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TOXICOLOGIAL PROFILING OF A *SIDDHA* FORMULATION *NAAURUVI KUZHITHYLAM* IN EXPERIMENTAL RODENTS

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ABSTRACT
Naauruvi Kuzhithylam (NKT) is a *Siddha* herbal formulation indicated in literature for Respiratory ailments. This study was aimed to evaluate the Acute and Sub acute oral toxicity study of *Naauyurivi Kuzhithylam (NKT)* in albino mice and Wistar rats respectively to reevaluate the traditional claims for the safety of the test drug. It was observed that the formulation was found to be non-toxic at dosage of 5000mg/kg/po in mice and so it can be classified under category-5 of globally harmonized system of classification and labelling of chemicals. The biochemical and hematological parameters were analyzed and the Histopathological study was performed for 28 days repeated oral toxicity. The data were statistically analyzed using one-way analysis of variance (ANOVA) using INSTAT-V3 software package and the results were expressed in mean ± SD. Through this study the *Siddha* formulation *Naauruvi Kuzhithylam (NKT)* can be evidently accepted to be non toxic in nature and its safety is accredited.

KEYWORDS: *Naauruvi Kuzhithylam (NKT), Siddha,* Acute toxicity, Sub acute Toxicity, Traditional medicine, Herbal medicine.

INTRODUCTION
Traditional systems of medicines are renowned in India due to repository of immense flora and fauna that have been therapeutically used since ancient times. Around 20,000 medicinal plants have been recorded in India, among which 7,000–7,500 plants have been traditionally claimed for its medicinal values.[1] The *Siddha* system of medicine is one such age old system that owes its origin to the thoughts and practices of Tamil sages who belonged to Southern parts of India called *Siddhars*. The *Siddha* literature has a vast collection of herbal and mineral drugs for the treatment of various common ailments of humans.[2,3] Though these formulations have been time tested for their safety and efficacy, most of them have not been scientifically validated.

Naauruvi Kuzhithylam (NKT) is a single herbal formulation that has been mentioned in *Gunapaadam Mooligai Vaguppu* a Siddha literature on Materia medica for the treatment of cough, Bronchial Asthma and other respiratory ailments (*Kapha diseases*).[4] Through this research an effort has been made to explore acute and subacute oral toxicity of the Siddha formulation *Naauruvi Chooranam* in experimental rodents and to authenticate its safety behind its traditional use.

MATERIALS AND METHODS
Preparation of *Naauruvi Kuzhithylam*
The whole plant (*Samoolam*) of *Achyranthes aspera* (*Naauruvi)* was dried and soaked in Cow's urine for one day. After which it was again dried and kept in *Kuzhithylum* Apparatus and was placed in a pit. Cow dung cakes were arranged in a regular manner in the pit around the apparatus from top to bottom. Then the cow dung cakes were incinerated and the *kuzhithylam* was collected.[4]

In vivo Toxicity studies
The preclinical studies for acute toxicity were carried out in pharmacological laboratory department of Pharmacology and toxicology, Vel's University. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) (CPCSEA XIII/VELS/COL/18/CPCSEA/IAEC/23.09.11).

Experimental animals
36 Albino mice weighing (24-27gms) and 24 Wistar Albino rats weighing (100-150g) of either sexes were used for acute and subacute toxicity study respectively. All the animals were maintained in a controlled environment condition of temperature (24 ± 1^0C) an alternative 12h light/ dark cycles. They were reared at the animal house of. Animals were kept in cages and fed

on commercial pellets (Hindustan Lever Ltd, Mumbai, India and water *ad libitum.*

Preparation of Stock solution

The *Siddha* drug *NKT* was mixed uniformly in 2% CMC solution to achieve 200mg/ml as main stock solution and used in this study.

Acute toxicity study

The acute oral toxicity study was carried out as per the OECD-425. The animals were segregated into six groups consisting of six mice each. The animals were administered with a start dose of 500mg/kg and the dose was increased up to 5000mg/kg. Daily observations were made for physiological and behavioral responses and mortality for a period of 1-14 days. The LD50 was determined by observing the mortality rate in the drug treated groups. One-tenth ($1/10^{th}$) of the lethal dose was considered as therapeutic dose for further pharmacological study

Sub-acute toxicity studies

The rats were divided at random into a control group and three experimental groups with six animals in each group. The vehicle control group received 0.2% Carboxy methyl cellulose (CMC), whereas the experimental groups received *NKT* (250, 500, and 1000 mg/kg body weight, p.o.) administered by means of bulbed steel needle for 28 days. Body weights were recorded on days 1 and 28 after which the animals were fasted overnight after the dosage period, anaesthetized with diethyl ether, and then decapitated. Paired blood samples were collected into heparinized and nonheparinized tubes. The heparinized blood was used for hematological evaluation; the non-heparinized blood was allowed to coagulate, contents centrifuged, and the serum separated was analyzed for biochemical parameters of the experiment.

Determination of serum biochemical and hematological parameters

Chemical analysis carried out on serum to assay blood urea, creatinine, total protein, albumin, total bilirubin, alkaline phosphatase (ALP), aspertate amino transferase (APT) and alanine amino transferase (ALT) measured. At the end of the experiment, Body weight was also noted and all the rats were fasted for 12hrs, sacrificed under anesthetic condition. The hematological and serum biochemical parameters were determined.

The hematological parameters assayed included red blood cell (RBC) and white blood cell (WBC) counts inclusive of polymorpho nuclear leucocytes and lymphocytes, platelets, hematocrit and hemoglobin (Hb) estimation. Erythrocyte indices (mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH)) was determined from values obtained from red blood cell (RBC) count, Hb concentration and packed cell volume (PCV) values.

Histopathology

All the animals were sacrificed and were necropsied after collection of blood samples. and the vital organs such as liver, lungs, heart, kidney, spleen and brain were removed, weighed individually and fixed in 10% buffered formalin in labeled bottles. Organs were processed to study histological changes adopting paraffin method. The histopathological changes of these tissues were observed on gross and microscopic bases.

Statistical analysis

The significant differences were assessed using one-way analysis of variance (ANOVA) using INSTAT-V3 software package. $P > 0.05$ were considered significant .The results were expressed as mean ± SD.

RESULTS AND DISCUSSION

Table 1: Acute toxicity study.

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.	500	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-
2	1000	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-
3	2000	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	-
4	5000	+	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	-

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

Sub acute toxicity Study

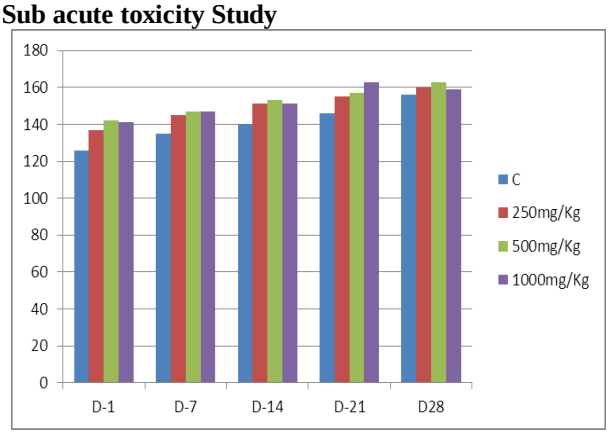


Fig-1: Changes in Body weight (gm) of *NKT* treated rodents.

The sub-acute toxicity study of *NKT* with the above doses did not reveal any toxicity symptoms as revealed in body weights (Fig-1) and also organ weights of rats. Body weight and organ weight changes serve as an indicator of adverse side effects since animals that survive cannot lose more than 10% of the initial body weight.[5] No significant decrease in body weight has found among the control and treated group. Instead the body weights of experimental and control rats were found to be increased significantly indicating that there was no loss of appetite and suggesting that *NKT* had no effect on the normal growth of rats, justifying the doses chosen.

Table 2: Effect of *NKT* on Hematological parameters.

Parameter	Control	250mg/kg	500 mg/kg	1000 mg/kg
RBC (mm^3)	5.67±0.41	5.34±0.46	4.90±0.38	5.66±0.42
HB (%)	14.54±0.35	14.67±0.46	14.82±0.42	14.84±0.41
Leukocyte($x10^3/mm^3$)	3.63±0.6	3.71±0.5	4.0±0.6	3.88±0.6
Platelets/ul	1.49±0.14	1.31±0.12	1.16±0.18*	1.08±0.22**
MCV (gl)	87.20±5.2	85.27±5.4	88.19±4.10	86.55±5.21
N	49.61±4.15	49.18±4.12	46.15±3.56	48.25±3.00
L	48.04±3.6	48.10±4.00	44.12±2.60	41.44±3.21**
M	4.00 ± 3.12	3.77 ± 3.00	3.04 ± 2.84*	3.01 ± 2.00*
E	2.36 ± 0.28	2.00 ± 0.19*	1.88 ± 0.26**	1.30 ± 0.12**
B	1.02 ± 0.62	0.82 ± 0.50	0.70 ± 0.34*	0.72 ± 0.30*
ESR (mm)	1±00	1±00	1±00	1±00
PCV	44.05±2.4	43.78±2.16	44.12±2.60	44.18±2.56
MCH pg	29.19±1.40	29.11±1.27	30.42±1.22	30.10±0.71
MCHC g/dl	34.64±0.5	33.77±0.5	35.00±1.54	35.18±2.00

Values are mean of 6 animals ± SEM (Dunnet't's test). *P<0.05; **P<0.01. N=6.

Table 2.1: Effect of *NKT* on Biochemical parameters of Liver function (LFT).

Dose (mg/kg)	Control	250mg/kg	500 mg/kg	1000 mg/kg
Total Bilirubin (mg/dL)	0.64±0.20	0.64±0.22	0.62±0.28	0.66±0.21
ALP (IU/L)	61±4.6	60.12±5.2	58.79±6.1	62.03 ±4.8
AST (IU/L)	89.12±8.0	90.18±10.05	90.57±6.00	91.1±6.24
ALT (IU/L)	89.30±8.0	87.21±5.8	88.8±7.12	90.2±6.46
Protein (g/dl)	4.55±0.58	5.0±0.32	4.97±0.28	4.78±0.40
Albumin (g/dl)	4.29±0.24	4.36±0.25	4.18±0.26	4.21±0.22

Values are mean of 6 animals ± SEM (Dunnett's test). ^{Ns}P>0.05 *vs. control group* N=6.

Table 2.2: Effect of *NKT* on Biochemical parameters of Renal function (RFT).

Dose (mg/kg)	Control	250mg/kg	500 mg/kg	1000 mg/kg
Urea (mg/dl)	35.18±4.2	34.65±4.4	35.12±5.6	34.82±4.7
Creatinine (mg/dl)	0.99±0.22	0.97±0.20	1.10±0.30	1.14±0.38
Uric acid (mg/dl)	5.17±1.21	5.30±1.42	4.98±1.28	4.90±1.36
Sodium	143.00±2.33	142.27±2.10	142.00±2.16	140.12±2.44
Potassium	7.10±0.72	7.24±0.68	6.67±0.60	5.17±0.60
Chloride	104.10±1.29	103.80±1.15	104.48±2.26	105.77±2.51

Values are mean of 6 animals ± SEM (Dunnet 't' test). *P<0.05 vs control group N=6.

Table 2.3: Effect of *NKT* on Lipid profile.

Dose (mg/kg)	Control	250mg/kg	500 mg/kg	1000 mg/kg
Cholesterol (mg/dl)	77.11±4.18	76.26±5.2	79.10±5.12	80.00±7.2
HDL (mg/dL)	15.00±2.56	14.42±2.34	14.48±2.30	14.10±2.08
LDL (mg/dL)	35.15±1.62	34.01±2.22	35.13±2.46	34.80±2.76
VLDL (mg/dl)	19.10±2.10	21.00±2.46	21.28±1.40	20.21±1.34
Triglyceride (mg/dl)	88.00 ± 27.12	89.40 ± 36.08	87.89 ±23.30	90.20 ± 33.52
Glucose (mg/dl)	103±10.8	101±12.4	104±10.4	103±12.46

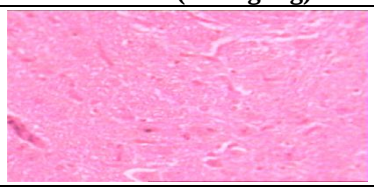
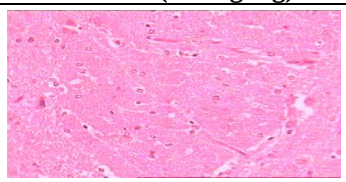
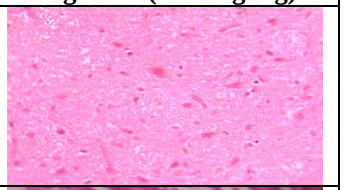
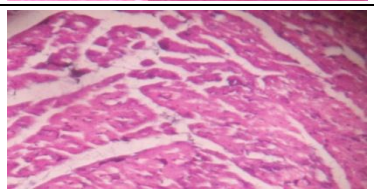
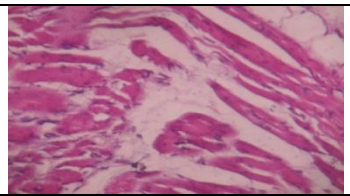
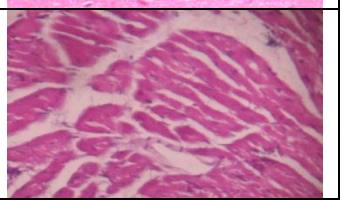
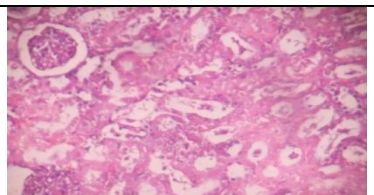
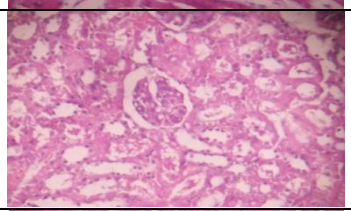
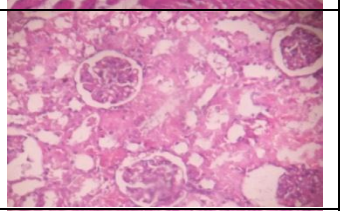
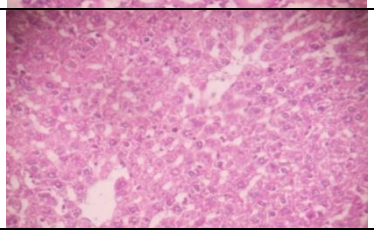
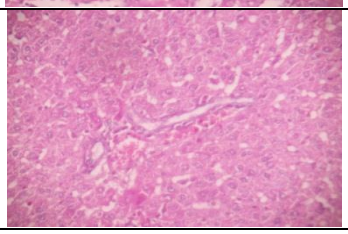
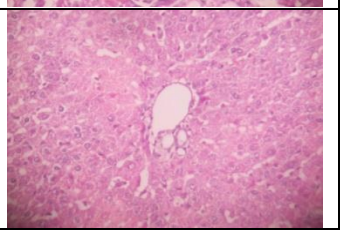
Values are mean of 6 animals ± SEM (Dunnet't' test). ^{Ns}P>0.05 vs control group N=6.

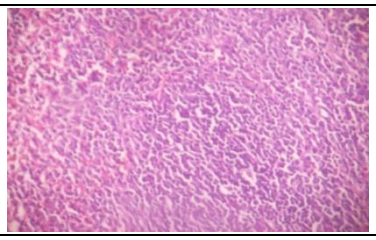
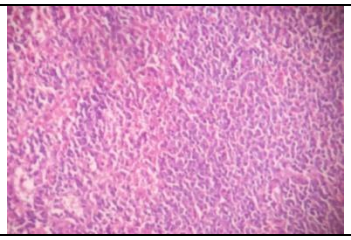
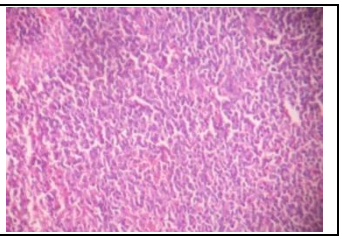
The trial drug *NKT* did not exhibit any significant toxicity as there was no abnormal behavioural changes or mortality after the oral administration of the drug up to a dose level of 5000 mg/kg body weight throughout period of study (Table-1). Therefore *NKT* falls under class 5 of of globally harmonized system (GHS) of classification and labeling of chemicals (LD50 > 5000 mg/kg).

Hematological and biochemical parameters play a decisive role in the drug induced toxicity Blood parameters analysis is highly relevant to risk evaluation in humans (91%) when assays involve rodents and nonrodents.[6] Damage to blood cells are detrimental to normal functioning of the body as blood forms the chief medium of transport for various drugs and xenobiotics in the body.[7] In the present study the Hematological (WBC, RBC, Hb, HCT, MCV, MCHC, PLT, MPV, DC, PLT CRIT, PDW%) parameters, platelets, Lymphocytes, Monocytes, Eosinophils and basophils were found to show significant alteration at higher doses of 500 and 1000mg/Kg but the values were found to be well within the clinical range for rats. This indicates that the test drug does not interefere with the production of blood cells (Hematopoeisis) and forecasts the associated safety when administered in the human body.

The biochemical parameters AST and ALT are regarded as "biomarkers" of liver injury and they indicate the intactness of liver cells and function.[8,9] The estimation of Serum electrolytes and renal parameters are sensitive indicators of nephrotoxicity.[10] The data analysis of the present results conclude that the test drug *NKT* is safe as it did not cause any significant rise of these enzymes ensuring that it did not cause any damage to the liver and renal parenchyma cells cause drug toxicity. There was also no change observed in the lipid profile of both control and treated groups indicating that the test drug does not cause any significant metabolic changes (Table 2-2.3).

Fig-3: Histopathological analysis.

Organs	Low dose (250mg/Kg)	Mid dose(500mg/Kg)	High dose(1000mg/Kg)
BRAIN			
HEART			
KIDNEY			
LIVER			

SPLEEN			

Histopathological analysis of the control and treated groups has shown normal morphological features of hepatocytes and renal architectures with no necrosis. Further the Heart, lung, brain and spleen of the treated rats also did not demonstrate significant changes in morphology indicating the preserved integrity of all these organs on treatment with *NKT* at low, mid and high dose treated groups.

CONCLUSION

The present preclinical study on aimed to validate the possible toxicity of the *Siddha* herbal formulation *Naauruvi Kuzhithylam (NKT)* in mice and rat models showed that the drug did not show any signs of behavioral abnormality or mortality in 14 days period of Acute toxicity study with LD50> 5000mg/kg and also showed no significant alteration in the hematological, biochemical parameters in the Sub-acute toxicity studies after repeated oral administration for 28 days. Hence the tested *Siddha* formulation *Naauruvi Kuzhithylam* may be considered as relatively safe for human consumption. Further testing on chronic toxicity and molecular studies may also be needed to confirm its safety potential in future.

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