oral Toxicity Study of Nilappanai Kizhangu Chooranam in Wistar Albino Rats

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Abstract

Nilappanai Kizhangu Chooranam (NKC) is a Poly herbal Siddha formulation (from the text Kannusaami Parambarai Vaithiyam) known for its Spermatogenic properties. However, scientific validation of its safety profile is essential. This study aims to evaluate the acute toxicity of Nilappanai Kizhangu Chooranam in Wistar albino rats. **Methods:** An acute oral toxicity study was conducted following OECD guidelines 423. Female Wistar albino rats were divided into control and test groups. The test group received a single dose of NKC at 2000 mg/kg body weight by oral gavage, while the control group received distilled water. The animals were observed for 14 days for clinical signs of toxicity, behavioral changes, body weight variations, and mortality. Hematological and biochemical parameters were analyzed at the end of the study, followed by histopathological examination of vital organs. **Results:** No mortality or significant behavioral changes were observed in Nilappanai Kizhangu Chooranam-treated rats. Body weight, food, and water intake remained normal. Hematological and biochemical parameters showed no significant deviations compared to the control group. Histopathological analysis revealed no major abnormalities in the liver, kidney, heart, or other organs. **Conclusion:** The results indicate that Nilappanai Kizhangu Chooranam is non-toxic at a dose of 2000 mg/kg in Wistar albino rats, suggesting a high safety margin for therapeutic use.

Keywords: NilappanaiKizhanguChooranam, acute toxicity, Wistar albino rats, Siddha medicine.

Introduction

Siddha Medicinal plants play a key role in the human health care. About 80% of the world population rely on traditional medicine which is based on plants[(1)]. The use of medicinal plants for healing purposes has been increasingly popular as they are believed as beneficial, free of side effects, and their efficacy and cost effectiveness[(2)]. These drugs are either single plant extracts or fractions or mixtures of extracts from different plants. These plant extracts are

standardized for their safety and efficacy[3]. The acute toxic class method was a stepwise procedure with the use of 3 animals of a single sex per step. Depending on the mortality and the moribund status of the animals, on average 2-4 steps maybe necessary to allow judgment on the acute toxicity of the test substance. Morbid animals or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed, and are considered in the interpretation of the test results in the same way as animals that died on 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%. Acute toxicity study was carried out as per OECD guideline (Organization for Economic Co - operation and Development, Guideline-423.

The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) under CPCSEA. This study evaluates acute and sub-acute toxicity profile of poly-herbal siddha formulation of SC in laboratory animals which will provide effective documentation for its safety aspect in human usage of this drug.

Materials and Methods

Methodology

Acute oral toxicity–OECD guidelines–423[4]

Selection of animal species

The preferred rodent species was rat, although other rodent species may be used. Healthy young adult animals of commonly used laboratory strain Swiss albino rat were obtained from Animal house of king‟s institute, Guindy, Chennai. Females should be nulliparous and non-pregnant. Each animal at the commencement of its dosing should be between 8 and 12 weeks old and its weight should fall in an interval within±20 % of the mean weight of the animals. The studies were conducted in the animal house of C.L. Baid Metha College of pharmacy, Thuraipakkam, Chennai.

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 hrs dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be grouped and tagged 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.

Experiment procedure

Administration of doses

NPKC was prepared as per the classical Siddha literature was suspended in 2% CMC with uniform mixing and was administered to the groups of Wistar albino rats. It was given in a single oral dose by gavages 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 hours prior to the administration of the test suspension. Finally, the number of survivors was noted after 24 hours 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

Since this NPKC has been under practice for long time and likely to be non- toxic, a limit test at one dose level of 2000 mg/kg body weight will be carried out with 6 animals (3 animals per step).

Duration of Study	:	48 hours
Evaluation	:	14 Days

Limit test

The limit test was primarily used in situations where the experimenter has information indicating that the test material was 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

- The animals were observed individually after dosing at least once during the first 30mins and periodically during the first 24 hours.

- Special attention: First 1-4 hours after administration of drug, and

- It was observed daily thereafter for a total of 14 days, except when they needed to be removed from the study and killed humanely for animal welfare reasons or are found dead.

Mortality

Animals will be observed intensively at 0.5, 2.0, 4.0, 6.0, 12.0, 24.0 and 48.0 hours following drug administration on day 1 of the experiment and daily twice thereafter for 14 days.

Body weight

Body weights will be recorded at day: -1, day 1, 2, 7 and 14 of the study

Cage-side observation

These include changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

Gross necropsy

All animals (including those which die during the test period are removed from the study) will be subjected to gross necropsy. Gross necropsy includes examination of the external surface of the body, all orifices, cranial, thoracic and abdominal cavities and their contents, brain, eye, thymus, lungs, heart, spleen, liver, kidneys, adrenals, testes and uterus of all animals

Histopathology

Microscopic examination will be carried out in organs to show the evidence of any toxicity in gross pathology.

Data and reporting

All the data were summarised in tabular form showing the animals used, number of animals displaying signs of toxicity, the number animals found dead during the test or killed for humane reasons, a description and the time course of toxic effects and reversibility, and necroscopic findings.

Test substance and Vehicle

In order to ensure the uniformity in drug distribution in the medium the suspension was made by mixing NPKC with 2% CMC solution and it was found suitable for dose accuracy.

Justification for choice of vehicle

The vehicle selected as per the standard guideline was pharmacologically inert and easy to employ for new drug development and evaluation technique.

28 Days repeated dose oral toxicity study of "*Nilappanai Kizhangu Chooranam*" on rats – (OECD-407 guidelines) [5]

Justification for Dose Selection

The results of acute toxicity studies in Wistar albino rats indicated that NPKC was non-toxic and no behavioral changes was observed up to the dose level of 2000 mg/kg body weight. On the basis of body surface area ratio between rat and human, the doses selected for the study were100mg/kg, 200 mg/kg and 400 mg/kg body weight. The oral route was selected for use because oral route was considered to be a proposed therapeutic route.

Preparation and administration of dose

NPKC 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 100, 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.

Methodology

Randomization, Numbering and Grouping of Animals

Ten rats (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

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.

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.

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.

Laboratory Investigations

Following laboratory investigations were carried out on day 29 in animal"s 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 rpm for 10 minutes. On 28th day of the experiment, 24 hours 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 faecal contamination. Toluene was 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 hours, 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 and Packed Cell Volume (PCV).

Biochemical Investigations

Serum was used for the estimation of biochemical parameters. Samples of control and experimental rats were analyzed for protein, bilirubin, urea, BUN, 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 on end of treatment 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, spleen, brain, heart, and lungs were recorded. The relative organ weight of each animal was then calculated as follows;

Relative organ weight =

$$\frac{\text{Absolute organ weight (g)}}{\text{Body weight of animal on sacrifice day (g)}}$$

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 hours and washed in running water for 24 hours. Samples were dehydrated in an auto technique 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, ovary, pancreas, brain, spleen and stomach, 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, haematology and blood chemistry were subjected to One-way ANOVA followed by Dunnet‟s multi comparision test using a computer software programme GRAPH PAD INSTAT-3 version.

Results

Acute Toxicity Reports

Findings of Acute toxicity studies of NPKC

Group	Day
Body weight	Normal
Assessments of posture	Normal
Signs of Convulsion	Absence (-)
Body tone	Normal
Lacrimation	Absence
Salivation	Absence
Change in skin color	No significant colour change
Piloerection	Normal
Defecation	Normal
Sensitivity response	Normal
Locomotion	Normal
Muscle gripness	Normal
Rearing	Mild
Urination	Normal

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. Diarrhea 18. Writhing 19 Respiration 20. Mortality.

Results of sub-acute oral toxicity 28-days repeated dose study in rats

Body Weight:

Table 1. Changes in body weight of NPKC after 28 days repeated oral dose toxicity studies.

Dose (mg/kg/day)	Days				
	0	7	14	21	28
Control	120.59±0.92	122.79±0.87	123.52±1.18	127.24±1.12	131.25±1.05
100	133.99±1.16*	133.22±1.75*	139.56±1.48*	142.08±1.92*	146.92±2.03*
200	138.76±1.12**	143.05±1.48	147.29±1.72	149.35±1.62**	154.49±1.99**

Values are expressed as mean ± S.D. N=3

Organ weight

Table 2: Changes in organ weight of NPKC

Organ	Control	100 mg/kg	200 mg/kg
Liver (g)	3.07±0.20	2.73±0.61	2.48±0.33
Heart (g)	0.32±0.04	0.29±0.05	0.27±0.05
Lung (g)	0.28±0.05	0.30±0.04	0.27±0.04
Spleen (g)	0.25±0.06	0.22±0.03	0.17±0.03
Brain (g)	0.37±0.05	0.56±0.52	0.49±0.05
Kidney (g)	0.76±0.05	0.81±0.10	0.74±0.07

*Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; N=3*

Haematological parameters

Table 3: Changes Observed In Haematological Parameters Of NPKC In Rats.

Parameter	Control	100mg/kg	200 mg/kg
RBC(x 10^6/mm^3)	8.29±0.43	8.36±0.43	9.49±0.46
PCV (%)	49.66±0.77	52.45±1.61	54.52±1.62
Hb (%)	15.13±0.39	15.41±0.44	15.83±0.54
WBC(x 10^3/mm^3)	11.75±0.85	11.21±0.63	10.83±0.68
Neutrophils (%)	23.29±0.73	22.85±0.74	21.51±0.41
Lymphocytes(%)	85.5±0.46	86.07±0.49	86.80±0.29
Eosinophils(%)	4.10±0.23	3.43±0.46	2.83±0.58
Platelets(x 10^3/mm^3)	425.73±1.35	438.40±1.65	440.87±1.80

*Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; N=3*

Biochemical parameters

Table 4: Changes Observed in Biochemical Parameters Of NPKC In Rats.

Parameters	Control	100 mg/kg	200 mg/kg
Glucose (mg/dl)	108.63±0.81	105.54±1.81	103.39±1.04
BUN (mg/dl)	22.06±1.55	22.87±1.49	24.89±0.89
Creatinine (mg/dl)	0.85±0.07	0.85±0.06	0.77±0.09
SGOT (U/L)	74.35±1.23	72.59±0.85	70.72±1.29
SGPT(U/L)	27.07±0.84	26.83±0.82	28.56±1.06
ALP (U/L)	104.63±1.14	101.17±1.71	99.84±1.50
Protein (g/dl)	8.58±0.68	7.87±0.89	7.44±0.81
Albumin (g/dl)	5.34±0.40	6.41±0.85	7.81±0.57
Total Cholesterol (mg/dl)	93.21±1.16	94.39±0.97	95.34±1.22
Triglycerides (mg/dl)	52.58±1.56	54.36±1.03	56.05±1.02

*Values are expressed as mean ±S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; N=3*

Urine Parameter

Table 5: Changes Observed In Urine Parameters Of NPKC In Rats.

Parameter	Control	100 mg/kg	200 mg/kg
Colour	Yellow	Yellow	Yellow
Transparency	Clear	Clear	Turbid
Specific gravity	1.01	1.02	1.04
pH	7.2	7.4	6.9
Protein	Nil	Nil	Nil
Glucose	Nil	Nil	Nil
Bilirubin	-ve	-ve	-ve
Ketones	-ve	-ve	-ve
Blood	Absent	Absent	Absent
RBCs	Nil	Nil	Nil
Epithelialcells	Nil	Nil	Nil
Casts	Nil	Nil	Nil

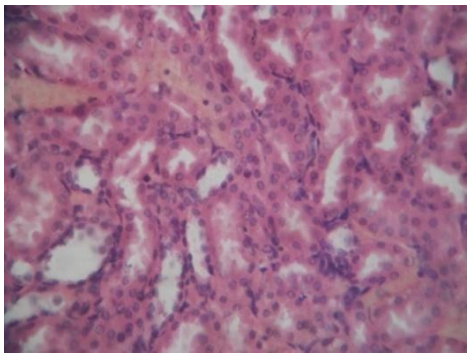
*Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; N=3*

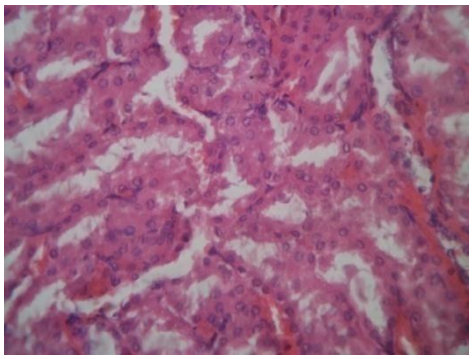
Histopathological reports

Histopathology Images of *Nilappanai kizhangu chooranam*

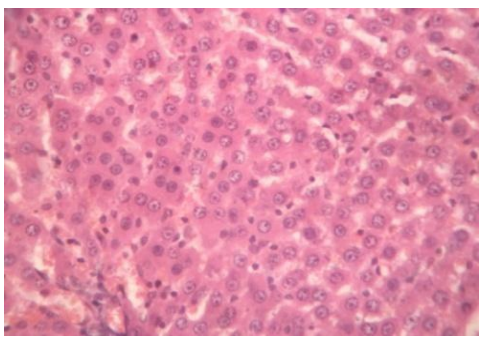
(100 mg/kg) **(200 mg/kg)**

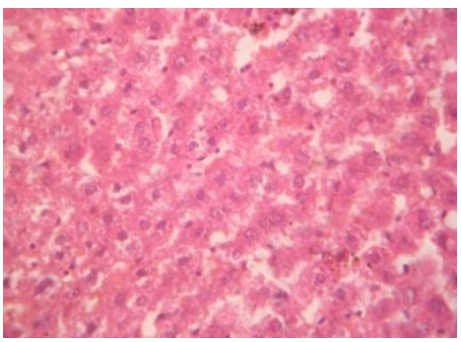
Kidney



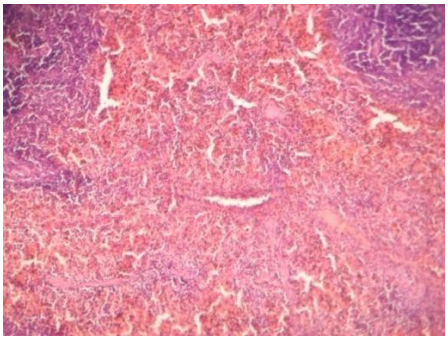


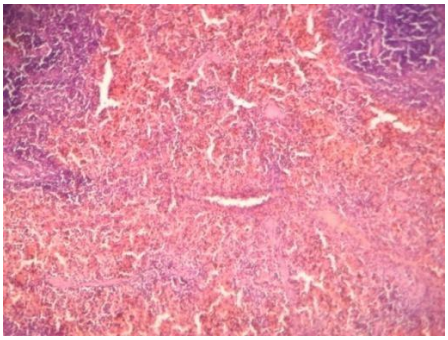
Liver





Spleen





Discussion

In the acute toxicity study, the rats were treated with different concentration of *Nilappanai kizhangu chooranam* from the range of 5mg/kg to 2000mg/kg which did not produce signs of toxicity, behavioural changes, and mortality in the test groups as compared to the controls when observed during 14 days of the acute toxicity experimental period. These results showed that a single oral dose of the extract showed no mortality of these rats even under higher dosage levels indicating the high margin of safety of this extract. In acute toxicity test the *Nilappanai kizhangu chooranam* was found to be non toxic at the dose level of 2000mg/ kg body weight.

In the evaluation of 28 days repeated oral dose toxicity study of the drug *Nilappanai Kizhangu Chooranam*, although the body weights were increased during treatment period, there were no significant different ($P > 0.05$) of body weights in male and female rats or between treatment and control groups. The RBC lymphocytes and neutrophils and coagulation parameters did not show any biologically or statistically significant differences between rats treated or controls. In hematological and biochemical examination, all the observations lie in the reference range. There is no change in the biochemical and haematological parameter when compared with the control groups

Furthermore no dose related histopathological changes were observed. Gross examination in necropsy and at microscopic examination revealed no changes that attribute to the administration of drug.

Compared with concurrent controls, rats fed with *trail drug Nilappanai Kizhangu Chooranam* showed no changes in clinical chemistry and hematology values at various dosages.

There were no significant different changes of organ-to-weight ratios in male and female rats or between treatment and control groups.

Treating Wister albino rats with *Nilappanai Kizhangu Chooranam* at levels of 100 and 200 mg/kg/day to male and female rats for 4 weeks did not cause death and was not associated with adverse effects in general condition, growth, body and organ weights, hematology and clinical chemistry values, nor did it cause abnormalities in necropsy and histopathology findings.

According to these results, *Nilappanai Kizhangu Chooranam* could be considered as no-observed-adverse-effect level (NOAEL) drug as it acts harmlessly under the current normal usage and this phenomenon is considered to be of no toxicological concern.

Conclusion

Based on OECD 423 the trail drug *Nilappanai kizhangu chooranam* is considered as non-toxic up to the dose of 2000mg/kg. The drug *Nilappanai Kizhangu Chooranam* was proved that it is free from toxicity through the acute and 28 days repeated oral toxicity study as per the OECD guidelines. In acute toxicity study there was no mortality of rats observed. In 28 days repeated oral toxicity study, the obtained results of

haematological, biochemical, urinary parameters were normal. The histopathological findings did not show any abnormalities.

By analyzing all those findings, it is proved that the drug *Nilappanai Kizhangu Chooranam* has high range of therapeutic value. And the safety of the drug for clinical use is ensured. So, it is confirmed that the herbal formulation *Nilappanai Kizhangu Chooranam* may never cause any adverse effects in clinical use. The preparation of the drug is cost effective too.

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